

**INTERACTIONS IN THE DYSTROPHIN-
ASSOCIATED PROTEIN COMPLEX
(DAPC) REGULATED BY
PHOSPHORYLATION**

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Abstract

Duchenne muscular dystrophy (DMD) is caused by mutations at the DMD locus leading to a lack of the dystrophin protein in muscle cells. Dystrophin associates with a group of membrane-associated proteins called the dystrophin-associated protein complex (DAPC) via an interaction with the membrane spanning glycoprotein β -dystroglycan. The exact functions of dystrophin and the DAPC have not been elucidated and little is known about the regulation of protein:protein interactions within this complex. However, the proteins are ideally placed to transduce signals from the extracellular matrix to the cytoskeleton and have been implicated in a wide variety of cell signalling events. The aim of this study was to identify novel phosphorylation events within the DAPC and determine their effects on the regulation of protein:protein interactions in the complex.

Upon treatment of C2/C4 cells (a mouse muscle cell line) with peroxyvanadate, a potent tyrosine phosphatase inhibitor, β -dystroglycan is tyrosine-phosphorylated. This phosphorylation event was initially detected by mobility shifts on SDS-polyacrylamide gels, and confirmed by immunoprecipitation and two-dimensional gel electrophoresis. The potential functional significance of this tyrosine phosphorylation was investigated using peptide 'SPOTs' assays. Phosphorylation of tyrosine in the 15 most C-terminal residues of β -dystroglycan disrupts its interaction with dystrophin. The tyrosine residue in β -dystroglycan's WW domain-binding motif PPPY appears to be the most crucial in disrupting the interaction. Therefore the β -dystroglycan/dystrophin interaction appears to be regulated by tyrosine phosphorylation.

Various methods were used to investigate the protein:protein interactions of β -dystroglycan. In a yeast two-hybrid assay β -dystroglycan was found to interact with actin. This novel interaction was further characterised by F-actin sedimentation assays and it was demonstrated β -dystroglycan facilitated the sedimentation of F-actin during low speed centrifugation. Furthermore, electron microscopy studies revealed that β -dystroglycan bundles actin filaments.

These studies highlight the importance of β -dystroglycan, not only in the regulation of the interaction between dystrophin and the rest of the DAPC but also its possible role in the regulation of the actin cytoskeleton. Understanding more about functions of dystrophin and the DAPC allows a greater insight into the pathogenesis of Duchenne muscular dystrophy.

Abbreviations

AD	Activation Domain
Amp	Ampicillin
ATP	Adenosine 5'-triphosphate
BD	(DNA) Binding Domain
β -Gal	β -Galactosidase
BLAST	Basic Local Alignment Search Tool
bp	Basepair
BSA	Bovine Serum Albumin
$^{\circ}$ C	Degrees Celsius
cDNA	Complementary Deoxyribonucleic Acid
cfu	Colony forming units
DAPC	Dystrophin-associated protein complex
cm	Centimetre
dATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
dTTP	Deoxythymidine 5'-triphosphate
dGTP	Deoxyguanosine 5'-triphosphate
ddNTP	Dideoxynucleoside 5'-triphosphate
dNTP	Deoxynucleotide 5'-triphosphate
DNA	Deoxyribonucleic Acid
DNase	deoxyribonuclease
DTT	Dithiothreitol
dTTP	Deoxythymidine Triphosphate
ECL	Enhanced Chemi-luminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic Acid
g	Gram(s)
g^{av}	Gravitational force
Gal	Galactose
GTP	Guanidine 5'-triphosphate
hrs	Hour(s)
HRP	Horse Radish Peroxidase
IPTG	Isopropyl- β -D-thiogalactopyranoside
kb	Kilobase pairs
kDa	KiloDalton
l	Litre(s)
M	Molar (moles per litre)
m A	Milliampere(s)
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
mM	Millimolar
m m	Millimetre(s)
MW	Molecular Weight

ng	Nanogram(s)
nm	Nanometre(s)
NMJ	Neuromuscular Junction
OD	Optical Density
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
pH	-Log ₁₀ [hydrogen ion concentration]
pmol	Picomole(s)
RNase	Ribonuclease
rpm	Revolutions Per Minute
SDS	Sodium Dodecyl Sulphate
SET	Sucrose/EDTA/Tris
TBE	Tris/Borate/EDTA (buffer)
TBST	Tris-buffered Saline/Tween-20
TE	Tris/EDTA (buffer)
TEMED	<i>N,N,N',N'</i> -tetramethyl-ethylenediamine
Tet	Tetracycline
Tris	Tris(hydroxymethyl)-amino-methane
Tris-HCL	Tris solution, pH adjusted with HCl
µg	Microgram(s)
µl	Microlitre(s)
U	Unit(s)
UAS	Upstream Activating Sequence
UV	Ultra Violet
V	Volt(s)
v/v	Volume Per Unit Volume
w/v	Weight Per Unit Volume
W	Watts
X-Gal	5-Bromo-4-chloro-3-indolyl-β- <i>D</i> -galactoside
YPDA	Yeast/Peptone/Dextrose/Adenine (medium)

Amino-acid Abbreviations

<u>Amino-acid</u>	<u>Three letter code</u>	<u>One letter code</u>
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any amino-acid		X

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CHAPTER 1
Introduction

Introduction

1.1. Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) is the most frequent lethal X-linked recessive disease with an incidence of 1 in every 3500 live male births (Emery, 1993). It is characterised by progressive muscle fibre degeneration and is caused by mutations at the DMD locus leading to a lack of dystrophin protein in muscle cells (Hoffman *et al.*, 1988). DMD patients are clinically normal at birth although the disease is characterised by elevated levels of the muscle isoform of creatine kinase (CK) in the serum. The first clinical sign of DMD is the delayed ability to walk and difficulty running due to the progressive weakening of the limb muscles. Wheelchair dependence is usually evident by the end of the first decade and the disease is fatal in the third decade resulting from the degeneration of diaphragm and cardiac muscles.

Becker muscular dystrophy (BMD) is a less severe and more variable form of muscular dystrophy which occurs in 1 male per 18500 (Bushby *et al.*, 1991). BMD patients have an abnormal dystrophin protein and/or altered levels of dystrophin expression usually due to in-frame deletions of the DMD gene.

Although the genetic defect underlying Duchenne and Becker muscular dystrophy has been known for some years (Hoffman *et al.*, 1987), the function of dystrophin and the sequence of events leading to the observed pattern of muscle fibre necrosis is unknown. It is hypothesised that dystrophin deficiency initiates a complex pathophysiological response which determines the clinical phenotype which develops. Early in the disease process, the absence of dystrophin causes elevated CK levels, grouped myofibre necrosis, pseudohypertrophy of muscles (apparent increased muscle mass due to replacement of muscle fibres with fibrotic

and adipose tissue) and muscle fibre size variability. The long-term effects of dystrophin deficiency include progressive fibrosis (excessive proliferation of connective tissue) and the failure of muscle regeneration due to inhibition by fibrotic proliferation or the exhaustion of the regenerative capacity of the cells. Both the fibrosis and lack of muscle regeneration lead to progressive fibre loss which causes gross muscle wasting and muscle weakness.

Neuronal nitric oxide synthase (nNOS) is associated with the sarcolemma of fast-twitch fibres in skeletal muscle (Kobzik *et al.*, 1994) as the result of its interaction with dystrophin (Brennan *et al.*, 1995). nNOS regulates and synthesises nitric oxide (NO) a major endogenous mediator involved in diverse developmental and physiological processes (Bredt & Snyder, 1992). nNOS has been found to be absent from the sarcolemma in patients with Duchenne muscular dystrophy and it is hypothesised that the disturbed regulation of NO contributes to the preferential necrosis of fast-twitch fibres in DMD.

One longstanding hypothesis is that the membrane defect in DMD leads to an abnormal influx of Ca^{2+} (Emery, 1993). Dystrophic muscle membranes undergo more frequent nonlethal tears (microdisruptions) than normal cell membranes, especially during exercise. Calcium entry through these microdisruptions leads to elevated calcium levels which alter the activity of the calcium leak channels. This is thought to contribute to the eventual necrosis of muscle cells in DMD due to increased calcium-dependent proteolysis (McCarter & Steinhardt, 2000)

Expression profiling using microarray technology has recently been used in an effort to to define biochemical cascades underlying the progressive pathophysiology of muscular dystrophies (Chen *et al.*, 2000). This work has provided insights into the disease processes involved and proteins

which may be involved in early stages of muscle fibre necrosis and inflammatory responses. There appears to be a persistent expression of developmentally regulated genes, suggesting that DMD muscle may assume a chronically dedifferentiated state. Many calcium-regulated signaling molecules are downregulated, which is thought to be a consequence of a negative-feedback loop caused by the chronic calcium influx through muscle membrane microdisruptions. More research using this method of expression profiling will no doubt lead to more understanding of the disease mechanisms of DMD and other muscular dystrophies.

1.1.1. Animal models of DMD

The *mdx* mouse is a naturally occurring animal model for DMD caused by a premature stop codon leading the generation of a dystrophin transcript only 27% of its full length. Overall, the *mdx* mouse has a milder pathology than humans. There is an assumption that the sarcolemmal membrane of mouse skeletal muscle endures less mechanical stress per unit area than in humans due the smaller size of the organism and the individual muscle fibres (Petrof *et al.*, 1993; Grady *et al.*, 1997b). This would account for the mild phenotype of the *mdx* mouse compared to the severity of DMD in humans.

There are many murine models for congenital muscular dystrophy (CMD) which is caused by a deficiency in the laminin $\alpha 2$ chain (Hillaire *et al.*, 1994). The absence of laminin $\alpha 2$ chain presumably leads to a disruption of the linkage between the sarcolemmal membrane and the extracellular matrix (Campbell, 1995). This dystrophic *dy/dy* mouse has a dysmyelination of the peripheral nervous system in addition to muscle degeneration (reviewed in Allamand & Campbell, 2000).

Mice with null mutations for utrophin (the ubiquitous homologue of dystrophin) also have a mild phenotype with no outward signs of muscular dystrophy (Deconinck *et al.*, 1997; Grady *et al.*, 1997a), suggesting that dystrophin and utrophin functionally compensate for one another. This potential compensation is demonstrated by mice deficient in both dystrophin and utrophin which have a severe phenotype comparable to DMD, including widespread loss of myocytes, myofibrosis and premature death. It has been suggested that this double knockout model is a more valid disease model for DMD due to its severity (Allamand & Campbell, 2000), but this is questionable. A disease model with utrophin present equates more accurately to the pathology of DMD patients.

The dystrophic golden retriever dog (GRMD) is perhaps a more attractive model for DMD because of its larger size (Cooper *et al.*, 1988) but there is phenotypic variability between litters, more ethical considerations than with mice, and their maintenance is more problematic and costly.

Inherited muscular dystrophy was first observed in the chicken in 1956, and when chicken dystrophin cDNA was sequenced, it was found to have a striking conservation of its C-terminal coding region with human dystrophin (Lemaire *et al.*, 1988). There has been very little research on the dystrophic chicken since the 1980s when it was used extensively in drug trials (Pisenti *et al.*, 1999).

1.2. Dystrophin

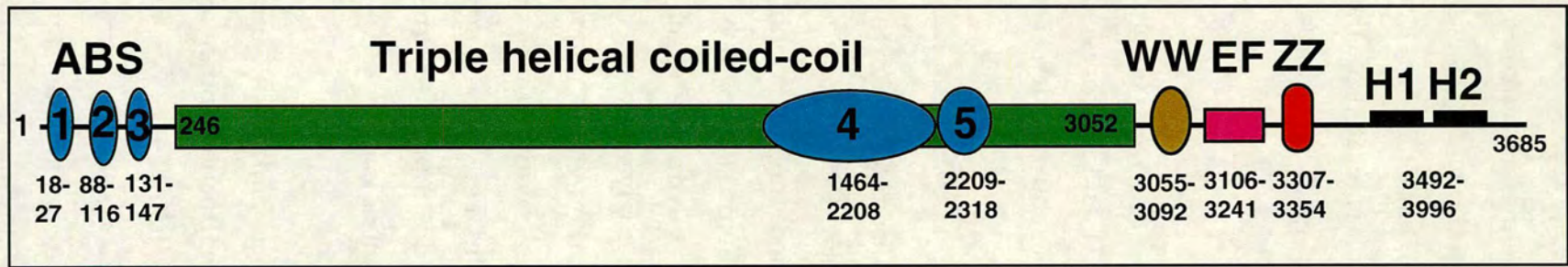
The gene responsible for the Duchenne muscular dystrophy phenotype was identified by positional cloning (Koenig *et al.*, 1987) and its protein product dystrophin subsequently characterised (Koenig *et al.*, 1988; Hoffman *et al.*, 1987). The DMD locus is located on the X chromosome at Xp21.1 and is the largest known gene covering 2.7 megabases and containing at least 79 exons.

The dystrophin protein is 427kDa in size, consists of at least eight identified protein domains (figure 1.1), and is expressed predominantly in skeletal, smooth and cardiac muscle and to a lesser extent in the brain (Chamberlain *et al.*, 1988).

In muscle cells dystrophin is localised to the cytoplasmic face of the sarcolemma via interactions at its C-terminus with a complex of membrane-spanning proteins termed the dystrophin-associated protein complex (DAPC) (section 1.3). DAPC proteins which dystrophin directly interacts with include dystrobrevin, syntrophin and β -dystroglycan. α -dystroglycan interacts with β -dystroglycan and extracellular matrix proteins, thus linking dystrophin to the extracellular matrix (ECM). At its N-terminus dystrophin interacts with the actin cytoskeleton. Consequently, dystrophin forms a link between the actin cytoskeleton and the extracellular matrix. The loss of dystrophin destroys this essential link and as a result leads to muscle degeneration.

The exact function of dystrophin is unknown, but it is proposed to stabilise myofibres during muscle contraction and relaxation (Petrof *et al.*, 1993). On the basis of dystrophin's homology to α -actinin and spectrin (Koenig *et al.*, 1988), the assumption was that dystrophin forms dimers. It has been suggested that dystrophin homodimers interact, forming an hexagonal array which confers flexibility to the membrane cytoskeleton

Figure 1.1. Domain Structure of Dystrophin



Domain structure of dystrophin

Representation of dystrophin domain structure indicating the principle domains. Numbers refer to amino-acid residues delimiting the domains or regions. Actin-binding sites ABS 1-3 represent the major actin-binding regions. The central triple helical coiled-coil region comprises over 70% of the protein and consists of 24 coiled-coil repeats separated by five proline-rich hinge regions. This region also contains ABS 4 (repeats 11-17) and ABS 5 (repeat 18). The WW domain (WW), EF hand region (EF) and ZZ domain (ZZ) are involved in binding β -dystroglycan. The C-terminal coiled-coil region comprises two α -helices (H1 & H2).

during muscle contraction and relaxation (Koenig & Kunkel, 1990). However, more recent evidence, including biochemical and electron microscopy studies, indicate that whole dystrophin does not form dimers in solution (reviewed in Winder, 1997).

In addition to its structural properties, dystrophin itself is phosphorylated and has links to signalling molecules via the DAPC (section 1.9) and has therefore been implicated in cell signalling events. However, it is not clear what the functional consequences of these events are.

1.2.1. Dystrophin isoforms and related proteins.

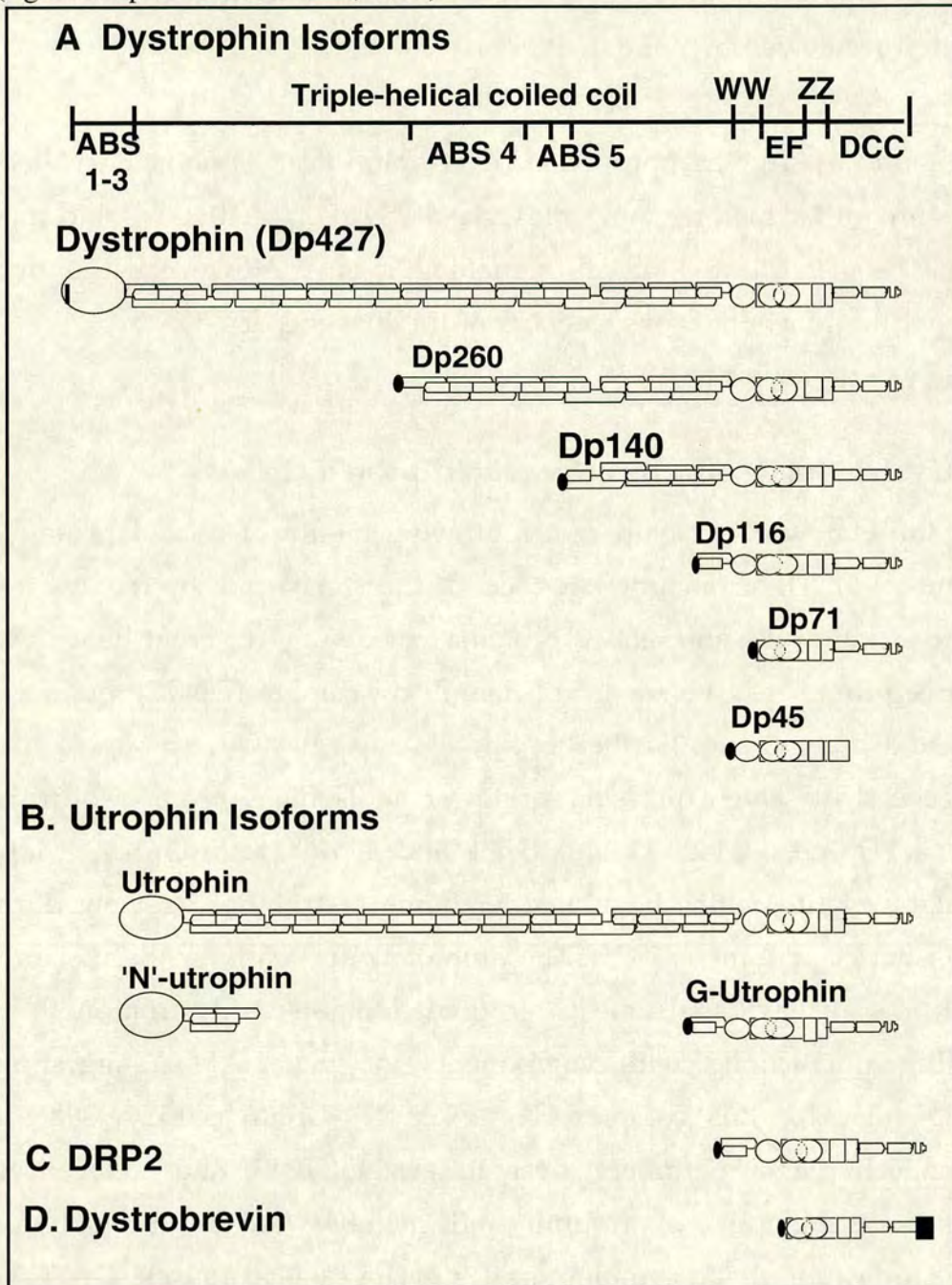
Dystrophin is now one member of a growing family of related proteins (see figure 1.2). These include products of the same gene by the use of alternative promoters, and related proteins encoded by different genes. At least three promoters give rise to full length dystrophin (Dp427) found in muscle and brain tissue. Further promoters near the C-terminus of the DMD gene drive the expression of lower molecular weight isoforms known as Dp260, Dp140, Dp116, Dp71 and Dp45 (according to their molecular weights) which have a wider tissue distribution (reviewed in Sadoulet-Puccio & Kunkel, 1996). The promoters are cell-type specific, and the isoforms all have the same C-terminal domains as dystrophin thus facilitating interactions with components of the DAPC, suggesting multiple roles for this complex. For example, Dp116 is expressed in Schwann cells of the peripheral nervous system (PNS) and co-localises with the dystroglycans, syntrophin and members of the sarcoglycan complex indicating that a complex similar to the skeletal muscle DAPC is formed in the PNS (Matsumura *et al.*, 1993).

Dystrophin also has alternatively spliced variants, the roles of which are unclear (reviewed in Amalfitano *et al.*, 1997). Dystrophin-related proteins include utrophin (the chromosome-6 encoded dystrophin related protein

Figure 1.2

Dystrophin isoforms and related proteins

(figure adapted from Winder , 1997)



A. Dystrophin structure and isoforms:

Translated products of the dystrophin gene.

Dp = dystrophin protein with number which denotes kDa.

Different shaped regions depict different domains. ABS= actin binding sites, WW = WW domain, EF= EF hands, ZZ= ZZ zinc finger domain, DCC = predicted coiled-coil domain.

B. Utrophin and isoforms: Utrophin is shorter than dystrophin by 2 triple helical coiled coil repeats.

C. DRP2: dystrophin related protein 2.

D. Dystrobrevin (87kDa acetylcholine receptor associated protein)

(DRP) (Love *et al.*, 1989)), dystrobrevin (Blake *et al.*, 1996) and DRP2 (Roberts *et al.*, 1996).

Both utrophin and dystrophin share considerable sequence, domain and functional homology with the cytoskeletal proteins spectrin and α -actinin, and together they form a broad family (Koenig *et al.*, 1988). All members of this family share an actin-binding domain, triple-helical coiled-coil repeat units and regulatory/membrane binding region. The homology between dystrophin and utrophin extends over their entire length, although utrophin is shorter by two triple helical coiled-coil repeats (Tinsley *et al.*, 1992). In non-muscle cells and at the neuromuscular junction (NMJ) of muscle cells, utrophin associates with a complex similar to the DAPC which has been termed the utrophin-associated protein complex (UAPC) (James *et al.*, 1996).

Whereas full length dystrophin isoforms (Dp427) are expressed predominantly in muscle, utrophin has a ubiquitous expression pattern (Tinsley *et al.*, 1992). During fetal muscle development, utrophin is expressed before dystrophin. It is localised to the sarcolemma and neuromuscular junctions (NMJ) until dystrophin is expressed and replaces utrophin at the sarcolemma, thus leaving utrophin localisation only at the NMJ (Clerk *et al.*, 1993). NMJs are highly developed structures which ensure communication between the central nervous system and contracting muscle fibres. Both dystrophin and utrophin are concentrated at the NMJ, dystrophin in the depths (crypts) and utrophin in the crests and upper parts of the postsynaptic folds of the muscle fibre membrane. Knockout mice studies have shown that utrophin is essential for the development of fully functional NMJs (Deconinck *et al.*, 1997; Grady *et al.*, 1997a), whereas neuromuscular transmission is normal in dystrophin-minus muscle and morphological alterations in the NMJ are slight (reviewed in Sealock & Froehner, 1997).

In DMD affected muscle, utrophin is overexpressed and appears to colonise the dystrophin-free space in *mdx* mice (Pons *et al.*, 1994) suggesting that utrophin could be upregulated in dystrophic tissue as a possible therapy for DMD/BMD sufferers (Tinsley & Davies, 1993). Utrophin has been found to functionally replace dystrophin in the *mdx* mouse (Bulfield *et al.*, 1984; Tinsley *et al.*, 1998) and alleviates the problem of the immune response elicited by dystrophin. For this reason, studies of utrophin as well as dystrophin have been in the forefront of research into understanding and generating cures for DMD. Additionally, utrophin's ubiquitous expression makes it more accessible to biochemical analysis than dystrophin, particularly because dystrophin has such a low abundance (Hoffman *et al.*, 1987) .

1.2.2. Dystrophin -primary structure.

Dystrophin has several predicted functional domains in its protein structure (figure 1.1). There is an N-terminal actin binding domain (reviewed in (Winder *et al.*, 1997)) and a large spectrin-like triple-helical coiled-coil domain which comprises over 70% of the protein (Koenig *et al.*, 1988). Towards the C-terminal end of the protein, there is a cysteine-rich region which contains a WW domain (Bork & Sudol, 1994; Andre & Springael, 1994), a pair of EF-hands (Tufty & Kretsinger, 1975), and ZZ zinc finger domain (Ponting *et al.*, 1996). At the most C-terminal domain there are two α helices which are predicted to form dimeric coiled-coils (Blake *et al.*, 1995).

1.2.3. Actin-binding properties of dystrophin

The amino-terminal 246 amino-acids of dystrophin have strong sequence similarity with the actin-binding domains of several well characterised F-actin cross-linking proteins (Koenig *et al.*, 1988) and dystrophin has been shown to bind F-actin in a large number of studies (reviewed in Winder

et al., 1997).

Dystrophin and utrophin are grouped into the calponin homology (CH) domain family (reviewed in Stradal *et al.*, 1998). This nomenclature is based on a 100 amino-acid sequence motif originally identified in a muscle regulatory protein, calponin (Castresana & Saraste, 1995). Dystrophin and utrophin have two pairs of CH domains, each pair comprising a CH1 domain and a CH2 domain in tandem. CH1 domains have an intrinsic ability to bind actin and the CH2 domain contributes to the overall stability of the interaction. Additionally, dystrophin and utrophin have three actin-binding sites at their N-termini (ABS-1, ABS-2, ABS-3) (Winder *et al.*, 1995). Experiments involving NMR, biochemical techniques using mutated residues/regions of dystrophin/utrophin, and proteolytic cleavage experiments have shown that the highly conserved ABS-1 and ABS-2 are contained within a CH1 domain and ABS-3 in a CH2 domain.

Interestingly, it has been shown that ABS1-3 bind non-muscle actin (platelet β -cytoplasmic actin) with an approximately four fold higher affinity than they bind muscle actin (skeletal muscle α -actin) (Winder *et al.*, 1995). This suggests that dystrophin is primarily associated with the cortical network of cytoplasmic β -actin rather than the contractile apparatus of muscle α -actin. Biochemical analysis of dystrophin actin-binding sites has revealed that they act as an intracellular anchor, playing no role in regulating actin re-organisation in the cell, just binding the F-actin cytoskeleton (Winder *et al.*, 1997).

1.2.4. The central rod domain of dystrophin

The central coiled-coil region of dystrophin comprises approximately 300kDa of the protein. This region is made up of 24 triple-helical coiled-coil repeats. The number of repeats varies between the members of the

dystrophin/spectrin family. In dystrophin, five regions in the central helical domain have been defined as “hinges” separating the coiled-coil repeats, and it has been suggested that they confer flexibility to the molecule (Koenig & Kunkel, 1990).

Another function of the rod domain may involve its actin-binding properties. Native dystrophin binds F-actin with a higher affinity than the isolated N-terminal actin binding domain. This supports the idea of rod domain involvement in actin-binding (Rybakova *et al.*, 1996). It has been shown that the DAPC can bind actin with a stoichiometry of 1 dystrophin: 24 actin monomers. If these 24 actin monomers were arranged in a single strand of a filament, dystrophin could be binding actin along more than half its length. Furthermore, another ABS has been found within repeats 12-16 of the central coiled-coil domain (ABS4) (Rybakova *et al.*, 1996) and yet another within repeat 18 (ABS5) (Winder, 1997). The ABS4/actin association is facilitated by electrostatic interactions between a cluster of basic repeats in ABS4 (whose delineation has been expanded to repeats 11-17) and the acidic actin filament (Amann *et al.*, 1998). Therefore, the dystrophin-actin interaction appears to be complex and multifaceted.

The importance of the N-terminus and rod domain of dystrophin is demonstrated by the failure of the dystrophin isoform Dp71 (figure 1.2) to correct the dystrophic phenotype. Dp71 comprises only the cysteine-rich and C-terminal domains of dystrophin and can restore the localisation of dystrophin-associated proteins to the cell membrane (Cox *et al.*, 1994; Greenberg *et al.*, 1994), but this is not sufficient to restore complete function. This strongly suggests that the N-terminus and rod domain are required for normal dystrophin function in skeletal muscle.

However, a transgenic *mdx* mouse expressing a dystrophin construct lacking amino-acids 45-273 had a benign phenotype (Corrado *et al.*, 1996).

Most mutations in the N-terminus of dystrophin lead to a severe BMD phenotype rather than a DMD phenotype. It is not clear whether the DMD patients which do have N-terminal defects or deletions have severe phenotypes because there is an important functional domain missing, or merely because an unstable truncated dystrophin protein is expressed.

Nevertheless, these seemingly conflicting results can be rationalised if the dystrophin:actin interaction occurs over a number of low affinity sites dispersed over the length of dystrophin's N-terminal domains and the rod domain. This redundancy illustrates why the N-terminus and rod domain are required, but no individual sequences in these regions appear to be essential for normal dystrophin function. It also explains why the whole dystrophin molecule binds actin at a higher affinity than individual ABS domains (Way *et al.*, 1992).

1.2.5. C-terminal domains of dystrophin

From a functional perspective, the carboxy-terminal domains of dystrophin are clearly the most important. Mutations in these domains have been found to cause the severest symptoms of muscular dystrophy (for review see (Amalfitano *et al.*, 1997). The importance of the carboxy-terminal regions is primarily because they associate with a group of membrane-associated proteins and glycoproteins called the dystrophin-associated protein complex (DAPC).

The domains in the C-terminal region of dystrophin include the WW, EF hand and ZZ domains which are involved in dystrophin's interaction β -dystroglycan, a transmembrane protein of the DAPC (discussed more fully in section 1.6.1). WW domains are protein:protein interaction domains which interact with proline-rich motifs in their ligands (see section 1.6.2). EF hand domains typically bind calcium (Tufty & Kretsinger, 1975) but it is a contentious issue whether dystrophin's two EF hand domains bind

calcium or not (Ervasti & Campbell, 1993; Koenig *et al.*, 1988; Milner *et al.*, 1992). However, it appears unlikely due to the lack of several crucial liganding residues. Nevertheless, it is still not possible to predict whether the EF hands in dystrophin play a structural or regulatory role (reviewed in Winder *et al.*, 1997).

The ZZ domain consists of two pairs of conserved cysteine residues which are predicted to form a zinc finger involved in protein:protein interactions (Ponting *et al.*, 1996). The ZZ domain has been shown to improve the dystrophin- β -dystroglycan interaction and mediates dystrophin binding to calmodulin (Anderson *et al.*, 1996; Vignano *et al.*, 2000). Calmodulin is the major regulator of calcium-dependent kinases (see section 1.9.3.2). The functional relevance of the dystrophin-calmodulin interaction is not established, but it appears that the binding of calmodulin and β -dystroglycan to dystrophin are mutually exclusive (Vignano *et al.*, 2000).

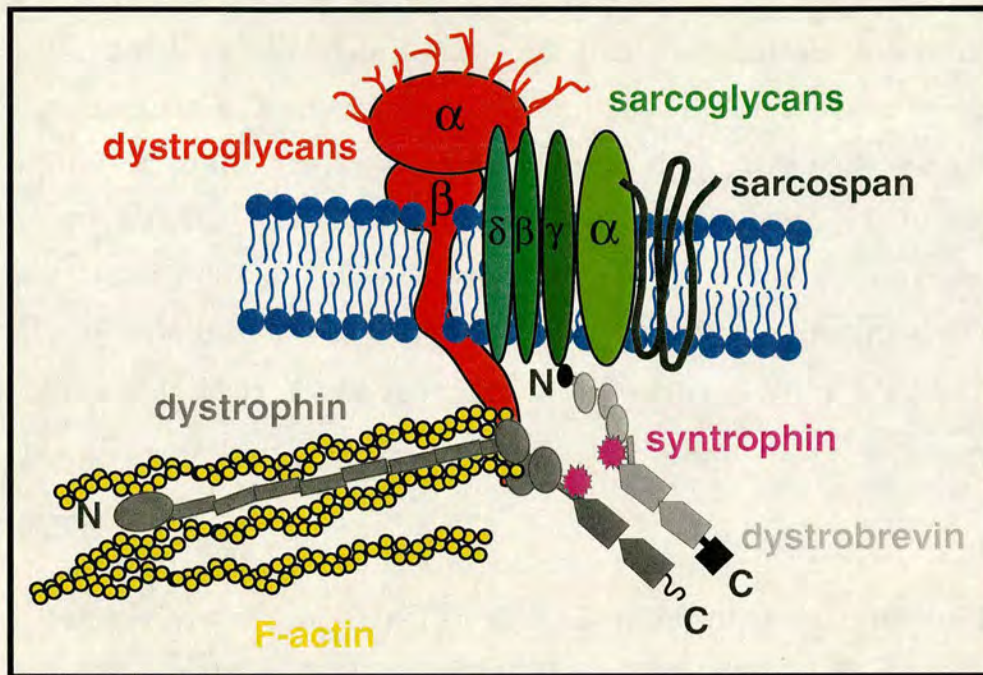
The final structures at the carboxyl terminal end of dystrophin are a pair of highly conserved helices, predicted to form parallel dimers (Blake *et al.*, 1995). Syntrophin, an intracellular protein of the DAPC binds to dystrophin near the N-terminal end of helix 1 (Suzuki *et al.*, 1994), and dystrophin interacts with dystrobrevin via coiled-coil interactions (Sadoulet-Puccio *et al.*, 1997).

1.3. The dystrophin-associated protein complex

The DAPC includes α - and β -dystroglycan, α -, β -, γ -, and δ - sarcoglycans, a syntrophin triplet, dystrobrevin and sarcospan (figure 1.3). Proteins of the DAPC are greatly reduced in dystrophic tissue presumably because they are no longer stabilised by dystrophin (Ervasti *et al.*, 1990). The tight association of the proteins of the DAPC has been demonstrated by co-purification from rabbit muscle membranes, co-sedimentation in sucrose density centrifugation, immunoprecipitation, co-localisation to the sarcolemma and protein crosslinking experiments (Ervasti *et al.*, 1990; Campbell & Kahl, 1989 ; Yoshida & Ozawa, 1990; reviewed in Campbell, 1995).

Dystrophin associates with the DAPC via its C-terminal interaction with the transmembrane glycoprotein β -dystroglycan (Suzuki *et al.*, 1992). α -dystroglycan is an extracellular glycoprotein which binds to merosin, the muscle isoform of laminin, thus linking dystrophin to the ECM (Ibraghimov-Beskrovnaya *et al.*, 1992).

The functions and the interactions of the dystrophin and DAPC proteins are not clearly defined. They are however, crucial in maintaining muscle cell integrity, as demonstrated by the severe defects when they are disrupted (Ervasti *et al.*, 1990). The C-terminal portion of dystrophin and therefore its interactions with other components of the DAPC, are particularly important for its function. This is demonstrated by deletions in this region give which rise to a severe dystrophic phenotype in which the DAPC fails to form (Koenig *et al.*, 1989), (Roberts *et al.*, 1992). However, not only mutations in the DMD locus cause muscular dystrophy - mutations in the sarcoglycans also cause distinct diseases called limb girdle muscular dystrophies (LGMD) (reviewed in Bushby, 1999).

Figure 1.3**The dystrophin-associated protein complex (DAPC)**

The DAPC includes α - and β -dystroglycan (100-200kDa, 43kDa), α -, β -, γ -, and δ -sarcoglycans (50, 43, 35, 35kDa), a syntrophin triplet (59kDa), dystrobrevin (87kDa) (N- and C-termini marked) and sarcospan (25kDa). Dystrophin (grey) (N- and C-termini marked) associates with the DAPC via its C-terminal interaction with the transmembrane glycoprotein β -dystroglycan (red). α -dystroglycan (red) is an extracellular glycoprotein which binds to merosin (not shown), the muscle isoform of laminin, thus linking dystrophin to the ECM. Dystrophin interacts with the actin cytoskeleton (yellow) via actin binding domains in its N-terminus and rod domain. Dystrobrevin and the c-terminus of dystrophin interact via their coiled-coil domains. Both proteins have a syntrophin-binding domain (syntrophin is shown in magenta) upstream from their first coiled-coil. The sarcoglycans are transmembrane proteins (green) which form a tight complex which also includes sarcospan (grey) which has multiple membrane spanning domains. Dystrobrevin is thought to associate with the sarcoglycan-sarcospan complex at its N-terminus, and there is evidence to suggest that δ -sarcoglycan lies in close proximity to the dystroglycan complex. Additional protein:protein interactions between DAPC proteins and proteins outside of the DAPC are not shown i.e. dystrobrevin and syntrophins associate with nNOS, α -dystroglycan interacts with the extracellular proteins laminin, perlecan and agrin. β -dystroglycan interacts with Grb2, rapsyn and caveolin-3.

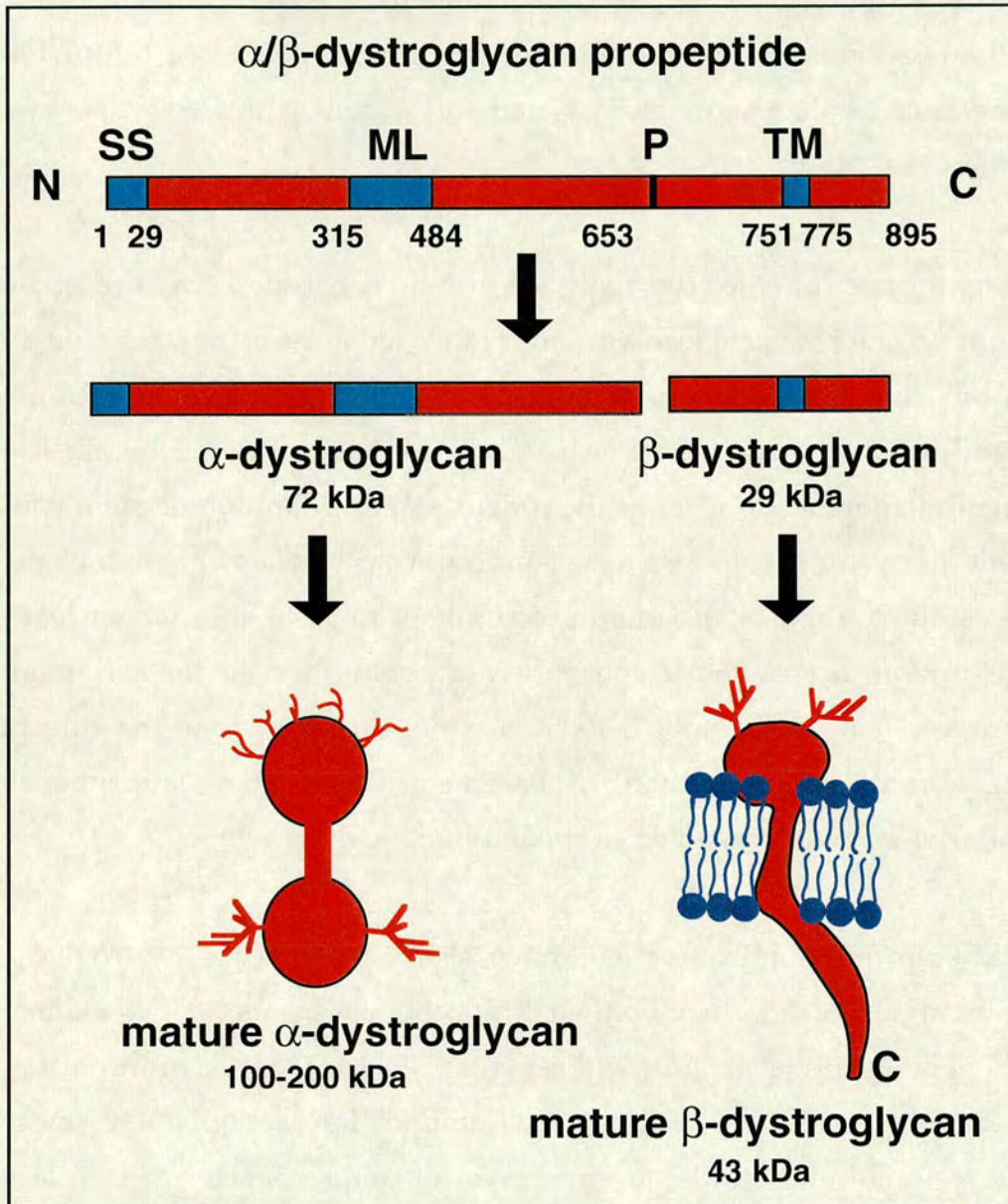
Animal models have been useful in attempts to dissect the functions of the individual DAPC components. Mice null for each of the DAPC proteins have been generated over the last few years (reviewed in Allamand & Campbell, 2000) and will be discussed in the context of the individual DAPC proteins below.

1.4. The Dystroglycans

The dystroglycan gene encodes both α -dystroglycan and β -dystroglycan as a single transcript which is post translationally cleaved into the two individual proteins (Ibraghimov-Beskrovnaya *et al.*, 1992), see figure 1.4. Both the proteins are heavily glycosylated and widely expressed (reviewed in Durbeej *et al.*, 1998).

The importance of the dystroglycans is demonstrated by the gene knockout in mice which leads to embryonic lethality at day 6.5 due to structural and functional perturbations of the Reichert's membrane (Williamson *et al.*, 1997). The Reichert's membrane is one of the earliest basement membranes to form in the rodent embryo. Embryonic stem cells deficient for dystroglycans also have a defect in early basement membrane synthesis due to the lack of laminin recruitment to basal sites which leads to a disruption in collagen V and perlecan organisation in the basement membrane (Henry & Campbell, 1998). It is not clear whether the role of dystroglycan in the formation of basement membranes is primarily structural or it is also involved in mediating signalling events.

Antibody perturbation experiments have also suggested that dystroglycan might be involved in kidney epithelial morphogenesis and salivary gland morphogenesis (reviewed in (Durbeej *et al.*, 1998)). The use of inhibitory antibodies to block the dystroglycan/laminin interaction in primary muscle cell cultures leads to myotubes of reduced size which lack spontaneous contractile activity, and myofibril disorganisation. These findings implicate the dystroglycans in a signalling pathway important for skeletal muscle maturation and maintenance, possibly involving laminin (Brown *et al.*, 1999).

Figure 1.4**Processing of the dystroglycan propeptide**(Ibraghimov-Beskrovnaya *et al.* 1992)

The dystroglycan gene encodes both α - and β -dystroglycan as a single 895 amino-acid polypeptide which is post-translationally cleaved and glycosylated. This propeptide has a signal sequence (SS), mucin-like region (ML) and transmembrane domain (TM) and is cleaved by an unknown protease at residue 653 (P) to yield α - and β -dystroglycan. The mature dystroglycans are glycosylated (over 50% of their mass comprises carbohydrate moieties). α -dystroglycan is a dumbbell shape as predicted by electron microscopy, β -dystroglycan is shown inserted into the lipid bilayer.

In order to elucidate more about the role of dystroglycans in muscle, chimeric mice were generated with skeletal muscle essentially devoid of dystroglycan (Cote *et al.*, 1999). Interestingly, in muscle cells of these mice, the composition and structure of their basement membrane appeared normal, suggesting that dystroglycan is not required for the development of the basement membrane in skeletal muscle. The chimeric mice lacking dystroglycan developed muscular dystrophy and in fact, resemble the severely affected dystrophin/utrophin double knockout mouse (Grady *et al.*, 1997b), although dystroglycan deficient muscle has more severely disrupted neuromuscular synapses. Presumably there is a residual amount of dystroglycan in the dystrophin/utrophin double knockout which facilitates the partial function of the NMJs. The study by Cote *et al.* (1999) also showed that the dystroglycan complex is necessary for localisation of dystrophin and the sarcoglycan complex at the sarcolemma. Therefore, it can be concluded from this research that the dystroglycan complex is essential for the maintenance of the integrity of the sarcolemma and the development and/or stability of NMJs, and its absence leads to muscular dystrophy .

No clinical effects of dystroglycan mutations have been identified, presumably because the consequences are too profound for survival. However, a deficiency of β -dystroglycan has been noted in one patient (Salih *et al.*, 1996). The normal expression of α -dystroglycan suggests there was either a mutation in the β -dystroglycan coding region or a primary mutation in another protein which affects the stability of β -dystroglycan. In addition, the correct localisation of all the DAPC components apart from β -dystroglycan in this case, suggests that dystrophin has another anchoring site, possibly in the sarcoglycan complex.

The dystrophin-dystroglycan and utrophin-dystroglycan complexes can be thought of cell adhesion structures, linking the ECM to the actin cytoskeleton, and anchoring cells to the ECM. Dystroglycan has been

shown to serve as a cell adhesion receptor in primary endothelial cell cultures (Shimizu *et al.*, 1999). The phenotype of the dystroglycan-null mouse definitively demonstrates that dystroglycan is essential for the formation of the Reichert's basement membrane (Williamson *et al.*, 1997). It is also well established that dystroglycan is required for the clustering of agrin, laminin and perlecan on the surface of skeletal muscle cells (reviewed in Henry & Campbell, 1999). The β -dystroglycan-dystrophin interaction is well characterised, but it is becoming clear that β -dystroglycan has other roles because it has been shown to interact with other proteins, such as Grb2, caveolin-3 and rapsyn (section 1.6.4), although the exact function of these interactions has yet to be established.

1.5. α -dystroglycan

α -dystroglycan is a 100-200kDa widely expressed peripheral membrane protein which links to the ECM via several ligands. Therefore, it comprises the extracellular component of the DAPC and provides the main linkage of the DAPC to the basement membrane. It binds the ECM proteins laminin, perlecan and agrin which interact with carbohydrate groups on α -dystroglycan. In muscle, α -dystroglycan interacts with the laminin-2 (merosin) which is a muscle specific isoform of laminin. Defects in laminin-2 expression cause congenital muscular dystrophy (CMD) (Hillaire *et al.*, 1994) and there is a reduction in laminin-2 expression in a number of other muscular dystrophies (Hayashi *et al.*, 1993).

Dystrophin and utrophin are concentrated at the NMJ and it has been demonstrated that α -dystroglycan binds the NMJ protein agrin. Agrin is a basement membrane-associated proteoglycan which induces the formation of postsynaptic specialisations at the NMJ. One of the earliest events of postsynaptic differentiation involves agrin-induced clustering of acetylcholine receptors (AChRs) at nerve-muscle contacts (Nitkin *et al.*, 1987). When it was identified that agrin bound α -dystroglycan, it was thought that α -dystroglycan was the signal-transducing agrin receptor, thus facilitating synapse formation (Gee *et al.*, 1994). However, further investigation suggests that α -dystroglycan does not fulfil the criteria for an agrin-signalling receptor (Ruegg & Bixby, 1998). Firstly, α -dystroglycan binds to inactive muscle isoforms and active neuronal isoforms of agrin with a similar affinity (Sugiyama *et al.*, 1994) whereas a receptor for agrin would be expected to bind the nerve isoform (which induces postsynaptic specialisations) exclusively or with a much higher affinity than any other isoform. Additionally, two research groups have demonstrated that the blocking of agrin-dystroglycan binding *in vitro* has no effect on agrin-induced acetylcholine receptor clustering (Sugiyama *et al.*, 1994;

Campanelli *et al.*, 1994). The physiological role of α -dystroglycan in synapse formation is now unclear but it may function more as a structural, rather than signalling component of postsynaptic apparatus assembly (reviewed in (Ruegg & Bixby, 1998)), as shown by the disruption of NMJs in chimeric mice with muscles devoid of dystroglycan (Cote *et al.*, 1999).

Additionally, perlecan is implicated in acetylcholinesterase (AChE) clustering at the NMJ (Peng *et al.*, 1999). Like agrin, perlecan is a heparan sulphate proteoglycan which resides on the surface of muscle cells and therefore is a likely candidate to localise AChE to the cell surface. The dystroglycan complex which mediates a transmembrane linkage between the ECM and the cytoskeleton could easily be part of the machinery for the sequestration and clustering of AChE and other ECM components in skeletal muscle during synaptogenesis. Perlecan and the laminins have an overlapping binding site on α -dystroglycan which raises the possibility of competitive interactions, but the biological significance of this is unclear. It is possible that different glycosylation patterns of α -dystroglycan could specify different ligand affinities (reviewed in Henry & Campbell, 1999).

α -dystroglycan has also recently been identified as a receptor for human pathogens including *Mycobacterium leprae* and several types of arenaviruses (Rambukkana *et al.*, 1998; Cao *et al.*, 1998). α -dystroglycan is therefore an important mediator of microbial pathogenesis and could be a useful target for therapeutic intervention.

1.6. β -dystroglycan

β -dystroglycan is a 43kDa glycoprotein with a N-terminal transmembrane domain and an unstructured C-terminal cytoplasmic tail. β -dystroglycan is considered to form the essential link between dystrophin and the rest of the DAPC. β -dystroglycan also binds other members of the dystrophin family, namely utrophin, Dp71 and Dp260 (Jung *et al.*, 1995).

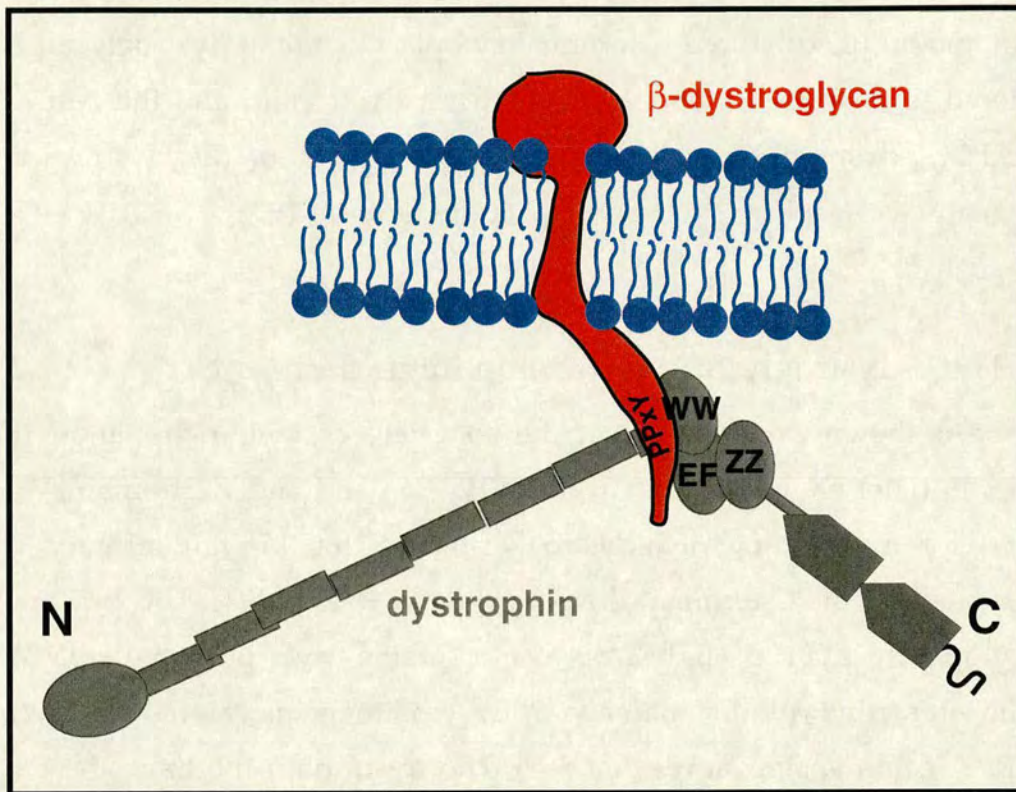
1.6.1. The β -dystroglycan -dystrophin interaction

It has been shown previously that the complete cysteine-rich region of dystrophin (later shown to contain the WW, EF hand and ZZ domains) is required for the binding of β -dystroglycan and this binding affinity is strengthened by the C-terminal domain (Suzuki *et al.*, 1994). The proline-rich cytoplasmic tail of β -dystroglycan contains two potential WW domain-interacting motifs, namely PPEY (commencing at residue 828) and PPPY (commencing at residue 889). The dystrophin binding site was subsequently localised to a 15-20 amino-acid proline-rich region at the extreme C-terminus of β -dystroglycan (amino-acids 876/881-895) by peptide-binding studies and fusion protein pulldown assays (Jung *et al.*, 1995; Rosa *et al.*, 1996). This region is necessary and sufficient for binding to dystrophin and the PPPY motif is now well characterised as a WW domain binding motif (see figure 1.5).

Rentschler *et al.* (1999) have now shown that this WW-domain mediated interaction, is necessary, but not sufficient for binding of dystrophin to β -dystroglycan. The interaction also requires the adjacent EF-hand regions (Rentschler *et al.*, 1999). This requirement is explained by the crystal structure of the dystrophin WW domain/EF-hand region complexed to a β -dystroglycan peptide, which shows that the two EF-hand regions form a composite surface which is required for dystrophin to bind to the PPxY motif in β -dystroglycan (Huang *et al.*, 2000).

Figure 1.5

The dystrophin- β -dystroglycan interaction



In muscle cells, the C-terminus of dystrophin interacts with the cytoplasmic domain of β -dystroglycan via its WW, EF-hand and ZZ domains. The WW domain of dystrophin binds to a PPxY motif in the proline rich region of β -dystroglycan. The WW domain is necessary but not sufficient for the binding of dystrophin to β -dystroglycan. The interaction also requires the adjacent EF-hand region. In addition, the ZZ domain appears to stabilize the interaction.

The EF hand region stabilises the fold of the WW domain and provides additional specificity for the recognition of β -dystroglycan. Therefore, both WW and the EF hands are required to stabilise and orientate the unstructured C-terminus of β -dystroglycan. This characteristic of the dystrophin WW domain, i.e. necessary but not sufficient for the interaction with its ligand, is unique amongst WW-domain mediated interactions

It is unclear whether the ZZ domains are essential for the dystrophin- β -dystroglycan interaction or not. Phage display analysis suggest that the UAPC cannot be formed if the ZZ domain is deleted from the c-terminus of utrophin (Vignano *et al.*, 2000). However, peptide assays suggest that the ZZ domain is not essential, but appears to significantly stabilise the dystrophin- β -dystroglycan interaction (Rentschler *et al.*, 1999).

1.6.2. WW domains

WW domains and their interaction with β -dystroglycan are the best characterised of dystrophin's C-terminal domains. WW domains are the smallest of all domains, comprising only 38-40 amino-acids and are involved in protein:protein interactions, mediated by proline-rich sequences in their ligands. They occur in single or multiple copies and are distributed in all species from yeasts and plants to mammals. WW domains were originally identified as highly conserved regions during sequence searches, independently by Bork and Sudol (1994) and Andre and Springael (1994). They derived their name WW due to two highly conserved tryptophan residues (single letter code W) spaced 20-22 amino-acids apart in the semi-conserved region of the 38-40 amino-acid domain (Bork & Sudol, 1994; Andre & Springael, 1994). Further studies have revealed that these conserved tryptophans are essential for the structure and function of the domain (reviewed in Sudol & Hunter, 2000).

Other features of WW domains are the presence of aromatic amino-acids between the two tryptophans, and the presence of a proline at position +2 in relation to the second tryptophan.

Similarly to other domains such as SH2, SH3 and PH domains, the WW domain is present in many different organisms and in a diverse range of proteins with seemingly unrelated functions, now reaching a total of 200. In addition to utrophin and dystrophin, these include the adapter protein Yes-associated protein (YAP), nuclear proteins such as the formin binding proteins (FBPs) and ubiquitin ligases such as Nedd4. Despite this diversity of proteins, a large proportion have a shared involvement in regulatory or signalling processes. Proteins which contain WW domains have been found to participate in cellular processes such as ubiquitin-mediated protein degradation, viral budding, RNA splicing, transcriptional activation and mitotic regulation (Bedford *et al.*, 2000). In addition to Duchenne muscular dystrophy, proteins involved in diseases such as Alzheimer's disease, Huntington's disease (Huntingtin interacting proteins) and Liddle's syndrome (amiloride-sensitive epithelial Na⁺ channel subunits) also contain WW domains (Kay *et al.*, 2000).

1.6.3. WW domain ligands and regulation

WW domains are often compared to SH3 domains due to their shared affinity for proline-rich ligands. Although the two domains resemble each other functionally, their structures are quite distinct. The different 3-dimensional structure of their ligand binding surfaces rules out direct competition. All WW domains so far characterised bind to a variety of short proline-containing motifs (summarised in table 1.1).

Table 1.1.**Classification of WW domains based on optimal ligand specificity**

Class	Motif	WW domains	Reference
I	PPXY	Dystrophin Nedd4 WW-3 YAP65	Rentschler <i>et al.</i> (99) Chen&Sudol (95)
II	PPLP	FBP11 FE65	Bedford <i>et al.</i> (97) Ernevoka <i>et al.</i> (97)
III	PPR	FBP30 FBP21 FE65	Bedford <i>et al.</i> (00)
IV	(pS/pT)P	Pin1 Nedd4 WW-2	Lu <i>et al.</i> (99)

The first WW domain-binding motif identified was named PY due to conserved prolines and a tyrosine in its sequence. This was originally characterised by Chen and Sudol (1995) who carried out a functional screen of a cDNA expression library and identified two ligands of the WW domain of Yes associated protein YAP65 (Chen & Sudol, 1995). They discovered a PPPPY motif in both ligands (WBP-1 and WBP-2) which the WW domain bound to. Site-directed mutagenesis showed that the second and third proline plus the tyrosine residue are essential for these ligands to bind YAP65 (PPPPY). Therefore the core consensus for WW ligands was denoted as PPXY, and termed PY. The WW domains in dystrophin and ubiquitin ligase Nedd4 also recognise the PPXY binding motif in their ligands.

On discovering that the WW binding motif contained a tyrosine residue, it was hypothesised that the WW/PY interaction could be regulated by tyrosine phosphorylation (Pirozzi *et al.*, 1997) in a manner analogous to the regulation of SH3 domain interactions when the ligand contains a tyrosine within the PxxP SH3-binding motif. Subsequently it has been shown biochemically that phosphorylation of the tyrosine residue in the PY motif disrupts its interaction with WW domains in its binding partner. These interactions include YAP65 and its ligand (Chen *et al.*, 1997), and utrophin with β -dystroglycan (James *et al.*, 2000).

As more WW-binding proteins were found, variations in the PY motif were revealed. In fact, four distinct binding motifs are now defined as WW domain-binding (table 1.1). The PPLP motif was found during a screen for binding partners of the WW domain in FBP11 (Bedford *et al.*, 1997), and subsequently the neural adapter protein FE65 was also found to bind a PPLP motif in its ligands (Ermekova *et al.*, 1997). Another formin binding protein FBP21 (a spliceosome-associated protein) was originally thought to bind a proline, glycine and methionine-rich motif (Bedford *et al.*, 1998). This PGM motif was defined as another specific class of proline-rich WW domain ligands. However, further analysis of binding experiments revealed a PPR or RPP motif in all the fusion peptides examined, and FBP21 was re-designated into class III of WW domain-containing proteins. (Bedford *et al.*, 2000). Drawing on further studies, Bedford's group also place FE65 into class III of WW-containing proteins stating that FE65 binds the Pro-Arg motif more strongly than PPLP (Bedford *et al.*, 2000). FE65 does bind PPLP but a substitution of Arg or Lys for the Leu increases the affinity (Ermekova *et al.*, 1997) which may suggest a dual specificity interaction.

The mitotic prolyl isomerase Pin1 and WW domain 2 of ubiquitin ligase Nedd4 in the fourth class of WW domains interact with their target proteins in a phosphorylation-dependent manner (Lu *et al.*, 1999;

Ranganathan *et al.*, 1997). These proteins only recognise their ligands when a serine or threonine in the binding motif is phosphorylated.

The binding of classes I and IV WW domains to their ligands has been definitively shown to be regulated by phosphorylation within the binding motif. However, there is also evidence of regulation by phosphorylation of serines and threonines flanking the PPxY motif (Kay *et al.*, 2000), although this hypothesis has not been tested on the other classes of motifs.

1.6.4. Interactions of β -dystroglycan with other proteins

β -dystroglycan has an unstructured cytoplasmic tail which has multiple binding sites for interactions with other proteins. More specifically, β -dystroglycan has two potential WW domain-binding motifs and several potential SH3 domain-binding motifs, providing a high possibility that β -dystroglycan interacts with signalling molecules.

1.6.4.1. Grb2

β -dystroglycan has been shown to interact with growth factor receptor binding protein 2 (Grb2) via its SH3 domain binding site (Yang *et al.*, 1995). Grb2 functions as an adaptor protein through its SH2 and SH3 domains and is involved in signal transduction pathways and cytoskeletal organisation ((Lowenstein *et al.*, 1992), reviewed in (Chardin *et al.*, 1995)). Grb2 links receptor tyrosine kinases to GTP-binding protein signalling. Ras, Rac and Rho are small GTP-binding proteins which are involved in cytoskeletal organisation such as stress fibre formation at focal adhesions and ruffle induction. The dystroglycan complex may regulate cellular functions through Grb2, where signal transduction could be triggered by the extracellular matrix.

The Grb2/ β -dystroglycan interaction is mediated through β -dystroglycan proline-rich domains and Grb2 SH3 domains (Yang *et al.*, 1995). The interaction may be important for linking β -dystroglycan and the DAPC into signalling pathways, for example, the transduction of signals between the extracellular matrix and the cytoskeleton leading to cytoskeletal reorganisation. There are few clues as to what these signalling pathways may be because binding partners for the β -dystroglycan/Grb2 complex have remained elusive. However, in a recent study, a complex containing Grb2, focal adhesion kinase (FAK) and dystroglycan was isolated from bovine brain synaptosomes by laminin chromatography (Cavaldesi *et al.*, 1999). It is not clear whether FAK and dystroglycan are connected through Grb2 (possibly through SH2- and SH3-mediated interactions) but this finding does implicate β -dystroglycan in focal adhesion signalling pathways.

1.6.4.2. Caveolin-3

Caveolins are integral membrane proteins which comprise the principle protein component of caveolae, which are vesicular invaginations of the plasma membrane. The caveolin family consists of caveolins 1, 2 and 3. β -dystroglycan has been recently shown to interact with the muscle-specific form, caveolin-3 (Sotgia *et al.*, 2000). Caveolins are scaffolding proteins which are thought to be structurally involved in the formation of the caveolae membranes by facilitating the assembly of signalling complexes (Okamoto *et al.*, 1998). Caveolins have been shown to interact with signalling proteins such as heterotrimeric G-proteins, Ha-Ras, Src family tyrosine kinases and endothelial nitric oxide synthase (eNOS) and have been termed as "molecular velcro" holding these proteins in complexes (reviewed in Okamoto *et al.*, 1998).

Mutations in the caveolin-3 gene cause autosomal dominant limb-girdle dystrophy (Minetti *et al.*, 1998), but paradoxically, an upregulation of

caveolin-3 protein expression leads to a Duchenne muscular dystrophy phenotype (Galbiati *et al.*, 2000). In DMD-affected muscle, the caveolae increase in size and there is a significant structural change which may be indicative of muscle regeneration. This increase in density of the caveolae is matched by an upregulation of caveolin-3 expression both in DMD patients and the *mdx* mouse (Vaghy *et al.*, 1998; Repetto *et al.*, 1999). Along with this upregulation of caveolin-3 in transgenic mice, a downregulation of dystrophin and β -dystroglycan is observed (Galbiati *et al.*, 2000). The β -dystroglycan-caveolin-3 interaction is mediated by a WW-like domain. Caveolin-3 and dystrophin share exactly the same binding motif in β -dystroglycan. Infact, caveolin-3 has been shown to compete with dystrophin so that it can no longer bind β -dystroglycan (Sotgia *et al.*, 2000). Therefore, if caveolin-3 is upregulated in DMD, the dystrophin/ β -dystroglycan interaction may be compromised, thus contributing to the degradation of the DAPC. Interestingly, caveolin-3 interacts directly with nNOS (Venema *et al.*, 1997). The correct localisation of nNOS to the membrane is lost in DMD muscle which may be due to the changes in expression levels of caveolin-3.

Caveolin-3 was thought to be the 25kDa unidentified dystrophin-associated protein until sarcospan was identified. However, it is possible that both sarcospan and caveolin-3 are part of the DAPC and their visualisation as part of the complex depends on the extraction conditions used. Whether caveolin-3 is part of the DAPC or just transiently associated with it, its ability to recruit signalling proteins is an exciting prospect for signal transduction in the DAPC.

1.6.4.3. Rapsyn

Dystroglycans have been shown to co-localise with rapsyn and AChRs in a heterologous system (Apel *et al.*, 1995) and *in situ* (Cartaud *et al.*, 1998). Further investigation has identified a rapsyn binding site corresponding

to amino-acids 787-819 of the cytoplasmic domain of β -dystroglycan (Cartaud *et al.*, 1998).

Rapsyn is a NMJ protein which has a central role in clustering of acetylcholine receptors (AChR) at the postsynaptic membrane and organising many other protein components of the post-synaptic apparatus. It is thought to facilitate the association between AChRs and utrophin and β -dystroglycan (Fuhrer *et al.*, 1999). This is demonstrated in a rapsyn *-/-* knockout cell line whereby AChRs no longer associate with the UAPC. It is not clear what role the β -dystroglycan/rapsyn/AChRs association has, but because β -dystroglycan is also bound to utrophin at the NMJ, the utrophin-cytoskeleton link may be the main structural framework for anchoring AChR macroclusters at the developing NMJ. These multiple links between AChRs and the UAPC would allow their maximum stabilisation.

α -dystroglycan has also been linked with AChRs i.e. it has been shown to interact with agrin and laminin which are both involved in AChR clustering (section 1.5). Agrin induces tyrosine phosphorylation of the AChR β -subunit and it is thought that rapsyn is downstream of agrin because in rapsyn *-/-* knockout cells AChRs are not efficiently phosphorylated (Fuhrer *et al.*, 1999). α -dystroglycan may have a direct role in the stabilisation of NMJs or act indirectly via the β -dystroglycan/rapsyn interaction.

1.7. Other DAPC proteins

1.7.1. Syntrophins

Syntrophins are 58-60kDa intracellular dystrophin-associated proteins which were originally identified as proteins enriched at the postsynaptic apparatus of the *Torpedo* electric organ (Cartaud *et al.*, 1993; Froehner *et al.*, 1987). Syntrophins are a family of multi-domain proteins which are likely to function as modular adapter proteins involved in recruiting signalling proteins to the DAPC. The domain structure of syntrophins includes two pleckstrin homology (PH) domains, a PDZ domain and a syntrophin-unique (SU) domain (Adams *et al.*, 1995). To date, neuronal nitric oxide synthase (nNOS), voltage gated sodium channels (VGSCs), stress-activated protein kinase-3 (SAPK3) (-a member of the MAPK family) and microtubule-associated serine/threonine kinase have been identified as interactors of the syntrophin PDZ domain (Brenman *et al.*, 1996; Gee *et al.*, 1998; Lumeng *et al.* 1999; Schultz *et al.*, 1998; Hasegawa *et al.*, 1999).

Dystrophin has a syntrophin binding site near the N-terminal end of α -helix 1 (amino-acids 3447-3481) (Jung *et al.*, 1995). Dystrophin and dystrobrevin associate via coiled-coil interactions in their C-terminal domains and between them recruit two syntrophin molecules via their SU domains (Ahn *et al.*, 1996; Sadoulet-Puccio *et al.*, 1997). However recent evidence suggests that there are 2 syntrophin binding sites on each of dystrobrevin and dystrophin which could potentially facilitate the recruitment of four syntrophin molecules per DAPC (Newey *et al.*, 2000).

There are three syntrophin isoforms (α 1, β 1 and β 2) which are encoded by separate genes but have the same domain structure (Ahn *et al.*, 1996). α 1-syntrophin is predominantly expressed in skeletal and cardiac muscle whereas β 1- and β 2-syntrophin are more widely expressed. In muscle,

there is a distribution of α 1- and β 1-syntrophin at the sarcolemma and NMJs although β 1-syntrophin is confined to fast twitch fibres. β 2-syntrophin is restricted to the NMJ. (Peters *et al.*, 1997). In DMD, localisation of α 1- and β 1-syntrophin at the sarcolemma is lost but β 2-syntrophin remains present at the NMJ.

Disruption of the α 1-syntrophin gene does not cause a myopathy despite reduced levels of α -dystrobrevin-2 (Adams *et al.*, 2000). This suggests that syntrophin is not a major player in causing muscular dystrophies, or alternatively, that other syntrophin isoforms compensate for the lack of α 1-syntrophin. However, it is evident that α 1-syntrophin plays an important role in synapse formation. The neuromuscular junctions of the α 1-syntrophin null mice are disorganised with undetectable levels of utrophin and reduced levels of acetylcholine receptors and acetylcholinesterase (Adams *et al.*, 2000).

1.7.2. Dystrobrevin

Dystrobrevin is a dystrophin-related protein (figure 1.2) and a dystrophin-associated protein i.e. it is homologous to, and interacts with, the C-terminus of dystrophin. It was originally identified as a 87kDa postsynaptic membrane protein associating with syntrophin in the *Torpedo* electric organ, and is a substrate for tyrosine kinases (Wagner *et al.*, 1993).

Dystrobrevin and dystrophin interact via their two C-terminal coiled-coil domains (Sadoulet-Puccio *et al.*, 1997). Both proteins have a syntrophin-binding domain upstream from their first coiled-coil domain.

There are two dystrobrevin genes which encode α - and β -dystrobrevin. Only α -dystrobrevin is expressed in muscle and it is alternatively spliced.

α 1-dystrobrevin and α 2-dystrobrevin have a widespread expression pattern. In muscle they are localised primarily at the NMJ and sarcolemma, respectively. α 3-dystrobrevin lacks domains which interact with syntrophin and dystrobrevin and its expression is restricted to muscle (Nawrotski *et al.*, 1998).

In recent studies, transgenic mice were generated expressing dystrophins with C-terminal deletions. It was found that if the dystrobrevin and syntrophin binding sites are removed from dystrophin, the DAPC is still correctly formed (Crawford *et al.*, 2000). This suggests that dystrobrevin and/or syntrophin have additional binding sites to the DAPC and there is now biochemical evidence to show that the N-terminal half of dystrobrevin associates with the sarcoglycan-sarcospan complex (Yoshida *et al.*, 2000).

Mice deficient in α -dystrobrevin have no defects in sarcolemmal structure or depletion of other DAPC components, but still have a muscular dystrophy phenotype, with defects in NO-mediated signalling, thus implicating α -dystrobrevin in the signalling functions of the DAPC (Grady *et al.*, 1999). The fact that nNOS is missing from the sarcolemma despite the presence of syntrophins, suggests that syntrophin is not sufficient to localise nNOS to the sarcolemma. It is becoming clear that both dystrobrevin and syntrophin are involved in nNOS localisation (Grady *et al.*, 1999).

1.7.3. Sarcoglycans

α -, β -, δ - and γ -sarcoglycans are transmembrane proteins of the DAPC and are restricted to muscle except β -sarcoglycan which is expressed at low levels in other tissues. Together the sarcoglycans form an independent subcomplex within the DAPC and a mutation in one sarcoglycan gene

destabilises the whole sarcoglycan complex (Yoshida *et al.*, 1994).

Little is known about the functional or structural role of sarcoglycans but their importance is demonstrated by mutations in their genes which give rise to limb-girdle muscular dystrophies (Mizuno *et al.*, 1994). This suggests that, like dystroglycan, they are involved in membrane-matrix interactions. Evidence to support this theory is growing. For example, the merosin composition of muscle fibre basal lamina is altered in patients lacking α -sarcoglycan (Higuchi *et al.*, 1994; Yamada *et al.*, 1995). Yoshida *et al.* (1998) reported that the treatment of cultured muscle cells with antisense oligonucleotides to α -sarcoglycan inhibits their adhesion to substrata (Yoshida *et al.*, 1996) and went on to demonstrate that bidirectional signalling between sarcoglycans and the integrin adhesion system occurs in cultured myocytes (Yoshida *et al.*, 1998). From these findings, one hypothesis is that the sarcoglycans and dystroglycans mediate interactions of muscle cells with the basal lamina, which is crucial for membrane integrity (Ettinger *et al.*, 1997).

It has been suggested that the sarcoglycan complex stabilises dystroglycan interactions and thus strengthens the membrane-protecting system. This is demonstrated by the destabilisation of dystroglycan interactions in the skeletal muscle of sarcoglycan-deficient animal models, leading to impaired localisation of α -dystroglycan to the sarcolemma (Roberds *et al.*, 1993; Holt *et al.*, 1998) and an unstable DAPC in β -sarcoglycan deficient mice (Araishi *et al.*, 1999). How the sarcoglycans might stabilise dystroglycan binding interactions is not known but there is evidence to suggest that δ -sarcoglycan lies in close proximity with the dystroglycan complex (Chan *et al.*, 1998).

1.7.4. Sarcospan

The 25kDa protein sarcospan co-localises and co-purifies with the DAPC therefore demonstrating that it is an integral member of the DAPC. Additionally, it has dramatically reduced expression in muscle from patients with DMD suggesting that the localisation of sarcospan to the membrane is dependent on proper dystrophin expression. Sarcospan has been shown to interact tightly with the sarcoglycans, thus forming the sarcoglycan-sarcospan complex (Crosbie *et al.*, 1999). Consequently, sarcospan is also depleted in LGMD where the sarcoglycan complex is lacking.

Sarcospan has multiple sarcolemmal spanning domains and is a member of the tetraspanin superfamily (Crosbie *et al.*, 1997). Each member possesses four transmembrane domains and a large extracellular loop, and they are thought to play important roles in mediating transmembrane protein interactions (Maecker *et al.*, 1997). In addition, these transmembrane domains hold sarcospan firmly within the lipid bilayer and thus could provide a solid anchorage for the DAPC as a whole. However, sarcospan-deficient mice have no muscle abnormalities histologically which suggests that sarcospan is not required for normal functioning of the DAPC or is compensated for by another protein (Lebakken *et al.*, 2000).

Members of the tetraspanin family are known to interact with integrins (Hemler *et al.*, 1996). Sarcospan has not been shown to interact with integrins as yet but it is an exciting prospect which could link the DAPC to focal adhesions and possibly shed more light on the signalling between integrins and sarcoglycans.

1.8. Redundancy within the DAPC

The redundancy of the DAPC is highlighted by the different isoforms of proteins which are available to make up the DAPC. Not only can β -dystroglycan associate with dystrophin, utrophin, but also the alternatively spliced transcripts of dystrophin.

ϵ -sarcoglycan has a high homology to α -sarcoglycan (44% amino-acid identity) but is more widely expressed (McNally *et al.*, 1998; Ettinger *et al.*, 1997) and can take the place of α -sarcoglycan in the sarcoglycan complex.

A second syntrophin-binding site has recently been identified in both dystrophin and dystrobrevin, upstream from the first, in a region which can be alternatively spliced (Newey *et al.*, 2000). Therefore, it appears that there is a mechanism to confer flexibility to the DAPC by altering the number of syntrophin binding sites. This increases the potential for specialised signalling complexes in different locations and stages of development.

Syntrophin isoforms share only 50% identity which suggests that each isoform could recruit different signalling proteins to the DAPC (Peters *et al.*, 1997). Moreover, two further brain specific isoforms of syntrophins have been found which increases the number of differential associations in the DAPC even further (Piluso *et al.*, 2000). Differential association of dystrophin with certain syntrophin isoforms may play a role in tailoring the DAPC for a particular membrane specialisation in different tissues.

This redundancy of the DAPC components provides a variety of ways that the complex could be assembled and regulated pairings with different signalling capabilities could be produced in different tissue types.

1.9. Regulation of the DAPC by phosphorylation

The spatial organisation of dystrophin and the DAPC suggests that their role is mainly concerned with stabilisation of the sarcolemma of muscle fibres. However, there is evidence which also implicates the complex in the transduction of extracellular signals which may be involved in the modulation of protein:protein interactions and regulation of cytoskeletal organisation.

Dystrophin and the DAPC have been implicated in transducing intracellular signals for the following reasons: they colocalise at the membrane and could easily transduce signals from the ECM to the cytoskeleton, they have been linked to various signalling molecules, and there is evidence of direct phosphorylation of dystrophin and the DAPC components.

1.9.1. Evidence of phosphorylation of dystrophin

Inspection of the amino-acid sequence of the dystrophin molecule indicates that it contains a number of consensus sites for phosphorylation by different protein kinases. These include cAMP/cGMP-dependent kinases, protein kinase C (PKC), casein kinase II, p34^{cdc2} kinase and MAP Kinase (Luise *et al.*, 1993; Milner *et al.*, 1993; Shemanko *et al.*, 1995). This suggests that dystrophin may be a target for multi-site phosphorylation.

Dystrophin has been shown to be phosphorylated *in vitro* and *in vivo* by several types of protein kinases (table 1.2). Experiments have been carried out *in vivo* on cultured skeletal muscle cells (Milner *et al.*, 1993), isolated sarcolemmal vesicles (Luise *et al.*, 1993; Milner *et al.*, 1993; Madhavan & Jarrett, 1994; Shemanko *et al.*, 1995; Walsh *et al.*, 1995), the isolated DAPC (Madhavan and Jarrett, 1994) and muscle cell extract (Shemanko *et al.*, 1995).

Table 1.2.**Summary of studies demonstrating phosphorylation of dystrophin and the DAPC.**

Phosphoprotein (amino-acid region)	Residues phosphorylated	Enzymes implicated	Reference
Dystrophin	serine, threonine	endogenous cAMP/cGMP-dep kinases CaM kinase II Casein kinase II Protein kinase C	Luise <i>et al.</i> (1993) <i>in vitro</i>
Dystrophin (3485-3685)	serine, threonine	p34 ^{cdc2}	Milner <i>et al.</i> (1993) <i>in vitro</i>
Dystrophin (2618-3074) 175kDa 59kDa 50kDa	serine	"DAPC protein kinase" CaM kinase II	Madhavan & Jarrett (1994)
Dystrophin	serine, threonine	PKA, PKC, CK-II	Senter <i>et al.</i> (1995)
Dystrophin (3485-3685)	threonine	MAP kinase p42/p44	Shemanko <i>et al.</i> (1995)
Dystrophin (3485-3685)	serine, threonine	CK II (dephosphorylated by calcineurin)	Walsh <i>et al.</i> (1995)
Dystrophin Dystrobrevin syntrophin	serine, tyrosine	endogenous kinases	Wagner <i>et al.</i> (1993) Wagner & Haganir (1994)
α -sarcoglycan γ -sarcoglycan	tyrosine	Src-family?	Yoshida <i>et al.</i> (1998)
β -dystroglycan	tyrosine	?	James <i>et al.</i> (2000)
syntrophins β -dystroglycan? α -sarcoglycan?	not specified	?	Yamamoto <i>et al.</i> (1993)

The DAPC has “endogenous protein kinase activity” i.e. when purified DAPC preparations are made from skeletal muscle, there is co-purifying protein kinase activity (Luise *et al.*, 1993; Madhavan & Jarrett, 1994). Since the purification of the DAPC involves isolation of a sarcolemma-enriched microsome fraction, solubilising in detergent, affinity chromatography on a lectin agarose and finally ion-exchange chromatography, it seems unlikely that a protein kinase would co-purify with the DAPC unless it had some specific association with one or more constituents of the DAPC (Madhavan & Jarrett, 1994). A high affinity of the kinase for its substrates could explain the co-isolation.

1.9.2. Evidence of phosphorylation of DAPC proteins

Whilst incubating purified DAPC preparation with Mg^{2+} and [γ - ^{32}P] ATP, Madhavan and Jarrett (1994) found that not only was dystrophin phosphorylated but also proteins of 175 kDa, 59kDa and 50kDa which they tentatively identified as an unknown DAPC-associated protein, syntrophin and α -sarcoglycan respectively. However, they were cautious because CaM kinase II subunits are also 59kDa and 50kDa (Madhavan & Jarrett, 1994).

In another *in vitro* study using the DAPC and its co-purifying protein kinase activity only, in addition to dystrophin, there were phosphorylated proteins in the 40-60 kDa range which were immunoprecipitated with the dystrophin antibody. These were thought to be components of the DAPC (β -dystroglycan, sarcoglycans and syntrophins all fall into this molecular weight range) or more conservatively, proteolytic products of dystrophin (Senter *et al.*, 1995).

Two-dimensional gel electrophoresis of the DAPC indicated that the syntrophins, α -sarcoglycan and β -dystroglycan could be phosphorylated (Yamamoto *et al.*, 1993). Silver staining of the 2D gels revealed many

more spots than were expected from the results of one-dimensional SDS-PAGE, some of which disappeared after alkaline phosphatase treatment.

It has been demonstrated that α - and γ -sarcoglycans are tyrosine phosphorylated in response to cell adhesion (Yoshida *et al.*, 1998) and dystrobrevin was initially identified as a tyrosine phosphorylated protein associated with dystrophin and syntrophin in the *Torpedo* electric organ (Wagner *et al.*, 1993). To date, the kinases responsible for these tyrosine phosphorylation events have not been identified.

1.9.3. Links to signalling molecules

The DAPC has been linked to many signalling proteins, such as Grb2 (section 1.6.4.1), calmodulin and nitric oxide (NO). In addition, the syntrophins recruit a wide variety of signalling molecules via their PDZ domains (section 1.7.1).

1.9.3.1. Nitric oxide

NO is major endothelial-derived relaxing factor in the body. It is a short-lived free radical, therefore the regulation of signalling occurs largely at the level of NO biosynthesis i.e. via NO synthase (NOS). NO may function as a vasodilator in muscle, thus controlling blood flow so that the muscle gets more oxygen whilst it is contracting. nNOS is absent from the sarcolemma in DMD (Brenman *et al.*, 1995) and it is suggested that the membrane localisation of nNOS is necessary to regulate blood flow to the muscle during exercise and DMD pathogenesis is exacerbated if blood flow is not high enough (Sander *et al.*, 2000).

nNOS is tethered to the sarcolemma (in healthy muscle) via its PDZ domain interacting with the PDZ domain in α 1-syntrophin, but its localisation also appears to depend on α -dystrobrevin (Grady *et al.*, 1999)

and may involve its association with caveolin-3 (Venema *et al.*, 1997). It has been demonstrated by the dystrobrevin null mouse and the α 1-syntrophin-null mouse that reduced association of nNOS with the DAPC does not itself contribute significantly to the dystrophic process. Both null mice lack nNOS localisation to the sarcolemma but only the dystrobrevin null mouse has a dystrophic phenotype (Grady *et al.*, 1999; Kameya *et al.*, 1999).

Activation of nNOS is poorly understood but requires calcium/calmodulin (section 1.9.4.2) and nNOS homodimerisation (Klatt *et al.*, 1994). In the model proposed by Peters *et al.* (1997), the close pairing of syntrophin to dystrophin and dystrobrevin could facilitate activation of nNOS by localising it to the sarcolemma and spatially assisting dimerisation (Peters *et al.*, 1997).

1.9.3.2. Calmodulin

Dystrophin and the DAPC have also been associated with calmodulin, a ubiquitous intracellular Ca^{2+} receptor. Calmodulin mediates many Ca^{2+} -dependent processes, for example, the regulation of calcium-dependent kinases which phosphorylate serine and threonine residues in target proteins. A high affinity calmodulin binding site has been described in dystrophin (between residues 3293 and 3349) and it has been demonstrated that the ZZ domain in both dystrophin and utrophin mediates binding to calmodulin (Anderson *et al.*, 1996; Vignano *et al.*, 2000). In addition, there are two low affinity calmodulin-binding sites in the N-terminus of dystrophin and utrophin (Jarrett & Foster, 1995); Campanelli *et al.*, 1994; Winder & Kendrick-Jones, 1995). Syntrophin also has calmodulin binding sites, one in the PH domain and the other in the PDZ domain. Therefore, it is possible that calmodulin modulates the binding of syntrophins to signalling modules e.g. nNOS, which bind to the PDZ domain (Madhavan *et al.*, 1992; Iwata *et al.*, 1998).

The precise function of calmodulin in the DAPC is unknown but the interaction of calmodulin with the ZZ domain of dystrophin inhibits the dystrophin- β -dystroglycan interaction (Vignano *et al.*, 2000) and Ca^{2+} -calmodulin antagonises syntrophin binding to dystrophin *in vitro* (Newbell *et al.*, 1997). Conflictingly, calmodulin has been shown to inhibit the actin-dystrophin interaction in some assays (Jarrett & Foster, 1995; Campanelli *et al.*, 1994), but not in others (Bonet-Kerrache *et al.*, 1994; Winder & Kendrick-Jones, 1995).

1.9.4. Does the disruption of α -dystrobrevin cause signalling defects?

Further evidence to support the role of the DAPC in signal transduction was brought about by disrupting the α -dystrobrevin gene in mice (Grady *et al.*, 1999). The pathology of the α -dystrobrevin knockout mouse resembles that of the *mdx* mouse with a dystrophic phenotype developing within one month of age. Interestingly, the mice have very few structurally damaged muscle fibres and the structural components of the DAPC and the integrity of the sarcolemma is intact. This lack of structural abnormalities suggests that it is a disruption of signalling events which causes the phenotype. nNOS is absent from the sarcolemma in these mice. This signalling molecule is known to associate with dystrobrevin, but there are possibly more.

1.9.5. Functional consequences of phosphorylation of the DAPC

The DAPC clearly has associated kinase activity and there is evidence of the phosphorylation of dystrophin and α - and γ -sarcoglycans, and potentially, β -dystroglycan and syntrophin are phosphorylated. The association of DAPC proteins with signalling molecules such as nNOS, calmodulin and Grb2 also implicate the DAPC in the functioning of signalling pathways. Despite these extensive studies gathering evidence

for the phosphorylation of dystrophin and the DAPC, and attempts to identify the kinases involved, we are still no further forward in elucidating the functional consequences of these phosphorylation events. It is possible that phosphorylation events could purely modulate protein:protein interactions within the DAPC, controlling the assembly, disassembly and general maintenance of the DAPC.

In addition, phosphorylation events could modulate interactions between the DAPC and proteins which associate with it. For example, phosphorylation of dystrophin could modulate its interaction with actin. Senter *et al.* (1995) demonstrated that the phosphorylation of the rod region and residues near to dystrophin's ABS 1-3 had effects on dystrophin binding to actin. In addition, phosphorylation of dystrophin by PKA caused a 3-fold increase in actin binding, and phosphorylation by CKII and PKC inhibited actin binding (Senter *et al.*, 1995).

There is also the possibility that the DAPC is involved in the transduction of signals from the ECM to the cytoskeleton. This idea is reinforced by the findings that various signalling molecules associate with the DAPC (section 1.9.4). One putative role of dystrophin and the DAPC in transducing signals maybe the control of cytoskeletal organisation via links with the Ras/Rac/Rho signalling pathways. Evidence for this includes β -dystroglycan associating with Grb2 (Yang *et al.*, 1995), and with activated MAPK in non-muscle cells (M. James & S.J. Winder, unpublished results). In the study by Yoshida *et al.* (1998), α - and γ -sarcoglycans were tyrosine-phosphorylated in response to cell adhesion to the ECM (via integrins). The DAPC was also shown to have a role in recruitment of proteins to focal adhesions (Yoshida *et al.*, 1998) which strengthens the idea that phosphorylation events in the DAPC could be involved in cell adhesion by way of the assembly and disassembly of focal contacts.

1.10. Aims and objectives

Dystrophin and the DAPC play a critical role in protecting muscle cells from damage, as shown by the devastating consequences when one or more of the component proteins are absent. Despite extensive research, the functions and the interactions of the dystrophin and DAPC proteins are still not clearly defined. However, it is becoming increasingly evident that the DAPC does not simply play a structural role, but is a dynamic complex involved in signalling pathways.

The organisation of the DAPC is becoming clearer but still little is known about the regulation of the interactions within the DAPC. It is known that β -dystroglycan directly associates with dystrophin, as does syntrophin and dystrobrevin. It has recently come to light that dystrobrevin associates with the sarcoglycan-sarcospan complex and it is suggested that the sarcoglycan-sarcospan complex also stabilises dystroglycan localisation and dystroglycan interactions. But if components of the DAPC are involved in phosphorylation events, how does this phosphorylation affect the protein:protein interactions within the complex? To date, this question has not been adequately addressed.

The initial aims of this study were to identify and characterise phosphorylation events in the DAPC with the aim of elucidating their effect on the interactions within the DAPC, the prospect being that this information would broaden the wealth of knowledge about the organisation of the DAPC. In principle, this information could help to identify the likely functions of the DAPC and their role in muscular dystrophy pathogenesis.

During the course of this study, a phosphorylation event was identified - namely, the tyrosine phosphorylation of β -dystroglycan (chapter 5). The exact role of β -dystroglycan is ill-defined but is thought to be crucial in

linking dystrophin to the membrane-spanning components of the DAPC. The phosphorylation of β -dystroglycan was found to disrupt its interaction with dystrophin, thus demonstrating that at least one of the interactions within the DAPC is regulated by phosphorylation.

In order to shed light upon the functions of β -dystroglycan and its role in signal transduction, extensive studies were carried out to identify novel binding partners of β -dystroglycan. Both protein biochemistry techniques (chapter 3) and yeast two-hybrid assays (chapter 4) were used. It was discovered that β -dystroglycan binds to F-actin, and unlike dystrophin, has F-actin-bundling activity. This raises exciting possibilities for the role of β -dystroglycan and the DAPC in regulation of the cytoskeleton.

CHAPTER 2
Materials and Methods



Materials and Methods

2.1. Bacterial methods.

2.1.1. Bacterial strains and media

Table 2.1

Escherichia coli strains

Strain	Genotype	Reference/ source
JM109	<i>e14⁻ (McrA⁻) recA1 endA1 gyrA96 thi-1 hsdR17(r_K⁻m_K⁻) supE44 relA1 Δ(lac-proAB) [F'<i>traD36 proAB lacI^qZΔM15</i>]</i>	Stratagene
BL21(DE3)	<i>B F⁻ dcm ompT hsdS(r_B⁻m_B⁻) galλ(DE3)</i>	Stratagene
MC1066	<i>Δ(lacI POZYA) 74 galU galK strA^r leuB6trpC9830 pyrF74::Tn5(Kn^r) hsdR⁻</i>	P. Legrain, Institut Pasteur

JM109 cells were used for cloning, propagation of plasmid DNA and protein expression. BL21(DE3) cells were used for protein expression. MC1066 cells were used for the rescue of prey plasmids from yeast cells in two-hybrid screens (section 2.5.7.1).

Table 2.2**Bacterial Media**

Medium	Components
LB	1% (w/v) Bacto-tryptone 0.5% (w/v) Bacto-yeast extract 0.5% (w/v) NaCl pH adjusted to 7.2 with 5 M NaOH
2x TY (pH 7.4)	16g Tryptone 10 g Yeast extract 5 g NaCl 1 l Distilled water
M9 -L	0.1% (w/v) -L drop-out powder 10% (v/v) 10x M9 salts ^φ 0.2% (w/v) Glucose ^φ 2 mM MgSO ₄ ^φ 0.2 mM CaCl ₂ ^φ pH adjusted to 6.5 with 5M NaOH
SOB	2% (w/v) Bacto-tryptone 0.5% (w/v) Bacto-yeast extract 10 mM NaCl 10 mM MgSO ₄ 2.5 mM KCl 10 mM MgCl ₂ ^φ
SOC	SOB medium 20 mM Glucose

* for solid media, 2% ((w/v)) agar was added prior to autoclaving.

φ added after autoclaving.

2.1.2. Growth of Bacteria

E. coli strains were routinely grown at 37°C in LB medium or 2x TY (table 2.2) for protein expression. To maintain selection for plasmid DNA, transformed bacteria were grown in medium containing ampicillin (100µg/ml).

2.1.3. Transformation of *E. coli*

2.1.3.1. Preparation of Electro-competent Cells

E. coli cells were taken from a frozen glycerol stock and grown overnight at 37°C on solid 2x TY medium (table 2.2). A single colony from this plate was used to inoculate 10ml of 2x TY liquid medium and left to grow at 37°C overnight. This culture was used to inoculate 500 ml of LB liquid medium to an OD₆₀₀ of 0.1. The culture was incubated at 37°C until the OD₆₀₀ was 0.5-0.7. At this time, the cells were placed on ice for 15 minutes. From this point, the cells were always kept on ice and all solutions and containers used were pre-chilled. The cells were subjected to centrifugation for 10 minutes at 5000 $\times g_{av}$. The cells were gently resuspended in 100ml sterile 10% (v/v) glycerol and centrifuged for 20 minutes at 5000 $\times g_{av}$. The cells were resuspended in 5ml of 10% (v/v) glycerol and aliquoted into Eppendorf tubes on ice (100 μ l aliquots). Cells were then snap frozen on dry ice and stored at -70°C.

2.1.3.2. Transformation of Electro-competent *E. coli* Cells

50 μ l electro-competent cells (section 2.1.3.1) were thawed on ice, and mixed with the DNA to be transformed. Cells were then transferred to a pre-chilled electroporation cuvette (0.2cm electrode gap) on ice. Electroporation was performed using a Biorad Gene Pulser II set at 200 ohms resistance, 25 μ F capacity and 2.5kV voltage. Immediately upon electroporation, 1ml of SOB medium (table 2.2) was added and the cells transferred to an Eppendorf tube. Cells were allowed to recover for 60 minutes at 37°C in a shaking incubator. The cells were then spread onto solid medium (table 2.2) supplemented with ampicillin and incubated at 37°C overnight.

2.2. Nucleic Acid Methods

2.2.1. Plasmids and clones used in this work

Table 2.3.

Plasmids

Plasmid	Features	Reference/ Source
pUC18	General cloning vector: LacZ' gene, LacI gene, multiple cloning site, pBR322 ori, Amp ^R	PharmaciaBiotech
pcDNAII	T7 promoter, Sp6 promoter, multiple cloning site, Amp ^R , F1 origin, LacZ gene	Invitrogen
pCEP4F (-EBNA)	Mammalian expression vector with EBNA sequence removed. Multiple cloning site, CMV and SV40 promoters, OriP, Amp ^R , Cole1, TKpA.	Invitrogen modified by B. Christy
pCMV5	Mammalian expression vector: CMV promoter, multiple cloning site, SV40 ori, f1 ori, hGH, Amp ^R .	Invitrogen
pSJW	pBR322 with T7 insert, multiple cloning site, Amp ^R	Winder <i>et al.</i> (1995)
PinpointX-3a	For expression of biotin tagged fusion proteins: <i>In vivo</i> biotinylation tag, <i>tac</i> promoter, T7 promoter, multiple cloning site, Factor Xa protease recognition site, Amp ^R .	Promega
pGEX-5X-3	GST gene fusion vector: GST gene, <i>tac</i> promoter, <i>Lac I^q</i> gene, Factor Xa protease recognition site, multiple cloning site, pBR322 ori, Amp ^R .	PharmaciaBiotech

Plasmid	Features	Reference /source
pAS2ΔΔ	Gal4 DNA binding domain fusion shuttle and expression vector: Multiple cloning site, Amp ^R , colE1 ori., <i>PADH1</i> , Gal4 DNA binding domain sequence, <i>ADH1</i> transcriptional terminator, 2μ, <i>TRP1</i> . Deletion of <i>CYH2</i> by <i>EcoRV</i> partial digest and HA by <i>EcoRI-NdeI</i> digest.	Fromont-Racine <i>et al.</i> , (1997)
pACTII [stop]	Gal4 activation domain fusion shuttle and expression vector: Multiple cloning site, Amp ^R , colE1 ori., <i>PADH1</i> , Gal4 activation domain sequence, HA-epitope sequence, <i>ADH1</i> transcriptional terminator, 2μ, LEU2.	Fromont-Racine <i>et al.</i> , (1997)

Table 2.4.**Clones used in this work**

Clones	Features	Source/ Reference
pAS2ΔΔ-β-DG	β-dystroglycan cytoplasmic domain (a-a 781-895) cloned into <i>NdeI/SalI</i> sites of pAS2ΔΔ	This work
pcDNAII- mDG.clone2	part of the transmembrane region of β-dystroglycan and entire cytoplasmic domain including 3' untranslated region (a-a 757-895) cloned into <i>EcoRI/BstXI</i> sites of pcDNAII	D. Blake Dept. of Human Anatomy and Genetics, University of Oxford.
Pinpoint- β-DG	β-dystroglycan cytoplasmic domain (a-a 775-895) cloned into <i>HindIII/NotI</i> sites of PinpointXa-3 vector	This work
pSJW-β-DG	β-dystroglycan cytoplasmic domain (a-a 781-895) cloned into <i>NdeI/SalI</i> sites of pSJW	Ann Nuttall University of Edinburgh
pSJW-Δ-β-DG	β-dystroglycan cytoplasmic domain (a-a 781-880) cloned into <i>NdeI/SalI</i> sites of pSJW	Ann Nuttall University of Edinburgh
Dystrophin mini-gene	6.4kb fragment of dystrophin (bp 112-2200 & bp 7307-12101) cloned into the <i>SallI</i> site of pUC18	J. Tinsley, MRC Harwell. England <i>et al.</i> (1990)
pCEP4F(-EBNA)-DYS	Dystrophin WW-EF-ZZ domains (a-a 3055-3354) cloned into <i>HindIII/NotI</i> sites of pCEP4F(-EBNA)	This work

Clones	Features	Source/ Reference
pCMV5-HA-DYS	Dystrophin WW-EF-ZZ domains (a-a 3055-3354) cloned into <i>KpnI/SmaI</i> sites of pCMV5-HA	This work
Pinpoint-DYS	Dystrophin WW, EF and ZZ domains (a-a 3055-3354) cloned into <i>HindIII/NotI</i> sites of PinpointXa-3 vector	This work
pCMV5-HA-FHOS	HA tag and the entire FHOS coding region cloned into <i>EcoRI/BamHI</i> sites of pCMV5	J. Westendorf, Vanderbilt University, Nashville, USA. (Westendorf <i>et al.</i> (1999))
pCEP4F(-EBNA)-mJAB1	Coding region of full length JAB1 cloned into <i>HindIII</i> site of pCEP4F (-EBNA)	B. Christy, University of Texas Health Science Center, San Antonio, USA.
pGEX5X-3-JAB1	Full length JAB1 cDNA cloned into <i>EcoRI/SmaI</i> sites of pGEX5X-3	E. Bianchi, Scientific Institute, San Raffaele-DIBIT, Milan, Italy.

2.2.2. Preparation of Plasmid DNA

2.2.2.1. Small Scale Preparation of Plasmid DNA by Spin Column

Plasmid DNA was prepared using the QIAprep spin miniprep kit (Qiagen), following the manufacturers' guidelines. DNA was extracted from 3ml of *E. coli* culture, resuspended in 50µl of distilled water and stored at -20°C.

2.2.2.2. Large Scale Preparation of Plasmid DNA from cDNA library amplification

Library clones were scraped off LB-amp agar plates into LB-amp media (table 2.2). The resultant 600ml of media was centrifuged at 4°C for 30min at 5,000 $\times g_{av}$ to harvest the cells. The cell pellet was resuspended in 60ml of GTE (50mM glucose, 25mM Tris pH8, 10mM EDTA pH8) plus RNase (10mg/ml). 120ml of solution II (0.2 M NaOH, 1% (w/v) SDS) was added and mixed gently. 200ml of solution III (3M KOAc/2M glacial acetic acid) was added next. Cell debris and chromosomal DNA were pelleted by centrifugation at 2,000 $\times g_{av}$ for 10 minutes at 4°C. The supernatant was decanted to a fresh tube and incubated at 37°C for 30 min to allow RNase activity. An equal volume of phenol:chloroform: isoamyl alcohol (24:24:1) was added to the supernatant, mixed, and centrifuged at 2000 $\times g_{av}$ for 10 minutes. The aqueous layer was carefully removed and an equal volume isopropanol added to precipitate the plasmid DNA. The DNA was pelleted by centrifugation at 12,000 $\times g_{av}$ for 10 minutes at 4°C and the pellet was washed with 70% (v/v) ethanol and allowed to air dry. The dried pellet was fully resuspended in 5ml of 1x TE, pH8 (10mM Tris-HCl, pH 7.5, 1mM EDTA) and stored at -20°C.

2.2.3. Spectrophotometric Quantification of Nucleic Acids.

The concentration of DNA or RNA was determined by measuring the absorption of diluted solutions at 260nm using a Cecil CE 2040 spectrophotometer, and a quartz cuvette. For double-stranded DNA, an OD₂₆₀ value of 1.0 represents a DNA concentration of approximately 50mg/ml.

2.2.4. Restriction Digestion of DNA

Restriction enzymes were purchased from Boehringer Mannheim or New England BioLabs. DNA was digested in volumes of 20-100 μ l. These contained the requisite quantity of DNA and the appropriate buffer (as supplied by the manufacturer) at 1x concentration. Between one and five units of restriction enzyme per μ g of DNA were added, with the restriction enzyme volume kept below 10% of the total reaction volume. The digest was incubated at the recommended temperature, typically for a period of 2 hours.

2.2.5. Amplification of DNA using the Polymerase Chain Reaction

Specific regions of DNA were amplified using the polymerase chain reaction (PCR). A PCR reaction mix was set-up in an 0.5ml Eppendorf tube as follows:

Template DNA	10-30ng of plasmid DNA
Oligonucleotide primer 1	0.5 μ M
Oligonucleotide primer 2	0.5 μ M
dNTPs (dATP, dCTP, dGTP, dTTP)	200 μ M each
MgCl ₂	1.5-6 mM
10x Polymerase buffer	1x
Taq polymerase	5 units per 100 μ l
Distilled water	to 100 μ l

All PCRs were carried out in a Biometra T3 thermocycler programmed according to the annealing temperature of the oligonucleotide primers used. A typical cycling program is shown below:

25 cycles of:	94°C for 30 seconds
	T _d °C [#] for 30 seconds.
	72°C for 1 minute.

#, T_d °C is the temperature at which 50% of the primers are annealed to target sequence. It can be calculated by the equation: $4(G+C) + 2(A+T)$, for oligonucleotides below 20bp in length. The T_d °C of the oligonucleotide with the lowest value is used as the annealing temperature in the thermal cycling. It is often necessary to use an annealing temperature a few degrees lower than the T_d °C.

2.2.6. Oligonucleotides used in this work.

Table 2.5

Oligo.	Function	Sequence (5'-3')
5' DYS/ <i>Bam</i> HI	Dystrophin WWEFZZ primer with <i>Hind</i> III site	ATAAAGCTTACGTCTGTCCAGGGTC CGTGGGAG
5' DYS/ <i>Kpn</i> I	Dystrophin WWEFZZ primer with <i>Kpn</i> I site	ATAGGTACCACGTCTGTCCAGGGTC CCTGGGAG
3' DYS/ <i>Not</i> I	Dystrophin WWEFZZ primer with <i>Not</i> I site	CCGCGGCCGCTTAATAGTGCATTTT ATGGCCTTT
3' DYS/ <i>Sma</i> I	Dystrophin WWEFZZ primer with <i>Sma</i> I site	CCGCCCCGGTTAATAGTGCATTTTA TGGCCTTT
5' β -DG / <i>Bam</i> HI	β -dystroglycan cytoplasmic domain primer with <i>Hind</i> III site	CGCAAGCTTTATCGCAAGAAGCGT AAGGGCAAG
3' β -DG/ <i>Not</i> I	β -dystroglycan cytoplasmic domain primer with <i>Not</i> I site	TAAGCGGCCGCTTAAGGTGGAACA TACGGAGGTGG
JOE17	pAS2 $\Delta\Delta$ sequencing primer	AAGAAGCTTTCTAGAATGAAGCTA CTGTCTTCT
PinPoint Xa-3 seq	PinPointXa-3 sequencing primer	GCGGTCAGGGTCTCATCAAG
Seq F	pACTII forward sequencing primer	GGCTTACCCATACGATGTTC
Seq R	pACTII reverse sequencing primer	TGAGATGGTGCACGATGC

All oligonucleotides were purchased from Genosys Biotechnologies Ltd. (Cambridge).

2.2.7. Purification of PCR Products

DNA fragments generated by PCR (section 2.2.5) were purified from oligonucleotide primers, unincorporated nucleotides, polymerases and salts using the QIAquick PCR purification kit (Qiagen), following the manufacturer's guidelines. Purified DNA was typically eluted in 30µl of sterile, distilled water and stored at -20°C.

2.2.8. Agarose gel electrophoresis

Agarose gel electrophoresis of DNA fragments was typically performed with 1.5% (w/v) agarose gels. Gels were prepared by melting the appropriate amount of agarose in 0.5 x TBE buffer (0.045M Tris-borate, 0.001M EDTA pH8) and adding ethidium bromide to a final concentration of 0.5µg/ml. Samples to be analysed were loaded directly using agarose gel loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (w/v) glycerol). The gel was placed in 0.5x TBE and typically run at 100V for 30 minutes. 1kb DNA ladder and 100bp DNA ladder (New England BioLabs) were used as size markers.

2.2.9. Isolation of DNA from Agarose Gel Slices

2.2.9.1. Isolation of DNA from Agarose Gel Slices by Spin Column

DNA fragments were separated by agarose gel electrophoresis (section 2.2.8) and the bands visualised on an UV transilluminator. The band to be purified was excised with a clean razor blade. DNA was isolated from agarose gel slices using the QIAquick gel extraction kit (Qiagen), following the manufacturer's guidelines. DNA was typically eluted in 30µl of sterile, distilled water and stored at -20°C.

2.2.9.2. Isolation of DNA from Agarose Gel Slices by GeneClean kit

DNA fragments were excised from agarose gels as above (section 2.2.9.1). The GeneClean III kit (BIO 101) was used to extract the DNA, following the manufacturers' protocol. The DNA was typically eluted in 20 μ l of Elution solution (RNase/DNase/Pyrogen free water) and stored at -20°C.

2.2.10. Ligation of DNA Molecules

Ligation reactions were typically performed in a final volume of 10 μ l. The vector and insert were gel purified (section 2.2.9) and their concentration measured by their OD₂₆₀ (section 2.2.3). The amount of vector and insert to add to the ligation reaction was calculated using the following equations:

$$\begin{aligned}\mu\text{g insert} &= 180 \times 33.33 \times 10^{-5} \times \text{kb} \\ \mu\text{g insert} &= 60 \times 33.33 \times 10^{-5} \times \text{kb}\end{aligned}$$

The reactions usually contained contained approximately 100ng of vector DNA, the amount of insert DNA as calculated, 1x T4 ligase buffer and 5 units of T4 DNA ligase (Boehringer Mannheim). Reactions were incubated at 16°C for 16 hours and stopped by 15 minutes incubation at 65°C. Control ligations were set up as above but without insert DNA.

2.2.11. DNA Sequencing by PCR-mediated Cycle Sequencing

Plasmid DNA to be sequenced was prepared using the QIAprep spin columns (Section 2.2.7). Reactions were performed with the dRhodamine terminator cycle sequencing kit (Perkin Elmer) in a PTC-100 Hot Lid reactor (MJ Research). A reaction mix was set-up as follows:

Template DNA	0.5µg of double-stranded DNA or 180ng of PCR product
Primer	3.2pmol
Terminator reaction mix	8µl
Distilled water	make-up volume to 20µl

Twenty-five cycles as described below were performed:

Step 1:	96°C for 30 seconds
Step 2:	50°C for 15 seconds
Step 3:	60°C for 4 minutes

DNA was precipitated from the reaction mix by adding 50µl of ethanol and 2µl of 3 M NaOAc, pH 5.2, incubating on ice for 10 minutes followed by centrifugation at 18,000 xg_{av} for 30 minutes. The pellet was washed with 250µl of 70% (v/v) ethanol and dried under vacuum. Samples were run by Nicola Preston (University of Edinburgh) on an ABI PRISM 377 DNA sequencer and the sequence analysed using the Gene Jockey II program.

2.3. Cell culture

2.3.1. Growth of cell lines

C2/C4 (Yoshida *et al.*, 1996) (a generous gift from Dr. Simon Hughes, King's College, London), C2/C12 (Yaffe & Saxel, 1977) (European Collection of Cell Cultures), and C2/C7 (a re-cloned C2/C12 cell line generously donated by Dr. M. Buckingham, Institute Pasteur, Paris, France) myoblasts were maintained in DMEM media (Gibco BRL) supplemented with 20% (v/v) foetal calf serum (Gibco BRL) in 5% CO₂ atmosphere at 37°C. Confluent cells were passaged using 1% (v/v) trypsin in 1mM EDTA/PBS and seeded at dilutions 1:4 -1:10. In order to induce differentiation, the amount of foetal calf serum in the growth media of confluent or semi-confluent myoblasts was reduced to 2% for up to 9 days. Myoblasts fused to form myotubes from day 4.

2.3.2. Harvesting of cells in RIPA buffer

Cells were washed once in cold phosphate buffered saline (50mM sodium phosphate pH 7.2, 150mM NaCl) before being harvested in ice cold radio-immunoprecipitation assay (RIPA) buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM ethylene glycol-bis-(β -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1mM ethylenediaminetetraacetic acid (EDTA), 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1mM sodium orthovanadate, 100 μ M leupeptin, 1mM phenylmethylsulphonyl fluoride (PMSF), 100 μ M N-tosyl-L-phenylalanine chloromethyl ketone (TPCK). After harvesting, cells were briefly sonicated to shear the DNA, centrifuged (18,000 $\times g_{av}$, 20 min) and stored at -20°C until required.

2.3.3. Alternative extraction conditions

Alternative extraction buffers were tried to improve extraction of the DAPC, namely: 1% (w/v) Brij 98 (20 Oleyl ether), 1% (w/v) CHAPS, 1% (w/v) digitonin, 1% (v/v) Triton X-100, 1% (v/v) Triton X-100/ 0.1% (w/v) SDS, or 1% (v/v) Tween-20 in PBS pH 7.2, 2mM MgCl₂ plus protease inhibitors. When using these extraction buffers, the cells were incubated at 4°C for 1 hour in the buffers prior to harvesting. To make 1% (w/v) digitonin, 10% (w/v) stock of digitonin was prepared, and solubilised by heating to 98°C for 15 minutes followed by incubation on ice for 2 hours. The solution was centrifuged to remove any unsolubilised solid, and the supernatant was used.

2.3.4. Differentiation Timecourse

Approximately 1×10^6 cells per 20cm² dish were seeded in DMEM media supplemented with 20% (v/v) foetal calf serum. After the cells reached 50% confluency, the growth media was replaced with DMEM media supplemented with 2% (v/v) foetal calf serum. Dishes of cells were lysed in RIPA buffer at 24 hour intervals over the course of 9 days. Protein concentrations of the samples were measured using the BCA kit (section 2.3.9). Equal amounts of protein were separated by SDS-PAGE, western blotted with dystrophin, utrophin and β -dystroglycan antibodies (table 2.6).

2.3.5. Peroxyvanadate treatment

C2/C4 myotubes were treated with peroxyvanadate in order to inhibit the activity of tyrosine phosphatases (Volberg *et al.*, 1992). The cells were washed with serum-free DMEM and then incubated at 37°C in peroxyvanadate (2mM H₂O₂, 1mM sodium orthovanadate in serum-free DMEM media). Initially, a timecourse of peroxyvanadate treatment was carried out, and then treatment for 45 minutes was carried out in all subsequent experiments. Control cells which were not treated with peroxyvanadate were incubated for an equivalent time in serum-free media. After treatment, the cells were harvested in RIPA buffer (section 2.3.2).

2.3.6. Calyculin A treatment

C2/C4 myotubes were treated with 100mM calyculin A (Calbiochem) in order to inhibit the activity of type 1 and 2A serine/threonine phosphatases (Ishihara *et al.*, 1989). The treatment was carried out in the same manner as peroxyvanadate treatment (section 2.3.5) but for 5 minutes at room temperature.

2.3.7. Replating of myoblasts onto ECM components.

ECM components used were gelatin, laminin, and fibronectin (all 2µg/ml solutions). The solutions were pipetted onto tissue culture dishes and incubated overnight at 4°C. The next day, the excess liquid was pipetted off before using the dishes. Cells were trypsinised, resuspended in DMEM supplemented with 20% (v/v) FCS and seeded onto ECM components or tissue culture plastic as a control, for 1, 2 or 4 hours at 37°C and subsequently lysed in RIPA buffer.

2.3.8. Transfection of C2/C4 myoblasts

C2/C4 cells were grown to 70% confluency in 6-well tissue culture dishes for transfection experiments. DNA used included pCMV-HA and pCEP4-F vectors with FHOS, JAB1 or dystrophin WW-EF-ZZ domains as inserts (Table 2.5). 1-2µg of DNA was added to 100µl DMEM (solution A). 2-15µl Lipofectamine reagent (GIBCO BRL) was added to 100µl DMEM (solution B). Solutions A and B were gently mixed in a sterile Eppendorf tube and incubated at room temperature for 40 minutes. The C2/C4 cells were washed with serum-free DMEM. 0.8ml of DMEM was added to the DNA/Lipofectamine, and this mixture was gently pipetted into each well of washed C2/C4 cells. The transfection was allowed to take place over 6 hours at 37°C in 5% CO₂ atmosphere. Subsequently, 3ml DMEM supplemented with 20% (v/v) FCS was added to each well of cells and they were placed at 37°C overnight. Cells were lysed in RIPA buffer (section 2.3.2) or other extraction buffers (section 2.3.3) 24 or 48 hours after the start of the transfection, and analysed by SDS-PAGE and western blotting (sections 2.4.4 & 2.4.6).

2.3.9. Quantification of protein concentration

The protein concentration in cell extracts was quantified using the BCA protein assay (Pierce) following the manufacturers' instructions. A series of bovine serum albumin (BSA) standards were prepared by diluting 2mg/ml BSA in water to give a dilution series ranging from 20-2000µg/ml BSA. 2ml of BCA working reagent (WR) was added to each standard solution and incubated at 37°C for 30 minutes. The OD₅₆₂ of each sample was measured and the values used to plot a graph of BSA concentration versus OD₅₆₂. Protein samples of unknown concentration was treated in the same manner and the concentration was extrapolated from the standard plot.

2.4. Protein Biochemistry Techniques

2.4.1. Antisera.

Table 2.6

Primary Antibodies

Antibody name	Directed against	Dilutions for western blotting and [IP]	Source/ Reference
MANDAG2*	β -dystroglycan (a-a 881-895)	1:500 [1:10]	G.E. Morris (NE Wales Institute, Wrexham) (Helliwell <i>et al.</i> 1994)
NCL-b-DG clone 43DAG/8D5*	β -dystroglycan 15 a-a peptide of extreme C-terminus (a-a 881-895)	1:50 [1:5-1:10]	Dr. L.V.B Anderson (University of Newcastle) ^s
Poly β -DG 1710 ^o	β -dystroglycan 15 a-a peptide of extreme C-terminus (a-a 881-895)	- [1:10]	S.Winder (University of Glasgow)
NCL-DYS1*	Dystrophin mid rod domain (a-a 1181-1388). Clone Dy4/6D3	1:100 [1:10]	L. Anderson (University of Newcastle) ^s
NCL-DYS2*	Dystrophin C-terminal 17 a-a (3669-3685). Clone Dy8/6C5	1:10 [1:10]	L. Anderson (University of Newcastle) ^s
VIA4-2A3	Dystrophin C-terminus & part of rod domain	- [1:25]	Upstate Biotechnology
XIXC2-D11	Dystrophin a-a 1415-1494 (rod domain)	- [1:25]	Upstate Biotechnology
Dystrobrevin ^o	α -dystrobrevin-1 C-terminus	1:1000	D. Blake (Dept. of Human Anatomy and Genetics, University of Oxford). Blake <i>et al.</i> (1998)

Antibody name	Directed against	Dilutions for western blotting and [IP]	Source/ Reference
IVD3-1	α -sarcoglycan	- [1:25]	Upstate Biotechnology
NCL- δ -SC [‡]	δ -sarcoglycan clone δ -Sarc3/12C1	1:500	Dr. L.V.B Anderson (University of Newcastle) [§]
RAB5 [‡]	Utrophin C-terminal coiled-coil domain	1:10000	S. Winder (Winder & Kendrick-Jones 1995)
GRB2*	Rat growth factor receptor 2 (C-terminus)	1:5000	Transduction Laboratories
FAK*	chicken focal adhesion kinase	1:1000	Transduction Laboratories
MAPK NEB9102 [‡]	Non phosphorylated p42 and p44 MAP kinase	1:1000	New England BioLabs
Paxillin*	chick paxillin	1:10,000	Transduction Laboratories
PY20*	Anti-phosphotyrosine	1:1000	Transduction Laboratories
RC20 [¶]	Anti-phosphotyrosine coupled to biotin	1:500	Transduction Laboratories
SHC [‡]	3 human SHC isoforms	1:1000	Transduction Laboratories
v-Src (Ab-1)*	SH3 domain of pp60 ^{src}	1:40	Oncogene Research Products

Antibody name	Directed against	Dilutions for western blotting and [IP]	Source/ Reference
Talin*	chicken talin, clone 8d4	1:100	Sigma Aldrich
Vinculin	Human Vinculin hVIN-1 clone	1:1000	Sigma Aldrich
HA	HA tag clone 3F10	1:1000 [1:100]	Roche
JAB1*	Bacterially expressed full length JAB1. Clone SP682 (subclone F3)	1:2000 [1:100]	E. Bianchi (Scientific Institute San Raffaele - DIBIT, Milano, Italy) (Bianchi <i>et al.</i> 2000)
M2	Flag Tag	1:100 [1:10]	Sigma Aldrich

*mouse monoclonal

φ rabbit polyclonal

ψ recombinant

§ antibodies commercially available from Novacastra Laboratories Ltd.

IP = immunoprecipitation

Table 2.7**Secondary Antibodies**

Protein directed against	Details	Conjugate	Raised in	Dilution for Western blotting
Extravidin	Binds biotin	HRP	-	1:1000
Anti-mouse IgG	whole molecule	HRP	goat	1:1000
Anti-rabbit IgG	whole molecule	HRP	goat	1:1000
Anti-mouse IgM	μ -chain specific	AP	goat	1:10000
Anti-rabbit IgG	γ -chain specific	AP	mouse	1:10000
Anti-mouse IgG	whole molecule	AP	goat	1:30000
Anti-rat IgG	whole molecule	biotin	sheep	1:333*

all purchased from Sigma Aldrich except * (Vector Laboratories)

HRP = horseradish peroxidase

AP = alkaline phosphatase

2.4.2. Immunoprecipitation assays.

Cell extracts in RIPA (section 2.3.2) were initially cleared by incubation with protein A-Sepharose (Pharmacia) or protein G-Sepharose (Sigma Aldrich) for 1-2 hours at 4°C. The required amount of antibody (table 2.6) was added to the cleared extract and the cells were incubated overnight at 4°C. In order to precipitate the antibody-protein complex, 50 μ l of a 50% (v/v) slurry of protein A- or G- Sepharose was added per sample, and incubated for a further 1-2 hours at 4°C. The samples were centrifuged briefly to pellet the Sepharose beads, and the beads were washed 4 times with cold RIPA buffer followed by a wash in cold 0.6M LiCl. The final pellet was re-suspended in SDS-PAGE loading buffer.

2.4.3. Lambda phosphatase treatment

Lambda phosphatase (λ ppase) (New England BioLabs) was used to treat immunoprecipitates of β -dystroglycan. A cocktail of 200 units λ ppase, 1X λ ppase buffer, 2mM $MnCl_2$, and H_2O to 10 μ l was added directly to the washed protein A Sepharose beads. The β -dystroglycan immunoprecipitates were treated at 30°C for 40 minutes. The reaction was stopped by boiling in SDS-PAGE loading buffer for 2 minutes.

2.4.4. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Large SDS-polyacrylamide gels (16cm x 20cm x 1mm) were run using the BIO-RAD Protean II vertical electrophoresis cell and mini-gels (10cm x 10cm x 0.75/1mm) were run using Cambridge Electrophoresis mini-gel cells. The separating gels were 3%-15% or 7.5%-20% gradient gels with 3% or 5% stacking gels, respectively. The gels were made individually, or 10 at a time in a gel casting chamber. The separating gel solutions were prepared (see table 2.8), poured between two sealed plates using a gradient mixer and overlaid with water-saturated butanol. The gels were allowed to set at room temperature, the butanol was washed off with sterile, distilled water and the stacking gel was poured and comb put in place. After polymerisation was complete, the seal was removed from the plates, the comb was gently taken out and the wells were washed with distilled water. The gels were assembled in the appropriate electrophoresis apparatus and the chambers filled with 1x SDS-PAGE running buffer (table 2.9). Protein samples were mixed with an equal volume of 2x SDS loading buffer (table 2.9), boiled for 2 minutes and loaded onto the gels. Pre-stained markers (size range 26.5-180 kDa) (Sigma-Aldrich) were loaded onto gels. Broad range molecular weight protein markers (size range 6.5-175 kDa) (BIO-RAD) were additionally loaded on to gels to be stained. Mini-gels were typically run for 35-45 minutes at 400V and large gels for 127.5mA/hours per gel.

Table 2.8**Quantities of gel mixes for SDS-PAGE**

(a single 16cm x 20cm x 1mm gel)

	Separating gel				Stacking gel	
	3%	7.5%	15%	20%	3%	5%
Acrylamide mix*	1.5ml	3.75ml	7.5ml	10ml	1ml	1.66ml
Separating/ stacking gel buffer	3.75ml	3.75ml	3.75ml	3.75ml	2.5ml	2.5ml
50% (v/v) glycerol	0.84ml	0.84ml	1.68ml	1.68ml	-	-
Water	8.84ml	6.60ml	2.02ml	-	6.42ml	5.76ml
TEMED ^φ	7.5μl	7.5μl	7.5μl	7.5μl	20μl	20μl
10% (w/v) APS ^ψ	60μl	60μl	45μl	45μl	60μl	60μl
Bromophenol blue	-	-	+	+	+	+

* 30% (w/v) acrylamide/0.8% (w/v) bisacrylamide (Severn Biotech Ltd.)

^φ N,N,N',N'-tetramethylethylenediamine^ψ ammonium persulphate

Proteins separated by SDS-PAGE were either stained with Coomassie blue stain for 15-30 minutes (and the gels subsequently destained with destain (table 2.9)), silver stained (section 2.4.5) or the proteins subjected to western blotting followed by antibody binding and detection (sections 2.4.6 & 2.4.7). Western blotting conditions and antibody concentrations were fully optimised for the DAPC and other relevant proteins.

2.4.5. Silver staining

(Adapted from (Morrissey, 1981))

SDS-PAGE gels were soaked in 50% methanol/10% acetic acid (v/v) for 30 minutes and subsequently washed in water (3 x 20 minute washes). Gels were soaked in DTT (0.5mg/100ml) in water for 30 minutes, and then 0.1% (w/v) silver nitrate for 30 minutes. The silver nitrate was briefly rinsed off in water and gels rinsed twice with developer (3% (w/v) sodium carbonate, 0.05% (v/v) formaldehyde) and the silver stain was developed for 10-30 minutes. The colour development was stopped by 0.115M citric acid solution and the gels washed in water.

2.4.6. Western blotting

Proteins were transferred electrophoretically from the SDS-polyacrylamide gel to polyvinylidene difluoride (PVDF) membrane (Biotrace) using the BIO-RAD Trans-Blot cell transfer system. All transfers were performed in CAPS/methanol transfer buffer (table 2.9). Large gels were blotted for 1500mA.hours or 3000mA.hours to transfer large proteins such as utrophin and dystrophin. Mini-gels were blotted for 400-800mA.hours. The transfers were performed either in the 4°C controlled temperature room or at room temperature using a super cooling coil (BIO-RAD).

2.4.7. Antibody binding and detection

After protein transfer was complete, PVDF membranes were blocked in 5% (w/v) skimmed milk powder in Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, 150mM NaCl) with 0.5% (v/v) Tween-20 (TBST) for 30 minutes and incubated overnight at 4°C in appropriately diluted primary antibody (table 2.6) in TBST on a roller. The membranes were subsequently washed 3 times in TBST before addition of the appropriate secondary antibody (table 2.7) diluted in TBST. Membranes were incubated for 2 hours in secondary antibody at room temperature. After washing in TBST, blots incubated with alkaline phosphatase-conjugated secondary antibodies were developed using 0.4 mM nitro blue tetrazolium and 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate in AP buffer (100mM NaCl, 5mM MgCl₂, 100mM Tris-HCl, pH 9.5). Alternatively, for blots incubated with peroxidase-conjugated secondary antibodies, detection was carried out using Amersham ECL kit (AmershamPharmaciaBiotech), according to manufacturers' instructions.

Table 2.9**Solutions for SDS-PAGE and western blotting**

Separating gel buffer	1.5 M Tris-HCl, pH 8.8 4% (w/v) SDS
Stacking gel buffer	0.5M Tris-HCl pH 6.8 0.4% (w/v) SDS
2x SDS loading buffer	100 mM Tris-HCl, pH 6.8 0.85M β -mercaptoethanol 4% (w/v) SDS 0.2% (w/v) Bromophenol blue 20% (v/v) Glycerol
10x SDS-PAGE running buffer	2.5mM Tris 1.9 M Glycine 1% (w/v) SDS
Coomassie Blue stain	0.1% (w/v) Coomassie blue R250 10% (v/v) acetic acid 50% (v/v) ethanol 10% (v/v) methanol
Destain	10% (v/v) acetic acid 5% (v/v) methanol
Western blotting transfer buffer	10mM CAPS pH11 20% (v/v) methanol

2.4.8. Two-dimensional gel electrophoresis.

All 2-D gel electrophoresis equipment was purchased from AmershamPharmaciaBiotech. β -dystroglycan was immunoprecipitated from equal amounts of protein from peroxyvanadate treated or untreated C2/C4 cell extracts, prepared in RIPA buffer (section 2.3.2). The Sepharose beads were re-suspended in rehydration buffer (8M urea, 2% (w/v) CHAPS, 2% (v/v) ampholine, 20mM DTT) and incubated for 1 hour at room temperature on a rolling incubator. After centrifugation, the supernatant (400 μ l per strip) was used to rehydrate 180mm pH3-10 Immobiline Drystrips in an Immobiline DryStrip Reswelling tray, overnight at room temperature. Strips were then subjected to isoelectric focusing on an Amersham PharmaciaBiotech Multiphor System in gradient mode (1mA, 5 Watts and 300V for 0.01 hour, 300V for 3 hours, 3500V for 5 hours, 3500V for 12.5 hours). Strips were stored at -80°C prior to SDS-PAGE. Strips equilibrated in equilibration buffer (50mM Tris-HCL (pH 8.8), 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT) for 30 minutes were separated in the second dimension on reducing 3-15% SDS-polyacrylamide gels (with no stacking gel) for 150 mA/hours per gel and western blotted (section 2.4.6).

2.4.9. Production of recombinant proteins

Unless stated otherwise, recombinant proteins were produced as follows: Proteins were expressed by growing transformed *E.coli* BL21(DE3) or JM109 (table 2.1) at 37°C in 2x TY media (table 2.2) until the OD₆₀₀ reached 0.6, then inducing protein production for 2 hours with 0.5mM isopropyl-β-D-thiogalactopyranoside. Following harvesting by centrifugation (5000 $\times g_{av}$, 10 minutes), the cell pellet was stored at -70°C overnight. After thawing, the pellet was re-suspended in SET buffer (25% w/v sucrose, 50mM Tris-HCl, pH 8, 1mM EDTA, 100μM leupeptin, 1mM PMSF, 100μM TPCK, 10mg/ml STI) and the cell suspension treated with lysozyme (0.5mg/ml) for 10 minutes at room temperature, followed by DNase1 (1μg/ml) (with 10mM MgCl₂ and 1mM MnCl₂) for 10 minutes. The lysed cells were then sonicated and centrifuged (24,000 $\times g_{av}$, 20 min) to remove cell debris. The resultant supernatant was clarified by ultracentrifugation (95,000 $\times g_{av}$, 1 hr). Samples were taken throughout the expression process (pre-induction, post-induction, post-lysis, soluble fraction, insoluble fraction) and analysed by SDS-PAGE to monitor the expression of the fusion protein.

2.4.10. Generation of Pinpoint fusion-proteins for pulldowns and 'SPOTs' assays

2.4.10.1. Expression of Pinpoint fusion proteins

The limits of the dystrophin cysteine rich domains WW, EF and ZZ were identified by sequence alignment and secondary structure prediction (James et al., 2000). cDNAs encoding residues 3055-3354 of human dystrophin (encompassing the WW, EF and ZZ domains, see figure 1.1) and residues 775-895 of β -dystroglycan cytoplasmic domain (see figure 1.4 & appendix) were amplified by polymerase chain reaction (PCR) (section 2.2.5) from the dystrophin mini-gene (cloned in pUC18) and mDG.clone2 respectively (table 2.4). The 5' primers contained a *HindIII* restriction site and the 3' primers contained a stop codon and *NotI* restriction site (table 2.5) which facilitated the cloning of the PCR products into the unique *HindIII* and *NotI* restriction enzyme sites of the PinPoint Xa-3 vector (Promega) to produce the PinPoint-DYS and PinPoint- β -DG constructs. The inserts were checked for mismatches by sequencing with the Pinpoint sequencing primer (table 2.5, section 2.2.11). *E. coli* JM109 (as suggested by the manufacturer) was transformed with PinPoint Xa-3, PinPoint-DYS or PinPoint- β -DG to express PIN (biotin tag alone, as a control) and the fusion proteins DYS-PIN and β -DG-PIN respectively (section 2.4.10.1). All the aforementioned fusion proteins were localised in the soluble fraction post cell lysis. Clarified supernatants were used directly in pulldown experiments (section 2.4.13.3). PIN and DYS-PIN were also purified for use in 'SPOTs' assays (section 2.4.10.2).

2.4.10.2. Purification of expressed Pinpoint fusion proteins.

PIN and DYS-PIN were purified by incubation of the crude cell supernatant with Softlink soft release Avidin resin (Promega: 1ml resin per 2 litre culture) overnight, at 4°C. The resin was subsequently washed four times with lysis buffer (50mM Tris-HCL, pH 7.5, 50mM NaCl, 5% (v/v) glycerol) and protein eluted with 5mM free biotin in lysis buffer, overnight, at 4°C. The free biotin was subsequently removed by dialysis in lysis buffer. The purification was monitored by SDS-PAGE and western blotting using ExtraAvidin-HRP (SigmaAldrich) followed by detection with ECL.

2.4.10.3. Hybridisation of 'SPOTs' membranes with DYS-PIN fusion protein.

The 'SPOTs' membranes were a generous gift from Dr. Marius Sudol, Mount Sinai School of Medicine, New York). The 'SPOTs' technique of peptide synthesis on derivatised cellulose membrane was performed as previously described (Blankenmeyer-Menge *et al.*, 1990; Frank & Doring, 1988; Kramer *et al.*, 1993). All reagents and equipment, including amino acids, derivatised membranes, incubation trays and software ('SPOTs', release 1.0), were purchased from Cambridge research Biochemicals and Genosys Biotechnologies, Inc. 'SPOTs' membranes were moistened with ethanol and blocked overnight in 10% (w/v) skimmed milk powder in TBST, at 4°C. The membranes were washed with TBST and probed overnight with DYS-PIN protein or PIN alone (10µg/ml). The membrane was washed four times with TBST and incubated with Extravidin-HRP diluted 1:1000 in TBST for 2 hours. After four washes with TBST, the blot was developed using ECL.

2.4.10.4. Regeneration of `SPOTs' membranes

The `SPOTs' membranes may be stripped and reused. The membranes were washed three times for 10 minutes with each of the following: distilled water, stripping buffer A (8M Urea, 1% (w/v) SDS, 0.5% (v/v) β -mercaptoethanol), stripping buffer B (10% (v/v) acetic acid, 50% v/v ethanol), and finally, ethanol. The membranes were stored at -20°C in a Ziplock bag until next needed.

2.4.11. Expression of β -dystroglycan cytoplasmic domain

(Method developed and carried out by Ann Nuttall & S. J. Winder, University of Edinburgh).

A construct of β -dystroglycan cytoplasmic domain (amino-acids 781-895) was cloned into pSJW (tables 2.3 & 2.4). pSJW- β -DG was transformed into *E.coli* BL21(DE3) and expressed as previously described (section 2.4.9). β -dystroglycan was successfully expressed and subsequently purified by anionic exchange chromatography using DEAE Sepharose, followed purification through a hydroxyapatite column. β -dystroglycan was eluted from the DEAE Sepharose column using a NaCl concentration of 200-300mM and was collected from the flowthrough of the hydroxyapatite column. A construct of β -dystroglycan cytoplasmic domain less the last 15 amino-acids (amino-acids 781-880) was also made, and the protein expressed and purified using the same method as for the full length cytoplasmic domain.

2.4.12. Expression of GST-JAB1 fusion protein

A construct of full length Jun activation-domain binding protein (JAB1) cloned into pGEX-5X-3 was used (tables 2.3 & 2.4). The construct was transformed into *E.coli* BL21(DE3) cells and expressed as in section 2.4.9. The fusion protein generated was largely present in the insoluble fraction of the lysed cells and the expression conditions were modified to produce a more soluble fusion protein. The temperature of expression was lowered to 20°C and expression time lengthened to 5 hours.

The GST-JAB1 fusion protein was purified using glutathione Sepharose 4B resin (AmershamPharmaciaBiotech) and following the manufacturers' instructions for batch purification. Clarified bacterial supernatant containing the GST-JAB1 fusion protein was added to a 75% (v/v) slurry of glutathione Sepharose 4B resin (prewashed three times with ice-cold PBS) and incubated on a roller for 1 hour at 4°C. The resin was sedimented by centrifugation at 5000 xg_{av} for 5 minutes and the supernatant discarded. The resin was washed five times with PBS. The GST fusion protein was eluted with glutathione elution buffer (0.3% (w/v) reduced glutathione dissolved in 50mM Tris-HCl (pH 8.0)). The elution buffer was added and left to incubate for 10 minutes at 4°C before centrifugation to sediment the resin and removal of supernatant containing GST-JAB1 fusion protein. This step was repeated three times to ensure a high yield of GST-JAB1.

2.4.13. Pulldowns

2.4.13.1 Coupling of β -dystroglycan cytoplasmic domain to CNBr-activated Sepharose 4B

The coupling method to couple β -dystroglycan cytoplasmic domain to CNBr-activated Sepharose 4B (AmershamPharmaciaBiotech) was carried out using the manufacturers' instructions. 1g of CNBr-activated Sepharose 4B was swelled with 1mM HCl and washed with 1mM HCl to remove additives. 4.5mg of purified β -dystroglycan cytoplasmic domain (section 2.4.11) was dialysed into coupling buffer (0.1M NaHCO₃ pH 8.3, 0.5M NaCl) overnight at 4°C. The protein solution was made up to 5ml using coupling buffer, added to the HCl-washed CNBr-activated Sepharose 4B and rotated end-over-end at 4°C overnight. Any excess ligand was washed away with 5 gel volumes of coupling buffer. Any remaining active groups on the Sepharose were blocked by an overnight incubation with 0.1M Tris-HCl buffer, pH8 or 1M ethanolamine, pH8. The Sepharose was subsequently washed with 3 cycles of alternating pH, each cycle consisted of a wash with 0.1M sodium acetate, pH 4, 0.5M NaCl followed by 0.1M Tris-HCl, pH 8, 0.5M NaCl. The resulting CNBr-activated Sepharose coupled to the β -dystroglycan cytoplasmic domain was stored in 0.1M Tris, pH8 plus 1mM Azide as a 50% (v/v) slurry. The coupling efficiency was calculated using the concentration of the excess ligand and varied from 80-90% in the various couplings carried out.

2.4.13.2 Pulldowns using β -dystroglycan cytoplasmic domain coupled to CNBr-activated Sepharose 4B

As a control, and for pre-clearing cell extracts, the coupling process (section 2.4.13.1) was carried out without any protein added to the CNBr-activated Sepharose 4B.

Approximately 15 μ g of β -dystroglycan (coupled to Sepharose beads) was added to every 350 μ g of protein (quantified from cell extracts (section 2.3.9)). Myoblast or myotube extracts were pre-cleared for 1 hour with CNBr-activated Sepharose with no ligand coupled to it. Extracts were subsequently incubated for 4 hours or overnight at 4°C with CNBr-activated Sepharose coupled to the β -dystroglycan cytoplasmic domain. The beads were washed 4 times with RIPA buffer followed by a wash with 0.6M LiCl. SDS-PAGE sample buffer was added to the washed beads and boiled for 2 minutes. The supernatant was analysed by SDS-PAGE and western blotting (sections 2.4.4 & 2.4.6).

In further controls, the CNBr-Sepharose beads were blocked with skimmed milk powder (5% w/v) or BSA (5% w/v) for one hour prior to the pulldown assay. Other controls included washing the beads in high salt (the salt concentration of RIPA buffer was raised from 150mM to 300mM) or increasing the number of washes from 4 to 10. In some instances, the C2/C4 cell extracts were pre-cleared for 1 hour with control or β -dystroglycan-coupled CNBr-Sepharose beads.

2.4.13.3. Pulldowns using DYS-PIN and β -DG-PIN fusion proteins

Clarified *E.coli* supernatants containing DYS-PIN or β -DG-PIN fusion proteins (section 2.4.10.1.) were incubated with ImmunoPure immobilised Streptavidin (Pierce) for 2 hours at 4°C to capture the fusion proteins onto the Streptavidin beads. The beads were collected by centrifugation and washed 4 times in RIPA buffer and added to C2/C4 myoblast or myotube cell extracts (pre-cleared with the streptavidin beads) and incubated overnight at 4°C. The beads were collected by centrifugation and washed 4 times with cold RIPA buffer followed by a wash in cold 0.6M LiCl and SDS-PAGE sample buffer was added to the beads. After 2 minutes boiling, the supernatant was run on SDS-PAGE gels and western blotted (sections 2.4.4 & 2.4.6).

2.4.13.4. Pulldowns using GST-JAB1 fusion protein

Approximately 1 μ g of purified GST-JAB1 fusion protein was added to 350 μ g of C2/C4 cell extract (pre-cleared for one hour with glutathione Sepharose) and incubated overnight at 4°C. 50 μ l of a 50% (v/v) slurry of glutathione Sepharose was subsequently added per sample and the tubes incubated for a further 2 hours at 4°C. The samples were centrifuged briefly to pellet the Sepharose beads, and the beads were washed 4 times with cold RIPA buffer followed by a wash in cold 0.6M LiCl. The final pellet was re-suspended in SDS-PAGE loading buffer, boiled for 2 minutes and analysed by SDS-PAGE and Western blotting (2.4.4 & 2.4.6).

2.5. Yeast two-hybrid assay

2.5.1. Yeast strains and media

Strains were routinely grown at 30°C on YPDA medium (Table 2.11). To maintain selection for plasmid DNA, and/or for auxotrophic markers inserted on the genome, cells were grown in the appropriate drop-out medium (table 2.11).

Table 2.10

***Saccharomyces cerevisiae* strains used in this work**

Strain	Genotype	Reference/ Source
CG1945	MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 LYS2::GAL1 _{UAS} -GAL1 _{TATA} -HIS3 gal4-542 gal80-538 cyh ^r 2 URA3::GAL4 _{17-mers (x3)} - CYC1 _{TATA} -lacZ	CLONTECH Feilotter et al. (1994)*
PJ689-4A	MATa trp1-901, leu2-3, 112, ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ	P. James et al. (1996)
Y190	MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3, 112, gal4Δ gal80Δ, cyh ^r 2, LYS2::GAL1 _{UAS} - HIS3 _{TATA} -HIS3, URA3::GAL1 _{UAS} -GAL1 _{TATA} -lacZ	Flick & Johnson (1990)

*CG-1945 is a derivative of HF7c (Feilotter *et al.* 1994)

Table 2.11

Yeast Media

Medium	Components
YPDA	1% (w/v) Bacto-yeast extract 2% (w/v) Bacto-peptone 2% (w/v) Glucose 0.004% (w/v) Adenine sulphate
-W/-LWH dropout media	2% Yeast nitrogen base w/o amino acids 2% (w/v) Glucose 2x CSM -W*/CSM -LWH* 1x adenine
-LW dropout media	2% Yeast nitrogen base w/o amino acids 2% (w/v) Glucose 2x CSM -LWH* 1x Adenine 1x Histidine
-LWA dropout media	2% Yeast nitrogen base w/o amino acids 2% (w/v) Glucose 2x CSM -LWAKLU* 1x Lysine 1x Histidine 1x Uracil

‡ for solid media, 2% (w/v) agar was added prior to autoclaving.

*Complete supplement mixture (CSM) minus specific amino-acids (BIO 101, Inc). Prepared to manufacturers' instructions and adjusted to pH 7.5 with NaOH.

Table 2.12
Nutrients and Supplements

Nutrient	100x Stock solution (mg/ml)
Adenine *	6
Histidine	6
Leucine	8
Lysine	3
Tryptophan	8
Uracil *	3

* stored at room temperature.

Stock solutions of the growth supplements were filter sterilised, stored at 4°C and added to media after autoclaving.

2.5.2. Transformation of Yeast

Yeast cells were transformed using the method of Gietz *et al.* (1993).

A single colony of the yeast strain to be transformed was inoculated into 10ml of liquid medium (table 2.11) and grown overnight at 30°C. The following day, the cells were diluted into 50ml of fresh medium to an OD₆₀₀ of 0.1 and incubated at 30°C. Upon reaching an OD₆₀₀ ~0.5, the cells were harvested by centrifugation at 3,500 $\times g_{av}$ for three minutes. Cells were transferred to an Eppendorf tube washed twice with 1ml of LiOAc mix (100mM LiOAc, 10mM Tris (pH7.4), 1mM EDTA). The pelleted cells (pelleted by centrifugation at 3,000 $\times g_{av}$ for 1 minute) were finally resuspended in 0.5ml of LiOAc mix and were ready for transformation.

1-5 μ g DNA to be transformed was mixed with 0-200 μ g of single stranded salmon sperm carrier DNA (incubated at 90°C for 10 minutes prior to use). 100 μ l of yeast cells in LiOAc mix were added to the DNA followed by 700 μ l of PEG mix (40% (v/v) PEG₂₀₀₀ in LiOAc mix). The mixture was pipetted up and down with a P1000 Pipettman to mix and incubated at 30°C for 30 minutes on a rotating wheel. Cells were heat-shocked at 42°C for 15 minutes, then sedimented by centrifugation at 3,000 $\times g_{av}$ for 30 seconds. The pelleted cells were resuspended in 200 μ l of water and plated onto the appropriate solid medium (Table 2.11) to maintain selection for the transforming DNA. Plates were incubated at 30°C for 2-3 days.

2.5.3. The yeast two-hybrid library

The yeast two-hybrid library used was a MATCHMAKER human skeletal muscle cDNA library (CLONTECH). The library cDNA is $XhoI$ -(dT)₁₅ primed and cloned into the $XhoI/EcoRI$ restriction sites of the pACT2 vector (table 2.3) which contains a *LEU2* selection marker. The library is purchased already transformed into *E.coli* BNN132 as a frozen glycerol stock. The library has 3.5×10^6 independent clones and the cDNA clones range from 0.4-4kb with an average size of 2kb.

2.5.3.1. Library titering protocol

The library titre was calculated so that the amount of library stock amplified represents 2-3 times the number of independent clones in the library.

1 μ l of frozen glycerol stock culture of library was added to 1ml of LB broth (dilution A (1:10³)). 1 μ l of dilution A was removed and added to 1ml of LB broth (dilution B (1:10⁶)).

1 μ l of dilution A was added to 50 μ l of LB broth and plated into an LB agar plate. 100 μ l and 500 μ l aliquots of dilution B were also plated onto LB agar plates and incubated overnight at 37°C.

The next day, the number of colonies were counted in order to determine the titer of the library (colony forming units (cfu)/ml) using the following formulae:

Number of colonies from dilution A $\times 10^3 \times 10^3 = \text{cfu/ml}$

Number of colonies from dilution B/plating volume $\times 10^3 \times 10^3 \times 10^3 =$ cfu/ml.

	<u>Number of colonies</u>	<u>Library titer (cfu/ml)</u>
1 μ l dilution A	46	4.6×10^7
100 μ l dilution B	4	4×10^7
500 μ l dilution B	23	4.6×10^7

Therefore, the library titer is 4.6×10^7 cfu/ml.

The library has 3.5×10^6 independent clones and to get a representative amplification of the library, 2-3 times this needs to be plated. Plating 2x the library would give 7×10^6 colonies.

$$\begin{aligned}
 \text{ml of library to plate} &= \frac{\text{number of colonies required}}{\text{cfu/ml}} \\
 &= \frac{7 \times 10^6}{4.6 \times 10^7} = 0.152\text{ml}
 \end{aligned}$$

2.5.3.2. Amplification of the skeletal muscle cDNA library

0.152ml of cDNA library was diluted in LB and was plated onto fifty nine 25cm² LB agar plates supplemented with ampicillin (Table 2.2). The plates were incubated at 30°C for 48 hours. The colonies were subsequently scrapped off in 5ml LB-Amp and the plates rinsed with a further 5ml LB-Amp, and the colonies in LB-Amp incubated for 2 hours at 30°C in a shaking incubator. 5ml of the culture was taken to make glycerol stocks of the library (25% (v/v) glycerol was added to the culture and 1ml aliquots were frozen at -70 °C).

From the remainder of the culture, plasmid DNA was extracted from the cells using the large scale preparation of plasmid DNA protocol (section 2.2.2.2).

2.5.4. The bait plasmid.

β -dystroglycan cytoplasmic domain (amino-acids 781-895) (see appendix) was cloned into the shuttle vector pAS2 $\Delta\Delta$ (Table 2.3) using restriction sites *NdeI* and *Sall*. The cloning was checked by automated sequencing of the plasmid (section 2.2.11) using sequencing primer JOE17 (table 2.5) which binds to the GAL4 DNA-binding domain. Subsequently, this bait plasmid was transformed into the appropriate yeast strain (table 2.10) and propagated on -W drop-out medium (table 2.11).

The yeast cells carrying the bait plasmid were tested for expression of β -dystroglycan cytoplasmic domain by carrying out a crude extract of the total cellular protein of the cells (section 2.5.5). 10 μ l of this extract was run on SDS-PAGE gels and western blotted with MANDAG2 anti- β -dystroglycan antibody (table 2.6) to check for β -dystroglycan expression.

2.5.5. Crude Extraction of Total Cellular Protein from Yeast

10ml of the -W dropout media (table 2.11) was inoculated with a single colony of bait cells (β -DG/pAS2 $\Delta\Delta$ in a host strain) and incubated at 30°C overnight. 50ml of fresh medium was inoculated with the pre-culture to an OD₆₀₀ of 0.1 and incubated again at 30°C. Upon reaching an OD₆₀₀ of ~0.5, the cells were harvested by centrifugation at 3,500 xg_{av} for 5 minutes. The pelleted cells were resuspended in 150 μ l 2x SDS-sample buffer (table 2.9) with 1mM phenylmethylsulphonyl fluoride (PMSF) added immediately before use. The cell suspension was transferred to a fresh Eppendorf tube containing 80 μ l of glass beads (425-600 μ m) and homogenised for 1 minute at high speed in a Mini BeadBeater (BIOSPEC Products). Centrifugation (13,000 xg_{av} for 10 minutes) was subsequently carried out at 4°C. The supernatant was removed to a fresh tube and used for analysis by SDS-PAGE and western blotting with β -dystroglycan antibodies.

2.5.6. Screening for yeast two-hybrid interactors

2.5.6.1 Titration of 3-aminotriazole.

In order to eliminate false positives in the yeast two-hybrid screen, titration of 3-aminotriazole (3-AT) was carried out by plating transformants on -LWH media containing different 3-AT concentrations ranging from 0 to 40mM. The transformants were allowed to grow for up to 5 days and their growth carefully monitored over this time to see what concentration of 3-AT inhibited growth.

2.5.6.2. Calculation of Transformation Efficiency and number of transformants screened

Cells growing on -LW media represent diploid cells containing both bait and prey (library) plasmid DNA. Bait cells were grown in -W drop-out liquid medium to an OD₆₀₀ of approximately 0.5, and 0.5-1.5µg skeletal muscle library was transformed in using the standard transformation method (section 2.5.2). 1/20th of each transformation was plated onto -LW media. The number of colonies growing after two days on these plates were counted.

To calculate the transformation efficiency:

Transformation Efficiency (%)

= Number of colonies on -LW plate x dilution factor (20) x 100

To calculate the amount of transformants screened:

Amount of transformants screened

= colonies on -LW x dilution factor (20) x number of transformations.

Different amounts of carrier DNA were used in the transformations to elucidate the amount gave the highest transformation efficiency. The different strains (table 2.10) were also compared for transformation efficiency.

2.5.6.3 The yeast two-hybrid screen

Transformants containing both the bait plasmid and library plasmids were selected for on -LW media. In order to find interactors of β -dystroglycan, transformants were plated onto -LWH or -LWA media to select for colonies where the *HIS* or *ADE* reporter gene was activated. To double-check the result, colonies from -LWH plates were re-streaked onto -LWA dropout media to check for growth. Colonies from -LWA plates were re-streaked onto -LWH dropout media. Activation of the *LacZ* reporter gene was also checked by X-Gal filter lift assays.

2.5.6.4 X-Gal Filterlift Assay

Filterlift assay solution (0.27% (v/v) β -mercaptoethanol, 0.04% (w/v) X-Gal (in dimethyl formamide)) was prepared in Z-buffer (60 mM Na_2HPO_4 (7H₂O), 40mM NaH_2PO_4 (H₂O), 10mM KCl, 1mM MgSO_4 (7H₂O)) fresh from stock solutions immediately prior to use. Cells were transferred to Hybond-C extra filters (Amersham), and the filters immersed in liquid nitrogen for five seconds. Filters were placed (cell side-up) onto Whatman 3MM paper soaked in filterlift assay solution, incubated at 30°C and examined at regular intervals. The reaction was stopped by transferring the filter to Whatman 3MM paper soaked in 1M Na_2CO_3 for 1 minute, and then to Whatman 3MM paper soaked in distilled water for 1 minute.

2.5.7. Analysis of Positive Clones from Two-hybrid Screens

The His⁺/Ade⁺/LacZ⁺ colonies isolated by the yeast two-hybrid assay were analysed to identify the library plasmid responsible for the two-hybrid interaction. To achieve this the library plasmids were rescued from the yeast cells and sequenced.

2.5.7.1 Plasmid rescue from yeast cells

This method of analysis is dependent upon isolating the prey plasmid DNA. Yeast cells carrying a library plasmid were collected with a toothpick and added to 200 μ l of lysis buffer (2% (v/v) Triton X-1001% (w/v) SDS, 100mM NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA), 200 μ l of glass beads (150-212 μ m) and 200 μ l of phenol: chloroform: isoamyl alcohol (25:24:1). This mixture was vortexed for 5 minutes and subjected to centrifugation at 18,000 $\times g_{av}$ for 5 minutes. 160 μ l of the supernatant was removed and added to 500 μ l of ethanol and 80 μ l of 7.5 M NH₄OAc, placed on dry-ice for 10 minutes and subjected to centrifugation at 18,000 $\times g_{av}$ for 10 minutes at 4°C. The DNA pellet was washed with 70% (v/v) ethanol, dried under vacuum and resuspended in 10 μ l of distilled water. 1-2 μ l of the rescued plasmid DNA was transformed (section 2.1.3.2) into electro-competent MC1066 cells (table 2.1). The transformants were plated onto M9 -L medium (table 2.2) which allows growth of only those *E. coli* cells carrying a plasmid with the *LEU2* gene (i.e. library plasmid). Mini-prep. DNA was prepared (section 2.2.2.1) from single colonies of the transformants. The size of the genomic DNA inserts in these plasmids was determined by restriction digestion (section 2.2.4) with *Bgl*II (which cuts in the library adaptor sequence at both ends of the insert) and agarose gel electrophoresis (section 2.2.8). The identity of the insert was determined by DNA sequencing (section 2.2.11) with primers Seq F and Seq R (table 2.5) and subsequent analysis using the BLAST program (section 2.5.7.2).

2.5.7.2 Computer Analyses: BLAST

Protein database searches were performed on the NCBI server (<http://www.ncbi.nlm.nih.gov/BLAST>) using the BLAST program. DNA sequences analysed using Sequencher and full length sequences were acquired from the Genbank database.

2.5.7.3 Controls

Library plasmids which elicited an interaction with the bait plasmid were plasmid rescued (section 2.5.7.1) and transformed back into yeast cells (section 2.5.2) containing the bait plasmid and selected for on -LWH and -LWA dropout media to check that the reporter genes were still activated. The library plasmids were also transformed into yeast carrying no bait plasmid and the transformed cells plated into -LA and -LH dropout media to verify that the reporter genes were only activated in the presence of the bait plasmid.

2.6. F-Actin binding experiments

Actin experiments to verify the actin/ β -dystroglycan interaction brought to light in the yeast two-hybrid screen were carried out using monomeric actin purified from acetone powder by Tommy Jess (Winder Lab, University of Glasgow). β -dystroglycan cytoplasmic domain was expressed and purified as in section 2.4.11. The molar concentration of the proteins was determined by measuring their absorbance at 280nm and calculating their concentration from this using their extinction coefficients.

2.6.1. Sedimentation Assays

50 or 100 μ l of reaction mixes of concentrated G-actin was mixed at the desired molar ratio (final actin concentration 5 μ M) with a dilution series of β -dystroglycan cytoplasmic domain (concentrations of 10, 20, 40, 60, 80 and 100 μ M) and initiation mix (1mM EGTA, 2mM MgCl₂, 0.5mM ATP) were allowed to polymerise (overnight at 4°C or for 4 hours at room temperature) and subsequently subjected to high and low speed sedimentation assays. Controls included actin alone (i.e. 5 μ M actin, no β -dystroglycan) and the highest concentration of β -dystroglycan (100mM) alone (no actin). The centrifugation step were carried out in a TA-100.2 Ultracentrifuge rotor (Beckman) at 100,000 $\times g_{av}$ or 20,000 $\times g_{av}$ for 15 minutes at 4°C.

Following the centrifugation, the supernatant was carefully removed and added to an equal volume of 2x SDS-PAGE sample buffer (Table 2.9). 50 μ l or 100 μ l (whatever the assay volume used) of 2x SDS-PAGE sample buffer was then added to the F-Actin pellet fraction. This was incubated at room temperature for 10 minutes, to allow the pellet to completely dissolve. The pellet fraction was then transferred to a fresh tube and the original washed with a further 50 μ l or 100 μ l of 2x SDS-PAGE sample buffer. This was then added to the pellet fraction. Equal volumes of the supernatant and pellet fractions were then subjected to SDS-PAGE analysis. 15% (w/v) polyacrylamide gels were used and run as described (section 2.4.4). The gels were stained with Coomassie blue protein stain to visualise the proteins.

2.6.2. Falling Ball Assay

Viscosity measurements of F-actin with or without β -dystroglycan were carried out using microcapillary falling ball viscometry (MacLean-Fletcher & Pollard, 1980). Reaction mixes of actin and β -dystroglycan identical to those used in the co-sedimentation assays (section 2.6.1) were set up.

The samples were drawn up into 100 μ l capillary tubes. The capillary tubes were plugged at one end with plasticine and held vertically. The actin was left to polymerise, either overnight at 4°C or for 4 hours at room temperature. The velocity of a 0.64mm diameter stainless steel ball was measured by recording the time it took to travel 10cm down the capillary tube.

In some cases the falling ball and sedimentation assays were also carried out using KME buffer (100mM KCl, 2mM MgCl₂, 1mM EGTA, 0.5mM ATP) instead of initiation mix, as a polymerisation agent.

2.6.3. Electron microscopy of actin filaments.

Reaction mixes of 5 μ M actin and 60 μ M β -dystroglycan, identical to those used in the co-sedimentation assays (section 2.7.1), were allowed to polymerise (overnight at 4°C or for 4 hours at room temperature) and electron microscopy was carried out by Tommy Jess (Winder lab, University of Glasgow). In brief, the sample was pipetted onto a charged carbon coated disc and negatively stained with Nanovan stain before viewing under the electron microscope.

2.6.4. Computer Analyses: MPsearch

The β -dystroglycan cytoplasmic domain amino-acid sequence was divided up into 20 amino-acid peptides, each overlapping by 10 amino-acids:

- Peptide 1. RKKRKGKLTLEDQATFIKKG
- Peptide 2. EDQATFIKKGVPIIFADELD
- Peptide 3. VPIIFADELDDSKPPSSSM
- Peptide 4. DSKPPSSSMPLILQEEKAP
- Peptide 5. PLILQEEKAPLPPPEYPNQS
- Peptide 6. LPPPEYPNQSVPETTPLNQD
- Peptide 7. VPETTPLNQDTMGEYTPLRD
- Peptide 8. TMGEYTPLRDEDPNAPPYQP
- Peptide 9. EDPNAPPYQPPPPFTVPMEG
- Peptide 10. PPPFTVPMEGKGSRPKNMTP
- Peptide 11. KGSRPKNMTPYRSPPPYVPP

A protein-protein database search was carried out, using MP search (Edinburgh Biocomputing Systems Ltd) based on the Smith-Waterman algorithm. Available at <http://ambler.icmb.ed.ac.uk>. Any actin-binding proteins homologous to regions of β -dystroglycan were noted.

CHAPTER 3
Proteins associating with β -dystroglycan

Proteins associating with β -dystroglycan

3.1. Introduction

The transmembrane glycoprotein β -dystroglycan is an essential component of the DAPC because it forms a link between dystrophin and extracellular matrix. However, evidence suggests the role of β -dystroglycan stretches far beyond this function, since in addition to dystrophin, β -dystroglycan interacts with proteins such as Grb2, caveolin-3 and rapsyn (section 1.6.4). Despite these findings, it is still not clear what the exact functions of β -dystroglycan are and more research is required.

The objective of this study was to investigate interactions of β -dystroglycan with proteins outside of the DAPC, in addition to studying protein:protein interactions within the DAPC. The aim was to gain a greater understanding of the function of β -dystroglycan by identifying novel interacting proteins, and also to investigate whether the phosphorylation of β -dystroglycan regulates any of these interactions.

In the first instance methods were developed in order to carry out experiments investigating interactors of β -dystroglycan. This included characterisation of mouse muscle cell lines which were the model system of choice, and optimisation of techniques used to extract proteins from the cells.

3.2. Characterisation of C2 mouse muscle cell lines

Mouse skeletal muscle cell lines have been widely used as a model for studying myogenesis. Initially, the majority of studies carried out to gain more information about dystrophin involved the extraction of dystrophin and DAPC proteins from skeletal muscle. This method yields adequate amounts of the desired proteins but is lengthy and time consuming. Due to its naturally low abundance, bacterial expression and purification of dystrophin constructs has proved useful to produce high quantities of the protein for biochemical assays and structural studies. However, using a muscle cell line provides a more *in vivo* approach compared to the aforementioned methods. Growing cultured cells allows the treatment of endogeneous proteins with chemical reagents such as phosphatase inhibitors while the cells are still viable, and easier protein extraction conditions compared to those from whole muscles.

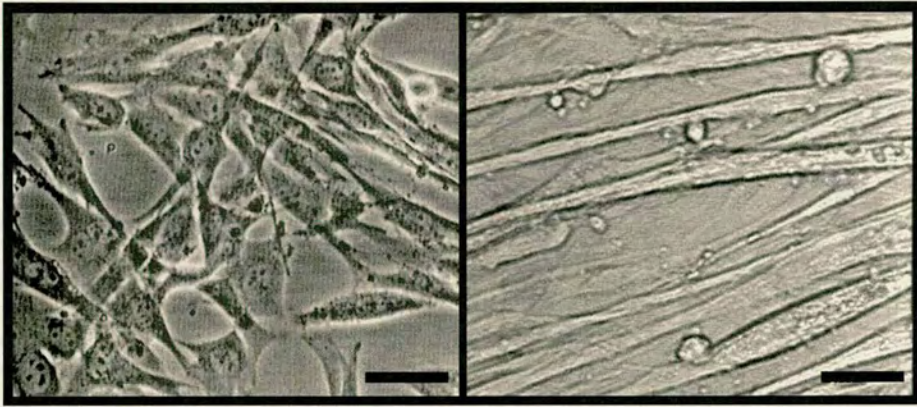
Three mouse skeletal muscle cell lines were assessed for their use in this study, namely C2/C12, C2/C7 and C2/C4 (section 2.3.1). On reaching confluency and/or reduction in serum levels, myoblasts spontaneously fuse to form multinucleated myotubes (figure 3.1A) and express proteins required specifically for differentiated muscle cells e.g. creatine kinase (Yaffe & Saxel, 1977). The cell lines were tested for their ability to fuse (this property is lost if cell lines are not maintained properly at low confluence), and for their expression patterns of dystrophin, β -dystroglycan and utrophin.

Whilst testing the cells for their expression of dystrophin, it was found in all three cell lines that dystrophin protein was not expressed in myoblasts but only in differentiated myotubes (figure 3.1B).

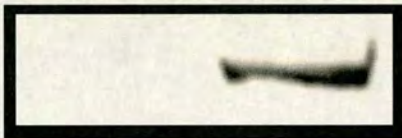
Figure 3.1

Dystrophin is expressed only in myotubes

A Myoblasts (day 1) Myotubes (day 9)



B Day 1 Day 9



IB: Dystrophin

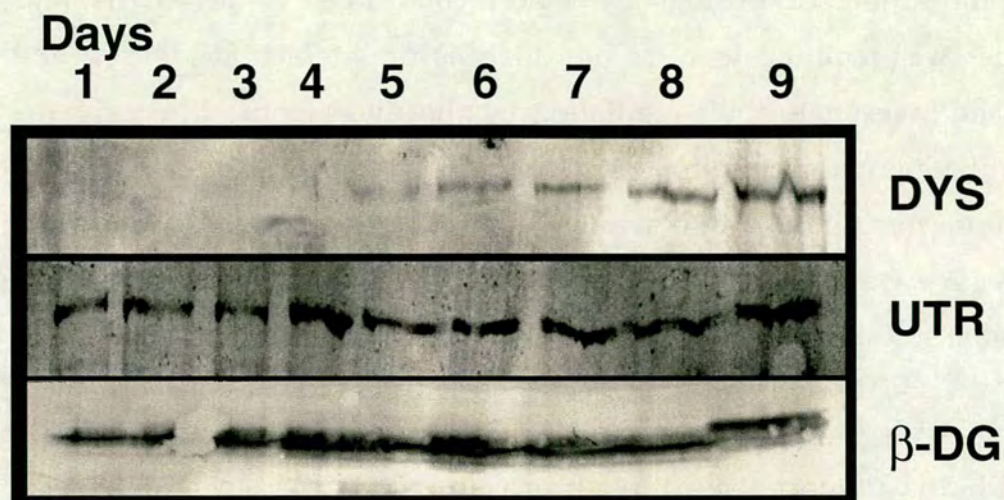
A. C2/C4 myoblasts on day 1 (left) compared to multinuclear myotubes after 9 days (right) in low serum differentiation medium. Scale bar = 50 μ m.

B. Western blot of myoblast extracts (day 1) and myotube extracts (day 9) blotted (IB) with DYS 1 and DYS 2 monoclonal antibodies (table 2.6). Dystrophin was only detected in differentiated myotubes, and not in myoblasts.

To determine more accurately when the expression of dystrophin commences, a timecourse of differentiation was carried out (section 2.3.4). The serum level in the growth media of the myoblasts was reduced from 20% (v/v) to 2% (v/v) to induce differentiation. Cell extracts were taken at daily intervals and western blotted for DAPC proteins.

During the differentiation timecourse of the C2/C4 cell line, it was found that β -dystroglycan and utrophin were expressed equally in myoblasts and myotubes, whereas dystrophin was only expressed in fairly advanced stages of differentiation (day 5) when myotubes are fully formed (figure 3.2). The predicted expression pattern of utrophin was that it is maintained at a steady level or decreases throughout differentiation as dystrophin takes over utrophin's localisation at the sarcolemma (Clerk *et al.*, 1993; Rigoletto *et al.*, 1995). It was found that in the C2/C4 cells, the overall expression levels of utrophin remained constant throughout differentiation.

The C2/C4 cell line demonstrated the predicted expression patterns of dystrophin, β -dystroglycan and utrophin (figure 3.2), and in addition, had higher expression levels of dystrophin compared to the other cell lines. Dystrophin has a very low abundance in any case (Hoffman *et al.*, 1987), and this higher expression of dystrophin in the C2/C4 cell line was considered an advantage for experiments involving the DAPC proteins, and it was the cell line of choice in subsequent experiments.

Figure 3.2**Differentiation timecourse of C2/C4 myoblasts**

The serum content in the growth media was reduced from 20% (v/v) to 2% (v/v) on day 1 when the myoblasts were approximately 50% confluent. Myoblast fusion first started to occur at days 3/4. Myotubes appeared fully formed by day 5. Cell extracts taken at daily intervals and equal amounts of protein loaded onto SDS-PAGE gels, and western blotted with antibodies DYS 1 and DYS 2 (dystrophin), NCL-43DAG/8D5 (β -dystroglycan) and RAB5 (utrophin) (see table 2.6).

Dystrophin is only expressed in fully fused myoblasts (day 5 onwards) whereas the ubiquitous utrophin and β -dystroglycan are expressed equally in myoblasts and myotubes. The C2/C4 cell line was considered to represent accurately the *in vivo* expression pattern of DAPC proteins.

3.3. Extraction conditions

The optimisation of protein extraction conditions of myoblasts and myotubes was required to carry out analysis of interactions within the DAPC and investigate their regulation by phosphorylation. The aim was to isolate a high concentration of dystrophin and DAPC proteins for use in immunoprecipitation and western blotting experiments. In addition, the objective was to isolate the DAPC and any associated proteins in an intact state so that protein:protein interactions could be investigated.

Dystrophin is a notoriously difficult protein to extract from muscle cells. This is because it is of very low abundance (0.002% of total skeletal muscle protein (Hoffman *et al.*, 1987)). For this study, it was necessary to extract sufficient amounts of dystrophin for visualisation by western blotting. Another complication is that certain members of DAPC are membrane proteins (β -dystroglycan and sarcoglycans) and others are cytoplasmic proteins (dystrophin, syntrophins). Therefore it is essential that conditions are optimised to accommodate both these characteristics.

A further requirement of methods used here was that they could facilitate a high throughput of small tissue culture samples (ranging from 200 μ l to 1ml). If expensive pharmacological reagents were to be used to dissect signalling pathways, large scale preparations would not be economically feasible. In addition, specialised tissue culture equipment is required for large scale growth of adherent cell cultures.

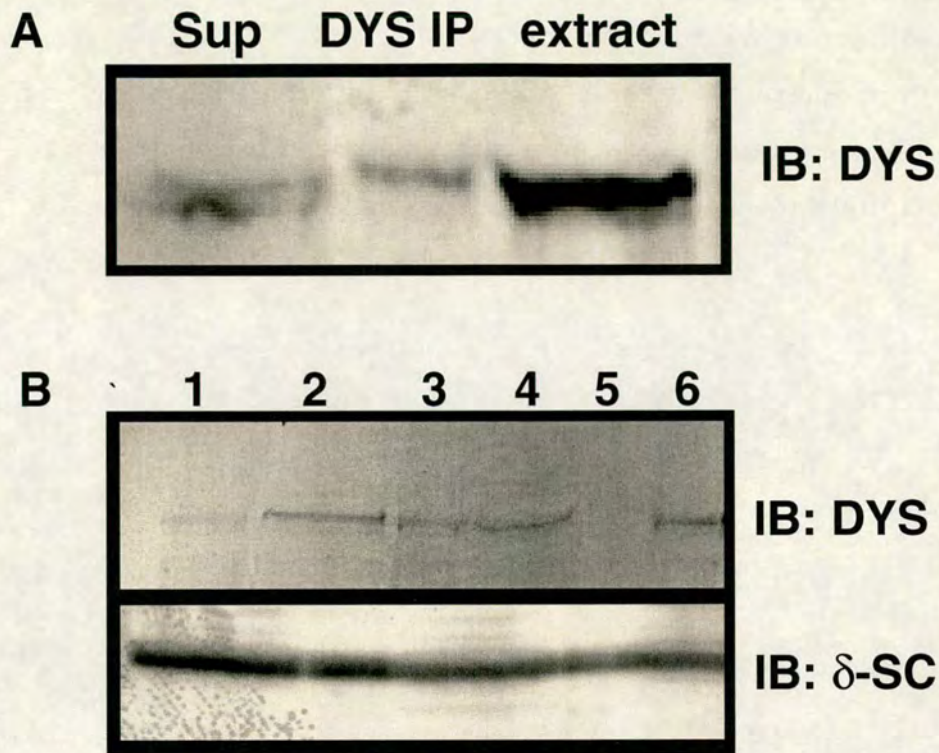
Several different extraction conditions were used in order to accomplish these aims. Little is known about the exact process by which each detergent works, therefore the detergent best used in a particular system is

usually worked out by trial and error and by surveying literature to gauge what has been successful previously. RIPA buffer has been used successfully to isolate DAPC proteins from rat L6 myotubes (Yoshida *et al.*, 1998) and was used in this study to lyse C2/C4 myoblasts and myotubes (section 2.3.2). RIPA buffer contains three detergents, namely 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate and 0.1% (w/v) SDS. SDS-PAGE electrophoresis of protein extracts, followed by western blotting with dystrophin antibodies demonstrated that RIPA buffer was far superior in extracting dystrophin than other buffers tested (data not shown). Further western blotting of DAPC proteins and utrophin showed that RIPA buffer adequately extracted all the proteins required. In addition, RIPA buffer delivered reproducible results and was easy to use. Therefore, RIPA buffer was considered as the best option and was used for the majority of extractions in this study.

3.4. Co-immunoprecipitation experiments

Using RIPA extraction buffer, co-immunoprecipitation experiments (section 2.4.2) were subsequently carried out to verify whether the DAPC was extracted as a whole entity. DYS 1 + DYS 2 monoclonal antibodies (table 2.6) were successful in immunoprecipitating dystrophin itself from RIPA extracts (figure 3.3A). Antibodies against representatives of the dystroglycan and sarcoglycan complexes (β -dystroglycan and δ -sarcoglycan) were also used to western blot dystrophin immunoprecipitates. In addition to DYS 1 & DYS 2, the evaluation of dystrophin antibodies VIA4-2A3 and XIXC2-D11 (table 2.6) was carried out. α -sarcoglycan antibody IVD3-1 was also analysed to clarify whether it has the ability to co-immunoprecipitate the whole DAPC. In the past, α -sarcoglycan antibodies have been shown to co-immunoprecipitate not only members of the sarcoglycan complex but other DAPC proteins too. For example, an α -sarcoglycan antibody was coupled to beads and incubated with partially purified DAPC complex and the immunoaffinity beads selectively absorbed dystrophin, sarcoglycans, and syntrophins (Ervasti *et al.*, 1990).

Using DYS1 + DYS2 antibodies, neither β -dystroglycan or δ -sarcoglycan were co-immunoprecipitated with dystrophin (data not shown), suggesting the DAPC was not remaining intact during extraction with RIPA buffer. However, VIA4-2A3 and XIXC2-D11 co-immunoprecipitated δ -sarcoglycan (figure 3.3B) but not β -dystroglycan. α -sarcoglycan antibody IVD3-1 also appeared to immunoprecipitate the whole DAPC because δ -sarcoglycan and dystrophin were co-immunoprecipitated with it, but again not β -dystroglycan.

Figure 3.3**Co-immunoprecipitation experiments****A. Immunoprecipitation of dystrophin**

Dystrophin was immunoprecipitated (IP) from RIPA extracted myotubes using DYS1 and DYS2 monoclonal antibodies (table 2.6). A sample of the immunoprecipitate (DYS IP), the supernatant from the immunoprecipitation (SUP) and untreated extract (extract) was analysed by SDS-PAGE and western blotted (IB) with DYS1 and DYS2. A small amount of dystrophin was immunoprecipitated and the supernatant contained the residual dystrophin.

B. Co-immunoprecipitation of dystrophin and δ -sarcoglycan

The same myotubes extracts were used to immunoprecipitate (IP) dystrophin with antibodies VIA4-2A3 and XIXC2-D11, and α -sarcoglycan with antibody IVD3-1. The immunoprecipitates were loaded in the even numbered lanes and the supernatants of the immunoprecipitates were loaded in the odd numbered lanes. All three immunoprecipitates were western blotted (IB) with dystrophin antibodies (DYS1 & DYS2) and δ -sarcoglycan antibody (NCL- δ -SC). Lanes 1 and 2 show α -sarcoglycan IP, Lanes 3 and 4 show dystrophin IP (VIA4-2 A3). Lanes 5 and 6 show dystrophin IP (XIXC2-D11). All three antibodies immunoprecipitated dystrophin and δ -sarcoglycan i.e. VIA4-2A3 and XIXC2-D11 co-immunoprecipitated δ -sarcoglycan and NCL- δ -SC co-immunoprecipitated dystrophin and δ -sarcoglycan.

β -dystroglycan antibodies (table 2.6) were also used in an attempt to co-immunoprecipitate dystrophin and Grb2, both of which have been shown previously to interact with β -dystroglycan (Jung *et al.*, 1995; Yang *et al.*, 1995). However, despite the use of several different β -dystroglycan antibodies, these interactions could not be demonstrated by co-immunoprecipitation.

3.5. β -dystroglycan interacts with at least three phosphoproteins

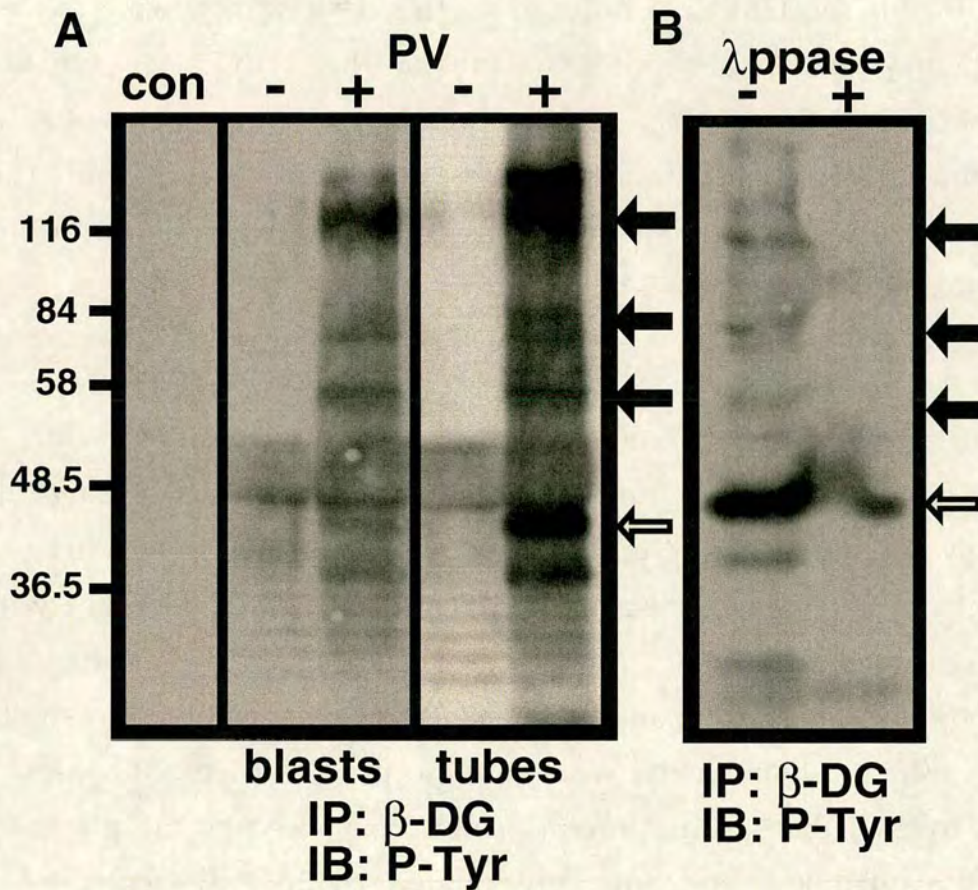
One of the initial aims of this study was to identify phosphorylation events within the DAPC. In order to do this, cell extracts were treated with peroxyvanadate in order to inhibit the activity of tyrosine phosphatases (Volberg *et al.*, 1992, section 2.3.5). Tyrosine phosphatase inhibitors preserve any transient phosphorylation events and this facilitates their visualisation by western blotting with anti-phospho tyrosine antibodies.

The first technique used to identify proteins associating with β -dystroglycan was co-immunoprecipitation experiments using β -dystroglycan antibodies (section 2.4.2). To elucidate whether β -dystroglycan is tyrosine phosphorylated and whether it associates with other proteins under these conditions, β -dystroglycan immunoprecipitates from peroxyvanadate treated myoblast/myotube extracts were western blotted with anti-phospho tyrosine antibodies. It was discovered that at least three proteins, recognised by the phosphotyrosine antibodies, co-immunoprecipitated with β -dystroglycan in peroxyvanadate treated cells (figure 3.4A). These phosphoproteins were in the approximate molecular weight ranges of 55kDa, 80-90kDa and 116-130kDa. This phenomenon was seen in both myoblast and myotube extracts and was highly reproducible. The phosphoproteins were not detected by anti-phospho tyrosine antibodies in untreated extracts or in the controls. The control immunoprecipitations contained no β -dystroglycan antibody and were used to detect any proteins non-specifically pulled down by the Sepharose beads.

-

Figure 3.4

Phosphoproteins co-immunoprecipitate with β -dystroglycan in peroxyvanadate treated myoblasts and myotubes.



A. Western blot of β -dystroglycan immunoprecipitates (β -DG IP) (immunoprecipitated using poly 1710 antibody) blotted (IB) with phosphotyrosine antibody PY20 (P-Tyr). The control (con) contained no 1710, therefore represents any non-specific pull-down by the protein A Sepharose, of which there was none. In peroxyvanadate-treated extracts (+PV), phosphoproteins of approximate molecular weights of 55kDa, 80-90kDa and 116-130kDa were co-immunoprecipitated with β -dystroglycan (shown by filled arrows). This was observed in myoblast extracts (blasts) as well as myotube extracts (tubes). The unfilled arrow points to β -dystroglycan (β -DG) which is detected by PY20 in peroxyvanadate treated extracts only.

B. Western blot of β -dystroglycan immunoprecipitates (β -DG IP) from myotube extracts, treated with peroxyvanadate, and treated (+ λ ppase) or untreated (- λ ppase) with λ phosphatase, and blotted with phosphotyrosine antibody RC20 (P-Tyr). The three groups of phosphoproteins (fainter in this experiment) in - λ ppase immunoprecipitates are no longer detected by RC20 after λ ppase treatment. A residual amount of β -dystroglycan still remains phosphorylated in the λ ppase treated immunoprecipitate.

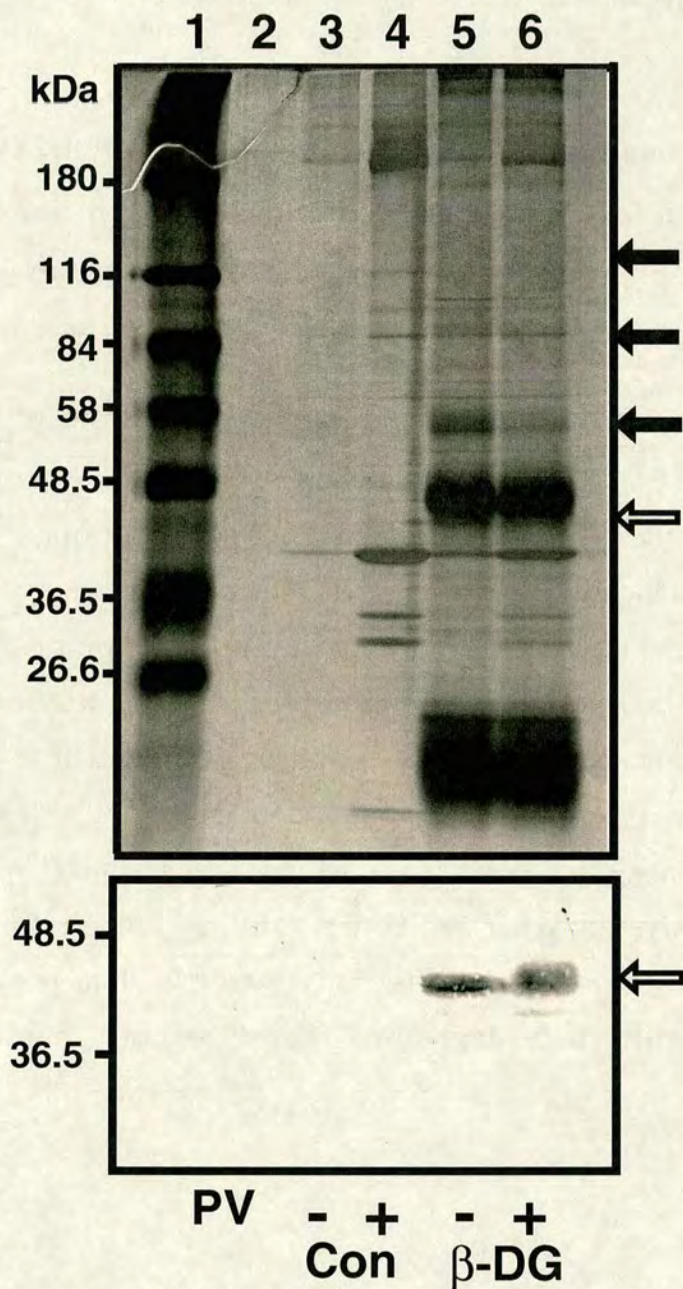
In addition, β -dystroglycan itself was also detected by phospho-tyrosine antibodies in extracts treated with peroxyvanadate. The tyrosine phosphorylation of β -dystroglycan is discussed more fully in chapter 5.

When β -dystroglycan immunoprecipitates are treated with lambda phosphatase (λ ppase) (section 2.4.3), a serine, threonine and tyrosine phosphatase, the three phosphoproteins were no longer detected by phospho-tyrosine antibodies (figure 3.4B). This confirmed that they were in fact phosphorylated proteins.

In an attempt to identify these phosphoproteins, the use of mass spectrometry or protein sequencing was considered. However, it can be observed on a silver stained gel (section 2.4.5) of the immunoprecipitates that there is a large number of proteins co-immunoprecipitating either non-specifically (in controls) or specifically with β -dystroglycan, and it is likely that more than three phosphoproteins are associating with β -dystroglycan (figure 3.5). Some of the proteins which are specifically immunoprecipitated by β -dystroglycan antibodies do so in both peroxyvanadate treated and untreated extracts. This suggests that the proteins are associated with β -dystroglycan, regardless of their phosphorylation state.

Figure 3.5

Silver staining of β -dystroglycan immunoprecipitates.



β -dystroglycan was immunoprecipitated (β -DG IP) from peroxyvanadate treated (+PV) and untreated (-PV) myotube extracts using polyclonal antibody 1710. The controls contained (con) no 1710 in order to detect any non-specific pulldown of proteins by the protein A Sepharose beads.

A. The immunoprecipitates were run on SDS-PAGE gels and silver stained. In the first lane is pre-stained molecular weight markers (SIGMA-Aldrich). No protein was loaded in the second lane. The controls were loaded into the third and fourth lanes, and β -dystroglycan immunoprecipitates in the fifth and sixth lanes.

β -dystroglycan was not clearly visible on the gel because it is obscured by the IgG heavy chain (unfilled arrow points to the location of β -dystroglycan, confirmed by western blotting with MANDAG2 -see **B**). The filled arrows denote the approximate location of phosphoproteins as seen on western blots (see figure 3.4). In the β -dystroglycan immunoprecipitates there are no proteins present in the 116-140kDa range, non-specific bands in the 80-90kDa range (i.e. also present in the controls) and a protein clearly associating with β -dystroglycan in the 55kDa range.

Representative of at least 5 similar experiments.

There appeared to be no proteins visible on the silver stained gel associating with β -dystroglycan in the 116-130 kDa range. This might be because they are present in very small amounts and the silver staining is not sensitive enough to detect them, but anti-phospho tyrosine antibodies are.

In the 80-90kDa range, many proteins appeared to be associated with β -dystroglycan, however, some of these proteins were also present in the controls, suggesting non-specific pulldown of proteins. In this range, there were small amounts of many proteins, therefore, it is difficult to gauge which proteins are specifically associating with β -dystroglycan.

In the 55kDa range, there is a protein specifically associating with β -dystroglycan, present in both peroxyvanadate treated and untreated extracts. Interestingly, there appears to be more of the protein in untreated extracts compared to treated.

β -dystroglycan itself is not clearly visible in the immunoprecipitates, this is because it is obscured by the large IgG Heavy chain band which is just above β -dystroglycan.

Due to the complex mixture of proteins seen in the silver stained immunoprecipitates, it was decided that mass spectrometry and protein sequencing were not viable options for identifying the phosphoproteins. There were many proteins visible on the silver stained gel but even so, these might not be the phosphoproteins, due to the difference in sensitivity of silver staining compared to immunoblotting.

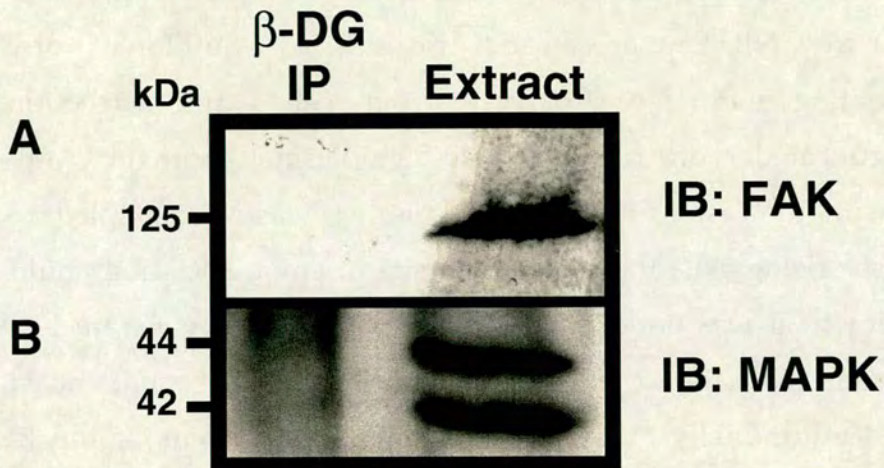
Using the approximate molecular weights of the three main phosphoproteins detected on western blots (figure 3.4), putative interactors of β -dystroglycan were tested by western blotting β -dystroglycan immunoprecipitates with antibodies directed against candidate proteins. The two groups of proteins investigated were signalling proteins and focal adhesion proteins. Many of these proteins are tyrosine phosphorylated, therefore, they were considered prime candidates for phosphoproteins associating with β -dystroglycan. In addition, proteins such as FAK (125kDa), vinculin (116kDa) and Src (60kDa) are in the molecular weight range of the phosphoproteins identified here.

Other focal adhesion/signalling proteins were also tested for association with β -dystroglycan, even if they were not in the molecular weight ranges of the phosphoproteins e.g. talin. The reason for this was that, if β -dystroglycan was associating with one member of the focal adhesion complex, many components of the complex may be pulled down in an immunoprecipitation experiment (Yoshida *et al.*, 1998). Additionally, some proteins migrate higher or lower than their molecular weight on SDS-PAGE gels, e.g. paxillin was considered as a viable candidate, it is 68kDa but runs at very diverse molecular weights on SDS-PAGE gels due to its different hyper-phosphorylated states.

Co-immunoprecipitation experiments proved unsuccessful and the focal adhesion proteins FAK, vinculin, paxillin and talin did not appear to co-immunoprecipitate with β -dystroglycan (a β -dystroglycan immunoprecipitate blotted with FAK antibody is shown in figure 3.6A). In western blots of β -dystroglycan immunoprecipitate blotted with an antibody against the signalling protein MAPK, it was unclear whether the two proteins were associated due to background on the blot (figure 3.6B).

Figure 3.6

Western blots of β -dystroglycan immunoprecipitates blotted with antibodies to focal adhesion and signalling proteins.



A. β -dystroglycan immunoprecipitate (β -DG IP) (using 8D5 monoclonal antibody) blotted (IB) with focal adhesion kinase (FAK) antibody. Extract alone was loaded onto the righthand lane. FAK (125kDa) was not co-immunoprecipitated with β -dystroglycan.

B. β -dystroglycan immunoprecipitate (using MANDAG2 monoclonal antibody) blotted (IB) with a MAPK kinase antibody which detects both the p42 and p44 isoforms of MAPK. The blot had some background and it is difficult to interpret if a small amount of MAPK is co-immunoprecipitated with β -dystroglycan.

3.6. Pulldowns using β -dystroglycan coupled to CNBr-Sepharose

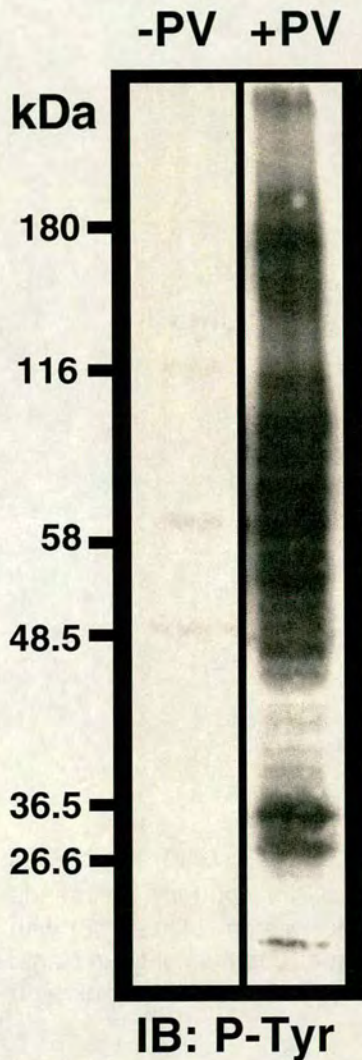
The rationale was to couple purified β -dystroglycan cytoplasmic domain (section 2.4.11) to CNBr-Sepharose and use this to “pulldown” any proteins associating with β -dystroglycan from cell extracts (section 2.4.13.2). A truncated construct of the cytoplasmic domain ($\Delta\beta$ -dystroglycan) with the last 15 amino-acids missing was also coupled to CNBr-Sepharose. Using this, the interaction site of any association could be narrowed down. It was hoped that this method would generate less non-specific bands than the co-immunoprecipitation experiments used previously, and additionally overcome the potential problem of the β -dystroglycan antibodies having the same epitope as the one confirmed protein: protein interaction site of β -dystroglycan. In this procedure, β -dystroglycan would be anchored by the N-terminal end of its cytoplasmic domain, thus leaving the rest of the domain free for interactions.

Initially, many proteins appeared to be pulled down with the β -dystroglycan cytoplasmic domain coupled to CNBr-Sepharose. Not only known interactors such as dystrophin, utrophin, Grb2, but also many of the candidate interactors of β -dystroglycan, such as focal adhesion proteins (vinculin, FAK, V-Src, paxillin), the DAPC protein dystrobrevin and signalling proteins SHC (SH2-Containing sequence) and MAPK. The large number of proteins associating with β -dystroglycan was deemed plausible: the DAPC has been shown to associate with focal adhesion complexes, and dystrophin co-immunoprecipitates with all the member proteins tested (Yoshida *et al.*, 1998), dystrobrevin is part of the DAPC (it interacts with the C-terminus of dystrophin (Sadoulet-Puccio *et al.*, 1997)) and β -dystroglycan has been shown to associate with MAPK in non-muscle cells (M. James & S. J. Winder, unpublished observation). However, a phospho-tyrosine western blot of the β -dystroglycan-CNBr-Sepharose

pulldown showed a tremendous number of proteins being pulled down (figure 3.7). It is unlikely that all these proteins are interacting with β -dystroglycan specifically, therefore this indicated that many proteins were non-specifically sticking to the Sepharose beads.

Figure 3.7

Western blot of pulldowns using β -dystroglycan cytoplasmic domain coupled to CNBr Sepharose beads.

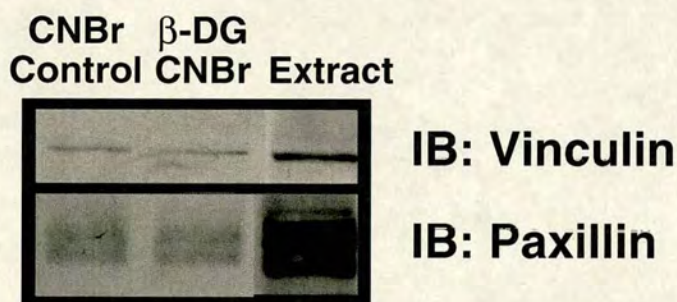


Proteins associating with β -dystroglycan were pulled down from peroxyvanadate treated (+PV) or untreated (-PV) myoblasts using the β -dystroglycan construct coupled to Sepharose beads. These pulldowns were western blotted (IB) with the phospho-tyrosine antibody RC20 (P-Tyr). The antibody detected proteins (phosphorylated on tyrosine residues) which were pulled down in the peroxyvanadate treated extracts. It is unlikely that β -dystroglycan interacts with so many proteins, therefore it was concluded that they were non-specific interactions.

On further inspection, many of these proteins pulled down by β -dystroglycan coupled to CNBr-Sepharose were being pulled down in the controls as well. This phenomenon can be observed in figure 3.8 where the control beads (CNBr-Sepharose beads subjected to the entire coupling process, but with no protein added) and β -dystroglycan-coupled CNBr-Sepharose beads pulled down comparative amounts of vinculin and paxillin. Similar results were noted for other proteins tested, such as dystrobrevin, SHC, MAP kinase and Grb2.

Figure 3.8

Western blot of pulldowns from myoblast extracts using β -dystroglycan coupled to CNBr Sepharose.



Myoblast extracts were incubated overnight with β -dystroglycan coupled to CNBr Sepharose (β -DG CNBr) or CNBr Sepharose with no protein coupled to it (CNBr control). The beads were washed thoroughly and loaded onto SDS-PAGE gels and western blotted (IB) with paxillin and vinculin monoclonal antibodies. Extract alone was loaded into the right hand lane. Comparable amounts of protein were pulled down by the β -dystroglycan coupled beads and the control beads.

Representative of at least 5 similar experiments.

In order to evaluate whether any of the interactions shown by the pulldowns were real, more stringent controls were carried out. The rationale was that if the amount of proteins pulled down non-specifically was reduced (background), then any specific interactions could be visualised more easily. A panel of control beads were used. This panel included batches of beads which were blocked with skimmed milk powder or BSA (5% (w/v)) before addition to the cell extracts. It was expected that if proteins were non-specifically sticking into the pores of the Sepharose, this could be prevented by the treatment with BSA or skimmed milk powder. To minimise non-specific ionic interactions, another control involved increasing the salt concentration in the RIPA buffer used in the post-pulldown washes from 150mM to 300mM. In the final control, the beads were washed 10 times with RIPA buffer instead of the usual 4 times.

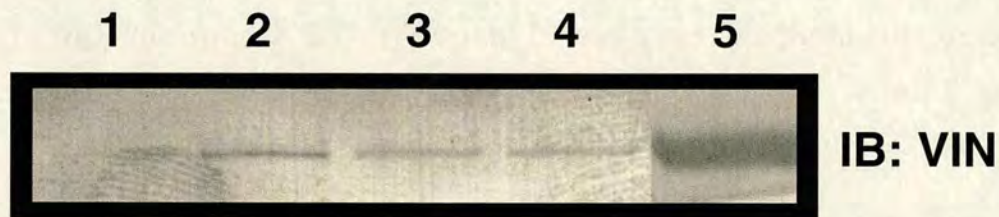
The non-specific interactions were not affected by any of these conditions except the blocking with skimmed milk powder which reduced the binding of paxillin significantly and the binding of Grb2 to a small extent. However, blocking with skimmed milk powder was not considered a viable option to reduce non-specific binding generally, because it did not affect all interactions equally.

In a final attempt to identify interactors with β -dystroglycan by this method, the extracts were precleared with blank beads prior to the experiment. The theory was that pre-clearing could remove any proteins that non-specifically bind the CNBr-Sepharose so that any further interactions with β -dystroglycan-coupled CNBr-Sepharose could be considered as genuine interactions. However the pre-clearing could also have a detrimental affect on the assay, i.e. by removing all the proteins that do in fact interact with β -dystroglycan. Figure 3.9 shows that there

were equal amounts of vinculin in the pre-cleared and non pre-cleared pulldowns. Blank beads were used for the clearing and the subsequent pulldown was carried out using blank beads (control) or β -dystroglycan-coupled beads. These results suggest that pre-clearing has no effect on the non-specific interactions, or if it does then there is still sufficient protein remaining to non-specifically bind the Sepharose in the pulldown step. Perhaps the short pre-clearing step (1 hour) does not allow non-specific binding to the same extent as the overnight pulldown step.

Figure 3.9

Pre-clearing extracts with blank CNBr Sepharose does not reduce non-specific binding.



Western blot of pulldowns from myoblast extracts.

Myoblast extracts 1 and 3 were pre-cleared with blank beads prior to the experiment. Samples 1 and 2 were incubated with blank CNBr Sepharose, 3 and 4 with β -dystroglycan cytoplasmic domain coupled to CNBr Sepharose. Any proteins pulled down with the beads were run on SDS-PAGE gels and western blotted (IB) with vinculin monoclonal antibody (VIN). Extract alone was loaded in Lane 5.

There is vinculin in the pre-cleared and non pre-cleared pulldowns suggesting that if pre-clearing does remove some of the protein, there is still sufficient remaining to non-specifically bind to the Sepharose. For example, in lane 2, the extract is pre-cleared with blank beads and subsequently incubated with blank beads and vinculin is still pulled down.

After detailed and thorough testing of this method, it was decided that it was not a reliable or successful procedure for analysing protein:protein interactions with β -dystroglycan. This was due to the high number of non-specific interactions caused by the CNBr-Sepharose.

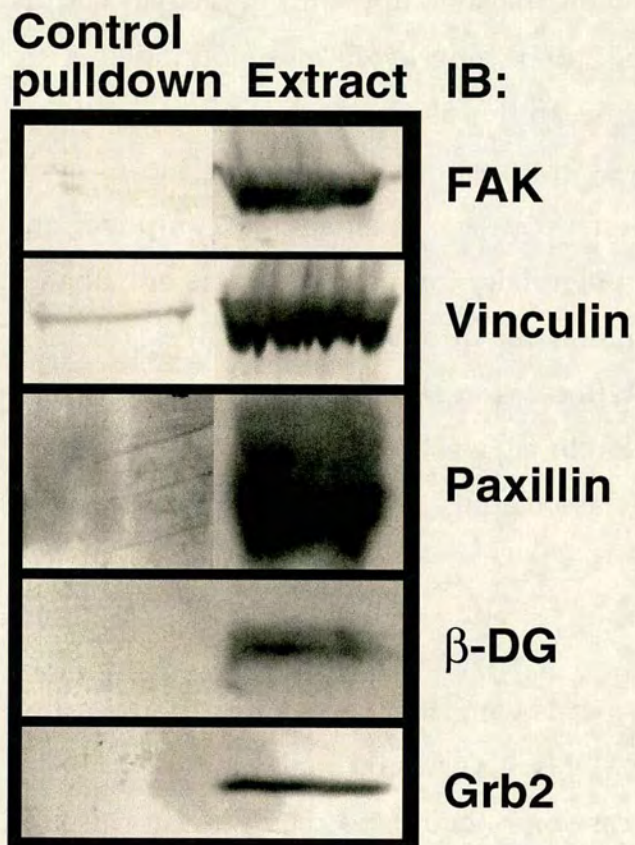
3.7. Pulldowns using biotin tag -fusion proteins and Streptavidin beads

Experiments using β -dystroglycan cytoplasmic domain coupled to CNBr-activated Sepharose had proved to be unreliable due to non-specific binding of proteins to the Sepharose. In this assay, the aim was to pull out any proteins associating with β -dystroglycan using a biotin tag (section 2.1.13.3). This method was chosen because it avoided the complications that the coupling procedure had potentially caused, and it was anticipated that using a tag to pull down interacting proteins would give a cleaner results than co-immunoprecipitation assays used previously. A fusion protein of β -dystroglycan with a biotin tag was made, added to cell extracts and pulled down (with any associating proteins) by Streptavidin immobilised onto Sepharose beads.

In this strategy, the Streptavidin beads were first checked for non-specific binding of proteins (figure 3.10). Only a very minimal amount of non-specific binding occurred in the case of vinculin and paxillin, but this was considered to be a low background, and not considerable enough to obscure the identification of any real interaction. Other proteins such as Grb2 and MAPK showed no non-specific interaction with the avidin beads, therefore the experiment was embarked upon.

Figure 3.10

Western blots of control pulldowns using Streptavidin-Sepharose beads.

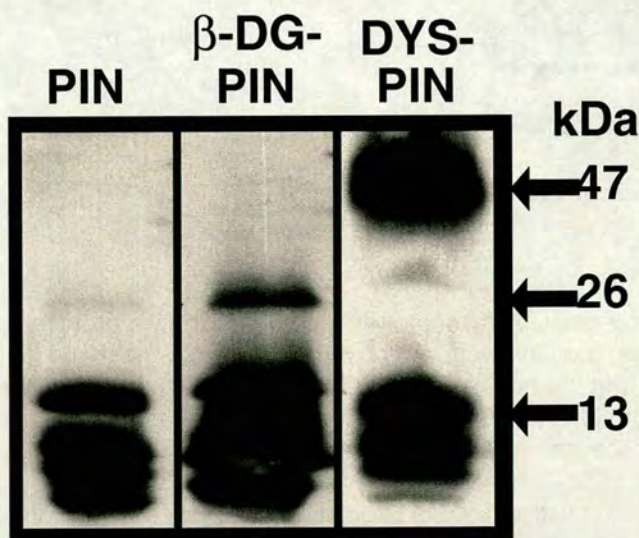


Before commencing experiments using Streptavidin immobilised on to Sepharose beads, the beads were incubated with myoblast extracts to check for non-specific protein interactions. The resultant pulldowns were run on SDS-PAGE gels (lefthand lane) with myoblast extract (righthand lane) and western blotted (IB) with FAK, vinculin, paxillin, β -dystroglycan (MANDAG2) or Grb2 antibodies (Table 2.6). A small amount of vinculin and paxillin was non-specifically pulled down with the beads but not a large enough quantity to disrupt the experiments. FAK, β -dystroglycan and Grb2 appeared not to non-specifically interact with the Sepharose beads.

A β -dystroglycan cytoplasmic domain construct (table 2.4) was cloned into the Pinpoint Xa-3 vector which facilitates the expression of biotin-tagged fusion proteins (table 2.3 & section 2.4.10.1). The fusion protein was successfully expressed in *E.coli*. (JM109 strain, table 2.1) and affinity purified from bacterial supernatants using Streptavidin beads (figure 3.11). The beads were thoroughly washed and subsequently added to myoblast or myotube extracts. It was predicted that any proteins interacting with β -dystroglycan would be pulled down with the fusion protein bound to the Streptavidin beads. However, none of the proteins tested were shown to interact with β -dystroglycan by this method (figure 3.12A). This still held true when the amount of fusion protein used was raised as high as 10 μ g per assay.

Figure 3.11

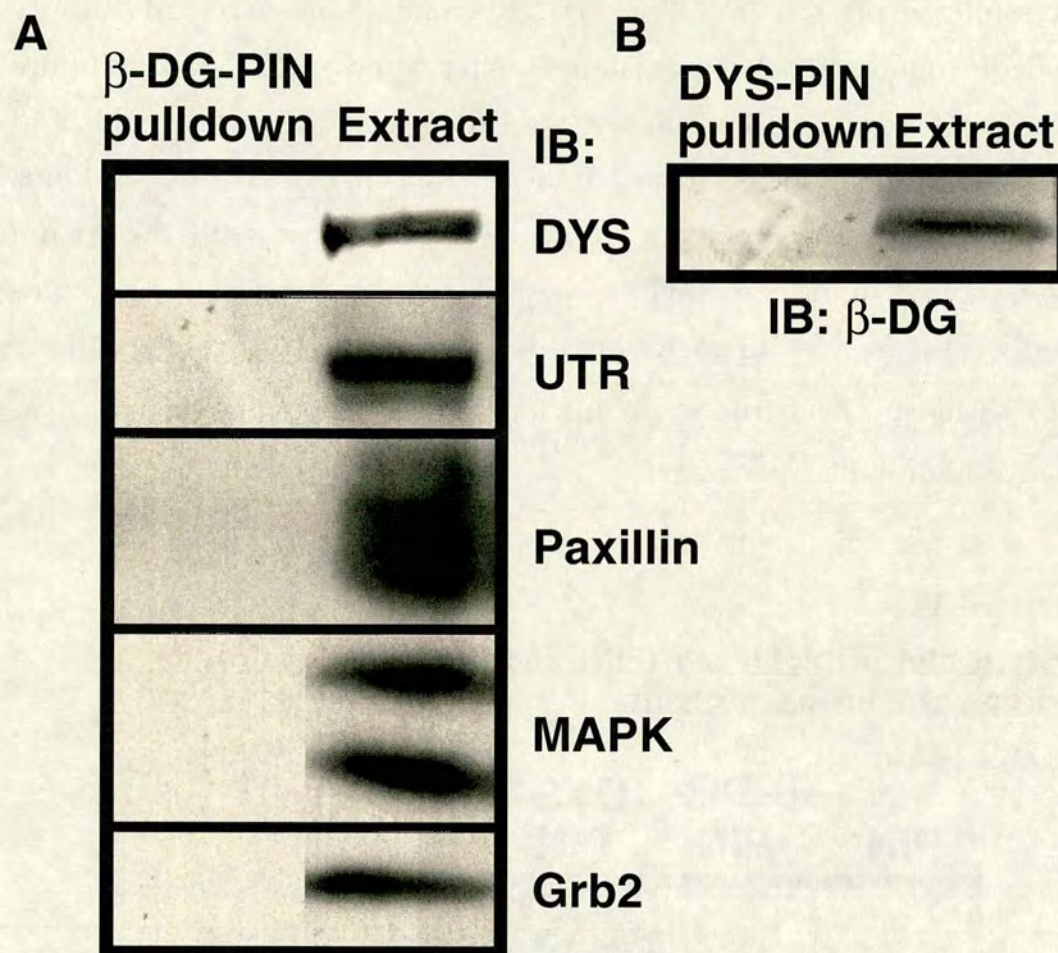
Western blot of biotin tag (PIN) and biotin tag-dystrophin/ β -dystroglycan fusion proteins



The fusion proteins were affinity purified from bacterial supernatants using streptavidin beads. The resultant proteins were run on SDS-PAGE gels, and detected with Extravidin-HRP. The lefthand panel shows the 13kDa biotin tag (PIN) and its breakdown products. The biotin tag alone can also be seen in the other two panels where the tag has separated from the fusion protein. The middle panel shows the 26kDa β -dystroglycan-PIN fusion protein. The righthand panel shows the 47kDa dystrophin-PIN fusion protein and its degradation products.

Figure 3.12

**Western blots of pulldowns using biotin tagged β -dystroglycan/
dystrophin fusion proteins**



A. β -DG-PIN was added to myoblast or myotube extracts to pulldown proteins interacting with dystrophin. Any interacting proteins were run on SDS-PAGE gels (lefthand lane) next to myoblast or myotube extract (righthand lane) and western blotted with antibodies against dystrophin (DYS1 & DYS2), utrophin (UTR) (RAB5), paxillin, MAP kinase (NEB9102), or Grb2 (Table 2.6). None of the interactions investigated were identified.

B. In an attempt to verify the dystrophin- β -dystroglycan interaction, DYS-PIN bound to streptavidin beads was added to myoblast or myotube extracts. The beads were subsequently washed and run on SDS-PAGE gels and blotted with a β -DG antibody (MANDAG2). The dystrophin- β -dystroglycan interaction could not be demonstrated by this method.

3.8. β -dystroglycan interaction with dystrophin

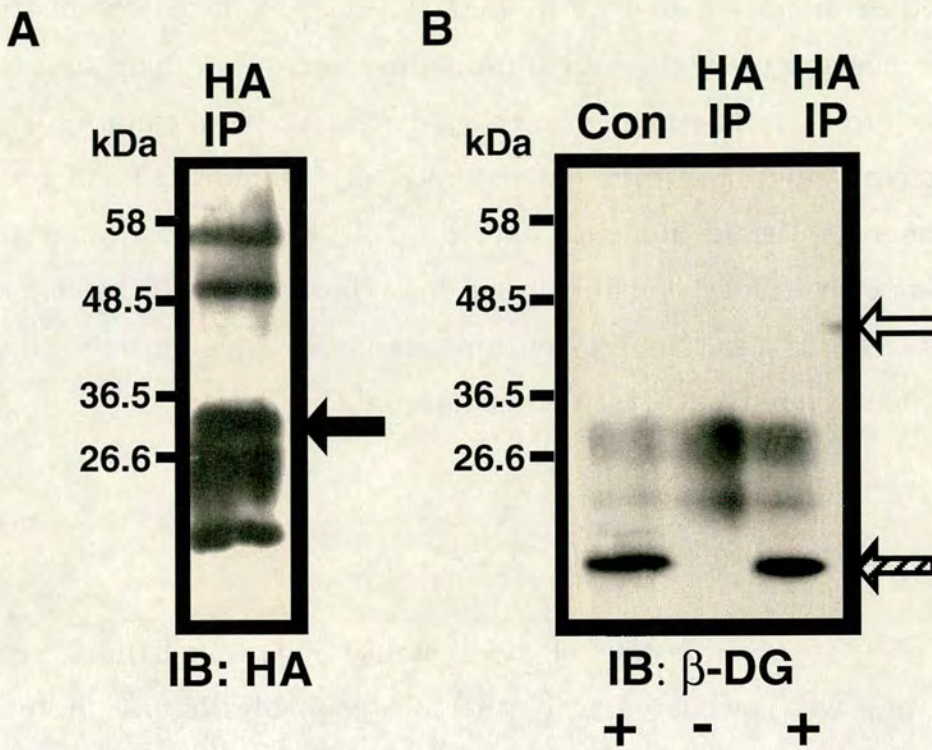
The β -dystroglycan/dystrophin interaction was thoroughly investigated in this study. It is the best characterised of interactions within the DAPC and in order to elucidate whether the the interaction is regulated by phosphorylation, it was essential to have a biochemical method showing the association of these two proteins, so that any changes in that association can be monitored during phosphorylation events.

An assay to show that phosphorylation of β -dystroglycan or dystrophin disrupts its interaction with dystrophin would involve demonstrating that the two proteins can no longer co-immunoprecipitate in peroxyvanadate treated extracts. However, no antibody has been found which co-immunoprecipitates these two proteins *per se*. Attempts with both anti-dystrophin antibodies and anti- β -dystroglycan antibodies were unsuccessful (section 3.4). Therefore, a construct of the WW, EF hands and ZZ domains of dystrophin (table 2.4) was made and cloned into the Pinpoint Xa-3 vector in the attempt to pull out β -dystroglycan from cell extracts using a biotin-tagged dystrophin fusion protein and Streptavidin beads (as above). β -dystroglycan showed no non-specific interaction with the Streptavidin beads, but neither was the dystrophin fusion protein able to pull out β -dystroglycan from the cell extracts (figure 3.12B).

The subsequent approach to show an association between dystrophin and β -dystroglycan involved expressing a tagged dystrophin protein in C2/C4 cells. This would enable the immunoprecipitation of the tagged protein using an antibody directed against the tag, and hopefully, the co-immunoprecipitation of β -dystroglycan. Once dystrophin and β -dystroglycan were successfully co-immunoprecipitated from cell extracts then this interaction could be investigated in peroxyvanadate treated cell

extracts. Myoblasts rather than myotubes were transfected because the static nature of myotubes may have deleteriously affected the transfection process. Additionally, there is no native dystrophin expressed in myoblasts, so any dystrophin expressed in the cell would be purely the tagged form. Initially a construct encompassing the WW, EF hands and ZZ domains of dystrophin was cloned into the pCEP4F vector which has CMV and SV40 promoters which express a FLAG tag fused to the cloned protein. pCEP4F-DYS was transfected into myoblasts using Lipofectamine reagent (section 2.3.8). As discussed further in section 4.4.5, the anti-FLAG antibody available (M2) was not reliable. The western blot of untransfected myoblast extracts and pCEP4-DYS transfected myoblast extracts showed exactly the same banding pattern. The anti-FLAG antibody did not appear to adequately immunoprecipitate FLAG-tagged proteins either, which prevented successful co-immunoprecipitation experiments using the FLAG tag.

As an alternative, the dystrophin construct was cloned into a pCMV-HA vector and transfected into myoblasts. HA-tagged dystrophin protein was successfully expressed, and the anti-HA antibody successfully immunoprecipitated the HA-tagged dystrophin protein (figure 3.13A). This immunoprecipitate was western blotted with a β -dystroglycan antibody, but β -dystroglycan did not co-immunoprecipitate with the HA-tagged dystrophin protein (figure 3.13B). Purified β -dystroglycan cytoplasmic domain was also added to the immunoprecipitation experiments to see if it associated with HA-tagged dystrophin. It did appear to do so, however, it was concluded that the purified β -dystroglycan was interacting non-specifically with the protein G Sepharose because it was also in the control immunoprecipitation (figure 3.13B).

Figure 3.13**HA-tagged dystrophin and β -dystroglycan do not co-immunoprecipitate****A. Western blot of HA-DYS immunoprecipitated by anti-HA antibody.**

pCMV-HA-DYS transfected myoblast extracts were immunoprecipitated with anti-HA antibody (HA IP) and western blotted (IB) with anti-HA antibody. The 34kDa HA-DYS protein was successfully immunoprecipitated from the transfected extracts (denoted by filled arrow).

B. β -dystroglycan does not co-immunoprecipitate with HA-DYS

HA-DYS was immunoprecipitated (IP) from pCMV-HA-DYS transfected myoblasts with anti-HA antibody, and western blotted with β -dystroglycan monoclonal antibody MANDAG2 (Table 2.6). Purified β -dystroglycan cytoplasmic domain was added (+) or omitted (-) from the immunoprecipitation experiment. In the control (Con), untransfected myoblasts were used. Native β -dystroglycan (molecular weight denoted by unfilled arrow) was not co-immunoprecipitated with HA-DYS. Purified β -dystroglycan (denoted by shaded arrow) appeared to immunoprecipitate with HA-DYS but was also immunoprecipitated in the control which suggests that it interacts non-specifically with the protein G Sepharose.

3.9. Discussion

The aim of the studies documented in this chapter was to identify proteins which interact with β -dystroglycan using protein biochemistry techniques. Several methods were thoroughly tested for their use in identifying proteins associating with β -dystroglycan, including co-immunoprecipitation, and different methods of "pull-downs" using β -dystroglycan cytoplasmic domain coupled to CNBr-Sepharose, and β -dystroglycan cytoplasmic domain fused to a biotin tag. Transfection experiments were also undertaken in order to demonstrate the interaction between β -dystroglycan and dystrophin.

3.9.1. Characterisation of C2 cell lines

Following the thorough testing of three mouse muscle cell lines, the C2/C4 cell line was considered to be the most suitable for use in this study. C2/C4 has efficient and consistent myoblast fusion, a higher expression of dystrophin protein than the other cell lines and displays the predicted expression pattern of DAPC proteins as *in vivo* (figure 3.2).

The predicted expression pattern of the ubiquitous proteins utrophin and β -dystroglycan is that they are expressed equally throughout all stages of differentiation. *In vivo*, it has been shown that dystrophin protein in foetal muscle samples (at 9 weeks of gestation), is barely detectable with antibodies on western blots and only distinct populations of larger myotubes are recognised by dystrophin immunostaining. However, this staining pattern increases and becomes more uniform with age and reaches the adult pattern of expression at 26 weeks of gestation (Clerk *et al.*, 1992). In cultured cells it has been previously shown that dystrophin mRNA is expressed only in fused myotubes and not myoblasts (Oronzi-

Scott *et al.*, 1988). In this study, it was found that dystrophin protein was not expressed in myoblasts but only in advanced stages of differentiation, which is in agreement with the described expression of dystrophin mRNA.

In addition to having high levels of myoblast fusion and the anticipated expression patterns of DAPC proteins, the C2/C4 cell line had consistent properties from batch to batch. For these reasons, it was the cell line of choice for use in this study.

3.9.2 . Co-immunoprecipitation experiments

Co-immunoprecipitation is a well established method for testing protein:protein interactions. In this study co-immunoprecipitation experiments were used to elucidate whether RIPA buffer permitted the extraction of the DAPC as a whole entity, and to gain understanding of protein:protein interactions within the DAPC, and attempt to identify proteins associating with β -dystroglycan outwith of the DAPC.

The co-immunoprecipitation of dystrophin and δ -sarcoglycan, but not β -dystroglycan, using dystrophin antibodies (VIA4-2A3 and XIXC2-D11) and the α -sarcoglycan antibody IVD3-1 (figure 3.3) suggests that dystrophin and the sarcoglycan complex are more tightly linked than β -dystroglycan with dystrophin or β -dystroglycan with the sarcoglycan complex. This is unexpected because β -dystroglycan and dystrophin are directly linked (Jung *et al.*, 1995), and it has been demonstrated that they have a tight association (Finn & Ohlendieck, 1997). Moreover, the sarcoglycan complex has not, to date, been shown to be directly linked to dystrophin.

It has been demonstrated that δ -sarcoglycan can be chemically crosslinked to the dystroglycan complex suggesting that they lie in close proximity (Chan *et al.*, 1998). In addition, studies on the β -sarcoglycan deficient mice have led to the hypothesis that the sarcoglycan complex stabilises the link between dystrophin and the dystroglycan complex (Araishi *et al.*, 1999). From these findings one would expect that there is a higher likelihood of the sarcoglycan complex co-immunoprecipitating with β -dystroglycan than dystrophin. However, although δ -sarcoglycan can be crosslinked to the dystroglycan complex, it has been demonstrated that α -sarcoglycan is less tightly associated with the rest of the sarcoglycan complex (Chan *et al.*, 1998). This may explain why the α -sarcoglycan antibody cannot co-immunoprecipitate β -dystroglycan but it does not explain why δ -sarcoglycan can be co-immunoprecipitated with dystrophin but β -dystroglycan cannot. The association between α -sarcoglycan and the dystroglycan complex may be weak and the α -sarcoglycan antibody possibly co-immunoprecipitates with dystrophin via dystrobrevin. Dystrobrevin has recently been shown to interact directly with the sarcoglycan-sarcospan complex (Yoshida *et al.*, 2000), and interacts with dystrophin, as demonstrated by co-immunoprecipitation data (Sadoulet-Puccio *et al.*, 1997). Therefore, dystrobrevin could act as a link between the sarcoglycans and dystrophin.

The co-immunoprecipitation assays were not effective in demonstrating other known protein:protein interactions with β -dystroglycan (i.e. β -dystroglycan with dystrophin and Grb2). One reason for this may be because that the best β -dystroglycan antibodies available are directed against the most C-terminal 15 amino-acids of β -dystroglycan cytoplasmic domain. This region contains the dystrophin/ β -dystroglycan, Grb2/ β -dystroglycan and caveolin-3/ β -dystroglycan interaction sites and is possibly where other proteins associate with β -dystroglycan as well. This means that the antibody may be competing off proteins which interact

with β -dystroglycan *in vivo*, or *vice versa* i.e. the β -dystroglycan:protein interaction is so strong that the antibody can not access the epitope.

In addition, the Grb2 binding site on β -dystroglycan overlaps with the dystrophin/ β -dystroglycan binding site. The two proteins have been shown to be in direct competition with one another *in vitro* for β -dystroglycan binding (Russo *et al.*, 2000). In addition, dystrophin competes with caveolin-3 for the WW-domain binding motif (PPxY) in β -dystroglycan (Sotgia *et al.*, 2000). The competitiveness of these interactions may explain why they could not be demonstrated by co-immunoprecipitation experiments. The dynamic nature of the interactions in the complex may make them difficult to capture by the co-immunoprecipitation process.

It was also unexpected that the dystrophin/ β -dystroglycan interaction could not be detected in C2/C4 cells transfected with a HA-tagged dystrophin construct (HA-DYS) (figure 3.13B). It is unlikely that the HA tag obscured any binding sites of the fusion protein because it is designed to be as small as possible to avoid any obstructions or interference with protein folding. The most probable reason for HA-DYS not associating with β -dystroglycan is that the HA-DYS protein was not folded properly in the cell. Like any protein:protein interaction, the dystrophin/ β -dystroglycan interaction is highly dependent on the structure of dystrophin because the interaction requires stabilisation by the orientation of EF hand regions as well as the WW domain-mediated interaction (Huang *et al.*, 2000). The HA-DYS fusion protein could also have been mis-localised so that was not in the correct compartment of the cell in order to interact with β -dystroglycan.

3.9.3. β -dystroglycan interacts with three phosphoproteins

However, during co-immunoprecipitation assays using β -dystroglycan antibodies, three phosphoproteins associating with β -dystroglycan were identified (figure 3.4). This suggests that if these proteins are directly interacting with β -dystroglycan, they are doing so at regions other than the C-terminal 15 amino-acids of β -dystroglycan (see above).

When the β -dystroglycan immunoprecipitates were analysed by silver staining, a complex mixture of proteins were observed (figure 3.5). Many of these proteins were thought to be contaminants from the anti-sera and proteins non-specifically interacting with the Sepharose. But it was also clear that more than just three proteins were specifically associated with β -dystroglycan. In addition, in the 116-130kDa range on the silver stained gel, no proteins appeared to associate with β -dystroglycan, contrary to the results obtained whilst western blotting β -dystroglycan immunoprecipitates with anti-phosphotyrosine antibodies. This highlights the sensitivity of the anti-phosphotyrosine antibodies compared to silver staining. The complex mixture of proteins viewed in the silver stained gels ruled out the prospect of using mass spectrometry or protein sequencing to identify the phosphoproteins associating with β -dystroglycan.

Focal adhesion complexes are specialised sites of cell adhesion which occur in many cell types in culture. ECM-integrin interactions occur at various cell-substrate linkages, for example, at neuromuscular junctions (NMJ), costameres and focal adhesion sites. Integrins are a large family of heterodimeric transmembrane proteins which, among other functions, mediate attachment to the ECM and are therefore involved in the regulation of many cellular functions. Ligand occupancy and clustering of integrins leads to the recruitment of signalling and actin-binding proteins

at focal adhesion sites, which in turn activate a variety of intracellular signalling events. Focal adhesions provide a model for studying the structural links and signalling between the ECM and the cytoskeleton, and the modulation of cell adhesion.

It was hypothesised that the three phosphoproteins found associating with β -dystroglycan were signalling or focal adhesion proteins. The DAPC has been shown previously to have links to focal adhesion proteins. Immunofluorescence studies have shown that dystrophin and utrophin co-localise with focal adhesion structures in tissue culture cells (James *et al.*, 1996; Belkin & Burridge, 1995; Belkin & Smallheiser, 1996). Yoshida *et al.* (1998) demonstrated that a dystrophin antibody co-immunoprecipitated focal adhesion proteins (FAK, vinculin, and talin) and integrin subunits, and an α -sarcoglycan antibody co-immunoprecipitated FAK (Yoshida *et al.*, 1998). β -dystroglycan itself has been associated with focal adhesions via the Grb2: FAK interaction in brain synaptosomes (Cavaldesi *et al.*, 1999), but a direct interaction has not been shown. Due to these previous studies, focal adhesion proteins were thought to be strong candidates for proteins which interact with β -dystroglycan.

MAP kinase was another candidate protein for interacting with β -dystroglycan in this study. There is evidence for MAP kinase (ERK) involvement in adhesion-mediated signalling and it has been demonstrated that active MAP kinase is targeted to newly forming focal adhesion complexes (Fincham *et al.*, 2000). In addition, Grb2 (which associates with β -dystroglycan) is involved in the Ras signalling pathway which leads to MAP kinase activation. β -dystroglycan has also been observed associating with MAP kinase in non-muscle cells (M. James & S. J. Winder, unpublished observation).

In order to identify whether focal adhesion and signalling proteins were in fact associating with β -dystroglycan, a range of antibodies directed against candidate proteins (table 2.6) were used to probe β -dystroglycan immunoprecipitates (figure 3.6). However, the findings proved inconclusive. The results often were difficult to analyse due to background. Therefore “pull-down” experiments were attempted.

3.9.4. Pull-down experiments

The pull-down experiments devised for this study using β -dystroglycan coupled to CNBr-Sepharose were not useful in identifying any proteins associating with β -dystroglycan. The Sepharose proved to be very “sticky” i.e. many proteins non-specifically and preferentially associated with the Sepharose rather than to β -dystroglycan. There appeared to be an equal amount of paxillin and MAPK pulled down in the controls compared to the β -dystroglycan-CNBr Sepharose pull-downs (figure 3.8). In the case of some proteins, there appeared to be even more protein in the control than in the experiment e.g. dystrobrevin, SHC. Overall, vinculin and SHC proved to be the “stickiest” of all the proteins analysed during the course of this study i.e. were pulled down in the largest quantities by the Sepharose beads.

Extensive attempts to minimise the non-specific binding to the CNBr-Sepharose were carried out. The use of ethanolamine rather than a Tris buffer to deactivate any remaining active groups after the coupling procedure did reduce the level of non-specific binding to some extent, but still the quantities were equal to the amount of protein pulled down by the CNBr-Sepharose coupled to β -dystroglycan. Other attempts to reduce non-specific binding included blocking the Sepharose beads with skimmed milk powder or BSA, pre-clearing the extracts before use, reducing the time that the extracts were incubated with CNBr-Sepharose

from overnight to 4 hours, or using more stringent washing conditions on the beads before analysis by SDS-PAGE and western blotting. However, no procedure succeeded in reducing non-specific binding to a level which made the assay workable. Whilst the controls were pulling down the same quantity of protein as the experiment, there was no way of detecting any real interactions by this method.

Interestingly, the Streptavidin-Sepharose used in pulldown experiments did not non-specifically interact with any proteins tested (figure 3.10). This suggests that the non-specific binding to the CNBr-Sepharose was due to its active groups, rather than the Sepharose itself -perhaps they were not fully deactivated despite the deactivation steps used in the protocol. It is also plausible that the purified β -dystroglycan cytoplasmic domain (which was coupled to the CNBr-Sepharose) interacts non-specifically with the Sepharose, but the tagged fusion protein (PIN- β -DG) does not. β -dystroglycan cytoplasmic domain has been observed in Sepharose-only controls, interacting non-specifically with protein A/G Sepharose (figures 3.13B & 4.5).

The assay using Streptavidin-Sepharose provided the desired lack of background, but still, protein interactions with β -dystroglycan were not observed, possibly because the conditions were not ideal and as the *in vivo* situation. Despite careful optimisation of extraction conditions of myoblasts and myotubes, the extraction conditions may not have been optimal to maintain protein:protein interactions. β -dystroglycan is a transmembrane protein, therefore, very gentle conditions will not solubilise it, but very stringent conditions will potentially disrupt protein:protein interactions. Therefore, a fine balance is required to maintain the *in vivo* situation as much as is attainable. In addition, some interactions are transient, or competitive e.g. Grb2 and dystrophin

competing for association with β -dystroglycan. This phenomenon demonstrates the dynamic nature of the DAPC and therefore its fragility when it comes to analysing the interactions biochemically.

β -dystroglycan associates with dystrophin in DAPC, but there is also evidence to suggest that there is another pool of β -dystroglycan which is not associated with the DAPC (Finn & Ohlendieck, 1998). During the extraction of proteins from myoblasts/myotubes using RIPA buffer, it appears that after sonication and centrifugation of the extracts, there are equal amounts of β -dystroglycan in the supernatant and the pellet (data not shown). It is possible that the β -dystroglycan in the soluble fraction is mainly β -dystroglycan which is not part of the DAPC. If this is the case, it is not surprising that β -dystroglycan antibodies can immunoprecipitate β -dystroglycan but do not co-immunoprecipitate any known associating proteins. This could also explain why the pulldowns show no proteins associating with β -dystroglycan because it would mean that the β -dystroglycan added to the extracts (in the form of the purified β -dystroglycan or fusion protein) is not competing with the β -dystroglycan pool which is part of the DAPC or other complexes.

Despite the limited success of the methods used, it has still been demonstrated here that β -dystroglycan associates with a number of phosphoproteins. It is possible that the phosphoproteins indirectly associate with β -dystroglycan via other interactors of β -dystroglycan. For example, the phosphoproteins observed may be focal adhesion proteins indirectly associating with β -dystroglycan. In this case, the “linker” protein connecting β -dystroglycan to the focal adhesions so far remains elusive. Subsequently, β -dystroglycan was used as bait in yeast two-hybrid screen in order to identify more of β -dystroglycan’s interactors (see chapter 4).

CHAPTER 4
Yeast Two-Hybrid Screen

A yeast two-hybrid screen using β -dystroglycan cytoplasmic domain as a bait to screen a skeletal muscle cDNA library.

4. 1. Introduction

The yeast two-hybrid assay is a highly sensitive technique that can be used to identify protein:protein interactions which are sometimes not revealed by other less sensitive methods (for reviews see Fritz & Green, 1992; Fields & Sternglanz, 1994; Brent & Finley, 1997). Additionally, because the assay is *in vivo*, proteins are present in the natural environment of the cell nucleus and not subjected to harsh chemical conditions. The aim of this screen was to identify interactors of β -dystroglycan which had so far proved difficult by biochemical methods, in particular unidentified phosphoproteins which had been shown to associate with β -dystroglycan in co-immunoprecipitation experiments (figure 3.4). These proteins also appear to associate with β -dystroglycan in pharmacologically untreated cells (see silver staining, figure 3.5) suggesting that the interactions are not necessarily phosphorylation-dependent, and therefore the yeast two-hybrid assay was considered to be a suitable method to potentially identify them.

The yeast two-hybrid assay was originally based on the observation that many eukaryotic transcriptional activators are composed of two distinct domains (DNA binding domain and transcriptional activating domain) which can be physically separated and can only function as an activator when brought together (Fields & Song, 1989; Chien *et al.*, 1991).

The yeast two-hybrid system utilises two plasmid-borne gene fusions that are co-transformed into a host yeast strain containing inducible reporter genes. The protein of interest (or "bait") is encoded as a gene fusion to DNA binding domain from either the GAL4 or LEXA protein. A second

protein, or library of proteins, is encoded as a gene fusion to a transcription activation domain. The interaction between the bait protein and a library protein results in the localisation of the transcription activation domain to the DNA of the host strain, activating transcription of the reporter genes and generating a phenotypic signal.

Uses of the yeast two-hybrid assay include identifying factors which interact with a protein of interest by screening a pool of clones (method used in this study), providing molecular evidence for a suspected interaction, and defining domains or individual amino-acids which are critical for a particular interaction.

The expectation was, that using the yeast two-hybrid assay in this study would substantiate known interactions with β -dystroglycan and additionally, identify new interacting proteins.

4.2. Setting up the yeast two-hybrid screen

4.2.1. The bait and the cDNA library.

β -dystroglycan cytoplasmic domain (amino-acids 781-895) was cloned 5' of the GAL4 DNA binding domain in the yeast two-hybrid bait vector pAS2 (section 2.5.4). The 5' vector-insert junction of β -DG/pAS2 was verified by DNA sequencing, confirming that β -dystroglycan was in frame. The β -dystroglycan monoclonal antibody MANDAG2 (table 2.6) was used to verify the expression of the GAL4- β -dystroglycan fusion protein by western blotting of proteins isolated from the host yeast strains transformed with β -DG/pAS2.

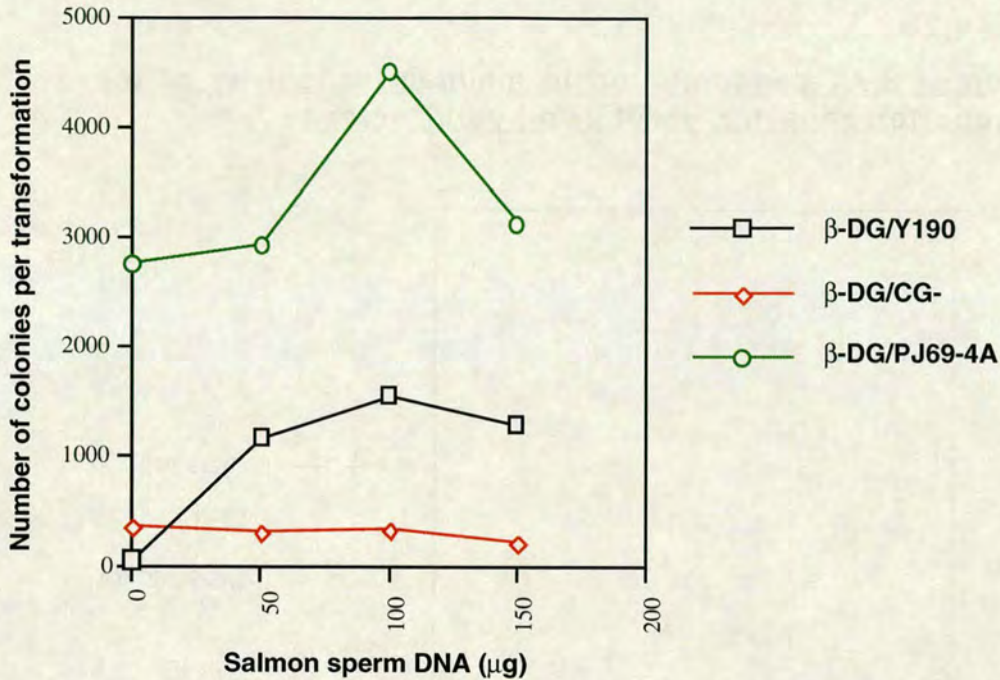
One factor which is critical for the success of a yeast two-hybrid screen is the quality of the library used. The MATCHMAKER human skeletal muscle cDNA library (section 2.5.3) was used for this yeast two-hybrid screen. This library has 3.5×10^6 independent clones, cloned into the pACT2 vector. 90% of these clones have an insert and the average insert size is 2kb. The titre of the library was calculated and the library re-amplified accordingly for this screen.

4.2.2. The *Saccharomyces cerevisiae* host strains

A good two-hybrid host strain should be extremely sensitive to small changes in reporter activity, therefore allowing detection of weak or transient interactions, but also providing a very low incidence of false positives. For this screen, β -DG/pAS2 was transformed into three host strains namely CG-1945, PJ49-6A, and Y190 (table 2.10) to compare their transformation efficiencies, and the relative sensitivities of their *HIS3* reporters during expression of the GAL4- β -dystroglycan fusion protein.

Some polypeptides are capable of activating the transcription of reporter genes when fused to a DNA-binding domain, even in the absence of an activation domain. The sensitivity (or "leakiness") of the *HIS3* reporter can be adjusted by adding a competitive inhibitor of the *HIS3* gene product, namely 3-aminotriazole (3-AT). For baits that activate *HIS3*, 3-AT is added at a level which makes the strain auxotrophic for histidine so that the library can be screened to detect interacting proteins that result in *further* activation of *HIS3*. However, if high concentrations of 3-AT are required, then the sensitivity of the system can be compromised.

The β -DG/pAS2 bait vector was transformed into the three yeast host strains to compare their suitability for this screen. Transformants were isolated using the tryptophan marker on pAS2 and grown up in media devoid of tryptophan (-W) ready for transformation with the skeletal muscle cDNA library. The leucine marker on the pACT2 plasmids allows the selection of library plasmids. The strains containing both bait and library plasmids were isolated on media lacking tryptophan and leucine (-LW) and the transformation efficiencies of the three strains were compared. The amount of salmon sperm DNA used as a carrier in the transformation was also varied because this can have an effect on the transformation efficiency. When the β -dystroglycan containing strains were transformed with 0.6 μ g library DNA, the β -DG/PJ69-4A strain clearly yielded the highest transformation efficiency (figure 4.1). β -DG/Y190 transformed reasonably well, but the transformation efficiency of β -DG/CG-1945 was very low indeed. In both PJ69-4A and Y190 it can be seen that using 100 μ g salmon sperm DNA as a carrier gave the optimum transformation efficiency.

Figure 4.1.**Comparison of three yeast two-hybrid host strains: transformation efficiencies and effect of different salmon sperm concentrations**

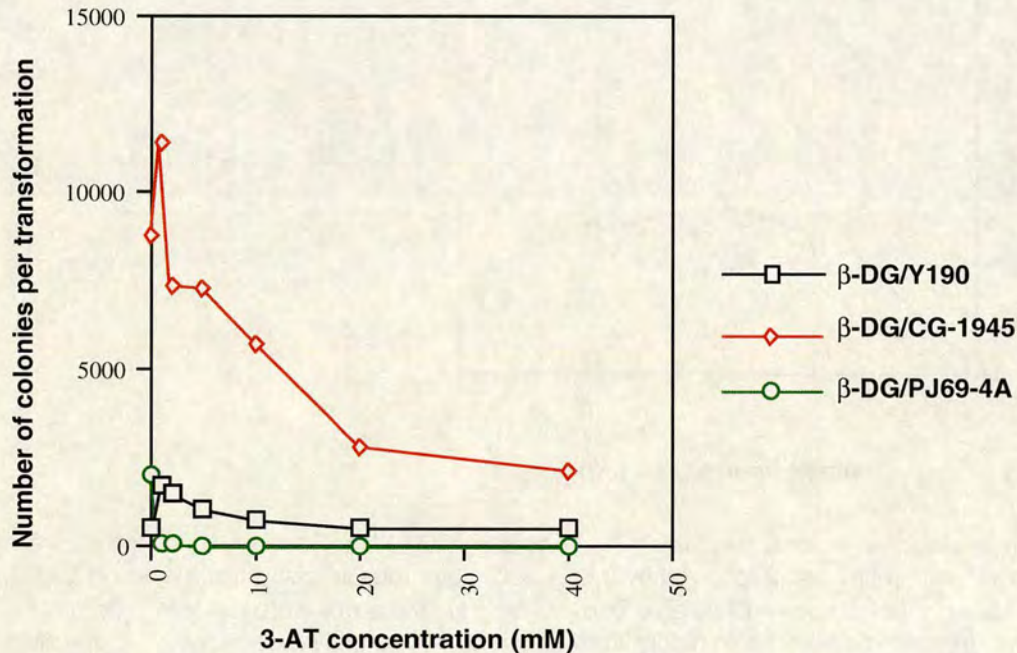
The three *Saccharomyces cerevisiae* host strains CG1945, PJ69-4A and Y190 were transformed with β -DG/pAS2 followed by 0.6 μ g of skeletal muscle cDNA library, using 0, 50, 100 or 150 μ g salmon sperm DNA as a carrier. The transformants were selected by plating onto -LW dropout media. The transformation efficiencies are shown as number of colonies per transformation (y-axis) against amount of carrier DNA used (x-axis). CG1945 had the poorest transformation efficiency, which was not affected by altering the amount of carrier DNA. PJ69-4A had by far the highest transformation efficiency. 100 μ g of salmon sperm DNA gave the optimum transformation efficiency in both PJ69-4A and Y190 strains.

After transforming the library into the three strains, the amount of 3-AT required to increase the stringency of the *HIS3* reporter was titrated (section 2.5.6.1). This was carried out by transforming library DNA into the β -dystroglycan containing strains and plating the transformants onto media containing different mM concentrations of 3-AT, but lacking tryptophan, leucine and histidine (-LWH). Figure 4.2 shows that β -DG/Y190 required an exceptionally high concentration of 3-AT (more than 40mM) to eliminate false positives. For β -DG/CG-1945, the growth of His⁺ colonies were significantly reduced by 3-AT concentrations over

10mM. β -DG/PJ69-4A required less than 1mM 3-AT to eliminate the growth of His⁺ colonies.

Figure 4.2

Titration of 3-AT concentration to maintain sensitivity of the HIS3 reporter gene for yeast two-hybrid screen



1.5 μ g library DNA was transformed into CG1945, PJ69-4A and Y190 strains containing β -DG/pAS2 and the transformants plated onto tryptophan, leucine and histidine dropout plates (-LWH) containing different mM concentrations of 3-AT. mM concentrations of 3-AT are shown on the X-axis and number of colonies per transformation on the y-axis. The titration of 3-AT was carried out to test whether β -DG/pAS2 activates the HIS3 reporter gene without the presence of an activation domain, and what concentration of 3-AT is required to inhibit this so that the strain remains auxotrophic for histidine.

The growth of His⁺ β -DG/Y190 colonies was very high and over 40mM 3-AT would be required to reduce this background. Growth of His⁺ β -DG/CG1945 colonies was reduced at 3-AT concentrations over 10mM. Growth of His⁺ β -DG/PJ69-4A colonies was totally abolished by less than 1mM 3-AT.

After the comparison of the three yeast strains, PJ69-4A was chosen for use in this yeast two-hybrid screen. This was because it facilitated the highest transformation efficiency when transforming library DNA into the strain containing β -DG/pAS2, and required the least amount of 3-AT to counteract leakiness of the *HIS* reporter gene. In fact, such a small

amount of 3-AT was required that it was excluded from the screen entirely.

4.3. Results of the yeast two-hybrid screen using PJ69-4A

The screen was carried out in three stages, each stage consisted of 20 individual transformations. A schematic of the screen is shown in figure 4.3. In total, approximately 900,000 transformants were screened (the transformation efficiency was calculated as in section 2.5.6.2). In stage 1, the transformations were plated onto -LWH dropout media and any His⁺ colonies were re-streaked onto -LWA dropout media. In stages 2 and 3, the transformations were plated onto -LWA dropout media and any Ade⁺ colonies were re-streaked onto -LWH dropout media. Any His⁺, Ade⁺ candidates were considered to represent real two-hybrid interactions. The prey plasmids responsible for the observed two-hybrid interactions were plasmid-rescued from these cells (section 2.5.7.1).

4.3.1. Clones isolated from the yeast two hybrid screen

Out of the approximately 900,000 transformants screened by the yeast two-hybrid assay, 150 His⁺ or Ade⁺ colonies were picked and restreaked onto media lacking Adenine or Histidine respectively. 78 of these colonies were His⁺ *and* Ade⁺ and the plasmids from these colonies were rescued and *Bgl*III digested. Out of these, 95% contained inserts. These 74 plasmids were subsequently sequenced and the sequences compared to sequence databases using BLAST (section 2.5.7.2). A summary of the sequencing results is shown in table 4.1.

Figure 4.3

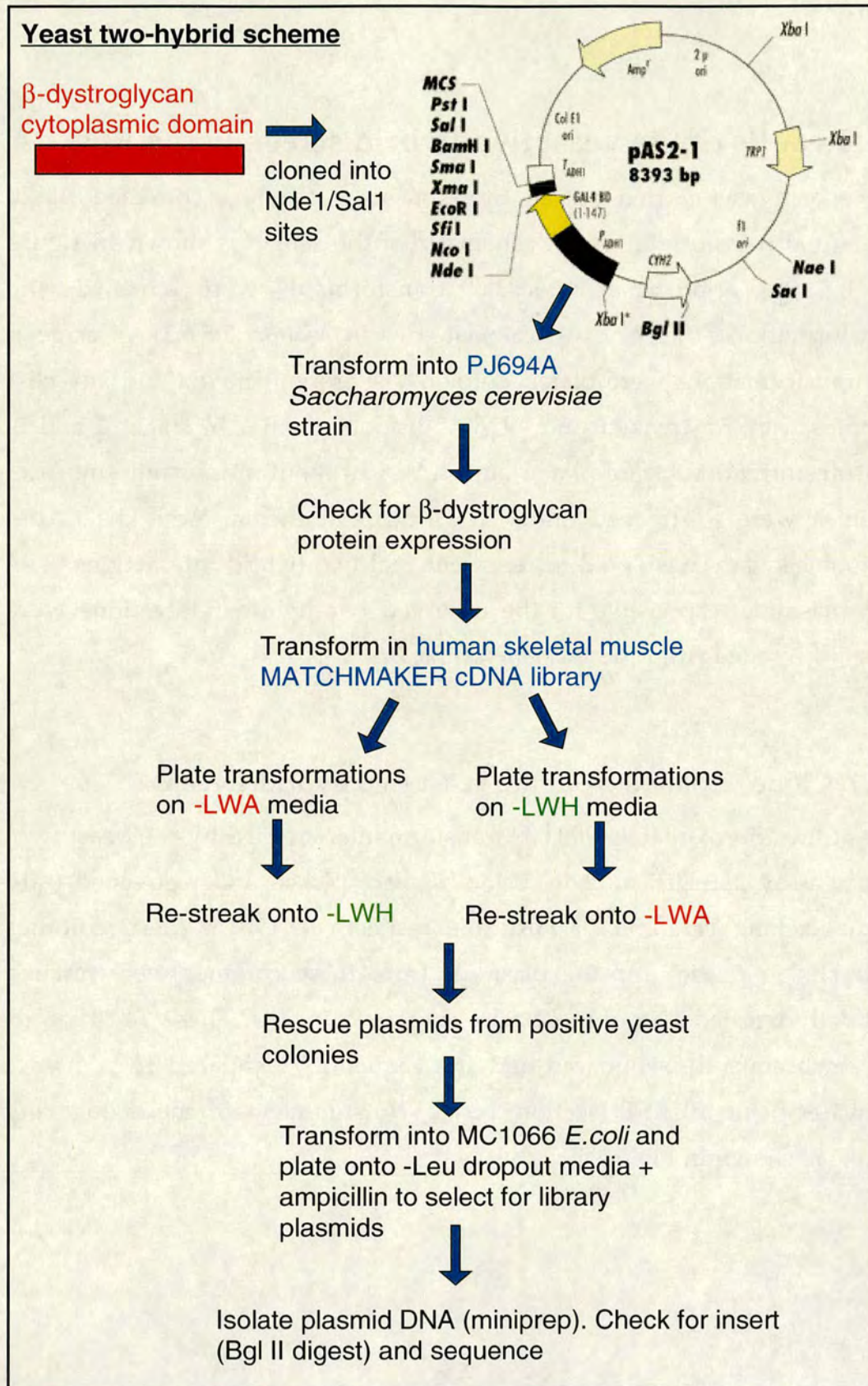


Table 4.1**Summary of library clones sequenced in the yeast two-hybrid screen**

Positive Clones	Frequency in screen
Nedd4-like ubiquitin-protein ligase (WWP1)	16
RAD6, huBC9,UBE21, E21 (ubiquitin-conjugating enzymes)	10
Rev7p/ MAD2-like 2/ MAD2B	7
Novel centrosomal protein RanBPM	6
Yes-associated protein 65kDa (YAP65)	4
Jun activation domain binding protein (JAB1)	3
Cytochrome C oxidase subunit II (mitochondrial)	3
Actin α1	2
Collagen type VI, α 3 (COL6A3)	2
FH1/FH2 domain containing protein (FHOS)	2
p51 delta / p53 homology	2
Acetyl-coenzyme A acetyltransferase 1	1
Adaptor-related protein complex mu1 subunit (CLAPM1)/ clathrin assembly protein 50 (AP50)	1
ATPase (Ca ²⁺ transporting)	1
BCL-2-associated athanogene 3 (BAG3)/ BAG family chaperone regulator 3/BCL-2 binding protein	1
Chromosome 4 clone C0483I23	1
Chromosome 16 BAC clone CIT987SK-A-962B4	1
Creatine kinase	1
DKFZp586K1922	1
Enoyl coenzyme A hydratase (mitochondrial)	1
Eukaryotic translation initiation factor 3, subunit 6/ subunit p48 (Int-6)	1
HIV type 1 enhancer binding protein 2 (HIVEP2)	1
HSPC147/ pt-wd mRNA for WD-40 repeat protein/ unr-interacting protein (UNRIP)/ <i>Mus musculus</i> serine-threonine kinase receptor-associated protein	1
Kallman syndrome 1 (KAL1) /ADMLX=putative adhesion molecule	1
Mitogen-activated protein kinase 7 (MAPK7)	1
Myoglobin	1
Skeletal muscle troponin T	1
Sterol regulatory binding transcription factor 1 (SREBF1)	1

As controls, the rescued library plasmids were transformed back into the β -DG/PJ69-4A strain to check whether the reporter genes were still activated by an interaction between the two plasmids (section 2.5.7.3). The library plasmids were also transformed into PJ69-4A with no β -DG/pAS2 present to confirm that the library plasmid alone did not activate the reporter genes.

4.3.2. Selection of interactors of β -dystroglycan to study in more depth

Sequencing of the 74 plasmids isolated from the yeast two-hybrid screen produced intriguing findings (table 4.1). Interestingly, the results did not include any known or previously described putative interactors of β -dystroglycan (sections 1.6.1, 1.6.4).

Despite the highly significant frequency of isolation, the top five proteins with the highest number of “hits” in the two-hybrid screen were disregarded for further analysis:

Nedd4-like ubiquitin-ligase WWP1 was pulled out of the skeletal muscle library 16 times with the β -dystroglycan bait. This is a highly significant result (22% of all clones sequenced) but was considered as a non-specific interaction and consequently discounted. WWP1 contains a type I WW motif which recognises a PPxY motif in its ligands, which is the same as the WW-binding motif in β -dystroglycan’s cytoplasmic domain, used in this screen. In biochemical peptide ligand assays, it has been shown that β -dystroglycan interacts with other WW domains apart from the one in dystrophin, including those from Nedd-4-like proteins (Pirozzi *et al.*, 1997). These may be non-specific interactions or perhaps WW-interacting proteins are regulated by the ubiquitin-mediated degradation pathway (see discussion, section 4.7.6). However, apart from involvement in the ubiquitin-mediated degradation pathway, the relevance of the β -

dystroglycan-WWP1 interaction is tenuous. The interaction of β -dystroglycan with YAP65 was also regarded as a non-specific WW-domain mediated interaction because like WWP1, YAP65 also interacts with the PPxY motif in its ligands.

Several different ubiquitin-conjugating enzymes were identified as interactors of β -dystroglycan in the yeast two-hybrid screen. One of these, RAD6, was found to self-activate in the controls i.e. the RAD6 clone activated the *HIS3* reporter gene without the presence of pAS2- β -dystroglycan. huBC9, UBE21 and E21 are highly homologous to RAD6, so presumably were self-activating *HIS3* as well. Therefore, these ubiquitin-conjugating enzymes were not considered as candidates for real interactors of β -dystroglycan.

Rev7p/MAD2-like protein was pulled out by β -dystroglycan cytoplasmic domain 7 times in the yeast two-hybrid screen. MAD2 (mitosis arrest-deficient-2) is a cell cycle mitotic spindle assembly checkpoint protein. This interaction was disregarded because MAD2 and MAD2-like proteins are frequently found as unlikely interactors in yeast two-hybrid screens. For example, recently it has been demonstrated that MAD2 interacts with tumour necrosis factor alpha convertase (TACE), a MHC- (major histocompatibility complex) encoded ubiquitin-like protein FAT10, the insulin receptor and oestrogen receptor- β (Nelson *et al.*, 1999; Liu *et al.*, 1999; O'Neill *et al.*, 1997; Poelzl *et al.*, 2000). These protein:protein interactions were all identified in yeast two-hybrid screens and the biological relevance is difficult to substantiate (Kevin Hardwick, personal communication).

Ran binding protein (RanBPM) is a novel centrosomal protein. It binds to Ran, a GTPase which has many roles in the cell. Ran has been implicated in nuclear transport, cell cycle control, and spindle assembly (reviewed in

Dasso, 2001) but it appears that RanBPM is involved in microtubule nucleation (Nakamura *et al.*, 1998). No obvious roles for RanBPM interacting with β -dystroglycan were deduced, therefore more biologically relevant interactions were tackled with the highest priority.

Out of the 74 plasmids sequenced, three proteins were chosen to study more fully, namely JAB1, FHOS and actin α 1. These proteins were chosen as potential genuine interactors of β -dystroglycan because some degree of biological relevance was ascertained. JAB1 is implicated in integrin-mediated signalling, as is the DAPC (Bianchi *et al.*, 2000, Yoshida *et al.*, 1998). FHOS is potentially involved in actin cytoskeleton reorganisation (Westendorf *et al.*, 1999; Tanaka, 2000). The actin cytoskeleton is linked to the DAPC via dystrophin and it would be a very important outcome if β -dystroglycan was also found to have a direct link to actin.

In the screen, the library plasmids encoding JAB1, FHOS and actin α 1 were all selected as positive clones (*Ade*⁺, *His*⁺) more than once (3x, 2x and 2x respectively). In addition, these plasmids did not activate the reporter genes alone, but activated the *GAL2-ADE* and *GAL1-HIS* reporter genes in conjunction with β -DG/pAS2. Furthermore, when checked by a β -galactosidase filter assay, the *GAL7-LacZ* reporter gene was also activated by pACT2-JAB1, pACT2-FHOS or pACT2-actin α 1 in association with β -DG/pAS2 (data not shown).

4.4. Characterisation of the JAB1- β -dystroglycan interaction

4.4.1. Jun activation-domain binding protein (JAB1).

JAB1 was isolated as an interactor of β -dystroglycan three times in the yeast two-hybrid screen. The three clones isolated all contained the entire 1.5kb coding sequence of the JAB1 gene. Human JAB1 was first described as a co-activator of the c-Jun and Jun D transcription factors (Claret *et al.*, 1996). JAB1 is also a component of the constitutive photomorphogenesis 9 (COP9) signalosome which is thought to be involved in modulating multiple signalling pathways in plants.

JAB1 was considered as a potential *in vivo* interactor of β -dystroglycan because it has recently been implicated in integrin-mediated signalling (Bianchi *et al.*, 2000) and is part of complex (COP9) involved in regulation of signalling pathways.

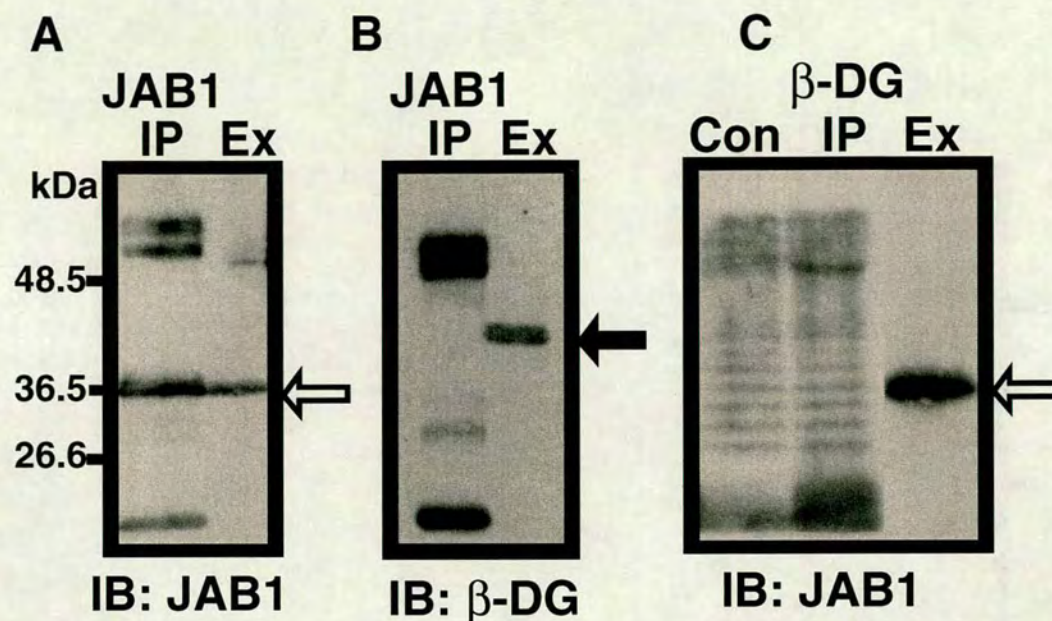
The DAPC also has links to integrin-mediated signalling: α - and γ -sarcoglycans are phosphorylated on tyrosine residues in response to integrin-dependent cell adhesion (Yoshida *et al.*, 1998); sarcospan is in the tetraspanin family and tetraspanins are known to associate with integrins (Hemler *et al.*, 1996); and β -dystroglycan has recently been shown to interact with β 4 integrin (Bittner *et al.*, 2001).

It was thought that a protein which is involved in integrin-mediated signalling, such as JAB1, could potentially associate with a member of the DAPC, a complex which is involved in cell adhesion. Therefore, a variety of experiments were carried out in order to elucidate whether the JAB1/ β -dystroglycan interaction occurs in C2/C4 myoblasts.

4.4.2. Co-immunoprecipitation experiments

Firstly, a JAB1 monoclonal antibody (table 2.6) was used to confirm that JAB1 protein is expressed in C2/C4 myoblasts and myotubes. A clear 37kDa band of JAB1 protein was detected when C2/C4 extracts were separated on SDS-PAGE gels and western blotted with JAB1 antibody (figures 4.4A & 4.4C, extract lanes). The JAB1 antibody also successfully immunoprecipitated JAB1 (figure 4.4A). The ability of β -dystroglycan antibody 1710 to immunoprecipitate β -dystroglycan is well characterised (chapter 5, (Ilsley *et al.*, 2001a)). Reciprocal immunoprecipitations (section 2.4.2) with JAB1 and β -dystroglycan antibodies, followed by western blotting showed no association between the two proteins (figures 4.4B & 4.4C).

It is possible that the two proteins were not co-immunoprecipitating because they are compartmentalised in different parts of the cell. Therefore purified β -dystroglycan cytoplasmic domain was added to the JAB1 immunoprecipitation experiments to elucidate whether this “free” β -dystroglycan could associate with JAB1. These JAB1 immunoprecipitates were subsequently western blotted with β -dystroglycan antibodies (figure 4.5). The purified β -dystroglycan protein appeared to co-immunoprecipitate with JAB1, but on further inspection, it was also present in the control immunoprecipitate (to which no JAB1 antibody was added). It is feasible that the purified β -dystroglycan protein was non-specifically interacting with the protein A Sepharose beads, possibly due to its high concentration in the mixture. Due to this occurrence, it was not possible to substantiate a real interaction between JAB1 and the purified β -dystroglycan protein by co-immunoprecipitation techniques. Therefore, alternative approaches were considered.

Figure 4.4. **β -dystroglycan and JAB1 do not co-immunoprecipitate.****A. JAB1 is immunoprecipitated by the JAB1 antibody.**

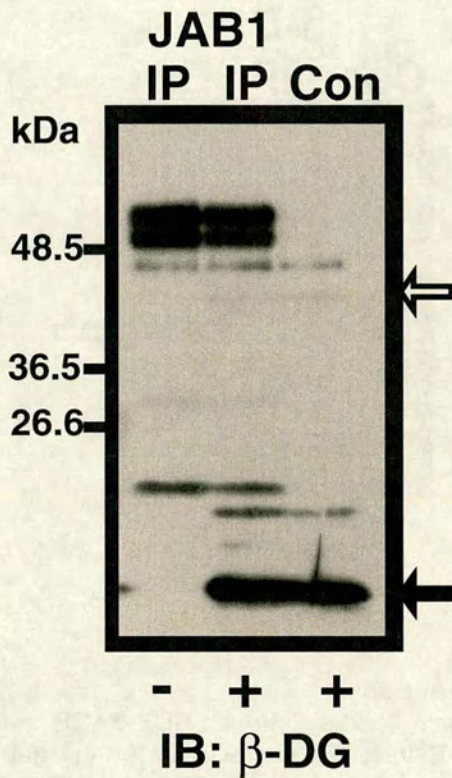
JAB1 was immunoprecipitated (IP) from myoblast extracts, loaded onto an SDS-PAGE gel and western blotted (IB) with JAB1 monoclonal antibody. Extract (Ex) was also run on the gel. JAB1 (37kDa) was clearly seen in the immunoprecipitate and the extract (shown by unfilled arrow).

B. JAB1 was immunoprecipitated (IP) from myoblast extracts, loaded onto an SDS-PAGE gel and western blotted (IB) with β -dystroglycan monoclonal antibody MANDAG2. β -dystroglycan (43kDa) (shown by an filled arrow in myoblast extract (Ex) was clearly not present in the JAB1 immunoprecipitate.

C. β -dystroglycan was immunoprecipitated from myoblast extracts using 1710 polyclonal antibody, loaded onto an SDS-PAGE gel and western blotted with JAB1 monoclonal antibody. The control (Con) was an immunoprecipitate prepared using 1710 pre-immune sera. JAB1 (37kDa) shown by an unfilled arrow in myoblast extract (Ex) was clearly not present in the β -dystroglycan immunoprecipitate or control.

Figure 4.5

Purified β -dystroglycan protein does not co-immunoprecipitate with JAB1.



JAB1 was immunoprecipitated from myoblast extracts and purified β -dystroglycan cytoplasmic domain was added to (+) or omitted (-) from the experiment. The immunoprecipitates were western blotted with β -dystroglycan monoclonal antibody MANDAG2. No native β -dystroglycan (43kDa) was co-immunoprecipitated (unfilled arrow) but the purified β -dystroglycan appeared to be co-immunoprecipitated (filled arrow). However purified β -dystroglycan was also pulled down in the control (no antibody) IP (Con) suggesting that it was non-specifically interacting with the protein A Sepharose.

4.4.3. "Pull-downs" using bacterially expressed GST-JAB1 fusion protein

GST-JAB1 fusion protein was expressed in *E.coli* cells transformed with pGEX5X-2-JAB1 (section 2.4.12). The protocol included an induction step of 2 hours at 37°C. However, this produced largely insoluble fusion protein which was present in the pellet of the bacterial cell extracts. Insolubility is a widely documented problem for a variety of proteins expressed in *E.coli*, many of which are only recoverable from insoluble inclusion bodies (Marston, 1986; Wilkinson & Harrison, 1991). In an endeavour to increase the solubility of the protein, induction was carried out at lower temperatures (30°C, 25°C and 20°C). Despite these reductions in temperature, the GST-JAB1 fusion protein remained largely insoluble. However, induction for 5 hours at 20°C produced enough soluble GST-JAB1 to carry out "pull-down" experiments. The GST-JAB1 fusion was purified using glutathione-Sepharose beads (see figure 4.6) and added to purified β -dystroglycan cytoplasmic domain protein (section 2.4.13.4). The rationale was that if JAB1 was associating with β -dystroglycan then β -dystroglycan would be pulled out with GST-JAB1 by glutathione-Sepharose beads. The pulldowns were western blotted with a β -dystroglycan antibody. Although purified β -dystroglycan was pulled down with JAB1-GST and the Sepharose beads, this phenomenon also occurred in a control pulldown using GST alone instead of GST-JAB1 (figure 4.7). Therefore, the pulling down of β -dystroglycan was not specific to an interaction with JAB1.

Figure 4.6

Coomassie staining of purified GST-JAB1.

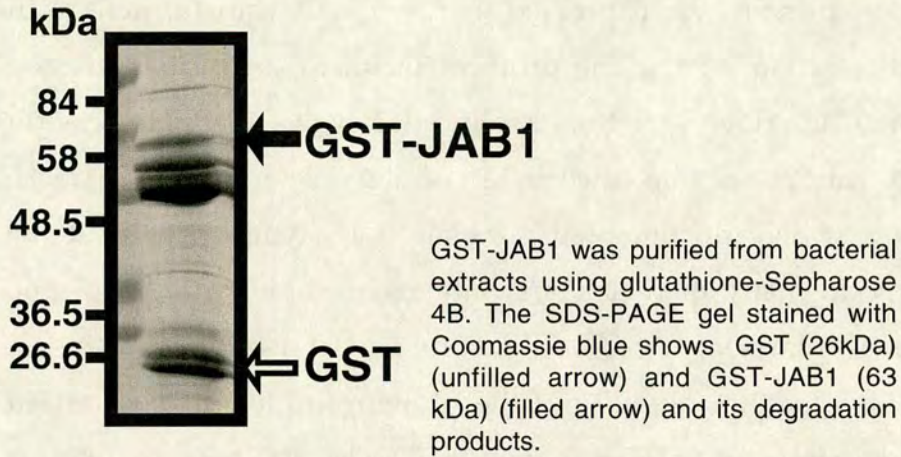


Figure 4.7

In vitro pulldowns using GST-JAB1 and purified β -dystroglycan cytoplasmic domain.



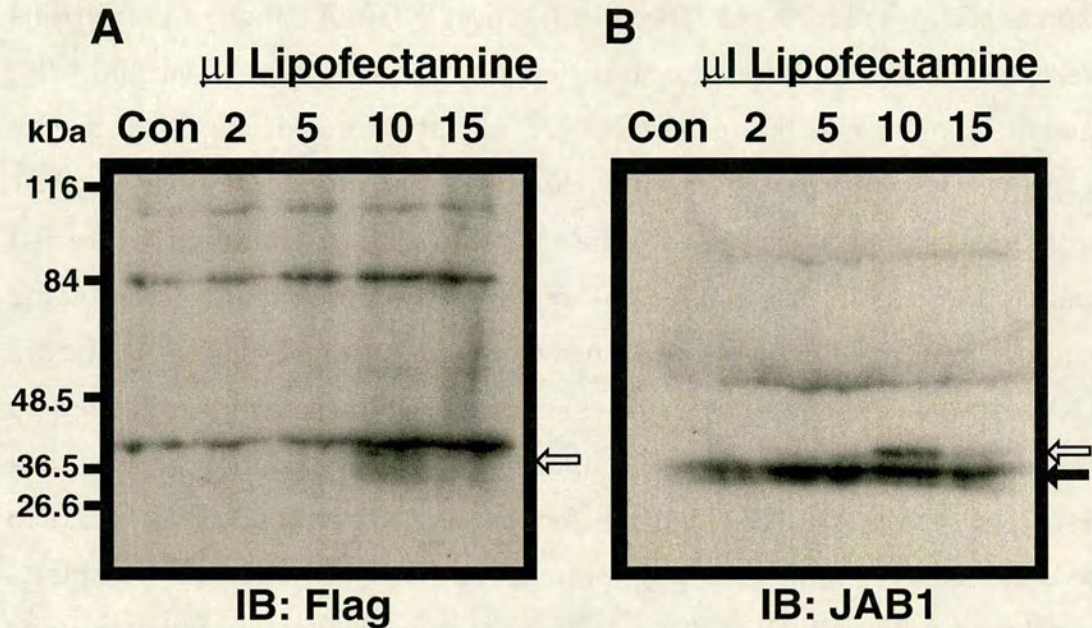
Purified β -dystroglycan cytoplasmic domain was incubated with GST (lanes 1 & 2) or GST-JAB1 (lanes 3 & 4) overnight. The GST or GST-JAB1 was subsequently pulled down using glutathione-Sepharose beads. The proteins associating with the beads (**B**) and the supernatants (**S**) were run on SDS-PAGE gels and western blotted with β -dystroglycan monoclonal antibody MANDAG2. The 13kDa β -dystroglycan protein appeared in the beads, pulled down with GST-JAB1 (shown by arrow) but it was also pulled down in the control (GST alone). This suggested that JAB1 was not specifically interacting with β -dystroglycan cytoplasmic domain. Excess β -dystroglycan was also present in the supernatants (**S**)

4.4.5. Transfection of JAB1 into myoblasts

In a subsequent experiment to demonstrate an interaction between JAB1 and β -dystroglycan, pCEP4F-JAB1 DNA was transfected into myoblasts to express a FLAG-tagged JAB1 protein (section 2.3.8). A range of conditions were tested to optimise the transfection procedure, e.g. varying the quantities of Lipofectamine and DNA, and the time of exposure to the reagents. Despite this, it was not clear whether the FLAG-tagged JAB1 protein was being expressed. The M2 monoclonal antibody directed against the FLAG tag (table 2.6) recognised approximately the same banding pattern of proteins in transfected and untransfected cells (figure 4.8A -compare control to other lanes) and none of these bands appeared to be same size as JAB1. This peculiarity of the M2 antibody was not restricted to this pCEP4F-JAB1 transfection. The M2 antibody was found to give exactly the same banding pattern on western blots of myoblasts transfected with pCEP4F-DYS (a dystrophin construct) as the western blots of pCEP4F-JAB1 transfected cell extracts in figure 4.8A (see section 3.8). However, on further inspection the myoblasts transfected with 10 μ l Lipofectamine and pCEP4F-JAB1, and western blotted with M2, an additional band around the molecular weight of a FLAG-tagged JAB1 was observed. Transfected extracts separated by SDS-PAGE and western blotted with JAB1 antibody showed the presence of JAB1 protein in all transfections (see figure 4.8B) but this was interpreted to be native JAB1 exclusively. Yet the myoblasts transfected with 10 μ l Lipofectamine had another slightly higher molecular weight protein which was detected by the JAB1 antibody which was potentially FLAG-tagged JAB1. Therefore, it was deduced that only the transfection using 10 μ l Lipofectamine and pCEP4F-JAB1 facilitated the expression of FLAG-tagged JAB1. But the co-immunoprecipitation of FLAG-tagged JAB1 and β -dystroglycan could not be tested fully because the M2 antibody did not immunoprecipitate FLAG-tagged proteins adequately.

Figure 4.8.

Western blots of myoblasts transfected with pCEP4F-JAB1.



Myoblasts transfected with 2 μ g pCEP4F-JAB1 DNA and 2, 5, 10 or 15 μ l Lipofectamine. Extracts from the cells were run on SDS-PAGE gels and western blotted (IB) with monoclonal antibodies M2 (anti-FLAG) or anti-JAB1 to detect the expression of FLAG-tagged JAB1.

A. The same pattern of expression was seen in the non-transfected control (Con) and the transfected cells, when blotted with M2. The M2 antibody only detected a protein of 37kDa (FLAG-tagged JAB1) in the 10 μ l Lipofectamine lane (denoted by unfilled arrow) which ran below the non-specific bands.

B. The JAB1 antibody did detect 37kDa JAB1 protein (shown by filled arrow) but this was interpreted as native JAB1. In the 10 μ l Lipofectamine lane only, a band running above native JAB1 (denoted by unfilled arrow) but still detected by the JAB1 antibody was observed. This band was running at the same molecular weight as the extra band detected by M2 in the 10 μ l Lipofectamine lane in 4.8A. Therefore this was presumed to be FLAG-tagged JAB1.

4.5. Characterisation of the FHOS- β -dystroglycan interaction

4.5.1. Formin Homology Overexpressed in Spleen (FHOS).

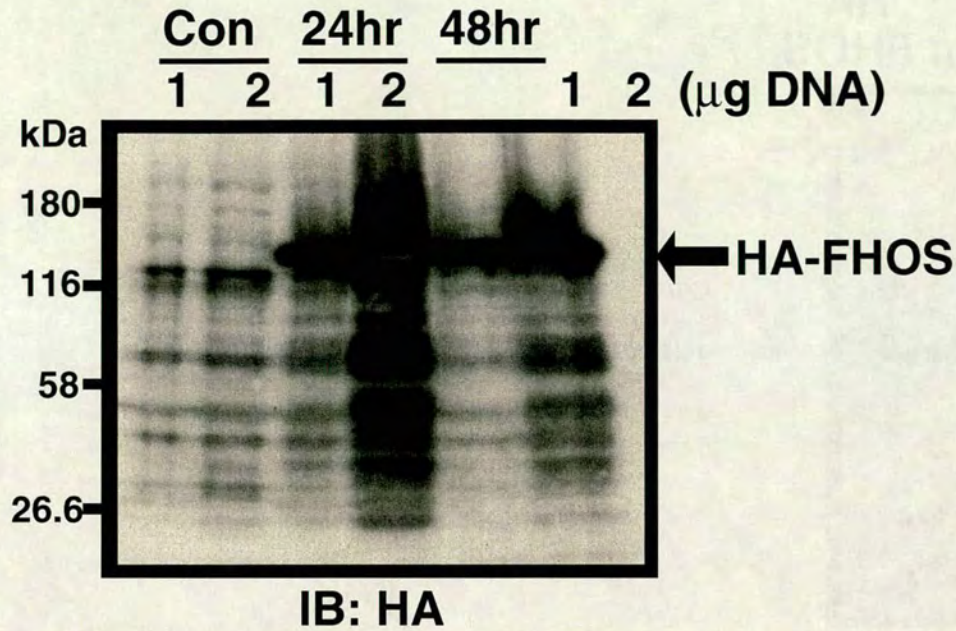
The C-terminal region of FHOS (amino-acids 943-1165) was identified twice as an interactor of β -dystroglycan in the yeast two-hybrid screen. FHOS was originally identified during a yeast two-hybrid screen of a B cell cDNA library, using AML-1B transcription factor as bait (Westendorf *et al.*, 1999). FHOS is a member of the formin family of proteins. Formin family members contain the Formin Homology domains FH1 and FH2 and are found in a diverse range of organisms. Proteins in the formin family include the gene products of *mDia* in mouse, *diaphanous* and *cappuccino* in *Drosophila*, *bni1* in *Saccharomyces cerevisiae* and *fus1* and *cdc12* in *Schizosaccharomyces pombe*. The founder members of this family, formin, were originally identified as transcripts of the *limb deformity* gene required for the proper formation of the two morphogenetic centres in mouse limb bud development (Woychik *et al.*, 1990). At least four major isoforms of formin are expressed during mouse embryogenesis and they are phosphoproteins localised largely in the nucleus.

The FH domains were first identified as regions of sequence homology in *diaphanous*, *bni1* and vertebrate formin (Castrillon & Wasserman, 1994). Many of the proteins with FH domains are involved in actin-dependent processes e.g. *diaphanous*, *cappuccino* and *bni1* mutations result in defects in cytokinesis, and the overexpression of truncated *mDia* causes abnormal organisation of the actin cytoskeleton. In fact, many of the proteins were identified genetically in the first instance via their defects in actin-mediated processes. Although FHOS was identified only recently and its function is unknown, it was a plausible candidate as an interactor of β -dystroglycan because other proteins in the formin family are involved in

reorganisation of the actin cytoskeleton. A potential function of β -dystroglycan and the DAPC is regulation of the cytoskeleton via signal transduction, therefore the FHOS/ β -dystroglycan interaction was considered to be relevant *in vivo*.

4.5.2. Transfection of FHOS into myoblasts

To confirm the FHOS/ β -dystroglycan yeast two-hybrid interaction, pCMV-HA-FHOS DNA was transfected into C2/C4 myoblasts to express HA-tagged FHOS protein *in vivo*. The transfection was successful and the expression of the 128kDa HA-tagged FHOS was confirmed by western blotting and immunoprecipitation experiments using a monoclonal HA antibody (table 2.6) (figures 4.9 & 4.10). Subsequently, the HA immunoprecipitate containing HA-tagged FHOS was western blotted with a β -dystroglycan antibody to determine whether β -dystroglycan and FHOS were interacting. Purified β -dystroglycan cytoplasmic domain was also added to the immunoprecipitation experiment to ascertain whether FHOS interacted with this in addition to, or instead of, native β -dystroglycan. Figure 4.11 shows that native β -dystroglycan was not present in the HA immunoprecipitate with HA-FHOS. The purified β -dystroglycan protein was in the immunoprecipitate, but controls revealed that the purified protein associating non-specifically with the protein G Sepharose beads, as seen previously (figures 3.13B & 4.5).

Figure 4.9**Expression of HA-tagged FHOS in C2/C4 myoblasts.**

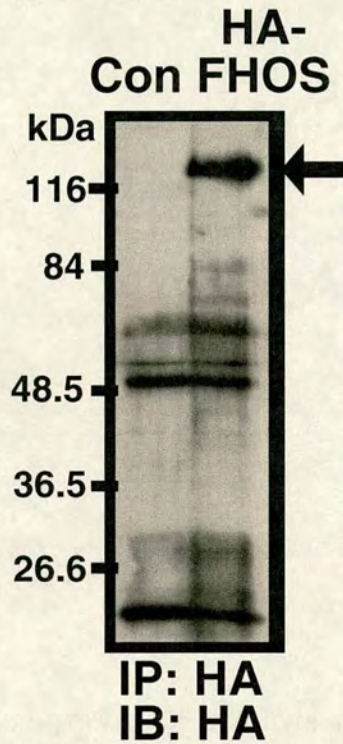
C2/C4 myoblasts were transfected with either 1 μ g or 2 μ g pCMV-HA FHOS DNA and Lipofectamine and analysed after 24 hours or 48 hours transfection. Proteins were extracted from the cells using RIPA buffer. The extracts were loaded onto SDS-PAGE gels and western blotted (IB) with anti-HA monoclonal antibody.

The controls (Con) were untransfected myoblasts.

HA-FHOS is 128kDa and can be seen in all the transfection experiments (shown by arrow), therefore 1 μ g of DNA and a 24 hour transfection time was considered to be sufficient for expression of HA-FHOS. There was no HA-FHOS expressed in the controls.

Figure 4.10

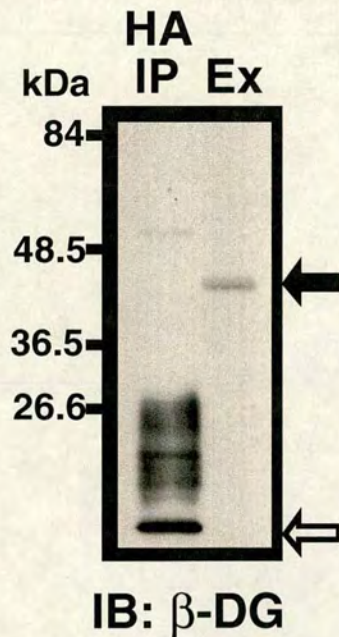
Western blot of HA-FHOS immunoprecipitated by anti-HA antibody.



pCMV-HA-FHOS transfected myoblast extracts and non-transfected extracts (Con) were immunoprecipitated (IP) with anti-HA antibody and western blotted with anti-HA antibody. The 128kDa HA-FHOS protein was clearly immunoprecipitated from the transfected extracts (shown by filled arrow).

Figure 4.11

β -dystroglycan does not co-immunoprecipitate with HA-FHOS .

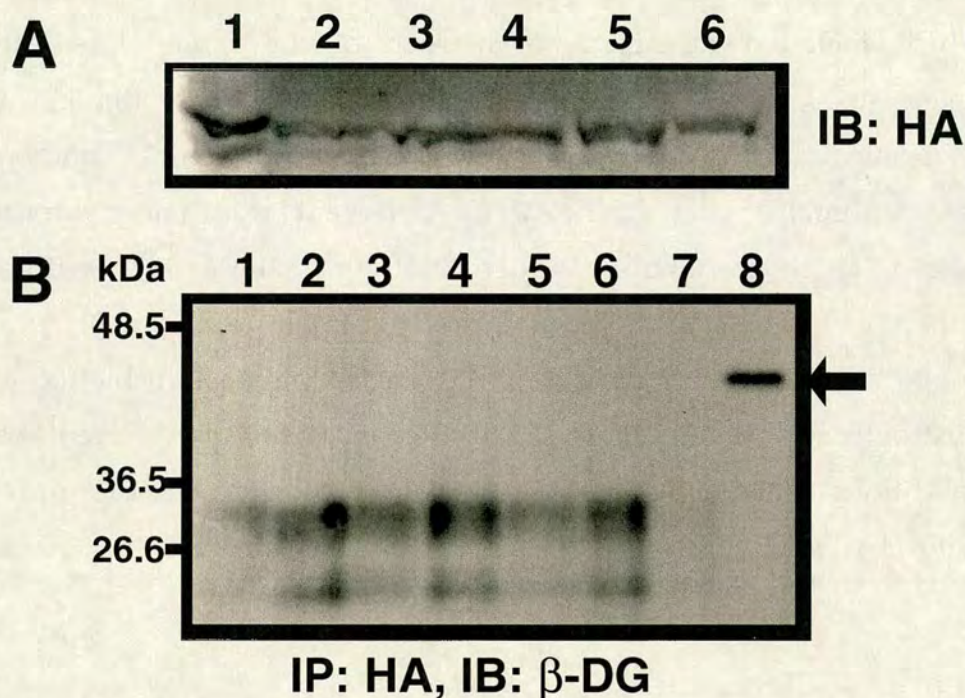


Western blot of HA-FHOS immunoprecipitated (IP) with anti-HA antibody from pCMV-HA-FHOS transfected myoblasts, and western blotted with β -dystroglycan monoclonal antibody MANDAG2. β -dystroglycan (43kDa) is present in the extract (Ex) (shown by filled arrow) but not in the immunoprecipitate with FHOS. Purified protein of β -dystroglycan cytoplasmic domain (13kDa) was also added during the immunoprecipitation experiment. This appears in the IP (unfilled arrow) but was later found to be non-specifically interacting with the protein G Sepharose rather than HA-FHOS.

It was speculated that the FHOS/ β -dystroglycan interaction was not observed in the transfected myoblast extracts due to the extraction conditions used. Therefore, to discount this possibility, pCMV-HA-FHOS transfected myoblasts were extracted with a panel of extraction buffers which included detergents from each of the three classifications of detergents, namely SDS and sodium deoxycholate (ionic), Brij 98, Digitonin, TritonX-100 and Tween-20 (non-ionic) and CHAPS (zwitterionic) (section 2.3.3). All the detergents adequately extracted HA-tagged FHOS (as shown by western blotting of the extracts with anti-HA antibody, figure 4.12A). Immunoprecipitations using anti-HA antibody were carried out using the different extracts, and western blotted with a β -dystroglycan antibody (figure 4.12B). Regardless of the different extraction conditions tested, β -dystroglycan did not appear to co-immunoprecipitate with HA-FHOS.

Figure 4.12

β -dystroglycan is not co-immunoprecipitated with HA-tagged FHOS under different extraction conditions.



A. Western blot of pCMV-HA-FHOS transfected myoblasts extracted under different conditions.

Myoblasts were transfected with pCMV-HA-FHOS and lysed 24 hours later with 1% (w/v) Brij 98 (lane 1), 1% (w/v) CHAPS (lane 2), RIPA buffer (lane 3), 1% (v/v) TritonX-100 (lane 4), 1% (v/v) TritonX-100/ 0.1% (w/v) SDS (lane 5) or 1% (v/v) Tween-20 (lane 6). The extracts were run on SDS-PAGE gels and western blotted with anti-HA antibody. The expressed HA-tagged FHOS protein was successfully extracted by all the detergents tested.

B. Co-immunoprecipitations

Myoblasts were transfected with pCMV-HA-FHOS and lysed 24 hours later with 1% (w/v) Brij 98 (lane 1), 1% (w/v) CHAPS (lane 2), RIPA buffer (lane 3), 1% (v/v) TritonX-100 (lane 4), 1% (v/v) TritonX-100/ 0.1% (w/v) SDS (lane 5) or 1% (v/v) Tween-20 (lane 6). The extracts were immunoprecipitated with anti-HA antibody, run on SDS-PAGE gels and western blotted with β -dystroglycan monoclonal antibody. Lane 7 is anti-HA immunoprecipitate from untransfected (control) myoblasts. The arrow points to β -dystroglycan in the myoblast extract loaded in lane 8.

No β -dystroglycan co-immunoprecipitated with HA-FHOS from the transfected myoblasts.

4.6. Characterisation of the actin α 1- β -dystroglycan interaction

4.6.1. Actin α 1

Skeletal muscle actin α 1 was identified twice in the yeast two-hybrid screen as a possible interactor of β -dystroglycan cytoplasmic domain. The actin clones isolated from the skeletal muscle cDNA library were approximately 1.4kb in length and contained the full length coding sequence of the ACTA1 gene (1374bp).

Actin α 1 is one of six closely related actin isoforms. Actins are highly conserved proteins which are involved in cell motility and cell structure. The 42kDa actin monomer (G-actin) self-assembles to form long helical filaments (F-actin) both *in vivo* and *in vitro*. Alpha actins are a major constituent of the contractile apparatus and α 1 actin expression is restricted to skeletal muscle.

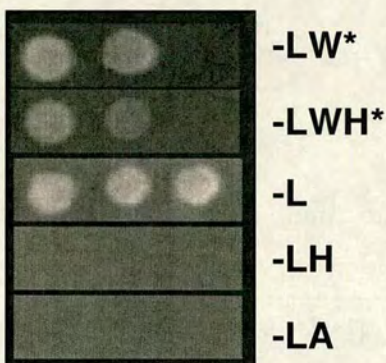
The interaction of β -dystroglycan with actin has not been tested previously, although dystrophin's actin-binding properties have been well documented (section 1.2.3). β -dystroglycan is part of a complex which interacts with actin (via dystrophin), therefore, it is plausible that β -dystroglycan could also interact with actin. Consequently, further experiments were carried out to substantiate this yeast two-hybrid result.

4.6.2. Controls to verify the actin α 1- β -dystroglycan interaction

The yeast two hybrid interaction of β -dystroglycan cytoplasmic domain and actin α 1 was verified by transforming pACT2-actin α 1 back into the β -DG/PJ694-A yeast strain to check that the activation of the reporter genes (*ADE* and *HIS*) could be reproduced (section 2.5.7.3). Evidence for the activation of the *HIS3* gene is demonstrated in figure 4.13, 2nd panel down. The lower two panels demonstrate that pACT2-actin α 1 alone (without pAS2- β -DG) cannot activate the *HIS* or *ADE* reporter genes.

Figure 4.13

Yeast two-hybrid controls: Growth of PJ69-4A *Saccharomyces cerevisiae* transformed with pACT2/actin α 1 and *pAS2 $\Delta\Delta$ / β -DG.



*PJ69-4A transformed with pACT2/actin α 1 and pAS2 $\Delta\Delta$ / β -DG grows on -LW media, showing that both plasmids are present (pACT2 has a *LEU* marker gene and pAS2 $\Delta\Delta$ has a *TRP* marker gene), and on -LWH media showing that the *HIS* reporter gene is activated due to the yeast two-hybrid interaction between actin α 1 and β -dystroglycan cytoplasmic domain.

When PJ69-4A is transformed with pACT2/actin α 1 only, the yeast grows on -L media but not on -LH or -LA media because neither the *HIS* or *ADE* reporter genes are activated. This shows that pACT2/actin α 1 does not self-activate.

4.6.3. Co-sedimentation assays

Sedimentation assays were carried out to determine whether β -dystroglycan was having an effect on the supermolecular structure of actin (section 2.6.1). In a high speed sedimentation assay (centrifugation over $20,000 \times g_{av}$) F-actin sediments. Any β -dystroglycan interacting with actin would be observed in the pellet with F-actin rather than in the soluble phase. In contrast, F-actin does not sediment under low speed sedimentation (less than $20,000 \times g_{av}$) unless complexed into supermolecular aggregates by a cross-linking protein (Meyer & Aebi, 1990).

G-actin was polymerised to F-actin in the presence of increasing concentrations of purified β -dystroglycan cytoplasmic domain (section 2.4.11) and centrifuged at $100,000 \times g_{av}$ for 15 minutes. Subsequently, equal volumes of the supernatant and pellet were analysed by SDS-PAGE. Figure 4.14B shows that F-actin sediments during high speed centrifugation in the actin-only control and β -dystroglycan remains in the soluble phase in the β -dystroglycan-alone control. However, when actin is polymerised in the presence of $100\mu\text{M}$ β -dystroglycan, some β -dystroglycan is found in the pellet with actin. This suggests that β -dystroglycan and F-actin are interacting in some way.

In the low speed co-sedimentation assay, F-actin and β -dystroglycan were centrifuged at $20,000 \times g_{av}$ and equal volumes of the supernatant and pellet analysed by SDS-PAGE. The gels were studied for increasing amounts of actin in the pellet with increasing β -dystroglycan concentration. This would show that β -dystroglycan was bundling or crosslinking actin. Figure 4.14A shows that in the actin-alone control, F-actin did not sediment during low speed centrifugation as expected. The majority of β -dystroglycan was also in the supernatant in the β -dystroglycan-alone control. However, when F-actin and $100\mu\text{M}$ β -dystroglycan were

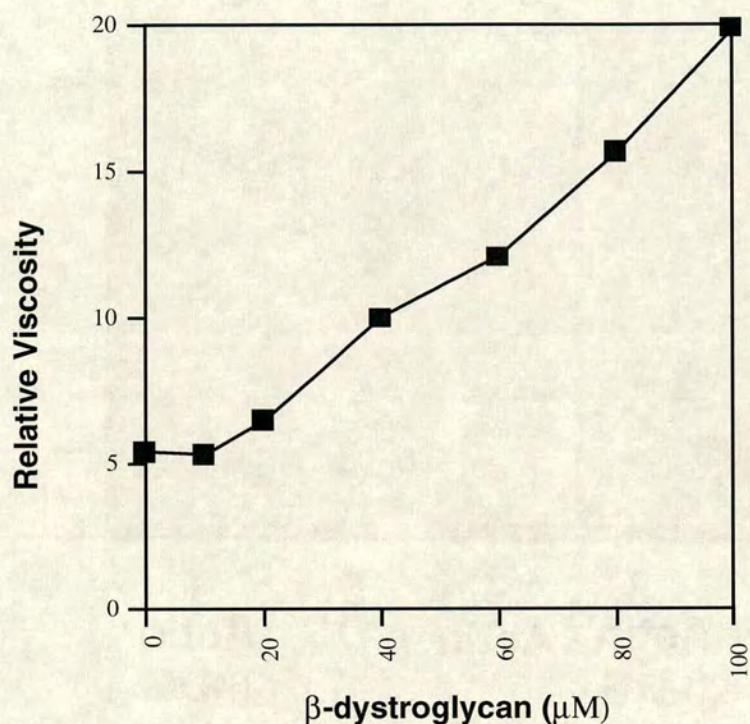
centrifuged together, a proportion of both proteins are found in the pellet. This suggests that β -dystroglycan causes F-actin to form supermolecular aggregates which can be pelleted by low speed centrifugation.

4.6.4. Microcapillary falling ball viscometry

Some proteins which bind to F-actin increase the viscosity of an F-actin solution *in vitro* (Ayscough, 1998). This increase in viscosity is caused by the crosslinking or bundling of actin filaments. If an actin-binding protein has two actin-binding sites (e.g. fimbrin) bundling of actin filaments can occur. If a protein binds actin at an exposed site on the filament and has the ability to self-associate (e.g. α -actinin) it should be capable of bundling or crosslinking actin filaments. The effects of actin-binding proteins on the viscosity of F-actin can be measured using microcapillary falling ball viscometry (section 2.6.2).

The effect of β -dystroglycan on the gelation of actin-filament solutions was examined by falling-ball viscometry. The speed of the steel ball was measured over a 10cm length of polymerised actin. β -dystroglycan increased the viscosity of F-actin 4 fold when present at a 2.5 :1 molar ratio with actin (figure 4.15). It was verified that the high concentration of β -dystroglycan itself was not causing the raise in viscosity by testing the viscosity of 100 μ M β -dystroglycan without F-actin. Therefore, the falling ball assay revealed that β -dystroglycan was potentially crosslinking or bundling F-actin.

Figure 4.15
Falling ball assay results



Viscosity measurements of F-actin polymerised in the presence of β -dystroglycan were carried out using microcapillary falling ball viscometry. G-actin (concentration 5μ M) was mixed with β -dystroglycan cytoplasmic domain (concentrations 10, 20, 40, 60, 80, 100μ M) and transferred to 100μ l capillary tubes. The velocity of a 0.64 mm diameter stainless steel ball was measured by recording the time (in seconds) it took to travel 10 cm down the capillary tube (relative velocity). With increasing concentration, β -dystroglycan increased the viscosity of F-actin. The viscosity of F-actin increased 4 fold when present at a 1:2.5 molar ratio with β -dystroglycan .

4.6.5. Electron microscopy studies

Electron microscopy studies revealed that β -dystroglycan was in fact bundling actin fibres, thus consolidating evidence from the co-sedimentation and falling ball assays that β -dystroglycan and F-actin interact in some fashion. Actin bundles are parallel arrays of closely packed actin filaments. Figure 4.16. clearly shows single actin filaments in the 5 μ M F-actin alone experiment (upper lefthand panel) and when 5 μ M F-actin is polymerised in the presence of 60 μ M β -dystroglycan, bundles of actin filaments are formed (upper righthand and lower panel).

4.6.5. Protein database searches to find homologous regions in β -dystroglycan cytoplasmic domain

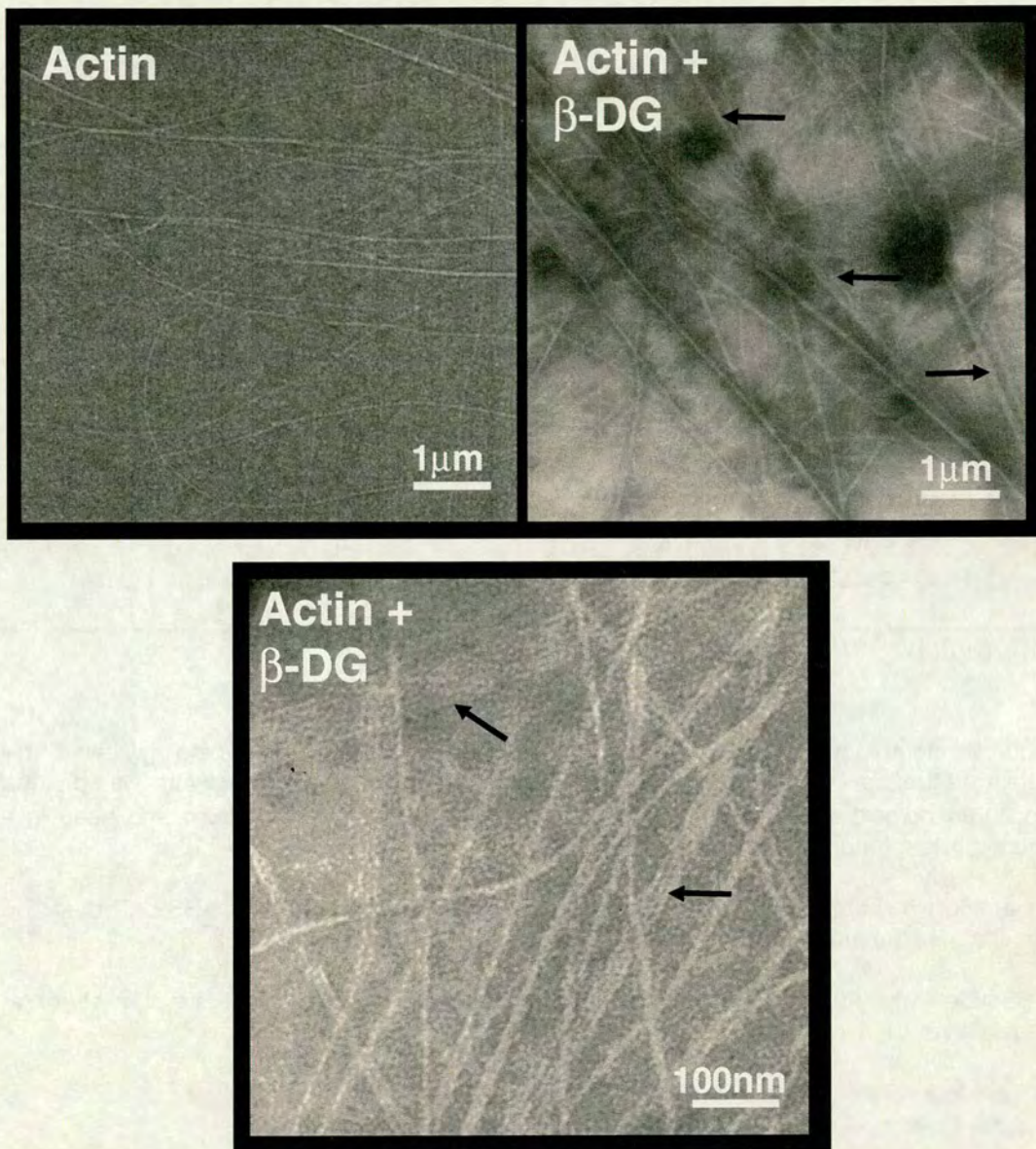
All actin-crosslinking proteins must have at least two actin-binding sites or form a dimer, where each site binds a separate actin filament. Since it was demonstrated that β -dystroglycan bundles F-actin, sequence homology searches were carried out to identify potential actin-binding sites in the protein sequence of β -dystroglycan. No significant matches were found in the database when the full length sequence of β -dystroglycan's cytoplasmic domain was used to search for homologous regions. Therefore, β -dystroglycan's cytoplasmic domain was broken down into smaller regions to search the database. The amino-acid sequence was divided up into 20 amino-acid sections, each overlapping by 10 amino-acids, and used in a protein:protein database search (MPsearch) (section 2.6.4). Many proline-rich proteins were identified due to the stretches of proline residues in β -dystroglycan e.g. VASP, WASP, proteins of the formin family (e.g. Formin, mDia, Cappuccino). Interestingly, peptide 1 (amino-acids 776-795) of β -dystroglycan had homologies to part of actin-binding site 2 (ABS2) in the N-terminal domains of utrophin and dystrophin (figure 4.17A). These shared identical amino-acids include the recognised actin-binding motif KLTL. Peptide 2 (amino-acids 786-805) also

had homology to amino-acids in dystrophin's central rod domain (amino-acids 1383-1402, coiled-coil number 10) (figure 4.17C). No actin-binding sites have been identified in this region of dystrophin although the rod domain does contain ABS4 and ABS5 (figure 1.1).

Peptide 1 of β -dystroglycan was also homologous to the actin-binding protein elongation factor 1 -alpha (EF-1 α) (figure 4.17B). EF-1 α is a member of a highly conserved family of proteins that catalyses the GTP-binding of aminoacyl-tRNA to ribosomes, thereby regulating the fidelity and rate of polypeptide elongation (reviewed in Condeelis, 1995). Numerous studies have demonstrated that EF-1 α binds and bundles actin filaments both *in vitro* and *in vivo*, and the bundling activity is potentially regulated by Ca²⁺-Calmodulin (Kurasawa *et al.*, 1996). The actin-binding sites of EF-1 α have not been defined, although the sites suggested are not in this region homologous to β -dystroglycan (amino-acids 263-282) (Liu *et al.*, 1996). However, this region is on the surface of the EF-1 α molecule and therefore it is possible that it is involved in actin-binding. In brief, at least three potential actin-binding regions were identified in the amino-acid sequence of β -dystroglycan's cytoplasmic domain. More biochemical and structural studies are required to delineate exactly where and how β -dystroglycan binds actin.

Figure 4.16.

β -dystroglycan bundles F-actin.



Electron microscopy of 5 μ M actin alone (upper panel) shows individual actin filaments (and a few in pairs). Electron microscopy of 5 μ M actin with 60 μ M β -dystroglycan (β -DG) (lower panels) shows bundles of actin filaments (indicated by arrows).

Figure 4.17**Homologies between β -dystroglycan and actin-binding proteins****A**

116	IVDGNH KLTL	GLLWSI ILHW	134	Utrophin
99	IVDGNH KLTL	GLIWN ILHW	118	Dystrophin
776	RKKRKG KLTL	EDQAT FIKKG	795	β -dystroglycan (peptide 1)

25% match

B

263	RLDRRSGKAL	EDDPKFIKTG	282	EF-1-alpha
776	RKKRKGKLT	EDQATFIKKG	795	β -dystroglycan (peptide 1)

45% match

C

1383	QESL TFIDKQ	LAAYI ADKVD	1402	Dystrophin
786	EDQAT FIKKG	VPIIF ADEL	805	β -dystroglycan (peptide 2)

35% match

Sequence homology searches were carried out to identify potential actin-binding sites in the protein sequence of β -dystroglycan. The cytoplasmic domain of β -dystroglycan was divided into 20 amino-acid sections, overlapping by 10 amino-acids (section 2.64) and used in a protein:protein database search.

A. Peptide 1 has amino-acids matches to dystrophin ABS2 and utrophin ABS2. This region also contains the motif KLTL which is recognised as a n actin-binding motif.

B. Peptide 1 also has amino-acids matches to the actin-binding protein EF-1-alpha, although this region of EF-1-alpha is not recognised as an actin-binding region.

C. Peptide 2 contains further amino-acids matches with dystrophin, in coiled-coil number 10. No actin-binding sites have been identified in this region of dystrophin.

4.7. Discussion

4.7.1. Selection of the appropriate yeast strain for the yeast two-hybrid screen

The expression of every bait protein elicits a different responses in yeast host strains. Therefore, three strains of *Saccharomyces cerevisiae* were characterised for their suitability in this screen.

The CG-1945 strain contains two reporter genes, *LacZ* and *HIS3* which are under the transcriptional regulation of the GAL4 and GAL1 promoters respectively. This provides a dual level of selection for interactions. Unfortunately, CG-1945 cells display bait-specific toxicity in some cases (usually characterised by the cells flocculating in liquid culture), which can be so severe that CG-1945 cannot be used for that particular bait.

The Y190 strain has *LacZ* and *HIS3* reporter genes which are both controlled by a GAL1 promoter. The *HIS3* reporter is sensitive but very leaky. This sometimes necessitates the use of high concentrations of 3-AT to counteract this "leakiness" and this may compromise the sensitivity of a screen.

PJ69-4A is a host yeast strain which was constructed in order to provide an extremely sensitive yeast two-hybrid assay with the elimination of false positives. Many of the host strains used in screens have a single promoter element to drive each reporter gene (e.g. Y190), whereas PJ69-4A has been constructed so that it contains three reporter genes (*HIS3*, *ADE2*, *LacZ*) each driven by a different reporter (GAL1, GAL2, GAL7). This reduces the incidence of promoter-specific false positives. In PJ69-4A, the GAL1-*HIS3* reporter construct used has a very stringent regulation compared to other constructs. In general, it only requires 0-2mM 3-AT in order to eliminate growth due to leakiness of the reporter. However, this GAL1-*HIS3*

reporter still produces a background of false positives but this is overcome by the GAL2-*ADE2* reporter used in conjunction with it, which is very stringent but remains very sensitive to weak interactions.

During the comparison of the three yeast host strains, it was concluded that PJ69-4A/ β -DG was the most appropriate for the screening of the skeletal muscle cDNA library. As for the other strains, Y190/ β -DG had a reasonable transformation efficiency but required very high amount of 3-AT, which creates the risk of lowering the overall sensitivity of the screen. CG-1945/ β -DG did not require a high concentration of 3-AT but the transformation efficiency was very low (perhaps due to bait toxicity, although no flocculation was observed in liquid cultures).

Transformations with PJ69-4A/ β -DG yielded high transformation efficiencies and required less than 1mM 3-AT. A high transformation efficiency is preferred because it facilitates the screening of more transformants at a time. The requirement for so little 3-AT made its use redundant in the screen, and therefore no sensitivity was compromised. Additionally, the use of 3-AT increases the time required for growth selection of positive clones from 2 days to 5 days, therefore, withdrawing its use shortened the timescale of the screen.

4.7.2. Putative interactors of β -dystroglycan identified in the yeast two-hybrid screen

From the results of the yeast two-hybrid screen using β -dystroglycan as a bait in a human skeletal muscle cDNA library, many proteins were identified as potential interactors of β -dystroglycan (table 4.1). The top five interactors were discounted because it was postulated that they were pulled out of the cDNA library due to non-specific WW domain-mediated interactions (WWP1, YAP65); or the library plasmids self-activated the reporter genes (ubiquitin-conjugating enzymes); or the

protein is a common yeast two-hybrid interactor (MAD2B); or no obvious biological relevance for the interaction could be predicted (RanBPM).

It was decided that three of the 74 sequenced interactors would be pursued further, namely JAB1, FHOS and actin α 1. These three proteins were thought to be sensible candidates as interacting partners of β -dystroglycan because they are potentially involved in cell adhesion via associations with integrin signalling (JAB1), or actin cytoskeleton reorganisation (FHOS) or part of the actin cytoskeleton itself (actin α 1).

4.7.3. Candidate interactor of β -dystroglycan : JAB1

4.7.3.1. The COP9 signalosome

Human JAB1 was first identified as a co-activator of the c-Jun and Jun D transcription factors (Claret *et al.*, 1996), but it is also a subunit (S5) of the COP9 signalosome (also termed the COP9 complex or JAB1-containing signalosome). The COP9 signalosome is a protein complex comprising 8 subunits which are highly conserved between plants and mammals (Wei *et al.*, 1998; reviewed in Wei & Deng, 1999).

The COP9 signalosome was originally isolated as a repressor of photomorphogenesis (light-activated development) in *Arabidopsis* (Chamovitz *et al.*, 1996). It is ubiquitous, but enriched in the nucleus. The function of the COP9 signalosome in mammalian cells is not clearly defined but the subunits are involved in diverse signalling events such as regulation of the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK)-mediated MAP kinase pathway (subunits S1 and S5), nuclear hormone receptor-mediated functions (S2) and cell cycle regulation (S6). The COP9 signalosome also has associated kinase activity which phosphorylates regulators of transcription (Seeger *et al.*, 1998). A kinase

must associate with the complex because none of the subunits have recognisable kinase domains. Consequently, the COP9 signalosome is thought to be an important cellular regulator involved in modulating multiple signalling pathways.

4.7.3.2. PCI/MPN domains

The subunits of the COP9 signalosome are closely related to subunits of the lid of the 19S regulatory particle of the 26S proteasome (which degrades ubiquitinated proteins) and the translation initiation complex 3 (eIF-3) (Glickman *et al.*, 1998). These three large multicomplexes all share PCI (Proteasome, COP9, Initiation factor 3) and MPN (Mpr1p and Pad1p N-terminal) domains (Hofmann & Bucher, 1998). Either a PCI or MPN domain is present in all 8 subunits of the COP9 signalosome, 7 of the 8 subunits of the proteasome lid and 5 of the 10 eIF2 subunits. The structural and functional roles of these domains are unknown. Although these complexes share similarities in their subunits, they are very distinct structural and functional entities. The presence of PCI and MPN domains in all these complexes suggests that they may have a role in macromolecular assembly e.g. they could homodimerise or heterodimerise to form a structural scaffold. Or perhaps they are docking sites for complexes to interact with common targets, such as ubiquitin, 26S base units (which link the lid of the 26S proteasome to the core) or other substrates. It is possible that all these complexes interact with the 26S proteasome through their MPN/PCI domains and fulfil their functions via protein degradation.

JAB1 has an N-terminal MPN domain and is closely related to p40 in the eIF3 complex and the POH (human homolog of *Saccharomyces cerevisiae* Pad-1) subunit of the 26S proteasome. Other motifs in the protein include five potential casein kinase II phosphorylation sites, two protein kinase C phosphorylation sites, four N-myristoylation sites and a

glycosaminoglycan attachment site (detected by PROSITE). JAB1 can be found in the nucleus as part of the COP9 signalosome or in a free monomeric form which is mostly cytosolic. Equilibrium between the two forms might be physiologically relevant and subject to regulation (Kwok *et al.*, 1998). The fact that JAB1 can be found in a free form in the cytoplasm, and not just part of the nuclear COP9-signalosome, creates the possibility that it is available to interact with β -dystroglycan in muscle cells.

4.7.3.3. JAB1 interacts with LFA-1 (α L/ β 2) integrin

JAB1 was recently found to interact with LFA-1 (α L/ β 2) integrin in a yeast two-hybrid screen (Bianchi *et al.*, 2000). The researchers wanted to identify molecules directly involved in transducing adhesion-mediated signals, and used the intracellular domain of LFA-1 β 2 integrin as bait in a lymphocyte DNA library. A fraction of JAB1 was also found to co-localise with LFA-1 at the membrane of COS7 cells by immunofluorescence. LFA-1 is thought to regulate JAB1 localisation because the engagement of LFA-1 caused an increase in the nuclear pool of JAB1, thus allowing enhanced binding of JAB1 to c-Jun.

In this study, JAB1 was considered as a relevant find in the yeast two-hybrid assay. It is involved in the regulation of signalling pathways (SAPK/JNK-mediated MAPK pathway) and is a component of a large signalling complex (COP9). Since little is known about the signalling role of the DAPC, it was considered that a JAB1/ β -dystroglycan interaction may shed light on the signalling pathways that the DAPC is potentially involved in. In addition, the JAB1/LFA-1 β 2 integrin interaction implicates JAB1 in the regulation of integrin-mediated adhesion. Considering that the DAPC acts as a cell adhesion structure, the possibility of a true JAB1/ β -dystroglycan interaction was regarded as an intriguing prospect.

Interestingly, in the yeast two-hybrid screen, a member of the eIF3 complex was also pulled out as a potential interactor of β -dystroglycan. This was subunit p48/mammary-tumour-associated protein INT-6 which has a C-terminal PCI domain, and is similar to the S2 subunit of the COP9 signalosome and subunit 9 of the 26S proteasome. This could be just a coincidence that another protein related to JAB1 was identified in the screen or it could strengthen a hypothesis of β -dystroglycan having links to the PCI/MPN domain-containing complexes.

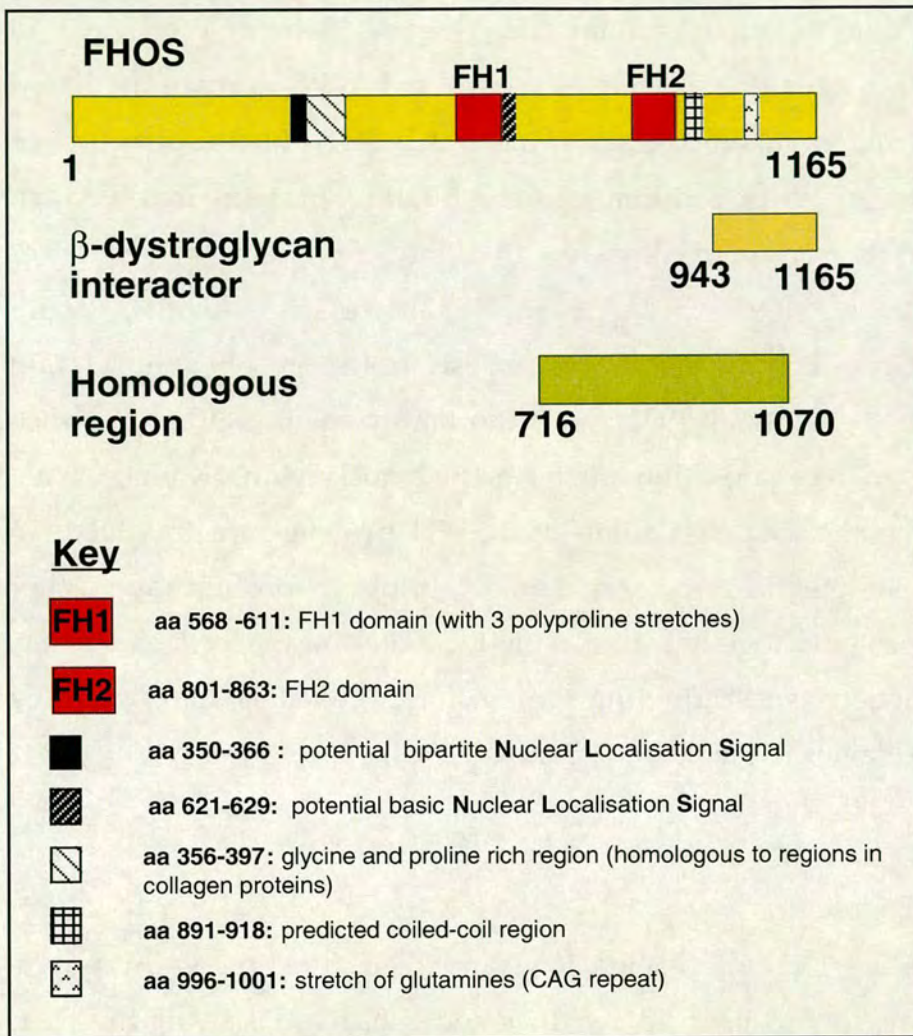
4.7.4. Candidate interactor of β -dystroglycan : FHOS

4.7.4.1. FHOS

FHOS contains a FH1 domain and a FH2 domain and is consequently classed as a member of the formin family. The FHOS open reading frame encodes 1165 amino-acids, and therefore a protein of approximately 128kDa (figure 4.18). The C-terminus of FHOS (amino-acids 716-1070) has significant homology to human diaphanous 1 and 2, murine p140mDia and formin, and *Drosophila* Diaphanous and Cappuccino proteins. The most conserved regions of FHOS with other FH proteins are in the FH1 and FH2 domains. In FHOS, the FH1 domain is small with only three poly-proline stretches. In addition, FHOS contains a predicted coiled-coil motif, a glycine and proline-rich segment homologous to regions in collagen proteins, a bipartite nuclear localisation signal (NLS) , a basic NLS and a stretch of glutamines encoded by a CAG nucleotide repeat. FHOS mRNA is expressed in every tissue tested to date, but most abundantly in spleen (Westendorf *et al.*, 1999). The cellular localisation of FHOS is predominantly in the cytoplasm. Unlike the formin, which are mainly localised in the nucleus, only a small amount of FHOS was observed in the nucleus by immunofluorescence studies (Westendorf *et al.*, 1999).

Figure 4.18

Open reading frame of FHOS showing domains and β -dystroglycan interacting region.



The open reading frame of FHOS encodes 1165 amino-acids (numbers on figure refer to amino-acid number). The key shows the various domains marked. The C-terminal end of FHOS (amino-acids 943-1165) labelled " β -dystroglycan interactor" interacted with β -dystroglycan in the yeast two-hybrid screen. Amino-acids 716-1070 labelled "homologous region" represent the region highly homologous to *Drosophila* Diaphanous and Cappuccino, human Diaphanous 1 and 2, murine p140mdia and Formin proteins.

4.7.4.2. FH1 domains

FH1 domains are 100 amino-acids long and characterised by a multiple repeats of 5-12 consecutive prolines. They are involved in protein:protein interactions due to their proline-rich regions which can bind to cytoskeletal and signalling proteins via SH3 and WW domain-mediated interactions (Ren *et al.*, 1993; Chan *et al.*, 1996). FH1 domains have been shown to interact with; profilin an actin-binding protein, involved in actin polymerisation and known to bind proline-rich sequences (Evangelista *et al.*, 1997; Chang *et al.*, 1997); cortactin, another actin-binding protein which appears to be a substrate for Src phosphorylation (Chan *et al.*, 1996; Wu *et al.*, 1991); and Rho family small G-proteins which are involved in reorganisation of the actin cytoskeleton (Kohno *et al.*, 1996). It is hypothesised that some of the FH proteins are involved in modification of the actin cytoskeleton by bringing profilin and other cytoskeletal proteins together at specific locations in the cell cortex, i.e. they may function as a scaffolding protein for cytoskeletal and signalling components (Frazier & Field, 1997; Tanaka, 2000).

4.7.4.3. FH2 domains

FH2 domains are 130 amino-acids long and their function is unknown. However, they have conserved residues and conserved spacing and it is speculated that they are involved in protein:protein interactions. The 200 amino-acid long intervening sequence between FH1 and FH2 is also conserved and it has been suggested that FH2 domain should be extended to include the sequences between the two domains (Frazier & Field, 1997). There are also stretches of sequence predicted to fold into coiled-coils which flank the FH region.

Interestingly, the FHOS clones (amino-acids 943-1165) which were identified as interactors of β -dystroglycan in the yeast two-hybrid screen

did not contain either of the FH domains or the coiled-coil region. The only motifs in this C-terminal end of FHOS are many phosphorylation sites (for Protein Kinase C and Casein kinase II, cAMP- and cGMP-dependent kinases), and two N-myristoylation sites (detected by PROSITE). However, the fact that proteins in the formin family have roles in organisation of the actin cytoskeleton prompted interest in the FHOS/ β -dystroglycan interaction and it was decided to investigate it further.

4.7.5. JAB1 and FHOS : results of characterising their interactions with β -dystroglycan

Unfortunately, neither the interaction of β -dystroglycan with JAB1 or FHOS could be substantiated *in vivo* by co-immunoprecipitation experiments of β -dystroglycan and endogenous JAB1 or β -dystroglycan and overexpressed HA-tagged FHOS from C2/C4 cell extracts. This could be due to problems with the immunoprecipitation experiments which have not proved entirely successful for identifying interactors of β -dystroglycan (see chapter 3). Or it is possible that an *in vivo* transcription-based assay such as the yeast two-hybrid assay is a highly sensitive technique and can detect weak interactions which other assays such as co-immunoprecipitation can not.

On the other hand, these two proteins selected may have been false positives in the yeast two-hybrid screen. Although the technique is continually evolving to reduce the amount of false positives, and the PJ69-4A strain used was constructed in a manner to minimise them, false positives still frequently occur. False positives are caused by plasmids which activate reporter gene expression but do not encode a protein which binds the target protein (the bait). This phenomenon often occurs if the library plasmid encodes a protein involved in transcription e.g. Sterol regulatory binding transcription factor 1 (SREBF1) and HIV type 1

enhancer binding protein 2 (HIVEP2) picked up in this yeast two-hybrid screen (table 4.1). Transcription can also be activated by a cDNA whose product can bind to the minimal GAL4 DNA-binding domain or sequences upstream of the reporter gene or just by non-specific binding to the DNA. But often, there is no explanation for the occurrence of a false positive.

One of the “contraindications” of embarking on a yeast two-hybrid screen is that although two proteins may interact in this assay, they may never actually encounter each other during normal conditions. This may be because the two proteins are not present in the same cellular compartment or during the same stage of the cell cycle or perhaps the domain or domains involved in the interaction are not exposed or accessible in the native protein.

Therefore, despite a putative biological relevance for the JAB1/ β -dystroglycan and FHOS/ β -dystroglycan interactions, the existence of these interactions could not be unequivocally proved in C2/C4 myoblasts. This may be due to the restrictions of the assays used or that the interactions do not exist *in vivo*.

4.7.6. β -dystroglycan: links to the ubiquitin-mediated degradation pathway?

During the course of the yeast two-hybrid screen, many proteins involved in the ubiquitin-mediated degradation pathway were identified as potential interactors of β -dystroglycan. For example, Nedd4-like protein ligase (WWP1) (an E3 protein) and ubiquitin-conjugating enzymes (E2) RAD6, E21, UBE21 and hUBC9. The frequency of these proteins in the screen was 36%.

4.7.6.1. The ubiquitin-mediated degradation pathway

Degradation of a protein by the ubiquitin-mediated degradation pathway involves three steps, namely the identification of the protein to be degraded, the marking of that protein by attachment of ubiquitin, and degradation of the tagged protein by the 26S proteasome. Ubiquitin or ubiquitin-like proteins are involved in a variety of fundamental cellular processes, some of which are non-proteolytic such as protein sorting, DNA repair, cell division, differentiation and development (reviewed in Ciechanover *et al.*, 2000; Wilkinson, 2000).

Ubiquitin is a highly conserved 76-residue protein which only exists in eukaryotic cells. It is activated by a ubiquitin-activating enzyme (E1). A ubiquitin-conjugating enzyme (E2) transfers the activated ubiquitin to its substrate, which is specifically bound to a member of the ubiquitin protein ligase family (E3). The modified protein is subsequently targeted to the proteasome for degradation.

The regulation protein ubiquitination is predicted to be controlled at the level of the E2 and E3 proteins. E3 ligase binds the substrate protein and E2, and serves as a scaffold protein. In most cases, an E3 recognises a subset of proteins that contain similar structural motifs and can be divided up into subtypes depending on what class of signals they recognise. HECT-domain proteins (homologous to E6-AP carboxyl terminus) comprise one of these subsets. The HECT domain has a conserved cysteine residue to which the activated ubiquitin protein is transferred to from E2. Nedd4-like protein ligase (WWP1) is a member of this subset and has a HECT domain in addition to its four WW domains. The PPxY motif of β -dystroglycan has been shown previously to interact with WW domains other than dystrophin's *in vitro* (Pirozzi *et al.*, 1997). Interestingly, these other WW domains were from proteins in the Nedd4-like family. However, these were purely biochemical assays and to date, no *in vivo*

evidence for these interactions has been demonstrated. Therefore it is assumed here that both the biochemical assays and the yeast two-hybrid screen gave rise to non-specific WW domain-mediated interactions, perhaps due to the high sensitivity of the assays. Nevertheless, the possible association of β -dystroglycan with the ubiquitin-mediated degradation machinery is discussed below.

4.7.6.2. Nedd4- a ubiquitin protein ligase

Nedd4 has 4 WW domains and a HECT domain which targets the kidney epithelial Na^+ channel. Liddle's syndrome, which manifests itself as a renal hypertension, is caused by an increased activity of the Na^+ channels because they are not targeted to the proteasome properly by Nedd4. This is because the WW domains of Nedd4 can no longer bind the β subunit of the Na^+ channels due to proline or tyrosine point mutations in their PPxY motifs, or deletions encompassing the PPxY motif (reviewed in Kay *et al.*, 2000). It is conceivable that other PPxY motifs are used as recognition sites of E3 ubiquitin ligases, such as β -dystroglycan recognition by Nedd4-like protein ligase (WWP1). This suggests that proteins which interact with WW domains may be regulated by ubiquitin-mediated degradation.

Ubiquitin signals on target proteins can be genetically programmed, acquired by phosphorylation or by binding to an adapter protein. Proteins which are damaged by oxidation or mutation or are misfolded or mislocalised are also substrates of the ubiquitin-mediated degradation pathway (reviewed in Laney & Hochstrasser, 1999). In the case of β -dystroglycan, it is possible that due to its overexpression in the yeast two-hybrid screen, or the potential instability of the protein, that it was targeted by proteins of the degradation pathway, or that β -dystroglycan is *usually* regulated by the ubiquitin-mediated degradation pathway, mediated by WW domain interactions between β -dystroglycan and the ubiquitin ligase WWP1. Another possibility is that a non-specific W W

domain-mediated interaction manifested itself in the yeast two-hybrid assay due to the sensitivity of the procedure.

4.7.7. Known interactors of β -dystroglycan were not identified in the screen

Disappointingly, previously characterised interactors of β -dystroglycan, dystrophin, Grb2, caveolin-3 and rapsyn (section 1.6.4) were not identified in this yeast two-hybrid screen.

These particular interactors may not have been revealed by this screen because only approximately 52% of the skeletal muscle cDNA library was screened. The reasons for this were time-constraints, and abundant results requiring further investigation which were generated from screening this percentage of the library.

However, it is not surprising that rapsyn was not identified as an interactor of β -dystroglycan - rapsyn is notoriously difficult to work with, and all the biochemical procedures tried, as well as yeast two-hybrid screens, have failed to identify its binding partners (reviewed in Colledge & Froehner, 1998).

There are several possible reasons why the dystrophin/ β -dystroglycan interaction was not identified in the yeast two-hybrid screen. Previously, dystrophin has proved problematic as a bait in yeast two-hybrid screens (M. James & S. J. Winder, personal communication). If cDNA boundaries used in constructs are not optimal, the cDNAs may be non-functional and/or lead to the expression of unstable or misfolded proteins. This may explain why overexpressed dystrophin WW, EF hands and WW domains in C2/C4 myoblasts did not appear to interact with native β -dystroglycan (section 3.8). In addition, a large protein such as dystrophin is unlikely to be included in one library clone, and this is likely to affect its interactions

and folding. Furthermore, the very low abundance of dystrophin in skeletal muscle raises the possibility that the protein was under-represented in the skeletal muscle cDNA library.

Unfortunately, the yeast two-hybrid screen shed no more light on β -dystroglycan's interactions with signalling and focal adhesion proteins. The dynamic nature of these interactions (e.g. Grb2 competes with dystrophin to interact with β -dystroglycan) potentially make them difficult to 'trap' by this method. Many fusions in cDNA libraries are non-functional due to low stability and incomplete or incorrect folding, but it is unlikely that all the interactors of β -dystroglycan were non-functional. It is more feasible that insufficient colonies were screened. Moreover, interactions mediated by phosphorylation can not be detected by the yeast two-hybrid assay unless the relevant tyrosine kinase is present in the yeast strain or is co-expressed. Therefore SH2-mediated interactions which require phosphorylated tyrosines in the ligand cannot be investigated. Involvement of β -dystroglycan itself in SH2-mediated interactions has not been demonstrated, but it may be indirectly involved due to SH2-mediated interactions in focal adhesion assembly/disassembly and other cell-adhesion induced signalling pathways. Alternatively, β -dystroglycan could be involved in other interactions (possibly WW-mediated), positively regulated by phosphorylation.

4.7.8. Candidate interactor of β -dystroglycan : Actin

Actin α 1 was identified in the yeast two-hybrid assay as a potential interactor of β -dystroglycan. In further biochemical assays, it was found that β -dystroglycan bound F-actin and increased its viscosity, thus substantiating the idea that β -dystroglycan interacts with actin.

If β -dystroglycan was bundling or crosslinking F-actin, one would expect it to cause F-actin to form supermolecular aggregates which sediment in low speed centrifugation, which is what was observed (figure 4.14A). In addition, β -dystroglycan significantly co-sedimented with F-actin during the high speed co-sedimentation assay. F-actin was pelleted as expected, and β -dystroglycan moved from the supernatant to the pellet, indicating that it interacts with F-actin (figure 4.14B). Subsequently, actin bundles were observed by electron microscopy of β -dystroglycan and F-actin (figure 4.16). This finding corroborates the results of the biochemical co-sedimentation assays and the falling ball assay, and provides direct evidence that β -dystroglycan bundles actin.

Bundles and meshworks of actin filaments form structures which act as a scaffold in cells. These scaffolds are involved in anchoring organelles and protein synthesis machinery in the cell, or supporting the plasma membrane or attaching cells to the ECM or to other cells. Large and small crosslinking proteins are thought to share similar actin-binding motifs despite causing actin to form different types of structures (reviewed in Matsudaira, 1991). Generally speaking, large flexible proteins crosslink actin filaments into networks or gels e.g. filamin (forms dimer of 240kDa) or spectrin (forms a tetramer of 920kDa) and small crosslinking proteins are more likely to pack actin filaments tightly into bundles e.g. EF-1 α (30kDa), fimbrin (68kDa) and possibly β -dystroglycan cytoplasmic domain (13kDa).

Actin-crosslinking proteins must have at least two actin-binding sites, or a single actin-binding site and act as a dimer. At this stage it is not clear where β -dystroglycan's actin-binding motif(s) are, although it does contain the actin-binding motif KLTL in the N-terminal region of the cytoplasmic domain, which it shares with ABS2 in dystrophin and utrophin. NMR studies predict that β -dystroglycan does not form dimers (J. Bramham & S. J. Winder, personal communication) which makes it unlikely that β -

dystroglycan only has one actin-binding domain and acts as a dimer to bundle actin.

There are two types of actin bundles. The first supports the finger-like shape of membrane projections such as microvilli, stereocilia and filopodia and sheet-like membrane extensions such as lamellipodia e.g. crosslinking by fimbrin, EF-1 α , villin. The second type of actin-bundle is characterised by its muscle sarcomere-like organisation and composition. Filaments in these bundles are crosslinked by proteins such as α -actinin and form stress fibres, the contractile belt and contractile ring. The bundles are anchored to specialised adhesion sites of the plasma cell membrane, for example, focal adhesion sites or immediate junctions. It is not apparent what type of actin bundles β -dystroglycan induces actin to form but the transfection of full length GFP-tagged dystroglycan into REF52 fibroblast cells causes the cells to produce prominent actin-rich extensions known as filopodia (Yun-Ju Chen & S. J. Winder, personal communication). The ability of β -dystroglycan to stimulate the production of filopodia is abolished if the intracellular domain of β -dystroglycan is removed from the GFP-construct. This indicates that β -dystroglycan causes actin to form the first type of bundles. However, β -dystroglycan's role as a cell adhesion molecule implies that it may be involved in focal adhesion assembly/disassembly. Therefore, one might expect β -dystroglycan to crosslink actin into the second type of bundles. Whatever the exact function, β -dystroglycan's association with actin implicate it as an important mediator in the control of the cytoskeleton.

In contrast to β -dystroglycan, dystrophin does not display actin filament bundling or crosslinking properties. In dystrophin/F-actin co-sedimentation assays, the DAPC was unable to effect the low speed sedimentation of F-actin, demonstrating that F-actin was not aggregated into supermolecular aggregates (Rybakova *et al.*, 1996). In addition,

electron microscopy studies of F-actin and the purified DAPC exhibited no bundling of F-actin. However, the significant amount of the DAPC in the pellet with actin after high speed centrifugation confirms that dystrophin does *bind* F-actin (Rybakova *et al.*, 1996). Dystrophin's inability to bind G-actin suggests that dystrophin is not involved in actin polymerisation kinetics, and does not appear to crosslink or bundle F-actin either. Therefore it is conceivable that it has a purely structural role (Winder *et al.*, 1995). However, it appears that dystrophin slows down the depolymerisation of actin filaments (Rybakova *et al.*, 1996). One hypothesis is that dystrophin binds along actin filaments, interacting with multiple actin monomers in a manner similar to other actin side-binding proteins (e.g tropomyosin). Therefore, dystrophin could prevent or retard the dissociation of actin monomers from the ends of the filaments to which it is bound, thereby stabilising the actin filaments (Rybakova & Ervasti, 1997). Further experiments are needed to find out what effect β -dystroglycan has on actin polymerisation kinetics, and it would be interesting to find out what the relationship is between dystrophin, β -dystroglycan and actin, i.e. do dystrophin and β -dystroglycan bind competitively or synergistically with actin?

Identifying actin as a potential interactor of β -dystroglycan in this two-hybrid assay, has significant implications for our understanding of the roles of the DAPC. It will be interesting to see what effect dystroglycan has on the actin dynamics in muscle cells, and whether it is the β -dystroglycan which is a member of the DAPC which crosslinks F-actin. β -dystroglycan could be interacting with actin in two ways, either as part of a multifaceted DAPC-actin interaction, or it could be a separate pool of β -dystroglycan interacting with actin, which is not part of the DAPC. The concept of β -dystroglycan interacting with actin has not been previously considered and it raises exciting possibilities for the role of β -dystroglycan as a cell adhesion molecule.

CHAPTER 5
**Investigating the phosphorylation of β -
dystroglycan and its effect on the β -
dystroglycan-dystrophin interaction**

Investigating the phosphorylation of β -dystroglycan and its effect on the β -dystroglycan-dystrophin interaction

5.1. Introduction

More and more evidence is emerging which demonstrates that the DAPC is a dynamic complex, interacting with signalling proteins (section 1.9.4) and potentially mediating signals between the outside and inside of cells. Therefore, the concept of the DAPC as a purely static structural support in muscle cells is gradually being challenged.

Dystrophin, dystrobrevin, α - and γ -sarcoglycans have already been identified as phosphoproteins. It is possible that more DAPC proteins are phosphorylated and one of the aims of this project was to identify novel phosphorylation events and elucidate the role of these phosphorylation events in the regulation of interactions within the DAPC.

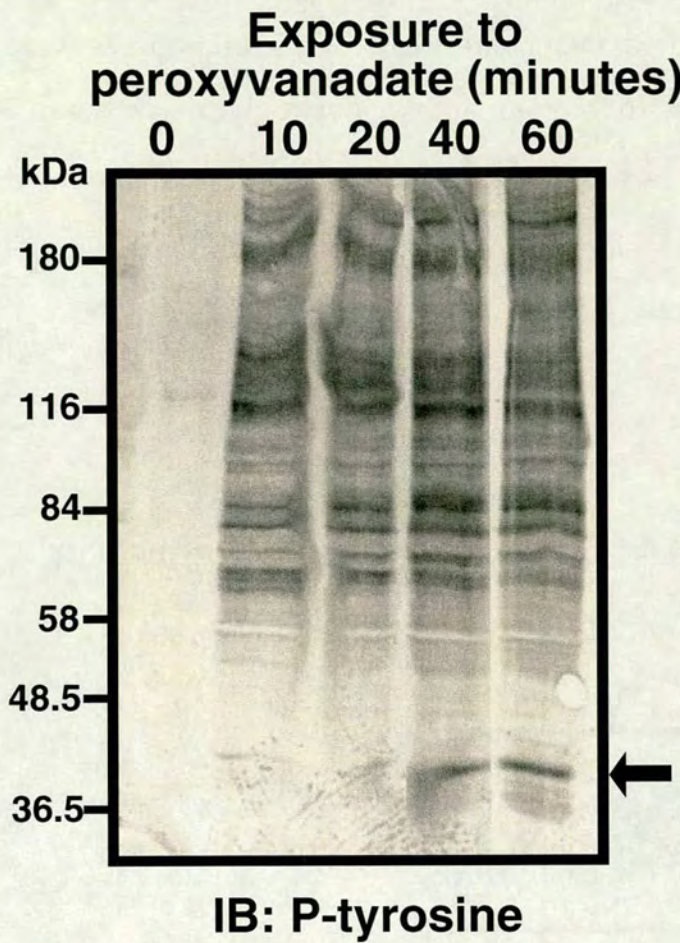
It became clear during β -dystroglycan immunoprecipitation assays that it is possible to maintain the otherwise transient phosphorylation of β -dystroglycan using the phosphatase inhibitor peroxyvanadate (section 3.5, James *et al.*, 2000). Considering that β -dystroglycan forms a link between dystrophin and the ECM, it was of great interest to identify whether its phosphorylation had any effect on protein:protein interactions within the DAPC, and in particular, the dystrophin- β -dystroglycan interaction.

This chapter incorporates investigations into the tyrosine phosphorylation of β -dystroglycan in muscle cells. In addition, a functional relevance to this phosphorylation was identified: experiments showing that phosphorylation of β -dystroglycan is important in the regulation of the dystrophin- β -dystroglycan interaction are also described in this chapter.

5.2. Treatment of C2/C4 cells with peroxyvanadate

Peroxyvanadate is a potent tyrosine phosphatase inhibitor which was used to capture phosphorylation events in C2/C4 cells. This enables the visualisation of phosphorylated DAPC proteins by western blotting with antibodies directed against phosphorylated residues. In a timecourse of exposure of myotubes to peroxyvanadate, myotubes were treated with peroxyvanadate and lysed at intervals (section 2.3.5). The resultant extracts were western blotted with the phospho-tyrosine antibody PY20 (figure 5.1). No proteins were recognised by PY20 in the untreated extracts but many proteins were phosphorylated during the first 10 minutes of peroxyvanadate treatment. A protein running at approximately the same electrophoretic mobility as β -dystroglycan was phosphorylated after approximately 40 minutes of exposure to peroxyvanadate. The phosphorylation of some proteins decreases after 40 minutes and after an exposure of 1 hour, cells round up and detach from the tissue culture flask, presumably due to the loss of cellular adhesion. Taking all this into consideration, in the proceeding experiments, cells were routinely treated for 45 minutes with peroxyvanadate in order to analyse phosphorylation events.

During immunoprecipitation experiments, it was noticed that anti-phosphotyrosine antibodies detected a protein running at the same electrophoretic mobility as β -dystroglycan in β -dystroglycan immunoprecipitates (section 3.5).

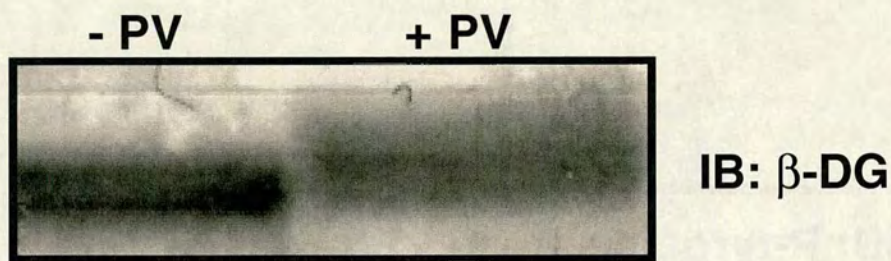
Figure 5.1**Timecourse of exposure of C2/C4 myotubes to peroxyvanadate.**

Myotubes were exposed to peroxyvanadate and lysed at intervals. The resultant extracts were western blotted (IB) with the phospho-tyrosine antibody PY20. No proteins were recognised by PY20 in the untreated extracts (0 minutes) but most proteins were phosphorylated by 10 minutes of peroxyvanadate treatment. However a protein running at approximately the same electrophoretic mobility as β -dystroglycan (shown by arrow) was not phosphorylated until 40 minutes of exposure to peroxyvanadate. The phosphorylation of some proteins decreased after 40 minutes (see 60 minutes lane).

It has been previously found that peroxyvanadate treatment of HeLa cells causes a retardation in electrophoretic mobility of β -dystroglycan when it is visualised by western blotting of SDS-PAGE gels (James *et al.*, 2000). In this study, when extracts of peroxyvanadate treated C2/C4 myotubes were run on SDS-PAGE and western blotted with β -dystroglycan antisera, a significant upward shift of β -dystroglycan was observed in the treated cells due to a retardation in electrophoretic mobility compared to β -dystroglycan in non-treated cells (Figure 5.2).

Figure 5.2

Peroxyvanadate treatment of β -dystroglycan causes a mobility shift.



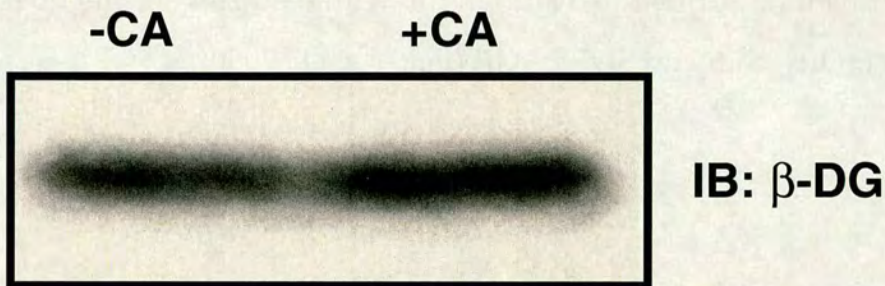
Peroxyvanadate treated (+PV) and untreated (-PV) myotube extracts western blotted (IB) with MANDAG2 (β -dystroglycan monoclonal antibody). An upward mobility shift of β -dystroglycan was observed in peroxyvanadate treated myotube extracts compared to β -dystroglycan from untreated extracts. This is indicative of a phosphorylation event.

5.3. Tyrosine phosphorylation β -dystroglycan

Mobility shifts are often indicative of a post-translational modification of a protein, such as a phosphorylation event. Treatment of the myotubes with calyculin A (section 2.3.6) (an inhibitor of type 1 and 2A serine/threonine phosphatases (Ishihara *et al.*, 1989)) did not cause any retardation in electrophoretic mobility of β -dystroglycan (figure 5.3). This suggests that if the modification is due to a phosphorylation event, it is caused by the phosphorylation of tyrosine residues rather than serine or threonine residues.

Figure 5.3

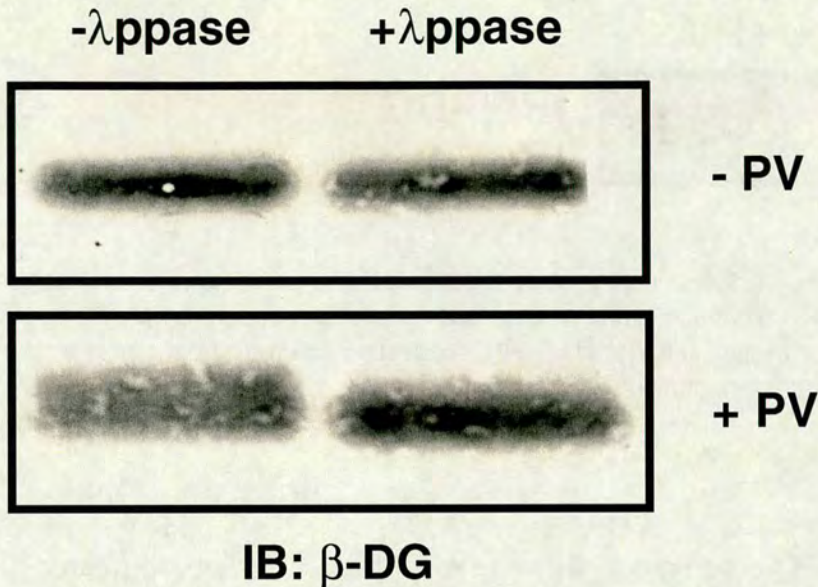
Calyculin A treatment of β -dystroglycan does not cause a mobility shift.



Calyculin A treated (+CA) and untreated (-CA) myotube extracts western blotted (IB) with MANDAG2 (β -dystroglycan monoclonal antibody).

Treatment of myotubes with Calyculin A did not cause any retardation in electrophoretic mobility. i.e β -dystroglycan from untreated extracts looked identical to β -dystroglycan from treated extracts. This suggests that the mobility shift of β -dystroglycan caused by treatment with peroxyvanadate is not due to a serine or threonine phosphorylation event.

To confirm further that the electrophoretic mobility shift was due to phosphorylation of β -dystroglycan, lambda phosphatase (λ ppase), a serine, threonine and tyrosine phosphatase, was used (section 2.4.3). When the β -dystroglycan immunoprecipitates from peroxyvanadate-treated cell extracts were treated with λ ppase, the electrophoretic mobility of β -dystroglycan was reduced to its previous levels i.e. equivalent to β -dystroglycan from untreated cells (figure 5.4). This strongly suggests that the mobility shift of β -dystroglycan in peroxyvanadate treated myotubes is due to a phosphorylation event. Peroxyvanadate has previously been shown to directly modify thiol groups in proteins (Mikalsen & Kaalhus, 1998). The reversal of the mobility shift of β -dystroglycan by λ ppase indicates that the modification of β -dystroglycan during peroxyvanadate treatment is caused by phosphorylation events rather than by modification of thiol groups. Furthermore, it has been previously shown that direct treatment of purified β -dystroglycan with oxidising agents does not result in mobility shifts on SDS-PAGE (James *et al.*, 2000).

Figure 5.4. **λ ppase abolishes the mobility shift of β -dystroglycan caused by peroxyvanadate treatment**

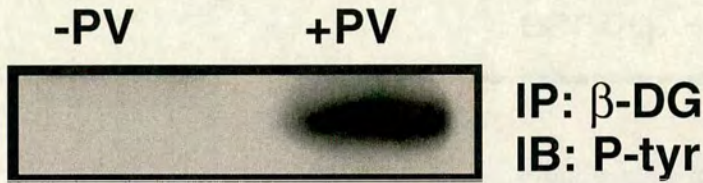
β -dystroglycan immunoprecipitates from peroxyvanadate treated (+PV) or untreated (-PV) myotube extracts were treated with λ ppase, and blotted (IB) with β -dystroglycan monoclonal antibody MANDAG2.

In peroxyvanadate untreated extracts, λ ppase has no effect on the electrophoretic mobility of β -dystroglycan, whereas in peroxyvanadate treated extracts, the mobility shift of β -dystroglycan is abolished by λ ppase, suggesting that it is caused by phosphorylation of β -dystroglycan.

To establish that this phosphorylation event was in fact tyrosine phosphorylation of β -dystroglycan, β -dystroglycan immunoprecipitates were western blotted with anti-phosphotyrosine antisera (figure 5.5). β -dystroglycan was detected by anti-phosphotyrosine antisera but only in extracts from peroxyvanadate treated myotubes. Therefore, it can be concluded from this that β -dystroglycan is tyrosine phosphorylated in peroxyvanadate treated myoblasts and myotubes.

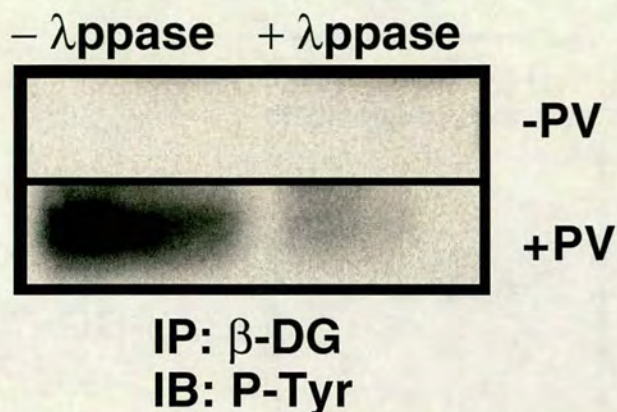
Figure 5.5

β -dystroglycan is recognised by anti-phosphotyrosine antibodies in peroxyvanadate treated myotube extracts.



β -dystroglycan immunoprecipitates (IP) blotted (IB) with anti-phosphotyrosine antibody PY20. PY20 recognises a band migrating at the same molecular weight as β -dystroglycan only in peroxyvanadate treated cell extracts (+PV), suggesting that β -dystroglycan is tyrosine phosphorylated under these conditions.

This was further confirmed by western blotting λ ppase-treated β -dystroglycan immunoprecipitates with anti-phosphotyrosine antisera. After λ ppase treatment, β -dystroglycan immunoprecipitated from peroxyvanadate treated cell extracts is no longer detected by anti-phosphotyrosine antibodies (figure 5.6). Therefore, when the mobility shift of β -dystroglycan is abolished by λ ppase treatment, β -dystroglycan is no longer tyrosine phosphorylated and is undetectable by anti-phosphotyrosine antibodies.

Figure 5.6.**Detection of β -dystroglycan by anti-phosphotyrosine antibodies is abolished by λ ppase treatment.**

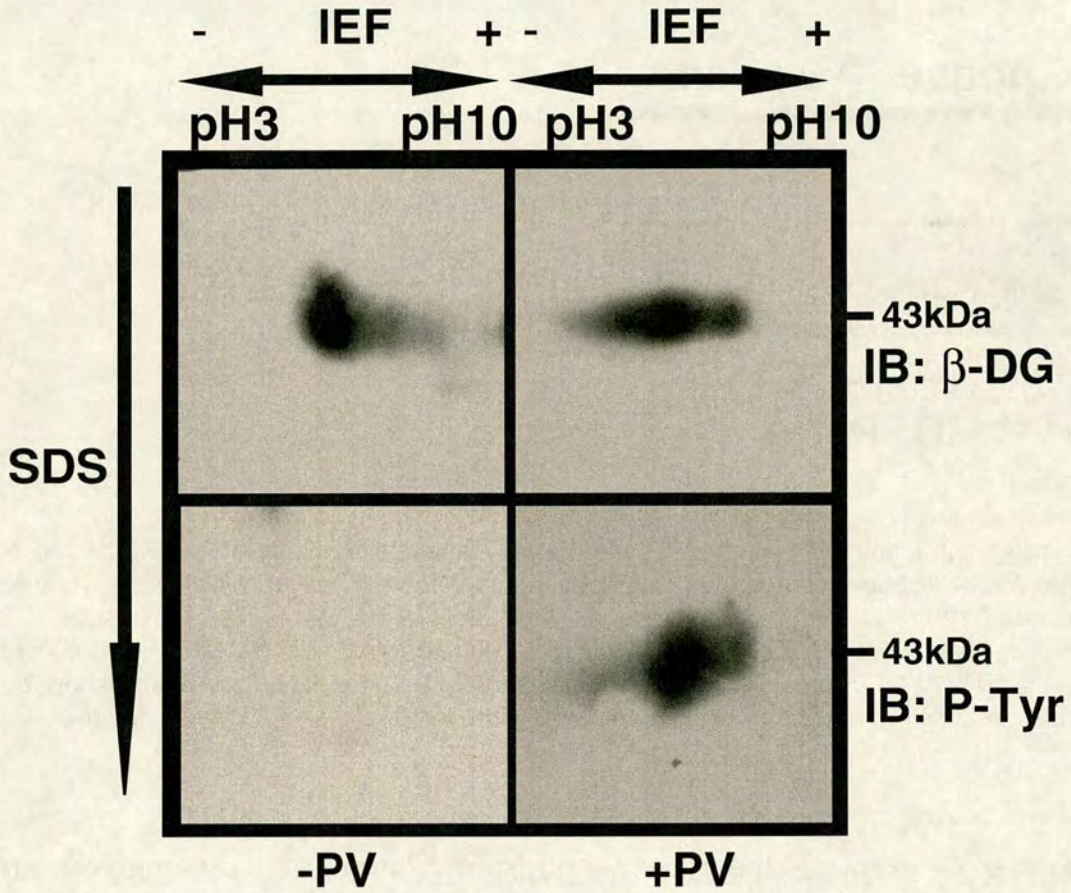
β -dystroglycan immunoprecipitates (IP) from peroxyvanadate treated (+PV) or untreated (-PV) myotube extracts were treated with λ ppase and blotted (IB) with anti-phosphotyrosine antibody PY20.

β -dystroglycan is not detected in peroxyvanadate untreated extracts. β -dystroglycan is only detected by PY20 in peroxyvanadate treated extracts but this detection is abolished by λ ppase treatment, presumably as β -dystroglycan is no longer tyrosine phosphorylated.

In order to examine the tyrosine phosphorylation of β -dystroglycan in more detail, two-dimensional gel electrophoresis was carried out on immunoprecipitates of β -dystroglycan from peroxyvanadate treated and untreated myoblast and myotube extracts (section 2.4.8). The immunoprecipitates were separated by isoelectric focusing, followed by SDS-PAGE, and western blotted with β -dystroglycan antibodies. In myotube extracts from untreated cells, β -dystroglycan appears as a single spot, whereas in peroxyvanadate treated cells, β -dystroglycan appears as two or possibly three spots, indicative of additional charged species, likely to be the result of phosphorylation (figure 5.7, upper panels).

Figure 5.7

Two-dimensional gel electrophoresis of β -dystroglycan from peroxyvanadate treated myotubes.



β -dystroglycan from untreated (-PV) or peroxyvanadate treated (+PV) myotube extracts was immunoprecipitated using 1710 polyclonal antibody, separated by isoelectric focusing (IEF) followed by SDS-PAGE and blotted (IB) with either β -dystroglycan monoclonal antibody MANDAG2 or phospho-tyrosine antibodies (P-Tyr).

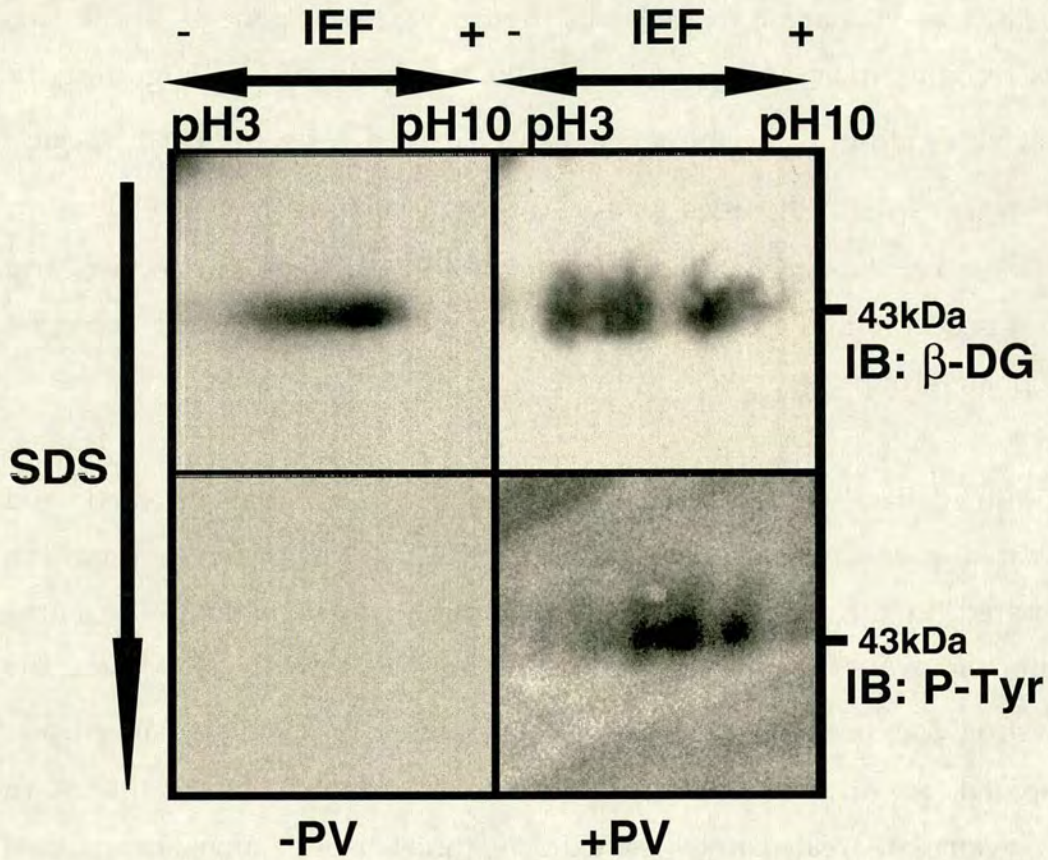
MANDAG2 recognises two or possibly three spots of β -dystroglycan as an ellipse in peroxyvanadate treated extracts (upper right panel) compared to one clear spot in untreated extracts (upper left panel). The phospho-tyrosine antisera detects two poorly defined spots migrating with the same mobility as the two most negatively charged β -dystroglycan species, but only in peroxyvanadate treated myotube extracts (lower right panel). No species are detected by the phospho-tyrosine antisera at this electrophoretic mobility in untreated extracts (lower left panel).

When β -dystroglycan immunoprecipitates separated by two-dimensional electrophoresis were blotted with anti-phosphotyrosine antibodies, two spots migrating in the same position as β -dystroglycan were detected, but only in peroxyvanadate treated cells (figure 5.7, lower panels). These two spots recognised by anti-phosphotyrosine antibodies were migrating in the same location as the most negatively charged β -dystroglycan species. This is consistent the idea that β -dystroglycan is phosphorylated on tyrosines: i.e. it is recognised by anti-phosphotyrosine antibodies, and migrates nearer to the cathode due to the addition of negatively charged phosphate groups.

Two-dimensional gel electrophoresis of peroxyvanadate treated and untreated myoblast extracts produced a slightly different spot pattern compared to the myotube extracts, although approximately the same phenomena were observed (figure 5.8). In untreated myoblast extracts, the β -dystroglycan antibody detected one or possibly two spots as an ellipse, compared to one clear spot in myotube extracts. In addition, in peroxyvanadate treated myoblast extracts, three distinct spots are detected by the β -dystroglycan antibody and the anti-phosphotyrosine antibody (compared to two or possibly three spots in myotube extracts). Therefore, as in the myotube extracts, there were more β -dystroglycan species in peroxyvanadate treated myoblast extracts compared to the untreated, presumably due to phosphorylation events. However, there is possibly one extra phosphorylated species in myoblast extracts compared to myotube extracts.

Figure 5.8

Two-dimensional gel electrophoresis of β -dystroglycan from peroxyvanadate treated myoblasts



β -dystroglycan from untreated (-PV) or peroxyvanadate treated (+PV) myoblast extracts was immunoprecipitated using 1710 polyclonal antibody, separated by isoelectric focusing (IEF) followed by SDS-PAGE and blotted (IB) with either β -dystroglycan monoclonal antibody MANDAG2 or phospho-tyrosine antibody PY20 (P-Tyr). MANDAG2 recognises three spots of β -dystroglycan in peroxyvanadate treated extracts (upper right panel) compared to one or possibly two spots (as an ellipse) in untreated extracts (upper left panel). The phospho-tyrosine antisera also detects three spots migrating with the same mobility as β -dystroglycan in peroxyvanadate treated myoblast extracts (lower right panel), but detects no β -dystroglycan in the untreated extracts (lower left panel).

5.4. Does the phosphorylation of β -dystroglycan affect its interaction with dystrophin?

The identification of the phosphorylation of β -dystroglycan on tyrosine residues raised the question of whether this phosphorylation is involved in the regulation of β -dystroglycan's protein:protein interactions. β -dystroglycan has been shown to interact with dystrophin, Grb2, rapsyn, caveolin-3 (sections 1.6.1. & 1.6.4) and three unidentified phosphoproteins (section 3.5). The dystrophin- β -dystroglycan interaction is the best characterised of these interactions, and is important for the stability of the DAPC (Rafael *et al.*, 1996) and was therefore studied further.

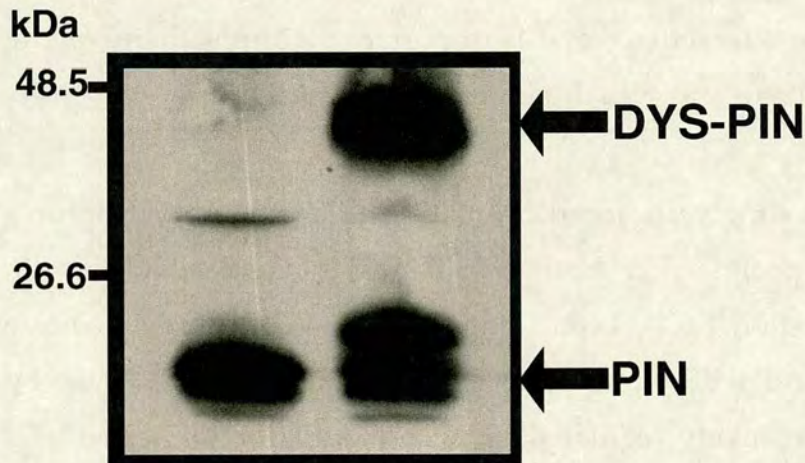
The dystrophin- β -dystroglycan interaction is mediated by dystrophin's WW domain and a proline-rich motif (PPxY) in the cytoplasmic domain of β -dystroglycan (section 1.6.1). Type 1 and Type IV WW domains have been shown to be regulated by phosphorylation events (table 1.1). Type IV WW domains are positively regulated by phosphorylation of serine and threonine residues in their ligands (Lu *et al.*, 1999; Ranganathan *et al.*, 1997) and type I WW domains are negatively regulated by phosphorylation of tyrosine in the PPxY motif of their ligands (Chen *et al.*, 1997; James *et al.*, 2000). In the work of James *et al.* (2000), it was demonstrated that the interaction between β -dystroglycan and utrophin is regulated by phosphorylation. Consequently, it was highly plausible that the interaction between β -dystroglycan and dystrophin is also regulated by phosphorylation. This could be highly significant for understanding more about the general functions of, and the regulation of interactions within, the DAPC.

To elucidate whether the tyrosine phosphorylation of β -dystroglycan affects its interaction with dystrophin, 'SPOTs' membrane binding assays were used. Peptide 'SPOT' blots of the β -dystroglycan cytoplasmic domain

were probed with purified dystrophin fusion protein comprising the WW, EF hands and ZZ domains fused to a biotin tag (DYS-PIN) (section 2.4.10). The purified DYS-PIN fusion protein and the purified biotin tag (used as a control) are shown in figure 5.9.

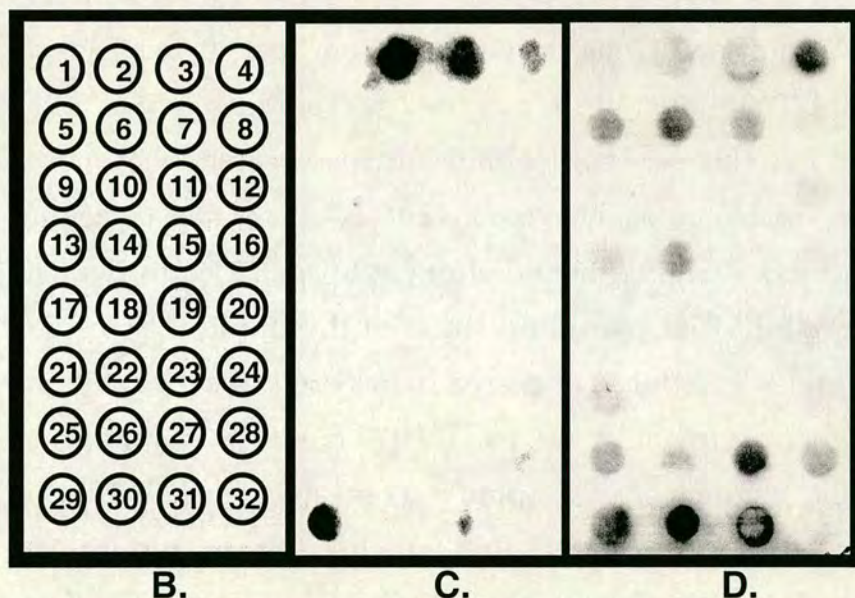
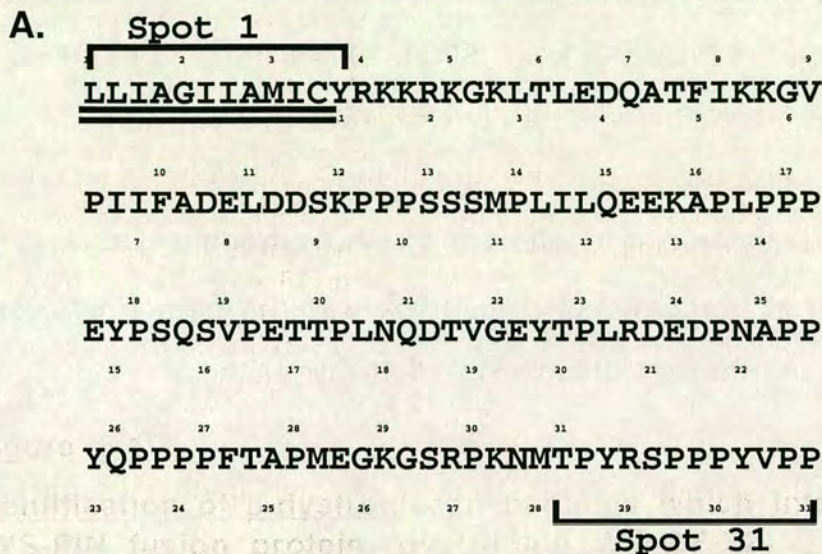
Figure 5.9

Expression of dystrophin-biotin fusion protein (DYS-PIN).



A construct of dystrophin was made comprising amino-acids 3055-3354, (encompassing the WW, EF hand and ZZ domains) and cloned into the PinPoint Xa-3 vector. This construct and the vector alone were bacterially expressed to produce a biotin tag (PIN 13kDa) and the DYS-PIN fusion protein (37.6kDa), which were purified using avidin resin. The purified proteins were western blotted with ExtraAvidin-HRP and developed with ECL. The biotin tag alone is shown in the left lane and DYS-PIN fusion in the right lane. The large PIN band in the right lane is as a result of proteolytic degradation of the fusion protein.

Initially, the interaction site between β -dystroglycan and dystrophin was confirmed by probing the cytoplasmic domain of β -dystroglycan with DYS-PIN fusion protein. A β -dystroglycan SPOT blot comprising the entire cytoplasmic domain of β -dystroglycan as 12 overlapping amino-acid peptides, offset by 4 amino-acids, was used (as shown in figures 5.10A and 5.10B).

Figure 5.10**Identification of β -dystroglycan peptides which interact with
DYS-PIN fusion protein (dystrophin WW-EF-ZZ)**

A. Scheme for the synthesis of peptides on the 'SPOTS' membrane. Twelve amino-acid long peptides representing part of the transmembrane region and the entire cytoplasmic domain of human β -dystroglycan (amino-acids 764-895) were synthesised on derivatised 'SPOTS' membranes with an offset of 4 amino-acids. The transmembrane region is denoted by a double underline. Numbers above and below the β -dystroglycan sequence denote the first and last amino-acid of the peptide, respectively, found on the corresponding spot. Brackets identify the sequences of spots 1 and 31. Spot 32 is a negative control where no peptide is synthesised. **B.** Numbered circles identify the location of each spot on the membrane. **C.** Control 'SPOTS' membrane described as above probed with PIN protein alone, and detected by ECL. The PIN protein bound weakly to spots 2, 3, and 29. **D.** 'SPOTS' membrane described as above probed with dystrophin (DYS-PIN) fusion protein, and detected by ECL. The DYS-PIN fusion bound strongly to spots 30 (PKNMTPYRSPPP) and 31 (TPYRSPPPYVPP) which contains a recognised WW-binding motif.

DYS-PIN bound strongly to spots 30 and 31 and weakly to spots 4, 27 and 29. Some non-specific binding to spots 2, 3, 29 and to a lesser extent spot 4 was revealed in a control experiment, in which the biotin tag alone (PIN) was incubated with the β -dystroglycan 'SPOTs'. From this it was inferred that DYS-PIN was interacting specifically with spots 4, 27, 30 and 31. The strongest binding to spots 30 and 31 is equivalent to the carboxyl terminal 16 amino-acids of β -dystroglycan interacting with dystrophin. This region of β -dystroglycan includes the PPxY motif which has been previously identified as a ligand for dystrophin's WW domain.

To ascertain whether the phosphorylation of residues in this 16 amino-acid region of β -dystroglycan affects its interaction with dystrophin, a separate SPOT blot comprising the peptides from spot 30 and 31 as a single peptide (KNMTPYRSPPPYVPP) was used for binding assays with DYS-PIN (figure 5.11). This series of peptides had sequential phospho amino-acids at all possible phosphorylation sites (i.e. serine, threonine and tyrosine residues). DYS-PIN bound strongly to spot 1, equivalent to spots 30/31 in figure 5.10. The phosphorylation of threonine⁸⁸⁴, tyrosine⁸⁸⁶ and serine⁸⁸⁸ (peptides 2 3, and 4) appeared to have no affect on this binding. However, when tyrosine⁸⁹² in the PPPY motif was phosphorylated (peptide 5) the binding of dystrophin was greatly reduced. This binding was further reduced when both tyrosine⁸⁸⁶ and tyrosine⁸⁹² were phosphorylated in peptide 6. Interestingly, in peptide 4 where only tyrosine⁸⁸⁶ is phosphorylated, binding of dystrophin is not affected, indicating that the phosphorylation of tyrosine⁸⁸⁶ is necessary but not sufficient, to completely abolish the interaction. These data therefore support the hypothesis that phosphorylation of β -dystroglycan on Tyr⁸⁹² is able to regulate β -dystroglycan's interaction with the C-terminus of dystrophin.

Figure 5.11**Phosphorylation of tyrosines in β -dystroglycan disrupt its interaction with dystrophin**

1. KNMTPYRSPPPYVPP
2. KNM_pTPYRSPPPYVPP
3. KNMTP_pYRSPPPYVPP
4. KNMTPYR_pSPPPYVPP
5. KNMTPYRSPPP_pYVPP
6. KNMTP_pYRSPPP_pYVPP
7. KNM_pTPYR_pSPPPYVPP
8. KNM_pTP_pYR_pSPPP_pYVPP

A 15 amino-acid peptide equivalent to spots 30/31 of β -dystroglycan cytoplasmic domain was synthesised containing the phospho-amino-acids pS, pT and pY at each possible position in the peptide, either singly or in multiples. The membrane was probed with dystrophin fusion protein (DYS-PIN). The binding of DYS-PIN was greatly reduced by phosphorylation of tyrosine⁶⁹² in the PPPY motif (peptide 5). This binding was further reduced when both tyrosine⁶⁸⁶ and tyrosine⁶⁹² were phosphorylated (peptides 6 and 8), but was not affected by serine/threonine phosphorylation (peptide 7).

5.7. Discussion

5.7.1. β -dystroglycan is tyrosine phosphorylated in muscle cells

Dystrophin and the DAPC, initially assumed to be proteins whose primary role is structural, have more recently been implicated in signalling events (section 1.9). Further studies have led to the reasoning the DAPC can actually be thought of as a dynamic complex, and spatially, it is ideally placed to transduce signals from the extracellular matrix to the inside of cells. Over the past few years there has been a growing body of evidence to suggest that the DAPC is involved in signalling pathways, predominantly the demonstration of the number of phosphoproteins in the DAPC. The phosphorylation of dystrophin on serine and threonine has been previously described (reviewed in (Michalak *et al.*, 1996; Winder, 1997)), dystrobrevin was originally identified as a phosphoprotein (Wagner *et al.*, 1993), and analysis of dystrophin and the DAPC using two-dimensional gel electrophoresis suggests that dystrophin, β -dystroglycan and potentially other components of the DAPC are phosphorylated (Yamamoto *et al.*, 1993). More recently, it has been demonstrated that α - and γ -sarcoglycans are phosphorylated and that β -dystroglycan is phosphorylated in non-muscle cells (James *et al.*, 2000). In this study, it has been definitively shown that β -dystroglycan is phosphorylated in muscle cells and this phosphorylation has an affect on protein:protein interactions within the DAPC.

This study describes the phosphorylation of β -dystroglycan on tyrosine residues in C2/C4 myotubes treated with peroxyvanadate. This was initially identified by mobility shifts on SDS-PAGE gels of β -dystroglycan from peroxyvanadate-treated myotube extracts (figure 5.2). It was subsequently demonstrated that β -dystroglycan from peroxyvanadate-treated myotube extracts is recognised by anti-phosphotyrosine antibodies

on both one and two-dimensional gels (figures 5.5, 5.7 & 5.8), and this recognition is abolished by lambda phosphatase treatment (figure 5.6).

It is curious that in the two-dimensional gel analysis, β -dystroglycan from myoblasts and myotubes exhibited different mobility characteristics. The presence of two species recognised by β -dystroglycan antibodies in untreated myoblasts (figure 5.8) suggests that there is a phosphorylated species caused by a peroxyvanadate-independent phosphorylation event. However, neither of these β -dystroglycan species were detected by anti-phosphotyrosine antibodies. Therefore, if one of these species is a phosphorylated form of β -dystroglycan, it must be phosphorylated on serines or threonines. It is also plausible that the presence of two β -dystroglycan species is due to different kind of post-translational modification e.g. differential glycosylation.

In addition, three phosphorylated species were detected by anti-phosphotyrosine antibodies in myoblasts compared to two in myotubes. It is possible that β -dystroglycan is more highly phosphorylated in myoblasts compared to myotubes. It is not unexpected that differentiated and undifferentiated cells have slightly different characteristics due to their specialised roles. For example, myoblasts are more motile than myotubes and may require more phosphorylation states.

5.7.2. Phosphorylation of β -dystroglycan disrupts its interaction with dystrophin

In order to characterise the dystrophin- β -dystroglycan interaction more fully, 'SPOT' blots comprising peptides of β -dystroglycan's cytoplasmic domain were probed with the DYS-PIN fusion protein. It was demonstrated that DYS-PIN bound to spots 4, 27, 30 and 31. The interactions with spots 30 and 31 were the strongest.

Spots 30 and 31 comprise the 16 most C-terminal amino-acids of β -dystroglycan. The amino-acid sequence of these peptides were subsequently used in the binding assay with phosphorylated residues (figure 5.11). This region of β -dystroglycan has been identified previously as being necessary for binding to dystrophin (Jung *et al.*, 1995) and Spot 31 (TPYRSPPPYVPP) contains the recognised WW-binding motif PPxY (Rentschler *et al.*, 1999). In addition, a dystrophin-GST fusion has also been observed to bind a similar peptide (Rentschler *et al.*, 1999) and James *et al.* (2000) showed that a utrophin-MBP (Maltose Binding Protein) fusion also bound the same sequence (James *et al.*, 2000). DYS-PIN bound strongly to the peptide (PKNMTPYRSPPP) (spot 30), although it does not include the whole PPxY motif. It is possible that the strong binding occurs because spot 30 overlaps with spot 31 and contains residues involved in the association of β -dystroglycan with the EF hand region of dystrophin (Huang *et al.*, 2000). Previous experiments with a dystrophin-GST fusion demonstrated strong binding to spot 31 only (Rentschler *et al.*, 1999). The finding that spot 30 is also bound may be due to differences in the limits of the constructs used (dystrophin residues 3055-3354 (this study) versus residues 3046-3447 (Rentschler *et al.*, 1999)).

It is notable that the putative WW-domain interacting motif PPEY in β -dystroglycan's cytoplasmic domain was not a substrate for DYS-PIN binding (figure 5.10). This motif is contained in peptides 15, 16 and 18 which have no signal after incubation with the DYS-PIN fusion protein. Therefore, this excludes the role of this motif in binding WW domains.

It was demonstrated that the phosphorylation of tyrosine residues within, and adjacent to β -dystroglycan's WW domain disrupt its interaction with dystrophin (figure 5.11). Although the WW-mediated interaction is necessary for the dystrophin- β -dystroglycan interaction, dystrophin's EF

hands and ZZ domain are also involved. It is likely that they have a more stabilising role, holding the two proteins in the correct configuration. It is probable that the WW domain is the main site by which the interaction is regulated, but the EF hands are also essential for the interaction (Rentschler *et al.*, 1999). In addition, from the recent crystal structure of dystrophin WW-EF hands region complexed with a β -dystroglycan peptide (Huang *et al.*, 2000), it is clear that the β -dystroglycan peptide binds a composite surface formed by both the WW domains and one of the two EF hands (figure 5.12).

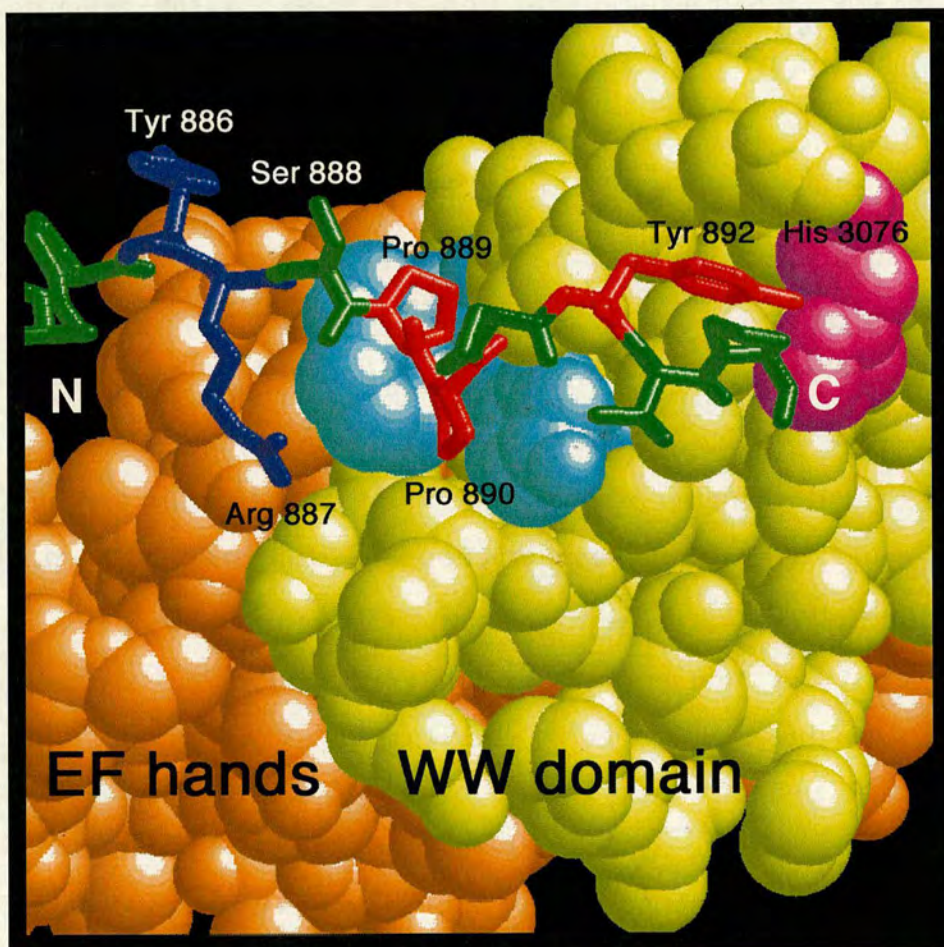
The crystal structure of dystrophin WW-EF hands region complexed with a β -dystroglycan peptide (Huang *et al.*, 2000) also strengthens the evidence for an interaction regulated by phosphorylation. The structure reveals the importance of tyrosine⁸⁹² in the interaction and from it, one can predict that phosphorylation of tyrosine⁸⁹² in the PPPY motif of β -dystroglycan would disrupt the interaction with the WW domain of dystrophin. Tyrosine⁸⁹² fits into a hydrophobic pocket of the WW domain and the interaction is co-ordinated by a hydrogen bond from the conserved histidine residue (His³⁰⁷⁶) of the WW domain. It is clear that the incorporation of a bulky phosphate group on tyrosine⁸⁹² would both disrupt a hydrogen bond and render the residue too large to fit into the WW domain pocket.

Chen *et al.* (1997) have described a similar phenomenon in the WBP-1/YAP65 interaction, in that phosphorylation of the tyrosine in the PPPY motif of WBP-1 abolishes its interaction with the WW domain of YAP65 in *in vitro* 'SPOTs' assays (Chen *et al.*, 1997). Phosphorylation of the tyrosine residue in β -dystroglycan's PPPY motif also disrupts its interaction with utrophin, as shown by 'SPOT' blot assays (James *et al.*, 2000).

Figure 5.12

The structure of dystrophin WW-EF region complexed with a β -dystroglycan peptide

(Figure adapted from Ilsley *et al.* (2001a))



Space filling representation of dystrophin's WW domain (yellow) and the region of the EF hand domain which comes into contact with β -dystroglycan (orange).

The bound β -dystroglycan peptide (amino-acids 885-893, PYRSPPYV) is represented in stick form and labelled at its N- and C-termini.

Proline⁸⁸⁹ and proline⁸⁹⁰ of the PPxY motif of β -dystroglycan insert into a concave hydrophobic surface formed by tyrosine³⁰⁷² and tryptophan³⁰⁸³ of the WW domain (space-filled, turquoise).

Tyrosine⁸⁹² also fits into a hydrophobic pocket of the WW domain and the interaction is coordinated by a hydrogen bond with Histidine³⁰⁷⁶ of dystrophin (space-filled magenta). Tyrosine⁸⁸⁶ also resides in a pocket of the dystrophin protein, but in the EF-hand region.

In addition, James *et al.* (2000) demonstrated that fusion proteins of utrophin WW, EF hand and ZZ domains failed to interact with β -dystroglycan *in vivo* in peroxyvanadate treated HeLa cells (James *et al.*, 2000).

In this study, it was also demonstrated that residues flanking the PPXY motif affect β -dystroglycan's interaction with dystrophin. The phosphorylation of tyrosine⁸⁸⁶ (i.e. spot 6, figure 6.11), which is N-terminal to the PPPY motif, affects the dystrophin- β -dystroglycan interaction. Like tyrosine⁸⁹², tyrosine⁸⁸⁶ also resides in a pocket of dystrophin protein, but in the EF hand region. Whilst phosphorylation of tyrosine⁸⁸⁶ alone is not sufficient to prevent binding, in conjunction with phosphorylation of tyrosine⁸⁹², binding of dystrophin to the β -dystroglycan peptide on the 'SPOT' blot is completely abolished.

Interestingly, in the study of the utrophin- β -dystroglycan interaction, the phosphorylation of tyrosine⁸⁸⁶ did not affect utrophin binding to β -dystroglycan, but the phosphorylation of serine⁸⁸⁸ in conjunction with tyrosine⁸⁸⁶ completely abolished it (James *et al.*, 2000). This demonstrates that there may be different mechanisms of regulation between β -dystroglycan and its binding partners, perhaps involving distinct kinases.

5.7.3. Which kinase phosphorylates β -dystroglycan?

The tyrosine kinase involved in the phosphorylation of β -dystroglycan is, as yet, unknown. Various serine/threonine kinases have been implicated in phosphorylating dystrophin, for example MAP Kinase, CaM kinase II, casein kinase II, p34^{cdc2}, protein kinase C (Luise *et al.*, 1993; Madhavan and Jarrett, 1994; Milner *et al.*, 1993; Shemanko *et al.*, 1995), (table 1.2). Importantly, the purified DAPC has been shown to have associated

endogenous serine/threonine kinase activity (Luise *et al.*, 1993; Madhavan and Jarrett, 1994) but the kinase(s) associated with the DAPC have not been identified. The tyrosine phosphorylation of α - and γ -sarcoglycans was inhibited by Herbimycin A (Yoshida *et al.*, 1998) which suggests the involvement of the Src family of kinases. However, β -dystroglycan is still receptive to phosphorylation in FAK knockout cells and Src knockout cells (James *et al.*, 2000) which excludes the possibility of these two kinases directly phosphorylating β -dystroglycan in HeLa cells. No other kinases have been tested, to date. Other candidate kinases could be proline-directed kinases such as the Cdk family, GSK-3, the MAP kinase family and p34^{cdc2}.

Sequence analysis of β -dystroglycan (PROSITE) does not provide any clues towards identifying the kinase involved either: there are no recognised tyrosine phosphorylation sites in the cytoplasmic domain, although there is one present in the extracellular domain (amino-acids 743-750). More investigations need to be carried out in order to determine the nature of the kinase involved, which in turn, may lead to the identification of signalling pathways that the DAPC is involved in.

5.7.4. Adhesion-dependent phosphorylation of β -dystroglycan?

The DAPC has previously been implicated in integrin-mediated adhesion events, in that α - and γ -sarcoglycans from myotubes were found to be phosphorylated on tyrosine residues in response to adhesion to extracellular matrix proteins fibronectin or collagen type 1 (Yoshida *et al.*, 1998). This phosphorylation was also induced by the RGDS (integrin recognition sequence) peptide, implying that the DAPC is involved in the integrin adhesion system (Yoshida *et al.*, 1998).

The tyrosine phosphorylation of β -dystroglycan in non-muscle cells is adhesion-dependent. This was first noticed due to a mobility shift on SDS-PAGE gels of β -dystroglycan from adherent HeLa cells compared to suspended HeLa cells, without the use of peroxyvanadate (James *et al.*, 2000). Additionally, phosphorylation occurred when HeLa cells were plated onto fibronectin or laminin substrates, suggesting an integrin-mediated event (James *et al.*, 2000).

The adhesion-dependent phosphorylation of β -dystroglycan was not evident in myoblast or myotube extracts in this study. C2/C4 myotubes did not survive the replating process, but myoblasts were re-plated onto gelatin, fibronectin, laminin and tissue-culture plastic alone (section 2.3.7). This was to identify if the adhesion of the myoblasts onto extracellular substrates stimulated the phosphorylation of β -dystroglycan. However, no mobility shifts of β -dystroglycan were observed on SDS-PAGE gels (data not shown), these being an indication of such a phosphorylation event. Treatment with peroxyvanadate after the replating process still caused a mobility shift of β -dystroglycan suggesting that the protein was still accessible to phosphorylation after replating. These preliminary experiments suggested that the phosphorylation of β -dystroglycan in C2/C4 cells is not stimulated by adhesion. Furthermore, Yoshida *et al.* (1998) did not identify adhesion-dependent phosphorylation of β -dystroglycan in their study (Yoshida *et al.*, 1998).

The possibility that the phosphorylation of β -dystroglycan is adhesion-dependent in non-muscle cells, but not in muscle cells, is intriguing. On a technical level, one reason for this may be that few myotubes survive trypsinisation and the subsequent maintenance in suspension. Or, this may be a genuine phenomenon caused by the static nature of the differentiated muscle cells compared to HeLa cells and/or a different role of the DAPC compared to the utrophin-associated protein complex.

However, these reasons do not explain why adhesion-dependent phosphorylation of β -dystroglycan is not observed in myoblasts either.

5.7.5 The dynamic nature of the DAPC

The dynamic nature of the β -dystroglycan/dystrophin interaction is illustrated by recent work on the β -dystroglycan/Grb2 interaction (Russo *et al.*, 2000). *In vitro* studies suggest that dystrophin and Grb2 compete in order to bind to β -dystroglycan which supports the hypothesis that the DAPC is actively turned over, but in a highly regulated manner. Disruption of this delicate balance by reduction or loss of dystrophin may explain some of the poorly understood mechanisms of disease progression in Becker and Duchenne muscular dystrophy.

In addition, it has been demonstrated that β -dystroglycan's interaction with the scaffolding protein caveolin-3 (section 1.6.4.2) is also a WW domain-mediated interaction, and dystrophin and caveolin-3 compete for the same PPxY motif on β -dystroglycan (Sotgia *et al.*, 2000).

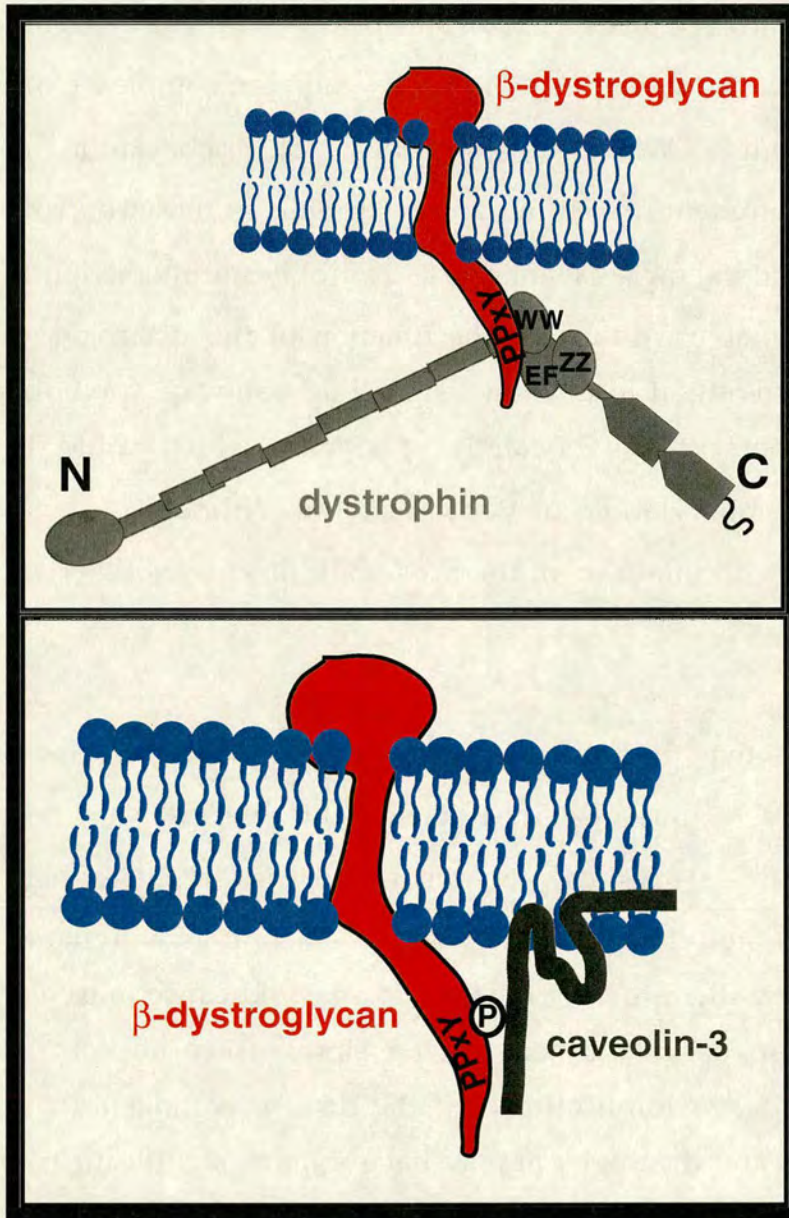
One proposed function of the caveolin-3/ β -dystroglycan interaction in healthy muscle is to competitively regulate the interaction and recruitment of dystrophin to the sarcolemma (Sotgia *et al.*, 2000). Dystrophin and caveolin co-localise at the sarcolemma, dystrophin has numerous caveolin-binding motifs and the two proteins co-immunoprecipitate. (Song *et al.*, 1996; Couet *et al.*, 1997). Therefore, caveolin-3 interacts with both β -dystroglycan and dystrophin, so could plausibly recruit them both to the sarcolemma, and keep them apart until they are assembled properly in the DAPC.

Interestingly, unlike dystrophin, caveolin-3 is able to bind phosphorylated β -dystroglycan. The WW domain of caveolin-3 is insensitive to the phosphorylation of tyrosine⁸⁹² in the PPxY motif of β -dystroglycan (Sotgia *et al.*, 2000) (figure 5.13). Therefore, the phosphorylation of β -dystroglycan may act as a switch to alter the affinity of the dystroglycan complex from dystrophin to caveolin-3. When β -dystroglycan phosphorylation is induced by the as yet unidentified stimulus, dystrophin is released from dystrophin, which would allow a caveolin-3/ β -dystroglycan interaction to take place. This presumably would alter the function of the dystroglycan complex, perhaps involving it in a distinct signalling pathway. Caveolin associates with members of the Src-family of kinases which could be involved in the phosphorylation of β -dystroglycan, although it does appear that caveolin is an inhibitor of the Src-family of kinases (Li *et al.*, 1996).

It has been suggested that disruption of the β -dystroglycan-dystrophin connection could lead to muscular dystrophy (Rentschler *et al.*, 1999). Discovering that the β -dystroglycan-dystrophin is regulated by phosphorylation (this study) suggests that this interaction is a dynamic one, and suggests that a disruption of the regulation could cause muscular dystrophy, rather than, or in addition to, the simple severing of the connection. This may have implications for the disease pathogenesis of muscular dystrophies and dystroglycan may have a more significant role than originally considered.

Figure 5.13

Dystrophin and caveolin-3 compete for the same binding site on β -dystroglycan



Dystrophin and caveolin-3 each have a WW-domain which binds to the PPXY motif in the cytoplasmic domain of β -dystroglycan. The two proteins compete for this site on β -dystroglycan. However, when β -dystroglycan is phosphorylated on the tyrosine in the PPXY motif, its binding to β -dystroglycan is abolished. Caveolin-3 is insensitive to this phosphorylation and is able to bind β -dystroglycan whether it is phosphorylated or not.

CHAPTER 6
Summary

Summary

In this thesis, work has been described which was carried out in order to understand more about the interactions of proteins within the dystrophin-associated protein complex (DAPC) and how they are regulated. The study has also involved the investigation into the interactions DAPC protein β -dystroglycan has with proteins outside of the DAPC.

The model of choice for this work was the C2/C4 mouse muscle cell line. This cell line was characterised for its use in these studies (i.e. differentiation properties, expression of DAPC proteins) and different protein extraction methods were tested to obtain the optimal conditions for extracting proteins of the DAPC. Efforts were made to isolate the whole DAPC and co-immunoprecipitate it as a whole entity so that interactions within the DAPC could be monitored. This proved to be difficult either due to the extraction conditions used or the antibodies available, or perhaps a combination of both. The co-immunoprecipitation of dystrophin and members of the sarcoglycan complex was successful, but unfortunately not the co-immunoprecipitation of dystrophin and β -dystroglycan.

β -dystroglycan was found to be phosphorylated on tyrosine residues when treated with the phosphatase inhibitor, peroxyvanadate. Immunoprecipitation of β -dystroglycan from peroxyvanadate treated cells also demonstrated that phosphoproteins are associated with β -dystroglycan. Silver staining of these immunoprecipitations suggest that some of these proteins are also associated with β -dystroglycan in unstimulated cells. Investigations are continuing in order identify these proteins This includes protein sequencing of proteins isolated from immunoprecipitations separated by SDS-PAGE.

A yeast two-hybrid assay using the β -dystroglycan cytoplasmic domain as a bait to screen a skeletal muscle cDNA library revealed that β -dystroglycan interacts with actin α 1. This interaction was subsequently substantiated by biochemical assays. Co-sedimentation experiments demonstrated that β -dystroglycan and F-actin were sediment together both at high and low speed centrifugation, suggesting that the two proteins interact and form supermolecular aggregates. Falling ball viscosity assays demonstrated that β -dystroglycan cytoplasmic domain increases the viscosity of F-actin at least 4-fold. Subsequent electron microscopy studies revealed that β -dystroglycan bundles actin filaments.

The association of the DAPC with the actin cytoskeleton via dystrophin is well characterised, but an interaction between β -dystroglycan and actin has not been shown previously. Dystrophin has been shown to bind F-actin but have no affect on actin dynamics, acting as an anchor linking to the actin cytoskeleton (Winder *et al.*, 1997). In contrast, it has been demonstrated here that β -dystroglycan bundles actin filaments and therefore potentially plays an active role in actin re-organisation in the cell.

With β -dystroglycan's potential role in cell adhesion, actin may link the dystroglycan complex to focal adhesions. For example, the phosphoproteins observed co-immunoprecipitating with β -dystroglycan may be focal adhesion proteins indirectly associating with β -dystroglycan. In this case, the "linker" protein connecting β -dystroglycan to the focal adhesions may be actin. Previous work has demonstrated that the DAPC is associated with focal adhesions either via dystrophin or α -sarcoglycan (Yoshida *et al.*, 1998) or Grb2 (Cavaldesi *et al.*, 1999) but the β -dystroglycan/actin interaction could further strengthen the link between the DAPC and focal adhesions. The fact that β -dystroglycan bundles actin could also explain why overexpression of β -dystroglycan causes a *cdc42*-like phenotype in fibroblast cells, i.e. actin arranged in a ring formation at

the periphery of the cell and filopodial projections of the cell membrane (Yun-Ju Chen & S.J. Winder, personal communication).

The role of β -dystroglycan in actin dynamics is being investigated by immunofluorescence studies of β -dystroglycan-GFP constructs and rhodamine-phalloidin-stained actin filaments (Yun-Ju Chen & S. J. Winder). In addition, an interaction between β -dystroglycan and β 4 integrin is also being pursued. A complex of plectin, integrin and β -dystroglycan has been isolated by co-immunoprecipitation experiments and investigations into the function of this complex continue (Bittner *et al.*, 2001).

Analysis of β -dystroglycan's protein:protein interactions continues. Currently a yeast two-hybrid screen is being carried out in our laboratory using the β -dystroglycan cytoplasmic domain lacking the WW domain as bait in a HeLa cell cDNA library. This study should shed light on which β -dystroglycan interactions are WW-domain dependent and also identify proteins which interact with β -dystroglycan in non-muscle cells. In addition, a yeast two-hybrid screen using a range of deletion constructs of the β -dystroglycan cytoplasmic domain could delineate the interaction site of β -dystroglycan and actin and other interactors of β -dystroglycan. The falling ball assay could also be carried out using purified proteins made with a range of β -dystroglycan deletion constructs to delineate potential actin-binding sites.

The significant role of the PPxY motif in β -dystroglycan has been illustrated in this study. For example, its importance in regulation of the β -dystroglycan/ dystrophin interaction, and how it affects β -dystroglycan's protein:protein interactions. It has been demonstrated that the phosphorylation of the tyrosine residue in this motif disrupts β -dystroglycan's interaction with dystrophin. The effect of the WW domain

on β -dystroglycan's protein:protein interactions was also shown in the yeast two-hybrid screen i.e. the high frequency of WW-mediated interactions, albeit non-specific.

To date, the structures of 6 WW domains (representing 3 different WW domain classes) have been solved (Huang *et al.*, 2000; Macias *et al.*, 2000; Verdecia *et al.*, 2000; Kanelis *et al.*, 2001). Despite the differences in ligand specificity and the diverse origins of these structures, they are almost completely superimposable (Ilsley *et al.*, 2001b).

The finding that the phosphorylation of the tyrosine residue within a WW-domain binding motif is not a new phenomenon but it is the first time that phosphorylation of flanking tyrosine residue has been shown to fully abolish the interaction. Phosphorylation of serine and threonine residues flanking the PPxY motif have been reported to positively or negatively regulate the WW-domain mediated interaction (James *et al.*, 2000; Kay *et al.*, 2000) but this is the first time that the involvement of a phosphorylated PPxY motif flanking tyrosine has been necessary to disrupt the interaction in conjunction with the tyrosine within the motif. Despite the high interest in the regulation of WW domain interactions, there is still no clear cut *in vivo* example of tyrosine phosphorylation regulating WW ligand complexes and it remains unclear as to what kinase might phosphorylate the PPxY motif and its flanking residues.

It is still not known what the function of the dystroglycans α and β are separately. Generation of the dystroglycan knockout mouse (Williamson *et al.*, 1997) and chimeric mice devoid of dystroglycan (Cote *et al.*, 1999) have been useful in understanding the essential functions of the dystroglycan complex as whole, but more studies are needed to elucidate the exact function of the separate α and β subunits. Separate α/β -dystroglycan knockout mice would provide an insight into separating out

the functions of these proteins. This would reduce the complexity and give a clearer idea of the roles of the α - and β -subunits in muscular dystrophies, branching epithelial morphogenesis, muscle differentiation, neuronal development and NMJ formation. Questions include: is the β -dystroglycan knockout lethal or is it just the connection between α -dystroglycan and the extracellular matrix which is essential for membrane formation? And presuming that β -dystroglycan has the important role in signalling that we think it does, what signalling problems would a β -dystroglycan knockout have?

New roles and binding partners are continually emerging for α - and β -dystroglycans and the role of dystroglycan as a link between the cytoskeleton and extracellular matrix is becoming increasingly complex. Some of these functions will undoubtedly involve dystroglycan as a member of the DAPC and give us a greater understanding of the part the DAPC plays in muscular dystrophies, which in turn, will lead to more progressive and effective cures for these diseases.

References

References

- Adams, M. E., T. M. Dwyer, L. L. Dowler, R. A. White, and S. C. Froehner. (1995).
Mouse α 1- and β 2-syntrophin gene structure, chromosome localisation, and homology with a discs large domain.
J. Biol. Chem., **270**:25829-25865.
- Adams, M. E., N. Kramarcy, S. P. Krall, S. G. Rossi, R. L. Rotundo, R. Sealock, and S. C. Froehner. (2000).
Absence of α -syntrophin leads to structurally aberrant neuromuscular synapses deficient in utrophin.
J. Cell. Biol., **150**:1385-1397.
- Ahn, A. H., C. A. Freener, E. Gussoni, M. Yoshida, E. Ozawa, and L. M. Kunkel. (1996).
The three human syntrophin genes are expressed in diverse tissues, have distinct chromosomal locations, and each bind to dystrophin and its relatives.
J. Biol. Chem., **271**:2724-2730.
- Allamand, V., and K. P. Campbell. (2000).
Animal models for muscular dystrophy: valuable tools for the development of therapies.
Hum. Mol. Gen., **9**:2459-2467.
- Amalfitano, A., J. A. Rafael, and J. S. Chamberlain. (1997).
Structure and mutation of the dystrophin gene.
In S. C. Brown and J. A. Lucy (eds.),
Dystrophin: gene, protein and cell biology, pp. 1-26.
Cambridge University Press.
- Amann, K. J., B. A. Renley, and J. M. Ervasti. (1998).
A cluster of basic repeats in the dystrophin rod domain binds F-actin through an electrostatic interaction.
J. Biol. Chem., **122**:28419-28423.
- Anderson, J. T., R. P. Rogers, and H. W. Jarrett. (1996).
 Ca^{2+} -calmodulin binds to the carboxyl-terminal domain of dystrophin.
J. Biol. Chem., **271**:6605-6610.
- Andre, B., and J.-Y. Springael. (1994).
WWP, a new amino-acid motif present in single or multiple copies in various proteins including dystrophin and the SH3-binding Yes-Associated Protein YAP65.
Biochem. Biophys. Res. Commun., **205**:1201-1205.
- Apel, E. D., S. L. Roberds, K. P. Campbell, and J. P. Merlie. (1995).
Rapsyn may function as a link between the acetylcholine receptor and the agrin-binding dystrophin-associated glycoprotein complex.
Neuron, **15**:115-126.
- Araishi, K., T. Sasaoka, M. Imamura, S. Noguchi, H. Hama, E. Wakabayashi, M. Yoshida, T. Hori, and E. Ozawa. (1999).
Loss of the sarcoglycan complex and sarcospan leads to muscular dystrophy in β -sarcoglycan-deficient mice. *Hum. Mol. Genet.*, **8**:1589-1598

Ayscough, K. (1998).

In vivo functions of actin-binding proteins.

Curr. Opin. Cell Biol., **10**:102-111.

Bedford, M. T., D. C. Chan, and P. Leder. (1997).

FBP WW domains and the Abl SH3 domain bind to a specific class of proline-rich ligands.

EMBO. J., **16**:2376-2383.

Bedford, M. T., R. Reed, and P. Leder. (1998).

WW domain-mediated interactions reveal a spliceosome-associated protein that binds a third class of proline-rich motif: the proline glycine and methionine-rich motif.

Proc. Natl. Acad. Sci. USA, **95**:10602-10607.

Bedford, M. T., D. Sarbassova, J. Xu, P. Leder, and M. B. Yaffe. (2000).

A novel Pro-Arg motif recognised by WW domains.

J. Biol. Chem., **275**:10359-10369.

Belkin, A. M., and K. Burridge. (1995).

Localization of utrophin and aciculin at sites of cell-matrix and cell-cell adhesion in cultured cells.

Exp. Cell Res., **221**:132-140.

Belkin, A. M., and N. R. Smallheiser. (1996).

Localization of cranin (dystroglycan) at sites of cell-matrix and cell-cell contact: recruitment to focal adhesions is dependent upon extracellular ligands.

Cell Adhesion Commun., **4**:281-296.

Bianchi, E., S. Denti, A. Granata, G. Rossi, J. Geginat, A. Villa, L. Rogge, and R. Pardi. (2000).

Integrin LFA-1 interacts with transcriptional co-activator JAB1 to modulate AP-1 activity.

Nature, **404**:617-621.

Bittner, R. E., H.-L. Huang, B. Nikolic, S. Ivanova, M. Seper, D. J. Blake, S. J. Winder, and G. Wiche. (2001).

Cytoskeleton-coupling of laminin receptors in normal and dystrophic muscle: multiple interactions between utrophin, plectin, β -dystroglycan and integrin β 4.

J. Cell Biol. (submitted).

Blake, D. J., R. Nawrotzki, N. Y. Loh, D. C. Gorecki, and K. E. Davies. (1998).

β -dystrobrevin, a member of the dystrophin-related family.

Proc. Natl. Acad. Sci. USA, **95**:241-246.

Blake, D. J., R. Nawrotzki, M. F. Peters, S. C. Froehner, and K. E. Davies. (1996).

Isoform diversity of dystrobrevin, the murine 87kDa postsynaptic protein.

J. Biol. Chem., **271**:7802-7810.

Blake, D. J., J. M. Tinsley, K. E. Davies, A. E. Knight, S. J. Winder, and J. Kendrick-Jones. (1995).

Coiled-coil regions in the carboxy terminal domains of dystrophin and related proteins: potentials for protein-protein interactions.

Trends Biochem. Sci., **20**:133-135.

Blankenmeyer-Menge, B., M. Nimtz, and R. Frank. (1990).

An efficient method for anchoring Fmoc-amino-acids to hydroxyl-functionalised solid supports.

Tetrahedron Lett., **31**:1701-1704

- Bonet-Kerrache, A., E. Fabbrizio, and D. Mornet. (1994).
N-terminal domain of dystrophin.
FEBS Letters, **355**:49-53.
- Bork, P., and M. Sudol. (1994).
The WW domain: a signalling site in dystrophin?
Trends Biochem. Sci., **19**:531-533.
- Bredt, D. S., and S. H. Snyder. (1992).
Nitric oxide, a novel neuronal messenger.
Neuron, **8**:3-11.
- Brenman, J. A., D. S. Chao, H. Xia, K. Aldape, and D. S. Bredt. (1995).
Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy.
Cell, **82**:743-752.
- Brenman, J. E., D. S. Chao, S. H. Gee, A. W. McGee, S. E. Craven, D. R. Santillano, Z. Wu, F. Huang, H. Xia, M. F. Peters, S. C. Froehner, and D. S. Bredt. (1996).
Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and α 1-syntrophin mediated by PDZ domains.
Cell, **84**:757-767.
- Brent, R., and R. L. Finley, Jr. (1997).
Understanding gene and allele function with two-hybrid methods.
Ann. Rev. Genet., **31**:663-704.
- Brown, S. C., A. Fassati, L. Popplewell, A. M. Page, M. D. Henry, K. P. Campbell, and G. Dickson. (1999).
Dystrophic phenotype induced in vitro by antibody blockade of muscle α -dystroglycan-laminin interaction.
J. Cell Sci., **112**:209-216.
- Bulfield, G., W. G. Stiller, P. A. Wight, and K. J. Moore. (1984).
X chromosome-linked muscular dystrophy (*mdx*) in the mouse.
Proc. Nat. Acad. Sci., **81**:1189-1192.
- Bushby, K. M., M. Thambyayah, and D. Gardner-Medwin. (1991).
Prevalence and incidence of Becker muscular dystrophy.
Lancet, **337**:1022-4.
- Bushby, K. M. D. (1999).
The limb-girdle muscular dystrophies -multiple genes, multiple mechanisms.
Hum. Mol. Genet., **8**:1875-1882.
- Campanelli, J. T., S. L. Roberds, K. P. Campbell, and R. H. Scheller. (1994).
A role for dystrophin-associated glycoproteins and utrophin in agrin-induced AChR clustering.
Cell, **77**:663-674.
- Campbell, K. P. (1995).
Three muscular dystrophies: loss of cytoskeleton-extracellular matrix linkage.
Cell, **80**:675-679.

- Campbell, K. P., and S. D. Kahl. (1989).
Association of dystrophin and an integral membrane glycoprotein.
Nature, **338**:259-262.
- Cao, W., M. D. Henry, P. Borrow, H. Yamada, J. H. Elder, E. V. Ravkov, S. T. Nichol, R. W. Compans, K. P. Campbell, and M. B. A. Oldstone. (1998).
Identification of α -dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus.
Nature, **282**:2079-2081.
- Cartaud, A., S. Coutant, T. C. Petrucci, and J. Cartaud. (1998).
Evidence for *in situ* and *in vitro* association between β -dystroglycan and the synaptic 43K rapsyn protein.
J. Biol. Chem., **273**:11321-11326.
- Cartaud, A., F. Stetzkowski-Marden, and J. Cartaud. (1993).
Identification of dystrophin-binding protein(s) in membranes from *Torpedo* electrocyte and rat muscle.
J. Biol. Chem., **268**:13019-13022.
- Castresana, J., and M. Saraste. (1995).
Does Vav bind to F-actin through a CH domain?
FEBS Lett., **374**:149-151.
- Castrillon, D., and S. Wasserman. (1994).
diaphanous is required for cytokinesis in *Drosophila* and shares domains of similarity with the products of the *limb deformity* gene.
Development, **120**:3367-3377.
- Cavaldesi, M., G. Macchia, S. Barca, P. Defilippi, G. Tarone, and T. C. Petrucci. (1999).
Association of the dystroglycan complex isolated from bovine brain synaptosomes with proteins involved in signal transduction.
J. Neurochem., **72**:1648-1655.
- Chamberlain, J. S., J. A. Pearlman, D. M. Muzny, R. A. Gibbs, J. E. Ranier, A. A. Reeves, and C. T. Caskey. (1988).
Expression of the murine Duchenne muscular dystrophy gene in muscle and brain.
Science, **239**:1416-1417.
- Chamovitz, D. A., N. Wei, M. T. Osterlund, X.-W. Deng, A. G. v. Arnim, J. M. Straub, M. Matsui, and X.-W. Deng. (1996).
The COP9 complex, a novel multisubunit nuclear regulator involved in light control of a plant developmental switch.
Cell, **86**: 115-121.
- Chan, D. C., M. T. Bedford, and P. Leder. (1996).
Formin binding proteins bear WWP/WW domains that bind proline-rich peptides and functionally resemble SH3 domains.
EMBO J., **15**:1045-1054.
- Chan, Y.-M., C. G. Bonnemann, H. G. W. Lidov, and L. M. Kunkel. (1998).
Molecular organization of sarcoglycan complex in mouse myotubes in culture.
J. Cell Biol., **143**:2033-2044.

- Chang, F., D. Drubin, and P. Nurse. (1997).
cdc12p, a protein required for cytokinesis in fission yeast, is a component of the cell division ring and interacts with profilin.
J. Cell Biol., **137**:169-182.
- Chardin, P., D. Cussac, S. Maignan, and A. Ducruix. (1995).
The Grb2 adaptor.
FEBS Lett., **369**:47-51.
- Chen, H. I., A. Einbond, S.-J. Kwak, H. Linn, E. Koepf, S. Peterson, J. W. Kelly, and M. Sudol. (1997).
Characterisation of the WW domain of human Yes-associated protein and its polyproline-associated ligands.
J. Biol. Chem., **272**:17070-17077.
- Chen, H. I., and M. Sudol. (1995).
The WW domain of Yes-associated protein binds a proline-rich ligand that differs from the consensus established for Src homology 3-binding modules.
Proc. Natl. Acad. Sci., **92**:7819-7823.
- Chen, Y.-W., P. Zhao, R. Borup, and E. P. Hoffman. (2000).
Expression profiling in muscular dystrophies: identification of novel aspects of molecular pathophysiology.
J. Cell Biol., **151**:1321-1336.
- Chien, C. T., P. L. Bartel, R. Sternglanz, and S. Fields. (1991).
The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest.
Proc. Natl. Acad. Sci. USA, **88**:9578-9582.
- Ciechanover, A., A. Orian, and A. L. Schwartz. (2000).
Ubiquitin-mediated proteolysis: biological regulation via destruction.
BioEssays, **22**:442-451.
- Claret, F. X., M. Hibi, S. Dhut, T. Toda, and M. Karin. (1996).
A new group of conserved coactivators that increase the specificity of AP-1 transcription factors.
Nature, **383**:453-457.
- Clerk, A., G. E. Morris, V. Dubowitz, K. E. Davies, and C. A. Sewry. (1993).
Dystrophin-related protein, utrophin, in normal and dystrophic human foetal skeletal muscle.
Histochem. J., **25**:554-561.
- Clerk, A., P. N. Strong, and C. A. Sewry. (1992).
Characterisation of dystrophin during development of human skeletal muscle.
Development, **114**:395-402.
- Colledge, M., and S. C. Froehner. (1998).
To muster a cluster: Anchoring neurotransmitter receptors at synapses.
Proc. Natl. Acad. Sci., **95**:3341-3343.
- Condeelis, J. (1995).
Elongation factor 1 α , translation and the cytoskeleton.
Trends. Biochem. Sci., **20**:169-170.

- Cooper, B. J., N. J. Winand, H. Stedman, B. A. Valentine, E. P. Hoffman, L. M. Kunkel, M. O. Scott, K. H. Fischbeck, J. N. Kornegay, R. J. Avery, and e. al. (1988).
The homologue of the Duchenne locus is defective in X-linked muscular dystrophy of dogs.
Nature, **334**:154-156.
- Corrado, K., J. A. Rafael, P. L. Mills, N. M. Cole, J. A. Faulkner, K. Wang, and J. S. Chamberlain. (1996).
Transgenic *mdx* mice expressing dystrophin with a deletion in the actin-binding domain display a "mild Becker" phenotype.
J. Cell Biol., **134**:873-884.
- Cote, P. D., H. Moukhles, M. Lindenbaum, and S. Carbonetto. (1999).
Chimaeric mice deficient in dystroglycans develop muscular dystrophy and have disrupted myoneural synapses.
Nature Genet., **23**:338-342.
- Couet, J., S. Li, T. Okamoto, T. Ikezu, and M. P. Lisanti. (1997).
Identification of peptide and protein ligands for the caveolin-scaffolding domain: Implications for the interaction of caveolin with caveolae-associated proteins.
J. Biol. Chem., **272**:6525-6533.
- Cox, G. A., Y. Sunada, K. P. Campbell, and J. S. Chamberlain. (1994).
Dp71 can restore the dystrophin-associated glycoprotein complex in muscle but fails to prevent dystrophy.
Nature Genet., **8**:333-339.
- Crawford, G. E., J. A. Faulkner, R. H. Crosbie, K. P. Campbell, S. C. Froehner, and J. S. Chamberlain. (2000).
Assembly of the dystrophin-associated protein complex does not require the dystrophin COOH-terminal domain.
J. Cell Biol., **150**:1399-1409.
- Crosbie, R. H., J. Heighway, D. P. Venzke, J. C. Lee, and K. P. Campbell. (1997).
Sarcospan, the 25-kDa transmembrane component of the dystrophin-glycoprotein complex.
J. Biol. Chem., **272**:31221-31224.
- Crosbie, R. H., C. S. Lebakken, K. H. Kolt, D. P. Venzke, V. Straub, J. C. Lee, R. M. Grady, J. S. Chamberlain, J. R. Sanes, and K. P. Campbell. (1999).
Membrane targetting and stabilization of sarcospan is mediated by the sarcoglycan complex.
J. Cell Biol., **145**:153-165.
- Dasso, M. (2001).
Running on Ran: nuclear transport and the mitotic spindle.
Cell, **104**:321-324.
- Deconinck, A. E., A. C. Potter, J. M. Tinsley, S. J. Wood, R. Vater, C. Young, L. Metzinger, A. Vincent, C. R. Slater, and K. E. Davies. (1997).
Postsynaptic abnormalities at the neuromuscular junctions of utrophin-deficient mice.
J. Cell Biol., **136**:872-894.
- Durbeej, M., M. D. Henry, and K. P. Campbell. (1998).
Dystroglycan in development and disease.
Curr. Opin. Cell Biol., **10**:594-601.

- Emery, A. E. H. (1993).
Duchenne Muscular Dystrophy. Oxford University Press, Oxford.
- England, S. B., L. V. B. Nicholson, M. A. Johnson, S. M. Forrest, D. R. Love, E. E. Zubrzyckagaarn, D. E. Bulman, J. B. Harris, and K. Davies. (1990).
Very mild muscular-dystrophy associated with the deletion of 46-percent of dystrophin.
Nature, **343**:180-182.
- Ermeikova, K. S., N. Zambrano, H. Linn, G. Minopoli, F. Gertler, T. Russo, and M. Sudol. (1997).
The WW domain of the neural protein FE65 interacts with proline-rich motifs in Mena, the mammalian homologue of *Drosophila* Enabled.
J. Biol. Chem., **272**:32869-32877.
- Ervasti, J. M., and K. P. Campbell. (1993).
A role for the dystrophin glycoprotein complex as a transmembrane linker between laminin and actin.
J. Cell. Biol., **112**:809-823.
- Ervasti, J. M., K. Ohlendieck, S. D. Kahl, M. G. Gaver, and K. P. Campbell. (1990).
Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle.
Nature, **345**:315-319.
- Ettinger, A. J., G. Feng, and J. R. Sanes. (1997).
 ϵ -sarcoglycan, a broadly expressed homologue of the gene mutated in limb-girdle muscular dystrophy 2D.
J. Biol. Chem., **272**:32534-32538.
- Evangelista, M., K. Blundell, M. Longtine, C. Chow, N. Adames, J. Pringle, M. Peter, and C. Boone. (1997).
Bni1p, a yeast formin linking cdc42p and the actin cytoskeleton during polarised morphogenesis.
Science, **276**:118-122.
- Feilotter, H. E., G. J. Hannon, C. J. Ruddell, and D. Beach. (1994).
Construction of an improved host strain for two hybrid screening.
Nucleic. Acid. Res., **22**:1502-1503.
- Fields, S., and R. Sternglanz. (1994).
The two-hybrid system: an assay for protein:protein interactions.
Trends Genet., **10**:286-292.
- Fields, S., and O. K. Song. (1989).
A novel genetic system to detect protein:protein interactions.
Nature, **340**:245-246.
- Fincham, V. J., M. James, M. C. Frame, and S. J. Winder. (2000).
Active ERK/MAP kinase is targeted to newly forming cell-matrix adhesions by integrin engagement and v-Src.
EMBO J., **19**:2911-2923.
- Finn, D. M., and Ohlendieck, K. (1998).
Oligomerisation of β -dystroglycan in rabbit diaphragm and brain as revealed by chemical crosslinking.
Biochim. Biophys. Acta **1370**, 325-336.

- Finn, D. M., and K. Ohlendieck. (1997).
Rabbit brain and muscle isoforms containing the carboxy-terminal domain of the 427kDa skeletal muscle dystrophin exhibit similar biochemical properties.
Neurosci. Lett., **222**:25-28.
- Flick, J. S., and M. Johnston. (1990).
2 systems of glucose repression of the GAL1 promoter in *Saccharomyces cerevisiae*.
Mol. Cell. Biol., **10**: 4757-4769.
- Frank, R., and R. Doring. (1988).
Simultaneous multiple peptide synthesis under continuous flow conditions on cellulose paper discs as segmental solid synthesis.
Tetrahedron, **44**:6031-6040
- Frazier, J. A., and C. M. Field. (1997).
Actin cytoskeleton: Are FH proteins local organizers?
Curr. Biol., **7**:R414-R417.
- Fritz, C. C., and M. R. Green. (1992).
Fishing for partners.
Curr. Biol., **2**:403-405.
- Froehner, S. C., A. A. Murnane, M. Tobler, H. B. Peng, and R. Sealock. (1987).
A postsynaptic Mr 58,000 (58K) protein concentrated at acetylcholine receptor-rich sites in *Torpedo* electroplaques and skeletal muscle.
J. Cell. Biol., **104**:1633-1646.
- Fromont-Racine, M., J.-C. Rain, and P. Legrain. (1997).
Towards a functional analysis of the yeast genome through exhaustive two-hybrid screens.
Nat. Genet., **16**:277-281.
- Fuhrer, C., M. Gautam, J. E. Sugiyama, and Z. W. Hall. (1999).
Roles of rapsyn and agrin in interaction of postsynaptic proteins with acetylcholine receptors.
J. Neurosci., **19**:6405-6416.
- Galbiati, F., D. Volonté, J. B. Chu, M. Li, S. W. Fine, M. Fu, J. Bermudez, M. Pedemonte, K. M. Weidenheim, R. G. Pestell, C. Minetti, and M. P. Lisanti. (2000).
Transgenic overexpression of caveolin-3 in skeletal muscle fibers induces a Duchenne-like muscular dystrophy phenotype.
Proc. Natl. Acad. Sci. USA., **97**:9689-9694.
- Gee, S. H., R. Madhavan, S. R. Levinson, J. H. Caldwell, R. Sealock, and S. C. Froehner. (1998).
Interaction of muscle and brain sodium channels with multiple members of the syntrophin family of dystrophin-associated proteins.
J. Neurosci., **18**:128-137.
- Gee, S. H., F. Montanaro, M. H. Lindenbaum, and S. Carbonetto. (1994).
Dystroglycan- α , a dystrophin-associated glycoprotein, is a functional agrin receptor.
Cell, **77**:675-686.
- Gietz, D., A. Stjean, R. A. Woods, and R. H. Schiestl. (1993).
Improved method for high-efficiency transformation of intact yeast cells.
Nucleic Acids Res., **20**:1425-1425.

- Glickman, M. H., D. M. Rubin, O. Cux, I. Wefes, G. Pfeifer, Z. Cjeka, W. Baumeister, V. A. Fried, and D. Finley. (1998).
A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3.
Cell, **94**:615-623.
- Grady, R. M., R. W. Grange, K. S. Lau, M. M. Maimone, M. C. Nicol, J. T. Stull, and J. R. Sanes. (1999).
Role for α -dystrobrevin in the pathogenesis of dystrophin-dependent muscular dystrophies.
Nature Cell Biol., **1**:215-220.
- Grady, R. M., J. P. Merlie, and J. R. Sanes. (1997a).
Subtle neuromuscular defects in utrophin-deficient mice.
J. Cell Biol., **136**:871-882.
- Grady, R. M., H. Teng, M. C. Nicholl, J. C. Cunningham, R. S. Wilkinson, and J. R. Sanes. (1997b).
Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: a model for Duchenne muscular dystrophy.
Cell, **90**:729-738.
- Greenberg, D. S., Y. Sunada, K. P. Campbell, D. Yaffe, and U. Nudel. (1994).
Exogenous Dp71 restores the levels of dystrophin-associated proteins but does not alleviate muscle damage in mdx mice.
Nature Genet., **8**:340-344.
- Hasegawa, M., A. Cuenda, M. G. Spillantini, G. M. Thomas, V. Buee-Scherrer, P. Cohen, and M. Goedert. (1999).
Stress-activated protein kinase-3 interacts with the PDZ domain of α 1-syntrophin.
J. Biol. Chem., **274**:12626-12631.
- Hayashi, Y. K., E. Engvall, H. E. Arikawa, K. Goto, R. Koga, L. Nonaka, H. Sugita, and K. Arahata. (1993).
Abnormal localisation of laminin subunits in muscular dystrophies.
J Neurol. Sci., **119**:53-64.
- Hemler, M. E., B. A. Mannion and F. Berditchevski. (1996).
Association of TM4SF proteins with integrins: relevance to cancer.
BBA- Rev. Cancer, **1287**:67-71.
- Henry, M. D., and K. P. Campbell. (1998).
A role for dystroglycan in basement membrane assembly.
Cell, **95**:859-870.
- Henry, M. D., and K. P. Campbell. (1999).
Dystroglycan inside and out.
Curr. Opin. Cell Biol., **11**:602-607.
- Higuchi, I., H. Yamada, H. Fukunaga, H. Iwaki, R. Okubo, M. Nakagawa, M. Osame, S. L. Roberds, T. Shimizu, K. P. Campbell, and K. Matsumura. (1994).
Abnormal expression of laminin suggests disturbance of sarcolemma extracellular-matrix interaction in Japanese patients with autosomal recessive muscular-dystrophy deficient in adhalin.
J. Clin. Invest., **94**:601-606.

Hillaire, D., A. Leclerc, S. Faure, H. Topaloglu, N. Chiannikulchai, P. Guicheney, L. Grinas, P. Legos, J. Philpot, T. Evangelista, M.-C. Routon, M. Mayer, J.-F. Pellissier, B. Estournet, A. Barois, F. Hentati, N. Feingold, J. S. Beckmann, V. Dubowitz, F. M. S. Tome, and M. Fardeau. (1994).

Localization of merosin-negative congenital muscular dystrophy to chromosome 6q2 by homozygosity mapping.

Hum. Mol. Genet., **3**:1657-1661.

Hoffman, E. P., K. H. Fischbeck, R. H. Brown, M. Johnson, R. Medori, J. D. Loike, J. B. Harris, R. Waterston, M. Brooke, L. Specht, W. Kupsy, J. Chamberlain, C. T. Caskey, F. Shapiro, and L. M. Kunkel. (1988).

Characterization Of dystrophin in muscle-biopsy specimens from patients with Duchenne's or Becker's muscular dystrophy.

New Eng. J. Med., **318**:1363-1368.

Hoffman, E. P., R. H. Brown. Jr., and L. M. Kunkel. (1987).

Dystrophin: the protein product of the Duchenne muscular dystrophy locus.

Cell, **51**:919-28.

Hofmann, K., and P. Bucher. (1998).

The PCI domain: a common theme in three multiprotein complexes.

Trends. Biochem. Sci., **23**:204-205.

Holt, K. H., L. E. Lim, V. Straub, D. P. Venzke, F. Duclos, R. D. Anderson, B. L. Davidson, and K. P. Campbell. (1998).

Functional rescue of the sarcoglycan complex in the BIO 14.6 hamster using δ -sarcoglycan gene transfer.

Mol. Cell, **1**:841-848.

Huang, X., F. Poy, R. Zhang, A. Joachimiak, M. Sudol, and M. J. Eck. (2000).

Structure of a WW domain containing fragment of dystrophin in complex with β -dystroglycan.

Nature Struct.Biol., **7**:634-638.

Ibraghimov-Beskrovnya, O., J. M. Ervasti, C. J. Leveille, C. A. Slaughter, S. W. Sernett, and K. P. Campbell. (1992).

Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix.

Nature, **355**:696-702.

Ilsley, J. L., M. Sudol, and S. J. Winder. (2001a).

The interaction of dystrophin with β -dystroglycan is regulated by tyrosine phosphorylation.

Cell. Signal., **13**: 625-632.

Ilsley, J. L., M. Sudol, and S. J. Winder. (2001b).

The WW domain: linking cell signalling to the membrane cytoskeleton.

Cell. Signal., (in press).

Ishihara, H., B. L. Martin, D. L. Brautigan, H. Karaki, H. Ozaki, Y. Kato, N. Fusetani, S. Watabe, K. Hashimoto, D. Uemera, and D. J. Hartshorne. (1989).

Calyculin-A and okadaic acid -inhibitors of protein phosphatase activity.

Biochem. Biophys. Res. Commun., **159**:871-877.

Iwata, Y., Y. Pan, T. Yoshida, H. Hanada, and M. Shigekawa. (1998).

α 1-syntrophin has distinct binding sites for actin and calmodulin.

FEBS Lett., **423**:173-177.

- James, M., N. t. Man, C. J. Wise, G. E. Jones, and G. E. Morris. (1996).
Utrophin-dystroglycan complex in membranes of adherent cultured cells.
Cell Motil. Cytoskel., **33**:163-174.
- James, M., A. Nuttall, J. L. Ilesley, K. Ottersbach, J. M. Tinsley, M. Sudol, and S. J. Winder. (2000).
Adhesion-dependent tyrosine phosphorylation of β -dystroglycan regulates its interaction with utrophin.
J. Cell Sci., **113**:1717-1726.
- James, P., J. Halladay, and E. A. Craig. (1996).
Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast.
Genetics, **44**:1425-1436.
- Jarrett, H. W., and J. L. Foster. (1995).
Alternate binding of actin and calmodulin to multiple sites on dystrophin.
J. Biol. Chem., **270**:5578-5586.
- Jung, D., B. Yang, J. Meyer, J. S. Chamberlain, and K. P. Campbell. (1995).
Identification and characterisation of the dystrophin anchoring site on β -dystroglycan.
J. Biol. Chem., **270**:27305-27310.
- Kameya, S., Y. Miyagoe, I. Nonaka, T. Ikemoto, M. Endo, K. Hanaoka, Y.-I. Nabeshima, and S. Takeda. (1999).
 α 1-syntrophin gene disruption results in the absence of neuronal-type nitric-oxide synthase at the sarcolemma but does not induce muscle degeneration.
J. Biol. Chem., **274**:2193-2200.
- Kanelis, V., D. Rotin, and J. D. Forman-Kay. (2001).
Solution structure of a Nedd4 WW domain-ENaC peptide complex.
Nat. Struct. Biol., **8**:407-412.
- Kay, B. K., M. P. Williamson, and M. Sudol. (2000).
The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains.
FASEB. J., **14**:231-241.
- Klatt, P., M. Schmid, E. Leopold, K. Schmidt, E. R. Werner, and B. Mayer. (1994).
The pteridine binding site of brain nitric oxide synthase. Tetrahydrobiopterin binding kinetics, specificity and allosteric interaction with the substrate domain.
J. Biol. Chem., **269**:13861-13866.
- Kobzik, L., M. B. Reid, D. S. Bredt, and J. S. Stamler. (1994).
Nitric oxide in skeletal muscle.
Nature, **372**:546-548.
- Koenig, M., A. H. Beggs, M. Moyer, S. Scherpf, K. Heindrich, T. Bettecken, G. Meng, C. R. Muller, M. Lindlof, H. Kaariainen, A. Delachapelle, A. Kiuru, M. L. Savontaus, H. Gilgenkrantz, D. Recan, J. Chelly, J. C. Kaplan, A. E. Covone, N. Archidiacono, G. Romeo, S. Liechtigallati, V. Schneider, S. Braga, H. Moser, B. T. Darras, P. Murphy, U. Francke, J. D. Chen, G. Morgan, M. Denton, C. R. Greenberg, K. Wrogemann, L. A. J. Blonden, H. M. B. Vanpaassen, G. J. B. Vanommen, and L. M. Kunkel. (1989).
The molecular-basis for Duchenne versus Becker muscular-dystrophy - correlation of severity with type of deletion.
Am. J. Hum. Genet., **45**:498-506.

Koenig, M., E. P. Hoffman, C. J. Bertelson, A. P. Monaco, C. Feener, and L. M. Kunkel. (1987).

Complete cloning of the Duchenne muscular-dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell*, **50**:509-517.

Koenig, M., and L. M. Kunkel. (1990).

Detailed analysis of the repeat domain of dystrophin reveals four potential hinge segments that may confer flexibility.

J. Biol. Chem., **265**:4560-4566.

Koenig, M., A. P. Monaco, and L. M. Kunkel. (1988).

The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein.

Cell, **53**:219-226.

Kohno, H., K. Tanaka, A. Mino, M. Umikawa, H. Imamura, T. Fujiwara, Y. Fujita, K. Hotta, H. Qadota, T. Watanabe, Y. Ohya, and Y. Takai. (1996).

Bni1p implicated in cytoskeletal control is a putative target of Rho1p small GTP-binding protein in *Saccharomyces cerevisiae*.

EMBO J., **15**:6060-6068.

Kramer, A., A. Schuster, U. Reineke, R. Malin, R. Volkmer-Engert, and J. Schneider-Mergener. (1993).

Simultaneous synthesis of peptide libraries on single cellulose membrane supports: examples for the identification of protein, metal and DNA binding peptide mixtures.

Peptide Res., **6**:314-319.

Kurasawa, Y., K. Hanyu, Y. Watanabe, and O. Numata. (1996).

F-actin bundling activity of Tetrahymena elongation factor 1 alpha is regulated by Ca²⁺ calmodulin.

J. Biochem., **119**:791-798.

Kwok, S. F., R. Solano, T. Tsuge, D. A. Chamovitz, J. R. Ecker, M. Matsui, and X. W. Deng. (1998).

Arabidopsis homologues of a JUN coactivator are present both in monomeric form and in the COP9 complex, and their abundance is differentially affected by the pleiotropic *cop/det/fus* mutations.

Plant Cell, **10**:1779-1790.

Laney, J. D., and M. Hochstrasser. (1999).

Substrate targeting in the ubiquitin system.

Cell, **97**:427-430.

Lebakken, C. S., D. P. Venzke, R. F. Hrstka, C. M. Consolino, J. A. Faulkner, R. A. Williamson, and K. P. Campbell. (2000).

Sarcospan-deficient mice maintain normal muscle function.

Mol. Cell. Biol., **20**:1669-1677.

Lemaire, C., R. Heilig, and J. L. Mandel. (1988).

The chicken dystrophin cDNA: striking conservation of the C-terminal coding and 3' untranslated regions between man and chicken.

EMBO. J., **7**:4157-4162.

- Li, S., J. Couet, and M. P. Lisanti. (1996).
Src tyrosine kinases, G α subunits and H-Ras share a common membrane-anchored scaffolding protein, caveolin.
J. Biol. Chem., **271**:29182-29190.
- Liu, G., J. Tang, B. T. Edmonds, J. Murray, S. Levin, and J. Condeelis. (1996).
F-actin sequesters elongation factor 1 α from interaction with aminoacyl-tRNA in a pH-dependent reaction.
J. Cell Biol., **135**:953-963.
- Liu, Y. C., J. Pan, C. Y. Zhang, W. F. Fan, M. Collinge, J. R. Bender, and S. M. Weissman. (1999).
A MHC-encoded ubiquitin-like protein (FAT10) binds noncovalently to the spindle assembly checkpoint protein MAD2.
Proc. Natl. Acad. Sci. USA, **96**:4313-4318.
- Love, D. R., D. F. Hill, G. Dickson, N. K. Spurr, B. C. Byth, R. F. Marsden, F. S. Walsh, Y. S. Edwards, and K. E. Davies. (1989).
An autosomal transcript in skeletal muscle with homology to dystrophin.
Nature, **339**:55-58.
- Lowenstein, E. J., R. J. Daly, A. G. Batzer, W. Li, B. Margolis, R. Lammers, A. Ullrich, E. Y. Skolnik, D. Bar-Sagi, and J. Schlessinger. (1992).
The SH2 and SH3 domain-containing protein Grb2 links receptor tyrosine kinases to Ras signaling.
Cell, **70**:431-442.
- Lu, P.-J., X. Z. Zhou, M. Shen, and K. P. Lu. (1999).
Function of WW domains as phosphoserine- or phosphothreonine- binding modules.
Science, **283**:1325-1328.
- Luisse, M., C. Presotto, L. Senter, R. Betto, S. Ceoldo, S. Furlan, S. Salvatori, R. Sabbadini, and G. Salviati. (1993).
Dystrophin is phosphorylated by endogenous protein kinases.
Biochem. J., **293**:243-247.
- Lumeng, C., S. Phelps, G. E. Crawford, P. D. Walden, K. Barald, and J. S. Chamberlain. (1999).
Interactions between β 2-syntrophin and a family of microtubule-associated serine/threonine kinases.
Nature Neurosci., **2**:611-617.
- Macias, M. J., V. Gervais, C. Civera, and H. Oschkinat. (2000).
Structural analysis of WW domains and design of a WW prototype.
Nature Struct. Biol., **7**:375-379.
- MacLean-Fletcher, S. D., and T. D. Pollard. (1980).
Viscometric analysis of the gelation of acanthamoeba extracts and purification of the two gelation factors.
J. Cell Biol., **85**:414-428.
- Madhavan, R., and H. W. Jarrett. (1994).
Calmodulin-activated phosphorylation of dystrophin.
Biochemistry, **33**:5797-5804.

- Madhavan, R., L. R. Massom, and H. W. Jarrett. (1992).
Calmodulin specifically binds three proteins of the dystrophin-glycoprotein complex.
Biochem. Biophys. Res. Commun., **185**:753-759.
- Maecker, H. T., S. C. Todd, and S. Levy. (1997).
The tetraspanin superfamily: molecular facilitators.
FASEB. J., **11**:428-442.
- Marston, F. A. O. (1986).
The purification of eukaryotic polypeptides synthesised in *Escherichia coli*.
Biochem. J., **240**:1-12.
- Matsudaira, P. (1991).
Modular organization of actin crosslinking proteins.
Trends Biochem. Sci., **16**:87-92.
- Matsumura, K., H. Yamada, T. Shimizu, and K. P. Campbell. (1993).
Differential expression of dystrophin, utrophin and dystrophin- associated proteins in
peripheral nerve.
FEBS Lett., **334**:281-285.
- McCarter, G. C., and R. A. Steinhardt. (2000).
Increased activity of calcium leak channels caused by proteolysis near sarcolemmal ruptures.
J. Membrane Biol., **176**:169-174.
- McNally, E. M., C. T. Ly, and L. M. Kunkel. (1998).
Human ϵ -sarcoglycan is highly related to α -sarcoglycan (adhalin), the limb girdle muscular
dystrophy 2D gene.
FEBS Lett., **422**:27-32.
- Meyer, R. K. and U. Aebi. (1990).
Bundling of actin-filaments by alpha-actinin depends on its molecular length.
J. Cell Biol., **110**:2013-2024.
- Michalak, M., S. Y. Fu, R. E. Milner, J. L. Busaan, and J. E. Hance. (1996).
Phosphorylation of the carboxyl-terminal region of dystrophin.
Biochem. Cell. Biol., **74**:431-437.
- Mikalsen, S. O., and O. Kaalhus. (1998).
Properties of pervanadate and permlybdate: connexin 43, phosphatase inhibition and
thiolreactivity as model systems.
J. Biol. Chem., **273**:10036-10045.
- Milner, R. E., J. L. Busaan, C. F. B. Holmes, J. H. Wang, and M. Michalak. (1993).
Phosphorylation of dystrophin.
J. Biol. Chem., **268**:21901-21905.
- Milner, R. E., J. I. Bussan, and M. Michalak. (1992).
Isolation and characterisation of different C-terminal fragments of dystrophin expressed in
Escherichia coli.
Biochem. J., **288**:1037-1044.

- Minetti, C., F. Sotgia, C. Bruno, P. Scartezzini, P. Broda, M. Bado, E. Masetti, M. Mazzocco, A. Egeo, M. A. Donati, D. Volonte, F. Galbiati, G. Cordone, F. D. Bricarelli, M. P. Lisanti, and F. Zara. (1998).
Mutations in the caveolin-3 gene cause autosomal dominant limb-girdle muscular dystrophy.
Nature Genet., **18**:365-368.
- Mizuno, Y., S. Noguchi, H. Yamamoto, M. Yoshida, A. Suzuki, Y. Hagiwara, Y. K. Hayashi, K. Arahata, I. Nonaka, S. Hirai, and E. Ozawa. (1994).
Selective defect of sarcoglycan complex in severe childhood autosomal recessive muscular dystrophy muscle.
Biochem. Biophys. Res. Comm. **203**:979-983.
- Morrissey, J. H. (1981).
Silver stain for proteins in polyacrylamide gels - a modified procedure with enhanced uniform sensitivity.
Anal. Biochem., **117**:307-310
- Nakamura, M., H. Masuda, J. Horii, K. Kuma, N. Yokoyama, T. Ohba, H. Nishitani, T. Miyata, M. Tanaka, and T. Nishimoto. (1998).
When overexpressed, a novel centrosomal protein, RanBPM, causes ectopic microtubule nucleation similar to gamma-tubulin.
J. Cell. Biol., **143**:1041-1052.
- Nawrotski, R., N. Y. Loh, M. A. Ruegg, K. E. Davies, and D. J. Blake. (1998).
Characterisation of α -dystrobrevin in muscle.
J. Cell. Sci., **111**:2595-2605.
- Nelson, K., J. Schlondorff, and C. P. Blobel. (1999).
Evidence for an interaction of the metalloprotease-disintegrin tumour necrosis factor alpha convertase (TACE) with mitotic arrest deficient 2 (MAD2), and of the metalloprotease-disintegrin MDC9 with a novel MAD2-related protein, MAD2 beta.
Biochem. J., **343**:673-680.
- Newbell, B. J., J. T. Anderson, and H. W. Jarrett. (1997).
Ca²⁺-calmodulin binding to mouse a1 syntrophin: syntrophin is also a Ca²⁺-binding protein.
Biochemistry, **36**:1295-1305.
- Newey, S. E., M. A. Benson, C. P. Ponting, K. E. Davies, and D. J. Blake. (2000).
Alternative splicing of dystrobrevin regulates the stoichiometry of syntrophin binding to the dystrophin protein complex.
Curr. Biol., **10**:1295-1298.
- Nitkin, R. M., M. A. Smith, C. Magill, J. R. Fallon, Y. M. Yao, B. G. Wallace, and U. J. McMahan. (1987).
Identification of agrin: a synaptic organising protein from *Torpedo* electric organ.
J. Cell Biol., **105**:2471-2478.
- O'Neill, T. J., Y. Y. Zhu, and T. A. Gustafson. (1997).
Interaction of MAD2 with the carboxyl terminus of the insulin receptor but not with the IGFIR - Evidence for release from the insulin.
J. Biol. Chem., **272**:10035-10040.

- Okamoto, T., A. Schlegel, P. E. Scherer, and M. P. Lisanti. (1998). Caveolins, a family of scaffolding proteins for organising "preassembled signaling complexes" at the plasma membrane. *J. Biol. Chem.*, **273**:5419-5422.
- Oronzi-Scott, M., J. E. Sylvester, T. Heiman-Patterson, Y. J. Shi, W. Fieles, H. Stenman, A. Burghes, P. Ray, R. Worton, and K. H. Fischbeck. (1988). Duchenne muscular dystrophy gene expression in normal and diseased human muscle. *Science*, **239**:1418-1420.
- Peng, H. B., H. Xie, S. G. Rossi, and R. L. Rotundo. (1999). Acetylcholinesterase clustering at the neuromuscular junction involves perlecan and dystroglycan. *J. Cell Biol.*, **145**:911-921.
- Peters, M. F., M. E. Adams, and S. C. Froehner. (1997). Differential association of syntrophin pairs with the dystrophin complex. *J. Cell Biol.*, **138**:81-93.
- Petrof, B. J., J. B. Shrager, H. H. Stedman, A. M. Kelly, and H. L. Sweeney. (1993). Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc Nat Acad Sci USA*, **90**:3710-3714.
- Piluso, G., M. Mirabella, E. Ricci, A. Belsito, C. Abbondanza, S. Servidei, A. A. Puca, P. Tonali, G. A. Puca, and V. Nigro. (2000). γ 1- and γ 2-syntrophins, two novel dystrophin-binding proteins localised in neuronal cells. *J. Biol. Chem.*, **275**:15851-15860.
- Pirozzi, G., S. J. McConnell, A. J. Uveges, J. M. Carter, A. B. Sparks, B. K. Kay, and D. M. Fowlkes. (1997). Identification of novel human WW domain containing proteins by cloning of ligand targets. *J. Biol. Chem.*, **272**:14611-14616.
- Pisenti, J. M., M. E. Delany, R. L. Taylor, U. K. Abbott, H. Abplanalp, J. A. Arthur, M. R. Bakst, C. Baxter-Jones, J. J. bitgood, F. A. Bradley, K. M. Cheng, R. R. Dietert, J. B. Dodgson, A. M. Donoghue, A. B. Emsley, R. J. Etches, R. R. Frahm, R. J. Gerrits, P. F. Goetinck, A. A. Grunder, D. E. Harry, S. J. Lamont, G. R. Martin, P. E. McGuire, G. P. Moberg, L. J. Pierro, C. O. Qualset, M. A. Quershi, F. T. Shultz, and B. W. Wilson. 1999. Avian genetic resources for biological research. *Avian genetic resources at risk: an assessment and proposal for conservation of genetic stocks in the USA and Canada*, Vol. 20, pp. 15-24. Genetic resources conservation programme, University of California division of agriculture and natural resources, Davis CA, USA.
- Poelzl, G., Y. Kasai, N. Mochizuki, P. W. Shaul, M. Brown, and M. E. Mendelsohn. (2000). Specific association of estrogen receptor beta with the cell cycle spindle assembly checkpoint protein, MAD2. *Proc. Natl. Acad. Sci., USA*. **97**:2836-2839.
- Pons, F., A. Robert, J. F. Marini, and J. J. Leger. (1994). Does utrophin expression in muscles of mdx mice during postnatal development functionally compensate for dystrophy deficiency? *J. Neurol. Sci.*, **122**:162-170.

- Ponting, C. P., D. J. Blake, K. E. Davies, J. Kendrick-Jones, and S. J. Winder. (1996). ZZ and TAZ: new putative zinc fingers in dystrophin and other proteins. *Trends Biochem. Sci.*, **21**:11-13.
- Rafael, J. A., G. A. Cox, K. Corrado, D. Jung, K. P. Campbell, and J. S. Chamberlain. (1996). Forced expression of dystrophin deletion constructs reveals structure-function correlations. *J. Cell Biol.*, **134**:93-104.
- Rambukkana, A., H. Yamada, G. Zanazzi, T. Mathus, J. L. Salzer, P. D. Yurchenco, K. P. Campbell, and V. A. Fischetti. (1998). α -dystroglycan is a Schwann cell receptor for *Mycobacterium leprae*. *Nature*, **282**:2076-2079.
- Ranganathan, R., K. P. Lu, T. Hunter, and J. P. Noel. (1997). Structural and functional analysis of the mitotic rotamase Pin1 suggests substrate recognition is phosphorylation dependent. *Cell*, **89**:875-886.
- Ren, R., B. Mayer, P. Cicchetti, and D. Baltimore. (1993). Identification of a 10 amino-acid SH3 binding site in two Abl SH3 binding proteins and its presence in formins and a muscarinic acetylcholine receptor. *Science*, **259**:1157-1161.
- Rentschler, S., H. Linn, K. Deininger, M. T. Bedford, X. Espanel, and M. Sudol. (1999). The WW domain of dystrophin requires EF-hands region to interact with β -dystroglycan. *Biol. Chem.*, **380**:431-442.
- Repetto, S., M. Bado, P. Broda, G. Lucania, E. Masetti, F. Sotgia, I. Carbone, A. Pavan, E. Bonilla, G. Cordone, M. P. Lisanti, and C. Minetti. (1999). Increased number of caveolae and caveolin-3 overexpression in Duchenne muscular dystrophy. *Biochem. Biophys. Res. Comm.*, **261**:547-550.
- Rigoletto, C., A. Prella, P. Ciscato, M. Moggio, G. Comi, F. Fortunato, and S. Scarlato. (1995). Utrophin expression during human fetal development. *Int. J. Devl. Neurosci.*, **13**:585-593.
- Roberds, S. L., J. M. Ervasti, R. D. Anderson, K. Ohlendieck, S. D. Kahl, D. Zoloto, and K. P. Campbell. (1993). Disruption of the dystrophin-glycoprotein complex in the cardiomyopathic hamster. *J. Biol. Chem.*, **268**:11496-11499.
- Roberts, R. G., M. Bobrow, and D. R. Bentley. (1992). Point mutations in the dystrophin gene. *Proc. Natl. Acad. Sci. USA*, **89**:2331-2335.
- Roberts, R. G., T. C. Freeman, E. Kendall, D. L. P. Vetrie, A. K. Dixon, C. Shaw-Smith, Q. Bone, and M. Bobrow. (1996). Characterisation of DRP2, a novel human dystrophin homologue. *Nature Genet.*, **13**:223-236.
- Rosa, G., M. Ceccarini, M. Cavaldesi, M. Zini, and T. C. Petrucci. (1996). Localisation of the dystrophin binding site at the carboxyl terminus of β -dystroglycan. *Biochem. Biophys. Res. Commun.*, **223**:272-277.

- Ruegg, M. A., and J. L. Bixby. (1998).
Agrin orchestrates synaptic differentiation at the vertebrate neuromuscular junction.
Trends Neurosci., **21**:22-27.
- Russo, K., E. D. Stasio, G. Macchia, G. Rosa, A. Brancaccio, and T. C. Petrucci. (2000).
Characterisation of the β -dystroglycan-growth factor receptor 2 (Grb2) interaction.
Biochem. Biophys. Res. Commun., **274**:93-98.
- Rybakova, I. N., K. J. Amman, and J. M. Ervasti. (1996).
A new model for the interaction of dystrophin with F-actin.
J. Cell Biol., **135**:661-672.
- Rybakova, I. N., and J. M. Ervasti. (1997).
Dystrophin-glycoprotein complex is monomeric and stabilizes actin filaments *in vitro* through a lateral association.
J. Biol. Chem., **272**:28771-28778.
- Sadoulet-Puccio, H. M., and L. M. Kunkel. (1996).
Dystrophin and its isoforms.
Brain Pathol., **6**:25-35.
- Sadoulet-Puccio, H. M., M. Rajala, and L. M. Kunkel. (1997).
Dystrobrevin and dystrophin: an interaction through coiled-coil motifs.
Proc. Natl. Acad. Sci., **94**:12413-12418.
- Salih, M. A. M., Y. Sunada, M. Al-Nasser, C. O. Ozo, M. H. S. Al-Turaiki, M. Akbar, and K. P. Campbell. (1996).
Muscular dystrophy associated with β -dystroglycan deficiency.
Ann. Neurol., **40**:925-928.
- Sander, M., B. Chavoshan, S. A. Harris, S. T. Iannaccone, J. T. Stull, G. D. Thomas, and R. G. Victor. (2000).
Functional muscle ischemia in neuronal nitric oxide synthase-deficient skeletal muscle of children with Duchenne muscular dystrophy.
Proc. Natl. Acad. Sci. USA., **97**:13818-13823.
- Schultz, J., U. Hoffmuller, G. Krause, J. Ashurst, M. J. Macias, P. Schneider, J. Schneider-Mergener, and H. Oschkinat. (1998).
Specific interactions between the syntrophin PDZ domain and voltage-gated sodium channels.
Nature Struct. Biol., **5**:19-24.
- Sealock, R., and S. Froehner. (1997).
The dystrophin family, agrin and construction of the neuromuscular junction.
In J. A. Lucy and S. C. Brown (eds.),
Dystrophin: gene, protein and cell biology, pp. 139-162.
Cambridge University Press, Cambridge.
- Seeger, M., R. Kraft, K. Ferrell, D. Bech-Otschir, R. Dumdey, R. Schade, R. Gordon, M. Naumann, and W. Dubiel. (1998).
A novel protein complex involved in signal transduction possessing similarities to 26S proteasome subunits.
FASEB. J., **12**:469-478.

- Senter, L., S. Ceoldo, M. M. Petrusa, and G. Salviati. (1995).
Phosphorylation of dystrophin: effects on actin binding.
Biochem. Biophys. Res. Commun., **206**:57-63.
- Shemanko, C. S., J. S. Sanghera, R. E. Milner, S. Pelech, and M. Michalak. (1995).
Phosphorylation of the carboxyl terminal region of dystrophin by mitogen-activated protein (MAP) kinase.
Mol. Cell. Biochem., **152**:63-70.
- Shimizu, H., H. Hosokawa, H. Ninomiya, J. H. Miner, and T. Masaki. (1999).
Adhesion of cultured bovine aortic endothelial cells to laminin-1 mediated by dystroglycan.
J. Biol. Chem., **274**:11995-12000.
- Song, K. S., P. E. Scherer, Z. Tang, T. Okamoto, S. Li, M. Chafel, C. Chu, D. S. Kohtz, and M. P. Lisanti. (1996).
Expression of caveolin-3 in skeletal, cardiac and smooth muscle cells.
J. Biol. Chem., **271**:15160-15165.
- Sotgia, F., J. K. Lee, K. Das, M. Bedford, T. C. Petrucci, P. Macioce, M. Sargiacomo, F. D. Bricarelli, C. Minetti, M. Sudol, and M. P. Lisanti. (2000).
Caveolin-3 directly interacts with the C-terminal tail of β -dystroglycan.
J. Biol. Chem., **275**:38048-38058.
- Stradal, T., W. Kranewitter, S. J. Winder, and M. Gimona. (1998).
CH domains revisited.
FEBS Lett., **431**:134-137.
- Sudol, M., and T. Hunter. (2000).
NeW Wrinkles for an old domain.
Cell, **103**:1001-1004.
- Sugiyama, J., D. C. Bowen, and Z. W. Hall. (1994).
Dystroglycan binds nerve and muscle agrin.
Neuron, **13**:103-115.
- Suzuki, A., M. Yoshida, K. Hayashi, Y. Mizuno, Y. Hagiwara, and E. Ozawa. (1994).
Molecular organisation at the glycoprotein-complex-binding site of dystrophin: three dystrophin-associated proteins bind directly to the carboxyl-terminal portion of dystrophin.
Eur. J. Biochem., **220**:283-292.
- Suzuki, A., M. Yoshida, H. Yamamoto, and E. Ozawa. (1992).
Glycoprotein-binding site of dystrophin is confined to the cysteine-rich domain and the first half of the carboxy-terminal domain.
FEBS Lett. **308**:154-160.
- Tanaka, K. (2000).
Formin family proteins in cytoskeletal control.
Biochem. Biophys. Res. Commun., **267**:479-481.
- Tinsley, J., N. Deconinck, R. Fisher, D. Kahn, S. Phelps, J. M. Gillis, and K. Davies. (1998).
Expression of full-length utrophin prevents muscular dystrophy in *mdx* mice.
Nature Med., **4**:1441-1444.

Tinsley, J. M., D. J. Blake, A. Roche, U. Fairbrother, J. Riss, B. C. Byth, A. E. Knight, J. Kendrick-Jones, G. K. Suthers, D. R. Love, Y. H. Edwards, and K. E. Davies. (1992). Primary structure of dystrophin-related protein. *Nature*, **360**:591-593.

Tinsley, J. M., and K. E. Davies. (1993). Utrophin: a potential replacement for dystrophin? *Neuromus. Disorders*, **3**:537-539.

Tufty, R. M., and R. H. Kretsinger. (1975). Troponin and parvalbumin calcium binding regions predicted in myosin light chain and T4 lysozyme. *Science*, **187**:167-9.

Vaghy, P. L., J. Fang, W. R. Wu, and L. P. Vaghy. (1998). Increased caveolin-3 levels in mdx mouse muscles. *FEBS Lett.*, **431**:125-127.

Venema, V. J., H. Ju, R. Zou, and R. C. Venema. (1997). Interaction of neuronal nitric-oxide synthase with caveolin-3 in skeletal muscle. *J. Biol. Chem.*, **272**:28187-28190.

Verdecia, M. A., M. E. Bowman, K. P. Lu, T. Hunter, and J. P. Noel. (2000). Structural basis for phosphoserine-proline recognition by group IV WW domains. *Nature Struct. Biol.*, **7**:639-643.

Vignano, A. T. D., G. D. Zenzo, M. Sudol, G. Cesareni, and L. Dente. (2000). Contribution of the different modules in the utrophin carboxyl-terminal region to the formation and regulation of the DAP complex. *FEBS. Lett.*-**471**:229-234.

Volberg, T., Y. Zick, R. Dror, I. Sabanay, C. Gilon, A. Levitzki, and B. Geiger. (1992). The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions. *EMBO. J.*, **11**:1733-1742.

Wagner, K. R., and R. L. Haganir. (1994). Tyrosine and serine phosphorylation of dystrophin and the 58 kDa protein in the postsynaptic membrane of *Torpedo* electric organ. *J. Neurochem.*, **62**:1947-1952.

Wagner, K. R., J. B. Cohen, and R. L. Haganir. (1993). The 87K postsynaptic membrane protein from *Torpedo* is a protein-tyrosine kinase substrate homologous to dystrophin. *Neuron*, **10**:511-522.

Walsh, M. P., J. L. Busaan, E. D. Fraser, S. Y. Fu, M. D. Pato, and M. Michalak. (1995). Characterisation of the recombinant C-terminal domain of dystrophin: phosphorylation by calmodulin-dependent protein kinase II and dephosphorylation by type 2B protein phosphatase. *Biochemistry*, **34**:5561-5568.

- Way, M., B. Pope, R. A. Cross, J. Kendrick-Jones, and A. G. Weeds. (1992).
Expression of the N-terminal domain of dystrophin in *E.coli* and demonstration of binding to F-actin.
FEBS Lett., **301**:243-245.
- Wei, N., and X.-W. Deng. (1999).
Making sense of the COP9 signalosome a regulatory protein complex conserved from *Arabidopsis* to human.
Trends Genet., **15**:98-103.
- Wei, N., T. Tsuge, G. Serino, N. Dohmae, K. Takio, M. Matsui, and X.-W. Deng. (1998).
The COP9 complex is conserved between plants and mammals and is related to the 26S proteasome regulatory complex.
Curr. Biol., **8**:919-922.
- Westendorf, J. J., R. Mernaugh, and S. W. Hiebert. (1999).
Identification and characterisation of a protein containing formin homology (FH1/FH2) domains.
Gene, **232**:173-182.
- Wilkinson, D. L., and R. G. Harrison. (1991).
Predicting the solubility of recombinant proteins in *Escherichia coli*.
Bio-technol., **9**:443-448.
- Wilkinson, K. D. (2000).
Ubiquitination and deubiquitination: targetting of proteins for degradation by the proteasome.
Semin. Cell Develop. Biol., **11**:141-148.
- Williamson, R. A., M. D. Henry, K. J. Daniels, R. F. Hrstka, J. C. Lee, Y. Sunada, O. Ibraghimov-Beskrovnaya, and K. P. Campbell. (1997).
Dystroglycan essential for early embryonic development: disruption of Reichert's membrane in *Dag1*-null mice.
Hum. Mol. Genet., **6**:831-841.
- Winder, S. J. (2001).
The complexities of dystroglycan.
Trends Biochem. Sci., **26**:118-124.
- Winder, S. J. (1997).
The membrane-cytoskeleton interface: the role of dystrophin and utrophin.
J. Muscle. Res. Cell. Motil., **18**:1-13.
- Winder, S. J., L. Hemmings, S. K. Maciver, and e. al. (1995).
Utrophin actin binding domain: analysis of actin binding and cellular targetting.
J. Cell Sci., **108**:63-71.
- Winder, S. J., and J. Kendrick-Jones. (1995).
Calcium/calmodulin-dependent regulation of the NH2-terminal F-actin binding domain of utrophin.
FEBS Lett., **357**:125-128.

Winder, S. J., A. E. Knight, and J. Kendrick-Jones. (1997).

Protein structure.

In J. A. Lucy and S. C. Brown (eds.),

Dystrophin: gene, protein and cell biology, pp. 27-55.

Cambridge University Press, Cambridge.

Woychik, R. P., R. L. Maas, R. Zeller, T. F. Vogt, and P. Leder. (1990).

'Formins' : proteins deduced from the alternative transcripts of the *limb deformity* gene.

Nature, **346**:850-853.

Wu, H., A. B. Reynolds, S. B. Kanner, R. R. Vines, and J. T. Parsons. (1991).

Identification and characterisation of a novel cytoskeleton-associated pp60^{src} substrate.

Mol. Cell. Biol., **11**:5113-5124.

Yaffe, D., and O. Saxel. (1977).

Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle.

Nature, **270**:725-727.

Yamada, H., F. M. S. Tome, I. Higuchi, H. Kawai, K. Azibi, M. Chaouch, S. L. Roberds, T.

Tanaka, S. Fujita, T. Mitsui, H. Fukunaga, K. Miyoshi, M. Osame, M. Fardeau, J.-C. Kaplan, T.

Shimizu, K. P. Campbell, and K. Matsumura. (1995).

Laminin abnormality in severe childhood autosomal recessive muscular dystrophy.

Lab. Invest., **72**:715-721.

Yamamoto, H., Y. Hagiwara, Y. Mizuno, M. Yoshida, and E. Ozawa. (1993).

Heterogeneity of dystrophin-associated proteins.

J. Biochem., **114**:132-139.

Yang, B., D. Jung, D. Motto, J. Meyer, G. Koretzky, and K. P. Campbell. (1995).

SH3 domain-mediated interaction of dystroglycan and Grb2.

J. Biol. Chem., **270**:11711-11714.

Yoshida, M., H. Hama, M. Ishikawa-Sakurai, M. Imamura, Y. Mizuno, K. Araishi, E.

Wakabayashi-Takai, S. Noguchi, T. Sasaoka, and E. Ozawa. (2000).

Biochemical evidence for association of dystrobrevin with the sarcoglycan-sarcospan complex as a basis for understanding sarcoglycanopathy.

Hum. Mol. Genet., **9**:1033-1040.

Yoshida, M., and E. Ozawa. (1990).

Glycoprotein complex anchoring dystrophin to the sarcolemma.

J. Biochem., **108**:748-752

Yoshida, M., A. Suzuki, H. Yamamoto, S. Noguchi, Y. Mizuno, and E. Ozawa. (1994).

Dissociation of the complex of dystrophin and its associated proteins into several unique groups by *n*-octyl β -D-glucoside.

Eur.J. Biochem., **222**:1055-1061.

Yoshida, T., H. Hanada, Y. Iwata, Y. Pan, and M. Sigekawa. (1996).

Expression of a dystrophin-sarcoglycan complex in serum-deprived BC3H1 cells and involvement of α -sarcoglycan in substrate attachment.

Biochem. Biophys. Res. Commun., **225**:11-15.

- Yoshida, S., A. Fujisawa-Sehara, T. Taki, K.-I. Arai, and Y.-I. Nabeshima. (1996).
Lysophosphatidic acid and bFGF control different modes in proliferating myoblasts.
Journal of Cell Biology, **132**:181-193
- Yoshida, T., Y. Pan, H. Hanada, Y. Iwata, and M. Shigekawa. (1998).
Bidirectional signaling between sarcoglycans and the integrin adhesion system in cultured
L6 myocytes.
J. Biol. Chem., **273**:1583-1590

Appendix

β -dystroglycan amino-acid sequence**Extracellular domain**

655	GSIVVEWTNN
665	TLPLEPCPKE
675	QITGLSRRIAE
685	DNGQPRPAFT
695	NALEPDFKAT
705	SIAITGSGSC
715	RHLQFIPVAP
725	PGIPSSVTPPT
735	EVPDRDPEKS
745	SEDDVY

Transmembrane domain

751	LHTVIPAVVV
761	AAILLIAGII
771	AMICY

Intracellular domain

776	RKKRKGKLT
786	EDQATFIKKG
796	VPIFADELD
806	DSKPPSSSM
816	PLILQEEKAP
826	LPPPEYPSQS
836	VPETTPLNQD
846	TVGEYTPLRD
856	EDPNAPPYQP
866	PPPFTAPMEG
876	KGSRPKNMTP
886	YRS <u>PPPY</u> VPP

 β -dystroglycan amino-acid sequence

Separated into the extracellular (amino-acids 655-750), transmembrane (amino-acids 751-775) and intracellular domains (amino-acids 776-895). The WW-domain interacting motif PPXY is doubly underlined.

Published Papers

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The WW domain: linking cell signalling to the membrane cytoskeleton.

Cellular Signalling, (in press)

James, M., A. Nuttall, **J. L. Ilsley,** K. Ottersbach, J. M. Tinsley, M. Sudol, and S. J. Winder. (2000).

Adhesion-dependent tyrosine phosphorylation of β -dystroglycan regulates its interaction with utrophin.

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The interaction of dystrophin with β -dystroglycan is regulated by tyrosine phosphorylation

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Abstract

Dystrophin and the dystrophin-associated protein complex (DAPC) have recently been implicated in cell signalling events. These proteins are ideally placed to transduce signals from the extracellular matrix (ECM) to the cytoskeleton. Here we show that β -dystroglycan is tyrosine-phosphorylated in C2/C4 mouse myotubes. Tyrosine phosphorylation was detected by mobility shifts on SDS-polyacrylamide gels (SDS-PAGE) and confirmed by immunoprecipitation and two-dimensional gel electrophoresis. The potential functional significance of this tyrosine phosphorylation was investigated using peptide 'SPOTS' assays. Phosphorylation of tyrosine in the 15 most C-terminal amino acids of β -dystroglycan disrupts its interaction with dystrophin. The tyrosine residue in β -dystroglycan's WW-binding motif PPPY appears to be the most crucial in disrupting the β -dystroglycan–dystrophin interaction. β -dystroglycan forms the essential link between dystrophin and the rest of the DAPC. This regulation by tyrosine phosphorylation may have implications in the pathogenesis and treatment of Duchenne's muscular dystrophy (DMD). © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Dystrophin; Dystroglycan; Tyrosine phosphorylation; Regulation; Muscular dystrophy

1. Introduction

Duchenne's muscular dystrophy (DMD) is caused by mutations at the DMD locus leading to a total lack of dystrophin protein in muscle cells [1]. Dystrophin associates with a group of membrane-associated proteins called the dystrophin-associated protein complex (DAPC). The functions and the interactions of the dystrophin and DAPC proteins are not clearly defined. They are however, crucial in maintaining muscle cell integrity, as demonstrated by the severe defects when they are disrupted [2]. In skeletal muscle, dystrophin is proposed to act as a link between the actin cytoskeleton and the extracellular matrix (ECM), thus stabilising myofibres during muscle contraction and relaxation [3]. Additionally, dystrophin has been implicated in cell signalling events. Spatially, it is ideally placed to transduce signals from the ECM into the cell, and there is

evidence of direct phosphorylation of dystrophin and the DAPC proteins [4–8]. The functional consequences of these phosphorylation events are unknown although recently, the phosphorylation of α - and γ -sarcoglycans was shown to be induced by integrin-mediated cell adhesion in muscle cells [9]. Furthermore, we have shown that β -dystroglycan is tyrosine-phosphorylated in an adhesion-dependent manner in non-muscle cells, and this affects its interaction with utrophin, the ubiquitous homologue of dystrophin [10].

β -Dystroglycan provides the essential link between dystrophin and the DAPC. The dystroglycan gene encodes both β -dystroglycan and the extracellular α -dystroglycan, which interacts with laminin in the ECM [11]. The importance of the dystroglycans is demonstrated by the gene knockout in mice, which leads to embryonic lethality at Day 6.5 due to failure of the Reichert's membrane to form [12] and embryonic stem cells deficient for these proteins are unable to assemble basement membrane [13] (see Ref. [14] for review). No clinical effects of β -dystroglycan mutations have been identified, presumably because the consequences are too profound for survival.

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It has been shown previously that the cysteine-rich region of dystrophin is required for the binding of β -dystroglycan and the binding affinity is strengthened by the C-terminal domain [15–17]. More recently, Rentschler et al. [18] have shown that this interaction requires the WW and EF hand domains. The ZZ domains are not essential but significantly stabilise the interaction.

In this current study, we show that β -dystroglycan is tyrosine-phosphorylated in muscle cells and that phosphorylation is important in the regulation of the dystrophin– β -dystroglycan interaction.

2. Materials and methods

2.1. Cell culture

C2/C4 myoblasts [19] were maintained in DMEM media (Gibco BRL) supplemented with 20% foetal calf serum (Gibco BRL) in 5% CO₂ atmosphere at 37°C. In order to induce differentiation, the amount of foetal calf serum in the growth media of confluent or semiconfluent myoblasts was reduced to 2% for up to 9 days. Myoblasts fused to form myotubes from Day 4.

C2/C4 myotubes were treated with peroxyvanadate in order to inhibit the activity of tyrosine phosphatases [20]. The cells were washed with serum-free DMEM and then incubated in peroxyvanadate (2 mM H₂O₂, 1 mM sodium orthovanadate in serum-free DMEM media) for 45 min at 37°C. Cells were washed once in cold phosphate-buffered saline (50 mM sodium phosphate pH 7.2, 150 mM NaCl) before being harvested in ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM ethylene glycol-bis-(*b*-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 100 μ M leupeptin, 1 mM phenylmethylsulphonyl fluoride (PMSF), 100 μ M *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK). Cells that were not treated with peroxyvanadate were incubated for an equivalent time in serum-free media before being harvested in RIPA as above. After harvesting, cells were briefly sonicated to shear the DNA, centrifuged (15,000 rpm, 20 min), and stored at –20°C until assay.

The protein concentration in cell extracts was quantified using the BCA protein assay (Pierce) following the manufacturers' instructions.

2.2. Antisera

NCL-DYS1 and NCL-DYS2 were gifts from L. Anderson (University of Newcastle) and are directed against the mid-rod domain (amino acids 1181–1388) and C-terminus (amino acids 3669–3685) of dystrophin (commercially available from Novacastra Laboratories). MANDAG2 [21] was kindly provided by G.E. Morris (NE Wales Institute,

Wrexham) and is directed against the C-terminus of β -dystroglycan (amino acids 881–895). Polyclonal antisera against β -dystroglycan phosphopeptide CMTPYRSPPP-pYVPP (poly β -DG 1710) and human utrophin carboxy-terminal coiled-coil domain (residues 3204–3433) (RAB5) [22] were raised in rabbits using standard techniques. Antiphosphotyrosine monoclonals (PY20 and RC20) were from Transduction Laboratories. The following dilutions were effective for Western blotting: MANDAG2 (1:500), NCL-DYS1 (1:100), NCL-DYS2 (1:10), PY20 (1:1000), RC20 (1:500), and RAB5 (1:10,000).

2.3. Immunoprecipitation assays

Cell extracts in RIPA were initially cleared by incubation with protein A-Sepharose or protein G-Sepharose for 1–2 h at 4°C. The required amount of antibody was added to the cleared extract and the cells were incubated overnight at 4°C. In order to precipitate the antibody–protein complex, 50 μ l of a 50% slurry of protein A-Sepharose was added per sample, and incubated for a further 1–2 h at 4°C. The samples were centrifuged briefly to pellet the Sepharose beads, and the beads were washed four times with RIPA buffer. The final pellet was resuspended in SDS-polyacrylamide gels (SDS-PAGE) loading buffer. Immunoprecipitations of β -dystroglycan were carried out with the following antisera at the indicated dilutions: MANDAG2 (1:5), poly β -DG 1710 (1:10).

2.4. Lambda phosphatase treatment

Lambda phosphatase (λ ppase, New England BioLabs) was used to treat immunoprecipitates of β -dystroglycan. The β -dystroglycan immunoprecipitates were treated with 200 units of λ ppase at 30°C for 40 min. A cocktail of λ ppase, 1X λ ppase buffer, 2 mM MnCl₂, and H₂O to 10 μ l was added directly to the protein A-Sepharose beads. The reaction was stopped by boiling in SDS-PAGE loading buffer for 2 min.

2.5. SDS-PAGE and Western blotting

Samples from immunoprecipitation experiments were run on 7.5–20% acrylamide SDS-PAGE with a 5% stacking gel under reducing conditions [23] and blotted onto polyvinylidene difluoride (PVDF) membranes in 10 mM CAPS, 20% methanol, and processed for immunodetection as previously described [10].

2.6. Two-dimensional gel electrophoresis

β -Dystroglycan was immunoprecipitated from equal amounts of protein from peroxyvanadate-treated or untreated cell extracts, prepared as above. The Sepharose beads were resuspended in rehydration buffer (8 M urea, 2% CHAPS, 2% ampholine, 20 mM DTT) and incubated for 1 h at room temperature. After centrifugation, the supernatant

was used to rehydrate 180 mm pH 3–10 Immobiline Dry-strips (Amersham Pharmacia Biotech) overnight. Strips were then subjected to isoelectric focusing on an Amersham Pharmacia Biotech Multiphor System in gradient mode (1 mA, 5 W, and 300 V for 0.01 h, 300 V for 3 h, 3500 V for 5 h, 3500 V for 12.5 h). Strips were stored at -80°C prior to SDS-PAGE. Strips equilibrated in equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 1% DTT) for 30 min were separated in the second dimension on reducing 3–15% SDS-PAGE with no stacking gel. All gels were electroblotted onto PVDF and immunoblotted with antiphosphotyrosine or β -dystroglycan antisera as described above. The immunoblots were developed by ECL (Amersham Pharmacia Biotech).

2.7. Generation of dystrophin fusion protein

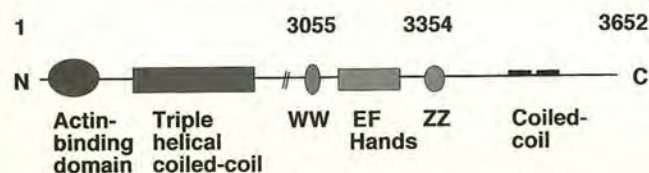
The limits of the dystrophin cysteine rich domains WW, EF, and ZZ were identified by sequence alignment and secondary structure prediction [10]. cDNA encoding residues 3055–3354 of human dystrophin (encompassing the WW, EF, and ZZ domains, see Fig. 1A) was amplified by polymerase chain reaction (PCR). The 5' primer contained a *Hind*III restriction site and the 3' primer contained a stop codon and *Not*I restriction site. PCR products were cloned into the unique *Hind*III and *Not*I restriction enzyme sites of

the PinPoint Xa-3 vector (Promega) to produce the DYS–PIN construct. *Escherichia coli* (JM109) was transformed with PinPoint Xa-3 (control; expression produces a biotin tag only (PIN)) or DYS–PIN and grown at 37°C until the OD_{600} reached 0.6, then induced with 0.5 mM isopropyl-*b*-D-thiogalactopyranoside for 2 h. Following harvesting by centrifugation (4200 rpm, 10 min), the cell pellet was stored at -70°C overnight. After thawing, the pellet was resuspended in SET buffer (25% w/v sucrose, 50 mM Tris-HCl, pH 8, 1 mM EDTA, 100 μM leupeptin, 1 mM PMSF, 100 μM TPCK, 10 mg/ml STI) and the cell suspension treated with lysozyme (0.5 mg/ml), followed by DNaseI (1 $\mu\text{g}/\text{ml}$). The lysed cells were then sonicated and centrifuged (14,000 rpm, 20 min) followed by ultracentrifugation (35,000 rpm, 1 h). Expressed protein was purified by incubation of the crude cell supernatant with Softlink soft release avidin resin (Promega: 1 ml resin/2 l culture) overnight, at 4°C . The resin was subsequently washed four times with lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% glycerol) and protein eluted with 5 mM free biotin in lysis buffer, overnight, at 4°C . The free biotin was subsequently removed by dialysis in lysis buffer. The purification was monitored by SDS-PAGE and Western blotting using ExtraAvidin-HRP (Sigma) followed by detection with ECL (see Fig. 1B).

2.8. Hybridisation of 'SPOTS' membranes with DYS–PIN fusion protein

SPOTS membrane synthesis was carried out as described previously [10,18]. A dystrophin WW–EF–ZZ-biotinylated tag fusion protein (DYS–PIN) was purified from bacterial lysates as above. SPOTS membranes were blocked overnight in 10% milk in TBST at 4°C and then probed overnight with DYS–PIN protein or PIN alone (10 $\mu\text{g}/\text{ml}$). The membrane was then washed four times with TBST and incubated with ExtraAvidin-HRP diluted 1:1000 in TBST for 2 h. After four washes with TBST, the blot was developed by ECL.

A. Schematic of dystrophin domains



B. Expression of dystrophin fusion protein

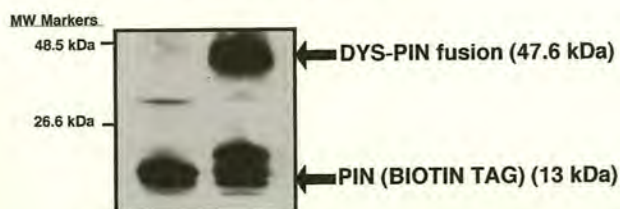


Fig. 1. (A) Schematic showing the protein domains of dystrophin. A construct was made comprising amino acids 3055–3354 (encompassing the WW, EF hand, and ZZ domains), fused to a biotin tag (PIN), 1–3652 across the top denotes amino acid numbers. (B) Expression of dystrophin construct fused to biotin tag. Amino acids 3055–3354 of dystrophin were cloned into the PinPoint Xa-3 vector. This construct and the vector alone were bacterially expressed to produce a biotin tag (PIN 13 kDa) and the DYS–PIN fusion (37.6 kDa), which were purified using avidin resin. The purified proteins were Western blotted with ExtraAvidin-HRP and developed with ECL. Biotin tag alone left lane and DYS–PIN fusion right lane. The large PIN band in the right lane is as a result of proteolytic degradation of the fusion protein.

3. Results

3.1. Characterisation of myotube differentiation

Mouse skeletal muscle cell lines have been widely used as a model for studying myogenesis. On reaching confluency and/or reduction in serum levels, myoblasts spontaneously fuse to form multinucleated myotubes and express muscle specific proteins. Several different mouse skeletal muscle cell lines were assessed for their use in this study. The C2/C4 cell line (a recloned C2/C12 line) [19] was chosen for its efficient myoblast fusion and the expected expression pattern of DAPC proteins. In a time course of differentiation, β -dystroglycan is expressed equally in myoblasts and myotubes, as is utrophin (the ubiquitous homologue of dystrophin) whereas dystrophin is only expressed

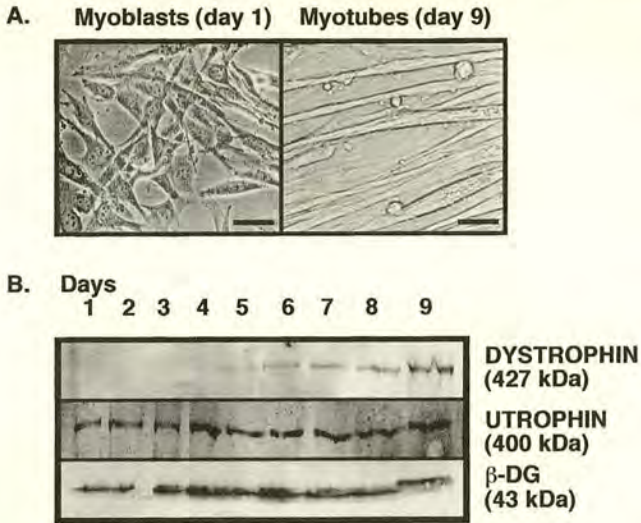


Fig. 2. Differentiation time course of C2/C4 myoblasts. (A) Myoblasts on Day 1 (left) compared to multinuclear myotubes after 9 days (right) in low serum differentiation medium. Scale bar = 50 μ m. (B) Western blots for dystrophin, utrophin, and β -dystroglycan in fusing myoblasts. The serum content in the growth media was reduced from 20% to 2% on Day 1 when the myoblasts were 50% confluent. Myoblast fusion first started to occur at Days 3 and 4. Myotubes appeared fully formed by Day 5. Dystrophin is only expressed in fully fused myoblasts (Day 5 onwards) whereas the ubiquitous utrophin and β -dystroglycan are expressed equally in myoblasts and myotubes.

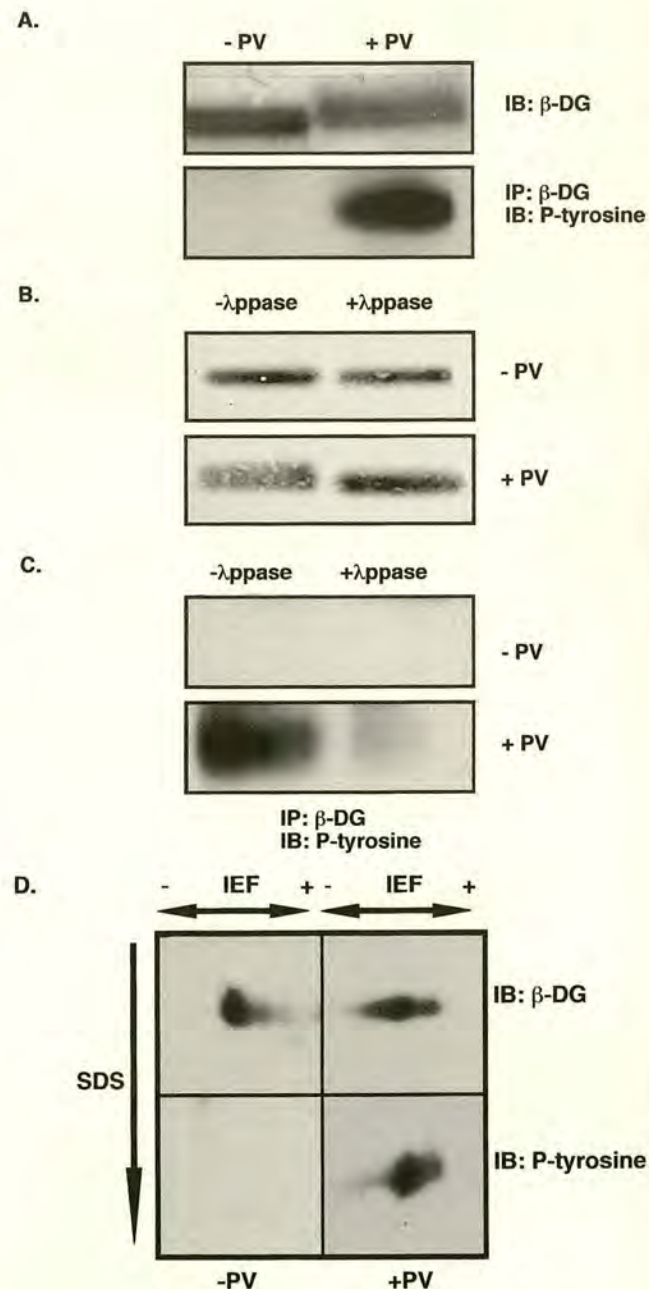
in fairly advanced stages of differentiation when myotubes are fully formed (see Fig. 2). Previously it has been shown that in cultured cells, dystrophin mRNA is expressed only in fused myotubes and not myoblasts [24].

Fig. 3. Phosphorylation of β -dystroglycan in C2/C4 myotube extracts. (A) Upper panels: Western blot of myotube extracts with the anti- β -dystroglycan monoclonal antibody MANDAG2. A distinct reduction in electrophoretic mobility of β -dystroglycan was observed in peroxyvanadate (+PV)-treated cell extracts as compared to untreated cell extracts (-PV) blotted for β -dystroglycan. Lower panels: Western blot of β -dystroglycan immunoprecipitates with antiphosphotyrosine antibody RC20. RC20 only recognises β -dystroglycan in the immunoprecipitations from peroxyvanadate-treated cell extracts, suggesting that β -dystroglycan is tyrosine-phosphorylated under these conditions. (B) Western blot of β -dystroglycan immunoprecipitates from either control (upper panel) or peroxyvanadate-treated myotube extracts (lower panel) with (+ λ ppase) or without (- λ ppase) lambda phosphatase treatment. In peroxyvanadate-untreated cell extracts λ ppase has no effect on electrophoretic mobility, whereas in peroxyvanadate-treated cells, the mobility shift is abolished by λ ppase treatment, suggesting that it is caused by phosphorylation of β -dystroglycan. (C) As (B), but β -dystroglycan immunoprecipitation was detected with antiphosphotyrosine antisera. λ ppase treatment abolishes the phospho tyrosine immunoreactivity on β -dystroglycan in peroxyvanadate-treated cells. (D) Two dimensional gel electrophoresis of β -dystroglycan from peroxyvanadate-treated myotubes. β -Dystroglycan immunoprecipitates were separated by isoelectric-focusing (IEF) followed by SDS-PAGE and blotted with β -dystroglycan monoclonal antibody MANDAG2. The immunoprecipitates from peroxyvanadate-treated cell extracts have three closely migrating spots (upper right) compared to one clear spot in untreated extracts (upper left). Antiphosphotyrosine antisera detects two poorly defined spots migrating with the same mobility as β -dystroglycan, and only in peroxyvanadate-treated myotube extracts.

At 9 weeks of gestation, dystrophin protein in foetal muscle samples is barely detectable with antibodies on Western blots and only distinct populations of larger myotubes are recognised by dystrophin immunostaining. However, the staining pattern increases and becomes more uniform with age, but does not reach the adult pattern of expression until 26 weeks of gestation [25]. This suggests that the dystrophin expression pattern in C2/C4 myotubes is equivalent to the *in vivo* situation.

3.2. β -Dystroglycan phosphorylation

We had previously found that peroxyvanadate treatment of HeLa cells caused a retardation in electrophoretic mobi-



lity of β -dystroglycan on Western blots [10]. When extracts of peroxyvanadate-treated C2/C4 myotubes were run on SDS-PAGE and Western blotted with β -dystroglycan antisera, a significant upward shift of β -dystroglycan was seen in the treated cells due to a retardation in electrophoretic mobility compared to β -dystroglycan in non-treated cells (Fig. 3A, upper panel). C2/C4 myoblasts and myotubes treated with peroxyvanadate for 45 min produced the maximum mobility shift of β -dystroglycan on SDS-PAGE. When exposed to peroxyvanadate for an hour or more, the cells start to round up and detach from the tissue culture flask (data not shown). Mobility shifts are indicative of a modification of the protein, for example, a phosphorylation event. Treatment of the myotubes with calyculin A (an inhibitor of Types 1 and 2A serine/threonine phosphatases

[26]) did not cause any retardation in electrophoretic mobility (data not shown), suggesting that if the modification is due to a phosphorylation event, it is phosphorylation of tyrosine residues rather than serine or threonine residues. To establish that this phosphorylation event was in fact tyrosine phosphorylation of β -dystroglycan, immunoprecipitates of β -dystroglycan were recognised on Western blots by anti-phosphotyrosine antisera (Fig. 3A, lower panels).

Peroxyvanadate has previously been shown to directly modify thiol groups in proteins [27]. To further confirm that the electrophoretic mobility shift was due to phosphorylation of β -dystroglycan, λ ppase, a serine, threonine, and tyrosine phosphatase, was used. When the β -dystroglycan immunoprecipitates from peroxyvanadate-treated cell extracts were treated with λ ppase the electrophoretic mobi-

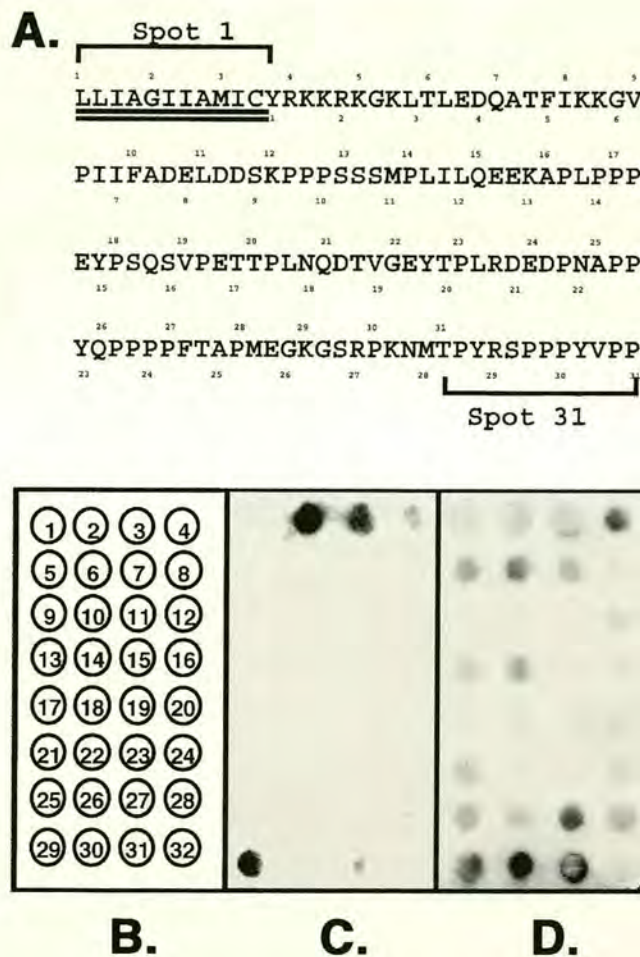


Fig. 4. Identification of β -dystroglycan peptides that interact with DYS-PIN fusion protein (dystrophin WW-EF-ZZ). (A) Scheme for the synthesis of peptides on the 'SPOTS' membrane. Twelve amino acid long peptides representing part of the transmembrane region and the entire cytoplasmic domain of human β -dystroglycan (amino acids 764–895) were synthesised on derivatised 'SPOTS' membranes with an offset of four amino acids. The transmembrane region is denoted by a double underline. Numbers above the β -dystroglycan sequence denote the first amino acid of the peptide found on the corresponding spot whilst numbers below the sequence denote the last amino acid of the peptide on the corresponding spot. Brackets identify the sequences of spots 1 and 31. Spot 32 is a negative control where no peptide is synthesised. (B) Numbered circles identify the location of each spot on the membrane. (C) Control 'SPOTS' membrane described as above probed with PIN protein alone, and detected by ECL. The PIN protein bound weakly to spots 2, 3, and 29. (D) 'SPOTS' membrane described as above probed with dystrophin (DYS-PIN) fusion protein and detected by ECL. The DYS-PIN fusion bound strongly to spots 30 (PKNMTPYRSPPP) and 31 (TPYRSPPPYVPP), which contains a recognised WW-binding motif.

lity of β -dystroglycan was returned to levels equivalent to β -dystroglycan from untreated cells (see Fig. 3B, lower panels). This strongly suggests that the mobility shift of β -dystroglycan in peroxyvanadate-treated myotubes is due to a phosphorylation event rather than a modification of thiol groups. This was confirmed in a second experiment where β -dystroglycan immunoprecipitates from control or peroxyvanadate-treated cells were Western blotted with antisera against phosphotyrosine before and after λ ppase treatment. As expected, λ ppase treatment of the β -dystroglycan immunoprecipitate from peroxyvanadate-treated cells resulted in the loss of almost all the phosphotyrosine immunoreactivity (Fig. 3C).

In order to examine the tyrosine phosphorylation of β -dystroglycan in more detail, two-dimensional gel electrophoresis was carried out on immunoprecipitates of β -dystroglycan from peroxyvanadate-treated and -untreated myotube extracts. The immunoprecipitates were separated by isoelectric focusing, followed by SDS-PAGE, and Western blotting with β -dystroglycan antibodies (see Fig. 3D, upper). In extracts from untreated cells, β -dystroglycan appears as a single spot, whereas in peroxyvanadate-treated cells, β -dystroglycan appears as two or possibly three spots, indicative of additional charged species, likely to be the result of phosphorylation. When β -dystroglycan immunoprecipitates separated by two-dimensional electrophoresis were blotted with the antiphosphotyrosine antibody PY20, the antibody recognised spots migrating in the same position as β -dystroglycan, but only in peroxyvanadate-treated cells (Fig. 3D, lower).

3.3. 'SPOTS' membrane binding assays

The phosphorylation of β -dystroglycan on tyrosine could potentially regulate the interaction between β -dystroglycan and dystrophin. To elucidate whether the tyrosine phosphorylation of β -dystroglycan affects its interaction with dystrophin, peptide 'SPOT' blots of the β -dystroglycan cytoplasmic domain were probed with purified dystrophin fusion protein comprising the WW, EF hands, and ZZ domains fused to a biotin tag (DYS-PIN). Firstly, the interaction site between β -dystroglycan and dystrophin was confirmed by probing the cytoplasmic domain of β -dystroglycan with DYS-PIN fusion protein. Fig. 4 shows a β -dystroglycan SPOT blot comprising the entire cytoplasmic domain of β -dystroglycan as 12 overlapping amino acid peptides, offset by four amino acids. Some non-specific binding to spots 2, 3, 29, and to a lesser extent to spot 4 was revealed in a control experiment, when the biotin tag alone (PIN) was incubated with the β -dystroglycan 'SPOTs'. DYS-PIN also bound weakly to spots 4 and 29, and to spot 27, however DYS-PIN bound very strongly to spots 30 and 31. This would suggest that the specific dystrophin/ β -dystroglycan interaction is restricted to spots 30 and 31, equivalent to the carboxyl terminal 16 amino acids of β -dystroglycan.

A. B.



1. KNMTPYRSPPPYVPP
2. KNM_pTPYRSPPPYVPP
3. KNMTP_pYRSPPPYVPP
4. KNMTPYR_pSPPPYVPP
5. KNMTPYRSPP_pYVPP
6. KNMTP_pYRSPP_pYVPP
7. KNM_pTPYR_pSPPPYVPP
8. KNM_pTP_pYR_pSPP_pYVPP

Fig. 5. The effect of β -dystroglycan phosphorylation on binding of β -dystroglycan peptides to DYS-PIN fusion protein. (A) A peptide equivalent to spots 30/31 synthesised containing the phospho amino acids pS, pT, and pY at each possible position in the peptide, either singly or in multiples. The membrane was probed with dystrophin (DYS-PIN) as described above. The binding of DYS-PIN was greatly reduced by phosphorylation of Tyr⁸⁹² in the PPPY motif (peptide 5). This binding was further reduced when both Tyr⁸⁸⁶ and Tyr⁸⁹² are phosphorylated (peptides 6 and 8), but not affected by serine/threonine phosphorylation (peptide 7).

To ascertain whether phosphorylation of residues in this region of β -dystroglycan affects its interaction with β -dystrophin, a separate SPOT blot comprising the peptides from spots 30 and 31 as a single peptide (KNMTPYRSPPPYVPP) was used for binding assays with DYS-PIN (Fig. 5A). A series of peptides was synthesised with phospho amino acids at all potential phosphorylation sites, i.e., serine, threonine, and tyrosine residues (see Fig. 5B). DYS-PIN bound strongly to spot 1, equivalent to spots 30/31 in Fig. 4A. The phosphorylation of Thr⁸⁸⁴, Tyr⁸⁸⁶, and Ser⁸⁸⁸ (peptides 2, 3, and 4) appeared to have no effect on this binding. However, when Tyr⁸⁹² in the PPPY motif is phosphorylated (peptide 5), the binding of dystrophin was greatly reduced. This binding is further reduced when both Tyr⁸⁸⁶ and Tyr⁸⁹² are phosphorylated (peptide 6). Interestingly, in peptide 4 when only Tyr⁸⁸⁶ is phosphorylated, binding of dystrophin was not affected, indicating that the phosphorylation of Tyr⁸⁹² in the PPPY motif is necessary to abolish the interaction. These data therefore support the conclusion that phosphorylation of β -dystroglycan on Tyr⁸⁹² is able to regulate its interaction with the dystrophin C-terminus.

4. Discussion

Dystrophin and the DAPC were initially proposed to be proteins whose primary role is structural. More recent studies have implicated the DAPC in signalling events, and thus it can now be thought of as a more dynamic complex. The complex is ideally placed to transduce signals from the ECM to the inside of cells. The phosphorylation of dystrophin on serine and threonine has been previously

described (reviewed in Refs. [28,29]) and analysis of dystrophin and the DAPC using two-dimensional gel electrophoresis suggests that dystrophin, β -dystroglycan, and potentially other components of the DAPC are phosphorylated [30]. β -Dystroglycan is phosphorylated in non-muscle cells [10] but β -dystroglycan has not previously been shown to be phosphorylated in muscle cells.

The WW domain of dystrophin is classified as a Class 1 WW domain and shares the PPxY ligand motif with other members of its class, including the Yes-associated protein kinase YAP65 and Nedd-4, a ubiquitin ligase [31]. The finding that the tyrosine residue in the PPPY motif was critical for binding to Class 1 WW domains [32,33], raised the question that tyrosine phosphorylation could regulate WW domain binding to its cognate ligand.

In this study, we have shown that the phosphorylation of tyrosine residues within and adjacent to β -dystroglycan's WW domain disrupts its interaction with dystrophin. The DYS–PIN fusion protein bound strongly to the peptide (TPYRSPPPYVPP) (spot 31), which contains a recognised WW-binding motif. A dystrophin–GST fusion has also been observed to bind a similar peptide [18] and James et al. [10] showed that a utrophin–MBP (maltose binding protein) fusion also bound the same sequence. This region of β -dystroglycan has been identified previously as being necessary for binding to dystrophin [16]. More recently, it has been shown that the PPPY motif in this peptide is required for β -dystroglycan binding to the WW domain of dystrophin [18]. DYS–PIN also bound strongly to the peptide (PKNMTPYRSPPP) (spot 30), possibly because it overlaps with spot 31 and contains residues involved in the association of β -dystroglycan with the EF hand region of dystrophin [34]. Previous experiments with a dystrophin–GST fusion demonstrated strong binding to spot 31 only [18]. The finding that spot 30 is also bound may be due to differences in the limits of the constructs used (dystrophin residues 3055–3354 [this study] versus residues 3046–3447 [18]).

The EF hands and ZZ domain of dystrophin are also involved in its interaction with β -dystroglycan but they probably have a more stabilising role, holding the two proteins in the correct orientation. The WW domain is likely to be the main site by which the interaction is regulated, but the EF hands are clearly also required for binding [18]. The recent crystal structure of dystrophin WW–EF hands region complexed with a β -dystroglycan peptide [34], clearly shows that the β -dystroglycan peptide binds a composite surface formed by both the WW domains and one of the two EF hands (see Fig. 6). The structure also reveals the importance of Tyr⁸⁹² in the interaction. From the structure, one can predict that phosphorylation of Tyr⁸⁹² in the PPPY motif of β -dystroglycan would disrupt the interaction with the WW domain of dystrophin. Tyr⁸⁹² fits into a hydrophobic pocket of the WW domain and the interaction is co-ordinated by a hydrogen bond from the conserved histidine residue (His³⁰⁷⁶) of the WW domain. Phosphorylation of

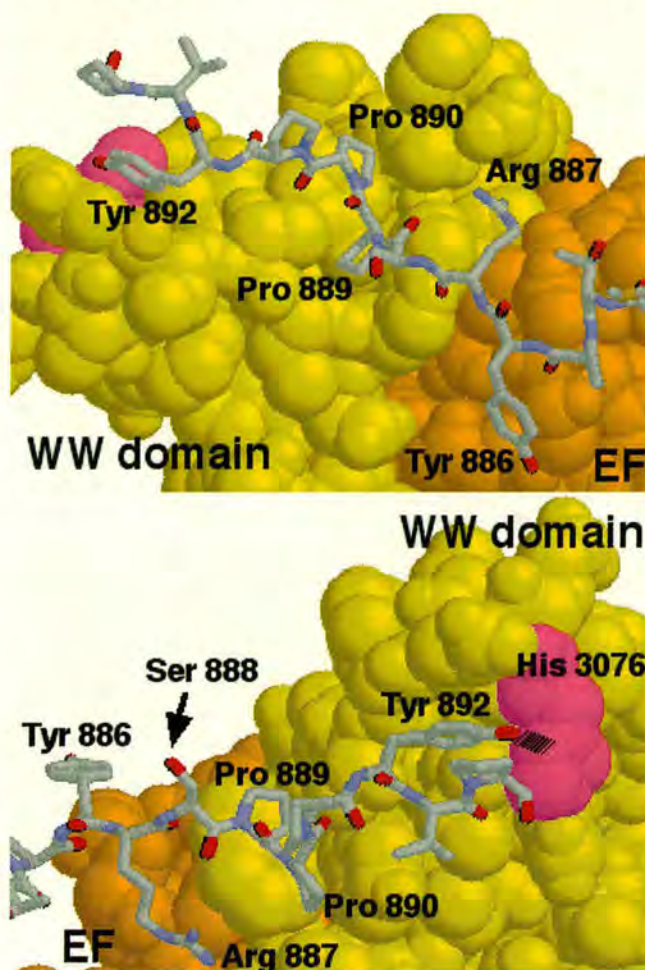


Fig. 6. Two different views of the structure of the dystrophin WW–EF hand region complexed with a β -dystroglycan peptide (from Ref. [34]). WW domain (yellow) and EF hand region (orange) are shown in a space filling representation, showing His³⁰⁷⁶ (magenta) in the WW domain. Only the region of the EF hand region that comes into contact with the β -dystroglycan peptide (shown in stick representation and CPK colouring) is shown for clarity. Residues in the β -dystroglycan peptide that make contact with dystrophin are labelled (upper panel). In the lower panel, Tyr⁸⁸⁶ and Ser⁸⁸⁸ are also labelled to indicate that they are not in close contact with dystrophin. The hydrogen bond between Tyr⁸⁹² and His³⁰⁷⁶ is represented as a dashed line.

Tyr⁸⁹² would break a hydrogen bond and render it too bulky to fit into the WW domain pocket. Tyr⁸⁸⁶, which is N-terminal to the PPPY motif, also appears to affect the dystrophin– β -dystroglycan interaction when it is phosphorylated (see Fig. 5, spot 6). Like Tyr⁸⁹² it also resides in a pocket of dystrophin protein, but in the EF hand region. Whilst phosphorylation of Tyr⁸⁸⁶ alone is not sufficient to prevent binding, in conjunction with phosphorylation of Tyr⁸⁹², binding to the peptide is completely abolished. Chen et al. have described a similar phenomenon in the WBP-1/YAP65 interaction, in that phosphorylation of the tyrosine in its PPPY motif of WBP-1 abolishes its interaction with the WW domain of YAP65 in *in vitro* ‘SPOTS’ assays [32]. Phosphorylation of the tyrosine residue in β -dystroglycan's PPPY motif also disrupts its interaction with utrophin, as

shown by 'SPOT' blot [10]. This latter study also demonstrated that fusion proteins of utrophin WW, EF hand, and ZZ domains also failed to interact with tyrosine-phosphorylated β -dystroglycan in HeLa cells [10].

The tyrosine phosphorylation of β -dystroglycan in non-muscle cells is adhesion-dependent [10] although in myotubes there does not appear to be adhesion-dependent phosphorylation of β -dystroglycan (data not shown) Ref. [9]. This may be due to the static nature of the differentiated muscle cells compared to HeLa cells, or a different role of the DAPC compared to the utrophin-associated protein complex. However, the DAPC has been implicated in integrin-mediated adhesion events previously, in that α - and γ -sarcoglycans from myotubes were found to be phosphorylated on tyrosine residues in response to adhesion. This phosphorylation was also induced by the RGDS (integrin recognition sequence) peptide, implying that the DAPC is involved with the integrin adhesion system [9]. This tyrosine phosphorylation was inhibited by Herbimycin A, which suggests the involvement of the Src family of kinases. More investigations need to be carried out in order to determine the nature of the kinase involved, which will, in turn, lead to a greater understanding of the regulation and function of dystrophin and the DAPC.

This study suggests that there is a role for tyrosine phosphorylation in regulating β -dystroglycan–dystrophin interactions. It has been suggested that disruption of the β -dystroglycan–dystrophin connection could lead to muscular dystrophy [18] but this study suggests that this interaction can be regulated in a dynamic way, but in muscular dystrophy, the regulation may be disrupted. This may have implications for the disease pathogenesis of muscular dystrophies. The dynamic nature of the β -dystroglycan–dystrophin interaction is further confirmed by recent work on the β -dystroglycan–Grb2 interaction [35]. The Grb2– β -dystroglycan interaction could facilitate the transduction of signals between the DAPC and extracellular proteins and other signalling pathways. In vitro studies suggest that dystrophin and Grb2 compete in order to bind to β -dystroglycan, which supports the hypothesis that the DAPC is actively turned over, but in a highly regulated manner. Disruption of this delicate balance by reduction or loss of dystrophin may explain some of the poorly understood mechanisms of disease progression in Becker's muscular dystrophy and DMD.

Acknowledgments

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References

- [1] Hoffman EP, Fischbeck KH, Brown RH, Johnson M, Medori R, Loike JD. *N Engl J Med* 1988;318:1363–8.
- [2] Ervasti JM, Ohlendieck K, Kahl SD, Gaver MG, Campbell KP. *Nature* 1990;345:315–9.
- [3] Petrof BJ, Shrager JB, Stedman HH, Kelly AM, Sweeney HL. *Proc Natl Acad Sci USA* 1993;90:3710–4.
- [4] Luise M, Presotto C, Senter L, Betto R, Ceoldo S, Furlan S. *Biochem J* 1993;293:243–7.
- [5] Madhavan R, Jarrett HW. *Biochemistry* 1994;33:5797–804.
- [6] Milner RE, Busaan JL, Holmes CFB, Wang JH, Michalak M. *J Biol Chem* 1993;268:21901–5.
- [7] Shemanko CS, Sanghera JS, Milner RE, Pelech S, Michalak M. *Mol Cell Biochem* 1995;152:63–70.
- [8] Walsh MP, Busaan JL, Fraser ED, Fu SY, Pato MD, Michalak M. *Biochemistry* 1995;34:5561–8.
- [9] Yoshida T, Pan Y, Hanada H, Iwata Y, Shigekawa M. *J Biol Chem* 1998;273:1583–90.
- [10] James M, Nuttall A, Ilsley JL, Ottersbach K, Tinsley JM, Sudol M, Winder SJ. *J Cell Sci* 2000;113:1717–26.
- [11] Ibraghimov-Beskrovnaya O, Ervasti JM, Leveille CJ, Slaughter CA, Sernett SW, Campbell KP. *Nature* 1992;355:696–702.
- [12] Williamson RA, Henry MD, Daniels KJ, Hrstka RF, Lee JC, Sunada Y, Ibraghimov-Beskrovnaya O, Campbell KP. *Hum Mol Genet* 1997;6:831–41.
- [13] Henry MD, Campbell KP. *Cell* 1998;95:859–70.
- [14] Winder SJ. *Trends Biochem Sci* 2001;26:118–24.
- [15] Suzuki A, Yoshida M, Hayashi K, Mizuno Y, Hagiwara Y, Ozawa E. *Eur J Biochem* 1994;220:283–92.
- [16] Jung D, Yang B, Meyer J, Chamberlain JS, Campbell KP. *J Biol Chem* 1995;270:27305–10.
- [17] Rosa G, Ceccarini M, Cavaldesi M, Zini M, Petrucci TC. *Biochem Biophys Res Commun* 1996;223:272–7.
- [18] Rentschler S, Linn H, Deininger K, Bedford MT, Espanel X, Sudol M. *Biol Chem* 1999;380:431–42.
- [19] Yoshida S, Fujisawa-Sehara A, Taki T, Arai K-I, Nabeshima Y-I. *J Cell Biol* 1996;132:181–93.
- [20] Volberg T, Zick Y, Dror R, Sabanay I, Gilon C, Levitzki A, Geiger B. *EMBO J* 1992;11:1733–42.
- [21] Helliwell TR, Nguyen thi Man, Morris GE. *Neuromuscular Dis* 1994;4:101–13.
- [22] Winder SJ, Kendrick-Jones J. *Anal Biochem* 1995;231:271–3.
- [23] Laemmli UK. *Nature* 1970;227:680–5.
- [24] Oronzi-Scott M, Sylvester JE, Heiman-Patterson T, Shi YJ, Fieles W, Stenman H. *Science* 1988;239:1418–20.
- [25] Clerk A, Strong PN, Sewry CA. *Development* 1992;114:395–402.
- [26] Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, Kato Y. *Biochem Biophys Res Commun* 1989;159:871–7.
- [27] Mikalsen SO, Kaalhus O. *J Biol Chem* 1998;273:10036–45.
- [28] Winder SJ. *J Muscle Res Cell Motil* 1997;18:1–13.
- [29] Michalak M, Fu SY, Milner RE, Busaan JL, Hance JE. *Biochem Cell Biol* 1996;74:431–7.
- [30] Yamamoto H, Hagiwara Y, Mizuno Y, Yoshida M, Ozawa E. *J Biochem* 1993;114:132–9.
- [31] Espanel X, Sudol M. *J Biol Chem* 1999;274:17284–9.
- [32] Chen HI, Einbond A, Kwak S-J, Linn H, Koepf E, Peterson S, Kelly JW, Sudol M. *J Biol Chem* 1997;272:17070–7.
- [33] Pirozzi G, McConnell SJ, Uveges AJ, Carter JM, Sparks AB, Kay BK, Fowlkes DM. *J Biol Chem* 1997;272:14611–6.
- [34] Huang X, Poy F, Zhang R, Joachimiak A, Sudol M, Eck MJ. *Nat Struct Biol* 2000;7:634–8.
- [35] Russo K, Stasio ED, Macchia G, Rosa G, Brancaccio A, Petrucci TC. *Biochem Biophys Res Commun* 2000;274:93–8.

The WW domain Linking cell signalling to the membrane cytoskeleton

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1. Introduction

Since the identification of the WW domain in 1994 [1,2], the WW domain has been implicated in mediating protein–protein interactions from viral budding through transcription to limb development and muscular dystrophy. This smallest of domains, comprising only 35 amino acids in a stable fold, occurs in single and multiple copies in signalling molecules and adaptors alike, in all species from yeast to plants and worms to man. The defining feature of the domain, from whence it derived its name, are two tryptophan residues (single letter code W) spaced 20–22 amino acids apart. In this short space of time the WW domain has become recognised as a major protein–protein interaction module widely distributed in all biological systems. Furthermore, the interactions of the WW domain have recently been shown to be regulated by phosphorylation giving the domain a status on a par with SH2 and SH3 domains (reviewed in Ref. [3]). In this review we will discuss the general background to the structure and ligand interactions of WW domains, and focus on the role of WW domains in linking and regulating specific membrane cytoskeletal connections.

2. WW domain ligands and structure

The WW domain comprises 30 amino acids in a compact short antiparallel β -sheet, with the limits of the β -sheets

being defined by two conserved tryptophan residues (Fig. 1). The core of the structure also contains other conserved aromatic residues, mainly tyrosine but also a highly conserved proline 2 residues c-terminal of the second conserved tryptophan. It is these conserved aromatic residues that are key to the specificity of WW domain interactions with its proline-containing ligands. The WW domain is the smallest protein domain that is stable as a triple stranded antiparallel β -sheet in the absence of disulphide bonds, tightly bound ions or ligands [4]. To date the structures of six WW domains, representing three different WW domain classes, have been solved [4–7]. Despite the differences in ligand specificity and the diverse origins of these structures, they are almost completely superimposable (Fig. 2A). A common feature of the ligand-binding site of all WW domain structures so far determined is the presence of stacked aromatic residues that provide a binding surface for a proline residue contributed by the WW domain ligand. The essential residues in the WW domain are a tyrosine from the central β -strand and the second conserved tryptophan at the end of the third β -strand, this is shown diagrammatically in Fig. 2B. The interaction with the WW domain is further strengthened by the stacking of other aromatic residues in the ligand, mainly proline and tyrosine, or by hydrophobic interactions with conserved residues, often histidine, in a surface formed by the loop between the c-terminus of the middle β -strand and the n-terminus of the third β -strand (Fig. 2B). The n-terminal conserved tryptophan is essential for the structure of the ligand-binding surface as a whole, which in essence is a shallow groove containing two hydrophobic regions. All WW domains that have so far been characterised bind to a variety of short proline-containing motifs with a tryptophan residue in the WW domain being essential for the interaction with a proline residue in the ligand. However, it is

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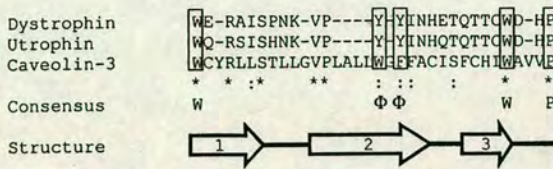


Fig. 1. Primary and secondary structure of WW domains. Sequence alignment of dystrophin, utrophin and caveolin-3 WW domains with important residues boxed. Other identical or similar residues are indicated by asterisks or colons, respectively. The overall consensus for all WW domains is shown below with the essential aromatic doublet indicated by the Greek letter Φ . At the bottom is a secondary structure cartoon with the three β -sheet structures represented as broad arrows and numbered 1-3, compare Fig. 2A.

the presence of other residues in the cognate ligand, and their phosphorylation state that further determines the type of WW interaction (see Table 1), and therefore WW domains have been classified according to the ligand with which they interact. In Class I WW domains, which have been studied most extensively (e.g., YAP65 and dystrophin), the first conserved tryptophan, in concert with an exposed tyrosine residue, forms a concave hydrophobic binding surface for the first two proline residues in the PPxY motif of the ligand that stack against the Trp and Tyr residues forming a hydrophobic buckle that presumably maintains the stable folded structure of the domain. The second conserved tryptophan of the WW domain is located on the convex side

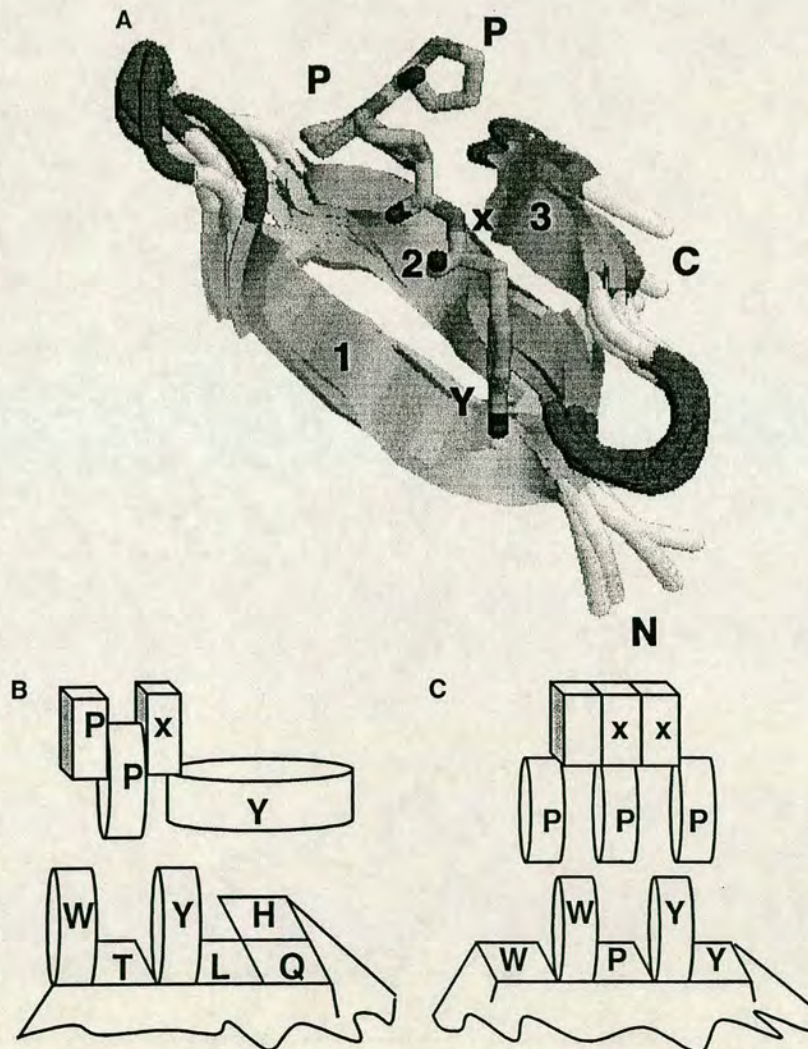


Fig. 2. Tertiary structure of WW domains. (A) Overlay of five WW domain structures using the Hierarchical Protein Structure Superposition programme LOCK; <http://gene.stanford.edu/LOCK/>. The five structures taken from the PDB (1E0L, 1E0M, 1E0N, 1F8A and 1EG4) were manually edited to represent the core of the WW domain fold and only the ligand for 1EG4 (dystrophin WW domain with β -dystroglycan peptide) is shown for clarity. N and C represents the amino and carboxyl termini of all five structures with major β -sheets (light grey) labelled as 1-3 and intervening turns dark grey. The stick representation of the bound peptide shows the Type I ligand from β -dystroglycan with the amino acids PPxY labelled accordingly from top (N-terminus) to bottom (C-terminus). (B) Schematic cartoon of WW domain structure (bottom) showing aromatic stacking of a Type I cognate ligand top, via the X-P groove. (C) Schematic cartoon of SH3 domain structure (bottom) showing aromatic stacking of an SH3 Class I ligand top, via two X-P grooves.

Table 1
Classification of WW domains based on optimal ligand specificity

Class	Motif	WW domains	References
I	PPxY	Dystrophin, Nedd4 WW-3, YAP65	[23,29]
II	PPLP	FBP11, FE65	[16,41]
III	PPR	FBP30, FBP21, FE65	[17]
IV	(S/T) _p P	Pin1, Nedd4 WW-2	[33]

of this region and stabilises the ligand-binding surface. The tyrosine residue of the PPxY motif is accommodated by a shallow concave surface formed by conserved Ile, Gln and His residues of the WW domain and forms a hydrogen bond with the His residue [5,8].

3. WW domains versus SH3 domains

WW domains resemble SH3 domains functionally due to their affinity for proline-rich ligands, but their structures are quite distinct. The SH3 domain was originally discovered as a 50-amino acid long region of homology shared between the Crk and Src gene products and phospholipase C- γ [9,10]. The SH3 domain mediates binding to proline-rich sequences with the minimum consensus sequence of PxxP and the specificity of this binding is obtained by unique sequences flanking the PxxP motif [11], see Fig. 2C. Investigations into the structure of peptide–SH3 complexes have shown that peptide ligands can bind in two orientations with respect to the SH3 domain [12,13]. The peptides can bind in either an N to C-terminal (Class I) or C to N-terminal (Class II) orientation relative to the SH3 domain. The orientation of the peptide is dictated by the location of a positively charged residue relative to the PxxP motif. This residue forms a salt bridge with an acidic residue in the SH3 domain. Therefore, Class I ligands have the motif +xxPxxP and Class II ligands have the motif PxxPx+, where + refers to a positively charged residue. Some SH3 domains, e.g., Src have the ability to bind both Class I and Class II ligands, the biological relevance of this phenomenon is not clear but may enable Src to bind different partners in multicomponent protein complexes. The structure of the SH3 domain is such that it has three shallow pockets for its ligand to bind (Fig. 2C). Two large pockets termed the ‘LP dipeptide pockets’ are parallel to each other and each accommodate

a proline from the PxxP motif plus an adjacent hydrophobic residue (A, I, L, V, P). The third surface is called the ‘specificity pocket’ and holds the positively charged residue of the ligand, i.e., N-terminus of Class I and C-terminus of Class II ligands. Despite similarities in WW and SH3 ligands, especially in Type II WW ligands, compare PPLP (Type II WW) with PxxP (SH3), the different three-dimensional structures of the ligand-binding surfaces in WW domains and SH3 domains preclude direct competition. A detailed comparison of the structures of SH3 and WW domains in complex with their polyproline ligands suggests that these evolutionarily unrelated domains have converged upon a similar solution for proline recognition. The rule of one or two ‘X-P grooves’: one for WW domains and one for SH3, provides an elegant and unifying mechanism by which SH3 and WW recognise their ligands [14]. However, overlapping Class III WW domain and SH3 domain ligands have been found in some proteins. It has been shown that the WW domain can compete with the Abl SH3 domain for a proline-rich region in formin and the two binding domains are contained within the same 10 amino acids [15,16]. Having these two domains in one protein could allow proteins to form different signalling complexes depending on their local environment, [17] or even raises the possibility that one of the domains regulates the other by the overlapping motifs being compatible and/or competitive.

4. WW domain–dystroglycan interactions

Dystroglycan is a heterodimeric membrane-associated protein comprising extracellular laminin-binding α - and transmembrane β -subunits, posttranslationally cleaved from a single precursor peptide (see Ref. [18] for review). The cytoplasmic tail of β -dystroglycan interacts with the cytoskeletal proteins dystrophin and utrophin that in turn interact with the actin cytoskeleton. Thus the dystrophin–dystroglycan and utrophin–dystroglycan complexes act as cell adhesion structures linking the extracellular matrix to the actin cytoskeleton. Perturbations in this link that arise through mutations in dystrophin lead to the severe muscle wasting disease Duchenne muscular dystrophy. Dystrophin and utrophin are large modular linker proteins each around 400 kDa and comprising at least six identified protein

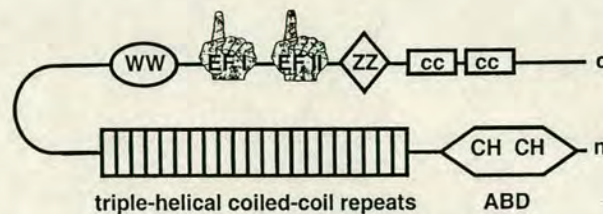


Fig. 3. Schematic representation of dystrophin/utrophin domain organisation showing from N to C terminus; actin binding domain (ABD) comprising two calponin homology domains (CH), spectrin-like triple-helical coiled-coil repeats, WW domain (WW), two EF hand regions (EF1, EF2), a ZZ domain zinc finger (ZZ) and two other predicted coiled coil regions (cc).

domains, some in multiple copies (see Ref. [19]), including a single WW domain at the start of the c-terminal 'cysteine-rich' region (Fig. 3). These Type I WW domains were amongst the first WW domains to be identified [1,2,20] (see Fig. 1). Type I WW domains interact with the ligand PPxY (Table 1) and the proline-rich cytoplasmic tail of β -dystroglycan contains two such potential motifs, one starting at residue 828 (PPEY) and one at residue 889 (PPPY). Peptide-binding studies and fusion protein pull-down assays identified the sequences around residue 889, in the C-terminal 10–15 residues of β -dystroglycan, as being necessary and sufficient for interactions with the dystrophin cysteine-rich region [21,22]. Furthermore, a more rigorous analysis of the dystrophin domain organisation needed for the WW domain mediated interaction with β -dystroglycan, revealed a requirement for the adjacent EF-hand region of dystrophin for ligand binding [23] (see Fig. 3). The apparent structural requirement for the dystrophin EF-hand region makes the WW domain of dystrophin unique among WW domains, in that this WW domain is not capable of supporting ligand binding on its own, but requires an 'accessory' domain. The reasons for this became clearer upon elucidation of the dystrophin WW domain/EF-hand region crystal structure [5], which revealed a contribution from the EF-hand region of dystrophin, to the binding of the dystroglycan peptide by stabilising interactions of the n-terminal of the PPPY motif. Closer examination of previous data also revealed that dystrophin WW domain alone was not sufficient to mediate the binding of β -dystroglycan, but required at the least the EF-hand region [21]. Furthermore, the more extensive analyses on dystrophin [23] and also on utrophin [24] not only revealed a requirement for the EF-hand region, but also the ZZ domain, a zinc finger region c-terminal of the EF-hand region [25] (see Fig. 3). The contribution of the ZZ domain to the β -dystroglycan interaction is further substantiated by the observations that calmodulin is able to interact with the ZZ domain of dystrophin [26] and utrophin ([27], and SJ Winder unpublished observations) in a calcium-dependent manner and that calcium-calmodulin is able to regulate the WW domain-mediated interaction between dystrophin or utrophin and β -dystroglycan [26,27].

5. Regulation of WW domain interactions

5.1. Phosphorylation

The identification of a tyrosine residue within the Type I WW domain binding motif leads to the hypothesis that the WW/PPxY interaction could be regulated by tyrosine phosphorylation [24,28–30], in a manner analogous to the regulation of SH3 domain interactions by tyrosine phosphorylation where the ligand contains a tyrosine within the PxxP motif. Subsequently, it has been shown biochemically that phosphorylation of the tyrosine residue in the PPxY motif disrupts its interaction with WW domains in its

binding partner [31]. Furthermore, it has been shown that adhesion-dependent or peroxyvanadate-induced tyrosine phosphorylation of the transmembrane adhesion receptor β -dystroglycan within a Type I WW domain ligand is able to regulate the interaction with its cognate WW domain in utrophin or dystrophin [24,32] (see below). More recently, with the identification of the Class IV WW domain ligand, it

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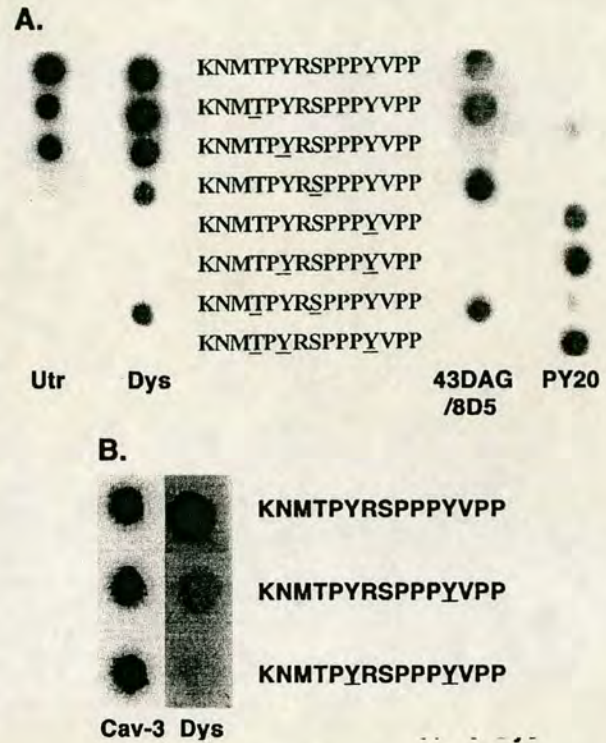


Fig. 4. SPOTs analysis of phosphorylation-regulated interactions between WW domains and β -dystroglycan. Binding of the utrophin (Utr; A, far left) and dystrophin (Dys; A, left) WW-EF-ZZ regions to a series of peptide spots comprising the carboxy-terminal 15 amino acids of β -dystroglycan with phospho-amino acid substitutions, indicated by underlines (A, centre). Utrophin binding to β -dystroglycan is sensitive to both serine and tyrosine phosphorylation [24] whereas dystrophin binding to β -dystroglycan, whilst also sensitive to tyrosine phosphorylation, is less sensitive to serine phosphorylation [5,32]. (A, right) Epitope mapping of the monoclonal antibody 43DAG/8D5 [42]. Like the β -dystroglycan monoclonal MAN-DAG2 [24], this antibody is also sensitive to tyrosine phosphorylation of β -dystroglycan. (A, far right) Detection with the anti-phospho tyrosine antibody PY20 confirms the presence of phospho-tyrosine residues in the peptide spots. Whilst a powerful method, there are limitations to the peptide SPOTs technique. Peptides are synthesised directly onto a solid support, thus sequences are not verified by direct sequencing and quantitation is not accurate. Unequivocal data on the role of serine phosphorylation on WW domain binding to β -dystroglycan awaits further verification in solution- or cell-based assays using rigorously defined peptides. Binding of caveolin-3 (Cav-3; B, left) and dystrophin (Dys; B, right) WW domain-containing constructs to peptide spots comprising the carboxy-terminal 15 amino acids of β -dystroglycan with phospho-tyrosine substitutions, indicated by underlines. Tyrosine phosphorylation inhibits the binding of dystrophin to β -dystroglycan but has no effect on caveolin-3 binding under these conditions. Caveolin-3 data from Ref. [40] (with permission) and dystrophin data from Ref. [32].

has been shown that phosphorylation of a serine or threonine in the binding motif is absolutely required for the interaction to take place. Thus the mitotic prolyl isomerase Pin1 and WW domain 2 of the ubiquitin ligase Nedd4 interact with their target proteins in a phosphorylation-dependent manner [33,34]. It is clear that the interaction between Type I WW domains and their cognate ligands can be negatively regulated by tyrosine phosphorylation and that Ser/Thr phosphorylation is required for Type IV interactions that can therefore be negatively regulated by dephosphorylation of the Ser or Thr residue. There is also biochemical evidence of regulation by phosphorylation of serine and threonine residues flanking Type I WW motifs [35], although physiological mediators of these phosphorylation reactions have not been identified *in vivo*.

5.2. β -dystroglycan

The tyrosine phosphorylation of the WW domain ligand in β -dystroglycan in nonmuscle cells is adhesion-dependent [24]. In myotubes, however, we and others have been unable to demonstrate adhesion-dependent phosphorylation of β -dystroglycan [32,36], but it has been possible to stimulate phosphorylation of β -dystroglycan pharmacologically using peroxyvanadate [32]. The lack of adhesion-stimulated β -dystroglycan phosphorylation in myotubes may be due to the static nature of the differentiated muscle cells compared to HeLa cells, or a different role for the dystrophin–dystroglycan complex in muscle as compared to the utrophin–dystroglycan complex in nonmuscle cells. Nevertheless, the dystrophin glycoprotein complex has previously been implicated in integrin-mediated adhesion events, in that α - and γ -sarcoglycans from myotubes were found to be phosphorylated on tyrosine residues in response to adhesion. This phosphorylation was also

induced by the RGDS (integrin recognition sequence) peptide, implying that the dystrophin glycoprotein complex is involved in the integrin adhesion system [36]. This tyrosine phosphorylation was inhibited by Herbimycin A, which suggests the possible involvement of the Src family of kinases. Studies carried out in HeLa cells failed to identify the kinase involved in the phosphorylation of the WW domain ligand in β -dystroglycan, but did rule out a role for Src and focal adhesion kinase [24]. Evidence for tyrosine phosphorylation regulating β -dystroglycan–dystrophin interactions has led to the suggestion that disruption of the β -dystroglycan–dystrophin connection could lead to muscular dystrophy [23]. Tyrosine phosphorylation of β -dystroglycan might imply that the dystrophin–dystroglycan interaction can be regulated in a dynamic way, but in muscular dystrophy due to perturbations in normal signalling processes this regulation may be disrupted. Disruption of this delicate balance by reduction or loss of dystrophin may explain some of the poorly understood mechanisms of disease progression in Becker and Duchenne muscular dystrophy.

Phosphorylation of tyrosine 892 in β -dystroglycan prevents the interaction with utrophin and dystrophin [24,32] (Fig. 4A). From the crystal structure of the WW domain and EF-hand region of dystrophin in complex with a β -dystroglycan peptide [5], it is clear that incorporation of a bulky phosphate group on tyrosine 892 would disrupt the hydrogen bond between tyrosine 892 of β -dystroglycan and histidine 3076 of the dystrophin WW domain, thereby disrupting the WW domain interaction [32]. Given the high degree of sequence similarity between dystrophin and utrophin in this region, (85% identical, 98% similar) it is extremely likely that the structure of utrophin in this region is the same and that the effect of tyrosine 892 phosphorylation would be mechanistically similar.

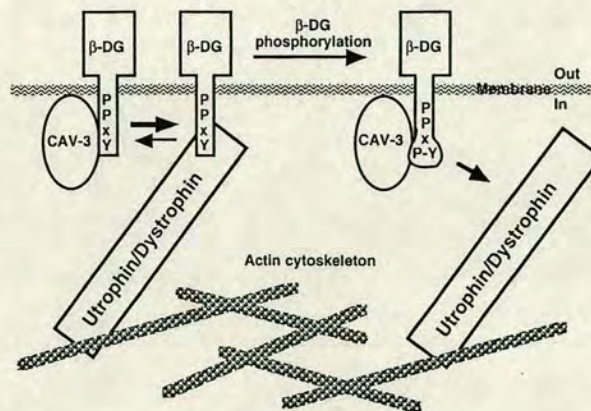


Fig. 5. Differential binding of caveolin-3 or dystrophin to β -dystroglycan is regulated by tyrosine phosphorylation: a model. Caveolin-3 (Cav-3) and dystrophin (and/or utrophin) are in competition for the same binding site in the c-terminus of β -dystroglycan (β -DG) with the equilibrium probably in favour of the dystrophin– β -dystroglycan interaction (left). However, on phosphorylation of β -dystroglycan on tyrosine residues [24,32] utrophin and dystrophin binding to β -dystroglycan is displaced allowing the binding of caveolin-3 to β -dystroglycan [40] (right).

5.3. Caveolin

Caveolin-3 is the principal structural protein of caveolae in striated muscle and is associated with the dystrophin–dystroglycan complex [37]. Mutations in caveolin-3 lead to a distinct muscular dystrophy phenotype known as limb–girdle muscular dystrophy-1C [38]. Paradoxically however, transgenic overexpression of caveolin-3 in striated muscle leads to a Duchenne muscular dystrophy phenotype [39]. A recent intriguing finding is that caveolin-3 also contains a WW-like domain (see Fig. 1) and that it too can interact with β -dystroglycan [40]. Furthermore, it was shown that caveolin-3 interacted with the same binding site on β -dystroglycan as dystrophin, and that caveolin-3 could compete with dystrophin for binding to β -dystroglycan. These biochemical findings helped explain the apparent paradox from the transgenic experiments, such that despite caveolin-3 being associated with the dystrophin–dystroglycan complex, by competing for binding sites on β -dystroglycan it excluded dystrophin binding to the extent that the link between the actin cytoskeleton and the extracellular matrix was disrupted leading to a Duchenne muscular dystrophy-like phenotype. From the point of view of WW domain biology, perhaps the most exciting finding was that the ability of the caveolin-3 WW domain to interact with β -dystroglycan was *insensitive* to the phosphorylation of tyrosine 892 in the β -dystroglycan PPPY motif [40] (see Fig. 4B). This leads to the possible situation whereby the phosphorylation of β -dystroglycan on tyrosine 892 would act as an effective switch to alter the affinity of the dystroglycan complex from dystrophin (or utrophin) to caveolin. Such that when β -dystroglycan is not phosphorylated, there is an equilibrium between dystrophin (or utrophin) and caveolins, probably in favour of dystrophin, for binding to β -dystroglycan. However, when β -dystroglycan is phosphorylated on tyrosine 892, either by cell adhesion [24] or other as yet unidentified stimuli [32], utrophin or dystrophin are released from β -dystroglycan allowing caveolin to bind, thus altering the composition, targeting and possible functions of the dystroglycan complex under these conditions (Fig. 5).

6. Summary

This simplest of structural domains, WW has grown in stature and complexity. In the 7 years since its identification it has become recognised as a crucial mediator of many protein–protein interactions. The mechanistic insight afforded by structural and biochemical analyses has revealed a complex set of interacting ligands and equally complex regulation of these interactions by posttranslational modifications such as phosphorylation. Most recently these studies have revealed competition between WW domains in caveolin-3 and dystrophin for the same

ligand in β -dystroglycan and differential regulation of binding by tyrosine phosphorylation that in turn regulates the connections between the extracellular matrix and the cytoskeleton. The WW domain may be structurally simple, but this belies the complexity of interactions in linking cell signalling to the membrane cytoskeleton.

Acknowledgments

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References

- [1] Bork P, Sudol M. Trends Biochem Sci 1994;19:531–3.
- [2] Andre B, Springael JY. Biochem Biophys Res Commun 1994; 205:1201–5.
- [3] Sudol M, Hunter T. Cell 2000;103:1001–4.
- [4] Macias MJ, Gervais V, Civera C, Oschkinat H. Nat Struct Biol 2000; 7:375–9.
- [5] Huang X, Poy F, Zhang R, Joachimiak A, Sudol M, Eck MJ. Nat Struct Biol 2000;7:634–8.
- [6] Verdecia MA, Bowman ME, Lu KP, Hunter T, Noel JP. Nat Struct Biol 2000;7:639–43.
- [7] Kanelis V, Rotin D, Forman-Kay JD. Nat Struct Biol 2001;8:407.
- [8] Macias MJ, Hyvonen M, Baraldi E, Schultz J, Sudol M, Saraste M, Oschkinat H. Nature 1996;382:646–9.
- [9] Stahl ML, Ferez CR, Kelleher KL, Kriz RW, Knopf JL. Nature 1988; 332:269–72.
- [10] Mayer BJ, Hamaguchi M, Hanafusa H. Nature 1988;332:272–5.
- [11] Yu H, Chen JK, Feng S, Dalgarno DC, Brauer AW, Schreiber SL. Cell 1994;76:933–45.
- [12] Feng S, Chen J, Yu H, Simmon J, Schreiber S. Science 1994;266: 1241–7.
- [13] Lim WA, Richards FM, Fox R. Nature 1994;372:375–9.
- [14] Zarrinpar A, Lim A. Nat Struct Biol 2000;7:611–3.
- [15] Chan DC, Bedford MT, Leder P. EMBO J 1996;15:1045–54.
- [16] Bedford MT, Chan DC, Leder P. EMBO J 1997;16:2376–83.
- [17] Bedford MT, Sarbassova D, Xu J, Leder P, Yaffe MB. J Biol Chem 2000;275:10359–69.
- [18] Winder SJ. Trends Biochem Sci 2001;26:118–24.
- [19] Winder SJ. J Muscle Res Cell Motil 1997;18:617–29.
- [20] Hofmann K, Bucher P. FEBS Lett 1995;358:153–7.
- [21] Jung D, Yang B, Meyer J, Chamberlain JS, Campbell KP. J Biol Chem 1995;270:27305–10.
- [22] Rosa G, Ceccarini M, Cavaldesi M, Zini M, Petrucci TC. Biochem Biophys Res Commun 1996;223:272–7.
- [23] Rentschler S, Linn H, Deininger K, Bedford MT, Espanel X, Sudol M. Biol Chem 1999;380:431–42.
- [24] James M, Nuttall A, Hsley JL, Ostersbach K, Tinsley JN, Sudol M, Winder SJ. J Cell Sci 2000;113:1717–26.
- [25] Ponting CP, Blake DJ, Davies KE, Kendrick-Jones J, Winder SJ. TIBS 1996;21:11–3.
- [26] Anderson JT, Rogers RP, Jarrett HW. J Biol Chem 1996;271:6605–10.
- [27] Vignano ATd, Zenzo GD, Sudol M, Cesareni G, Dente L. FEBS Lett 2000;471:229–34.
- [28] Pirozzi G, McConnell SJ, Uveges AJ, Carter JM, Sparks AB, Kay BK, Fowlkes DM. J Biol Chem 1997;272:14611–6.
- [29] Chen HI, Sudol M. Proc Natl Acad Sci USA 1995;92:7819–23.

- [30] Sudol M. *Prog Biophys Mol Biol* 1996;65:113–32.
- [31] Chen HI, Einbond A, Kwak S-J, Linn H, Koepf E, Peterson S, Kelly JW, Sudol M. *J Biol Chem* 1997;272:17070–7.
- [32] Ilsley JL, Sudol M, Winder SJ. *Cell Signal* 13 (in press).
- [33] Lu P-J, Zhou XZ, Shen M, Lu KP. *Science* 1999;283:1325–8.
- [34] Ranganathan R, Lu KP, Hunter T, Noel JP. *Cell* 1997;89:875–86.
- [35] Kay BK, Williamson MP, Sudol M. *FASEB J* 2000;14:231–41.
- [36] Yoshida T, Pan Y, Hanada H, Iwata Y, Shigekawa M. *J Biol Chem* 1998;273:1583–90.
- [37] Song KS, et al. *J Biol Chem* 1996;271:15160–5.
- [38] Minetti C, et al. *Nat Genet* 1998;18:365–8.
- [39] Galbiati F, et al. *Proc Natl Acad Sci USA* 2000;97:9689–94.
- [40] Sotgia F, et al. *J Biol Chem* 2000;275:38048–58.
- [41] Ermekova KS, Zambrano N, Linn H, Minopoli G, Gertler F, Russo T, Sudol M. *J Biol Chem* 1997;272:32869–77.
- [42] Cullen MJ, Walsh J, Nicholson LVB. *Acta Neuropathol* 1994; 87:349–54.

Adhesion-dependent tyrosine phosphorylation of β -dystroglycan regulates its interaction with utrophin

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SUMMARY

Many cell adhesion-dependent processes are regulated by tyrosine phosphorylation. In order to investigate the role of tyrosine phosphorylation of the utrophin-dystroglycan complex we treated suspended or adherent cultures of HeLa cells with peroxyvanadate and immunoprecipitated β -dystroglycan and utrophin from cell extracts. Western blotting of β -dystroglycan and utrophin revealed adhesion- and peroxyvanadate-dependent mobility shifts which were recognised by anti-phospho-tyrosine antibodies. Using maltose binding protein fusion constructs to the carboxy-terminal domains of utrophin we were able to demonstrate specific interactions between the WW, EF and ZZ domains of utrophin and β -dystroglycan by co-immunoprecipitation with endogenous β -dystroglycan. In extracts from cells

treated with peroxyvanadate, where endogenous β -dystroglycan was tyrosine phosphorylated, β -dystroglycan was no longer co-immunoprecipitated with utrophin fusion constructs. Peptide 'SPOTS' assays confirmed that tyrosine phosphorylation of β -dystroglycan regulated the binding of utrophin. The phosphorylated tyrosine was identified as Y₈₉₂ in the β -dystroglycan WW domain binding motif PPxY thus demonstrating the physiological regulation of the β -dystroglycan/utrophin interaction by adhesion-dependent tyrosine phosphorylation.

Key words: β -Dystroglycan, Adhesion, Tyrosine phosphorylation, WW domain

INTRODUCTION

Utrophin is a ubiquitous cytoskeletal protein forming a link between the actin cytoskeleton and the extracellular protein laminin via a membrane glycoprotein complex which includes α - and β -dystroglycans (Ervasti and Campbell, 1993; Matsumura et al., 1992; Tinsley et al., 1992; Winder et al., 1995b). In normal skeletal muscle, where utrophin is restricted to the myotendinous and neuromuscular junctions, utrophin binds to a multimeric protein complex comprising dystroglycans and sarcoglycans. This complex is indistinguishable from the dystrophin glycoprotein complex found in the rest of the sarcolemma (Matsumura et al., 1992), though there is differential distribution of the syntrophins between these complexes. In non-muscle tissues however, sarcoglycans, with the possible exception of β -sarcoglycan (Bonnemann et al., 1995; Lim et al., 1995), and dystrophin (except in neuronal tissues) are not expressed and utrophin associates specifically with α - and β -dystroglycan at the cell membrane (James et al., 1996; Matsumura et al., 1997). In non-muscle cells in culture, utrophin and dystroglycans are

associated with cell-cell and cell-matrix adhesion structures (Belkin and Burridge, 1995a,b; Belkin and Smallheiser, 1996; James et al., 1996; Khurana et al., 1995) and may therefore play a role in the organisation of focal adhesions or adherens junctions and may exist as distinct cell adhesion complexes in their own right. The assembly and disassembly of many adhesion structures are known to be regulated by tyrosine phosphorylation (Burridge and Chrzanowska-Wodnicka, 1996).

Both utrophin and dystroglycans have been demonstrated to play a key role in adhesion-mediated events. The dystroglycan gene encodes both α - and β -dystroglycan as a single transcript which is post-translationally cleaved (Ibraghimov-Beskrovnyaya et al., 1992). Disruption of dystroglycan function, either by antibodies (Durbeej et al., 1995) or gene knockout (Henry and Campbell, 1998; Williamson et al., 1997) has a profound effect on developmental processes that rely on cell adhesion. Antibodies which block α -dystroglycan binding to laminin in epithelial cells inhibit branching morphogenesis (Durbeej et al., 1995), whilst transgenic disruption of the dystroglycan gene (DAG1) in mice leads to embryonic lethality

at around day 6.5 due to the failure of Reichert's membrane to form (Williamson et al., 1997). Embryonic stem cells deficient for α - and β -dystroglycan are unable to assemble basement membrane (Henry and Campbell, 1998). A significant reduction of α - and β -dystroglycan in the sarcolemma is also symptomatic of mutations in the dystrophin gene which lead to Duchenne and Becker muscular dystrophies (Ervasti et al., 1990; Matsumura et al., 1993), resulting in the destabilisation of the membrane cytoskeleton and loss of sarcolemmal integrity, see Winder (1997) for review. Additionally, antisense depletion of utrophin in astrocytes markedly reduces cell-matrix interactions (Khurana et al., 1995).

Utrophin is the autosomal homologue of dystrophin, the gene affected in Duchenne muscular dystrophy (Tinsley et al., 1992). Like utrophin, dystrophin interacts with the cytoplasmic domain of β -dystroglycan through a cluster of domains in the carboxy terminus of dystrophin known collectively as the cysteine-rich region. This region comprises 3 recognised modules, a WW domain (Sudol, 1996b), a pair of EF hands (Koenig et al., 1988; Tufty and Kretsinger, 1975) and a ZZ domain (Ponting et al., 1996). WW domains are 30 amino acid modules containing conserved tryptophan residues that are involved in protein-protein interactions through proline-rich motifs in their cognate ligands. EF hands are highly conserved motifs involved in the binding of calcium or magnesium ions and may play a regulatory or a structural role. The ZZ domain is a putative zinc finger motif found in various functionally distinct proteins that is believed to mediate protein-protein interactions. It has been shown previously that the cysteine-rich region of dystrophin is required for the binding of dystrophin to β -dystroglycan and the C-terminal domain further increases the binding affinity (Jung et al., 1995; Rosa et al., 1996; Suzuki et al., 1994). Most recently it has been documented that the WW domain in concert with the EF hands binds to the C-terminal PPxY motif of β -dystroglycan and that this binding is enhanced by the presence of the ZZ domain (Rentschler et al., 1999).

In this current study we investigated the binding of the utrophin carboxy-terminal domains to β -dystroglycan using utrophin fusion proteins as probes to identify β -dystroglycan binding sites in utrophin. In addition we have examined the role of adhesion-dependent tyrosine phosphorylation of β -dystroglycan in regulating the interaction between β -dystroglycan and the carboxy terminus of utrophin. Using a phosphorylation state-specific monoclonal antibody we have mapped the site of tyrosine phosphorylation to the essential tyrosine in the β -dystroglycan WW domain binding motif PPxY and demonstrated that phosphorylation of this tyrosine regulates the β -dystroglycan-utrophin interaction.

MATERIALS AND METHODS

Cell culture

HeLa cells were maintained in RPMI 1640 media (Gibco BRL) supplemented with 5% foetal calf serum (Gibco BRL). In order to inhibit the activity of tyrosine phosphatases and effectively block the cells in a tyrosine phosphorylated state, peroxyvanadate treatment was carried out on confluent cultures of HeLa cells. Adherent or suspended cells were washed with serum-free RPMI and then incubated in serum-

free RPMI media) or 100 nM calyculin A (Calbiochem) for various times at 37°C. Cells were washed once in cold phosphate buffered saline (50 mM sodium phosphate, pH 7.2, 150 mM NaCl) before being harvested in ice-cold radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM ethylene glycol-bis-(β -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 100 μ M leupeptin, 1 mM phenylmethylsulphonyl fluoride (PMSF), 100 μ M *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)) for 30 minutes on ice. Cells which were not treated with phosphatase inhibitors were incubated for an equivalent time in serum-free media before being harvested in RIPA as above. After harvesting, cells were briefly sonicated to shear the DNA and stored at -20°C until assay. For studies with cells in suspension, HeLa cells were first removed from the culture vessel by PBS/EDTA treatment and resuspended in serum free RPMI 1640, with 2% bovine serum albumin to prevent non-specific adhesion, and peroxyvanadate as above. Cells were maintained at 37°C in a 50 ml tube on a tube roller for 1 hour. Cells that were still in suspension after one hour were decanted to a fresh tube, recovered by centrifugation and extracted in RIPA buffer as above, adapted from Renshaw et al. (1997).

Antisera

The anti- β -dystroglycan antibody 43DAG1/8D5 was a gift from L. Anderson (University of Newcastle) and MANDAG2 (Helliwell et al., 1994) was kindly provided by G. E. Morris (NE Wales Institute, Wrexham). Polyclonal antisera against bacterially expressed maltose binding protein (RAB4) and the utrophin carboxy-terminal coiled coil domain (RAB5), residues 3204-3433 of human utrophin (Winder and Kendrick-Jones, 1995) were raised in rabbits using standard techniques. RAB4 and RAB5 antisera were effective on western blots at dilutions of 1:5,000 and 1:10,000, respectively, and the utrophin antiserum did not recognise dystrophin in western blots of whole rat muscle extracts or in mouse C2C12 mouse myotubes (data not shown). Anti-phospho-tyrosine monoclonal (PY20) was from Transduction Laboratories.

Generation of utrophin fusion-proteins

The limits of the utrophin cysteine rich domains WW, EF and ZZ were identified by sequence alignment (Schultz et al., 1998; Sudol, 1996a; Thompson et al., 1997) and secondary structure prediction (Rost et al., 1994; Schultz et al., 1998). Specific utrophin cysteine-rich domains were amplified by polymerase chain reaction (PCR) using domain specific primers to the residues shown in Fig. 3A. 5' primers contained *Bam* HI and *Nde*I restriction sites and 3' primers contained a stop codon and *Sal*I restriction site. PCR products were cloned into the unique *Bam* HI and *Sal*I restriction enzyme sites of pMAL-C2 (New England BioLabs). Cultures of transformed *E. coli* (BL21 (DE3)) were grown at 37°C until the OD₆₀₀ reached 0.5, and induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside for 2 hours. Following harvesting by centrifugation at 4,000 g for 20 minutes the cell pellet was re-suspended in 50 ml column buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 100 μ M leupeptin, 1 mM PMSF, 100 μ M TPCK) and stored at -20°C overnight. After thawing, the cell suspension was sonicated in short 15 second pulses for a total time of 2 minutes before being centrifuged at 9,000 g for 30 minutes MBP fusions were purified by a single passage over an amylose resin (New England Biolabs) column. Bound fusion proteins were eluted using column buffer containing 10 mM maltose. Fusion containing fractions identified on Coomassie-stained SDS gels were pooled, dialysed against RIPA buffer overnight and then stored at -20°C until use. A human utrophin construct encompassing the WW, EF and ZZ regions (residues 2783-3199) fused to GST was generated by cloning a *Bam*HI and *Eco*RI digested PCR-generated fragment into the *Bam*HI and *Eco*RI sites of pGEX-2TK.

In vitro binding and immunoprecipitation assays

For immunoprecipitation of β -dystroglycan/MBP-fusion complexes, fusion proteins were used at a final concentration of 10 μ g/ml. Cell extracts in RIPA were initially cleared by incubation with Protein A-Sepharose for 60 minutes at 4°C. The required volume of each fusion was added to 100 μ l aliquots of cleared cells, the total volume of each assay was made up to 175 μ l using RIPA and 50 μ l RIPA containing 1% BSA was added to each tube. After 2 hours incubation at 4°C 10 μ l of 43DAG1/8D5 and/or MANDAG2 was added to each tube and the cells were incubated for a further hour at 4°C. In order to precipitate the antibody-protein complex, 50 μ l of a 20% slurry of Protein A-Sepharose was added per sample, and the cells were incubated for a further hour at 4°C. The samples were centrifuged briefly to pellet the Sepharose beads, and the beads were washed 4 times with RIPA. The final pellet was re-suspended in SDS-PAGE loading buffer. Immunoprecipitation of β -dystroglycan, phosphotyrosine containing proteins and utrophin were carried out with the relevant antisera at dilutions of 1:20, 1:200 and 1:20 respectively and recovered with Protein A-Sepharose as above.

SDS-PAGE and western blotting

Samples from immunoprecipitation experiments were run on 3-15% acrylamide SDS-polyacrylamide gels with a 3% stacking gel under reducing conditions (Laemmli, 1970) and blotted onto polyvinylidene difluoride (PVDF) membranes in 25 mM Tris, 198 mM glycine, 10% methanol. Membranes were blocked in 5% skimmed milk powder in Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 30 minutes and incubated overnight at 4°C in appropriately diluted primary antibody in 5% skimmed milk in TBS and washed 3 times in TBS with 0.5% Tween-20 before addition of the alkaline-phosphatase conjugated secondary antibody in 1% skimmed milk in TBS. Membranes were incubated for 2 hours in secondary antibody at room temperature. After washing in TBS/Tween, blots were developed using 0.4 mM nitroblue tetrazolium and 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 5 mM MgCl₂, 100 mM Tris-HCl, pH 9.5. Enhanced chemifluorescence detection of utrophin maltose binding protein fusion proteins was performed using a Vistra ECF western blotting kit (Amersham Life Sciences) according to the manufacturer's instructions, images were captured on a Storm 460 Phosphorimager in fluorescence mode and data quantified using ImageQuant software.

Two-dimensional gel electrophoresis

β -Dystroglycan was immunoprecipitated from equal amounts of protein (as determined by BCA assay; Pierce) from suspended or adherent and peroxyvanadate treated or untreated cell extracts, prepared as above. Equal amounts of immunoprecipitated complexes were subjected to first dimension isoelectric focusing on 180 mm, pH 3-10 Immobiline Drystrips (Amersham Pharmacia Biotech) on an Amersham Pharmacia Biotech Multiphor System according to the manufacturer's instructions. Equilibrated drystrips were then separated in the second dimension on reducing 3-20% SDS-polyacrylamide gels. All 4 gels were electroblotted onto PVDF and immunoblotted with a cocktail of MANDAG2 and 43DAG/8D5 (1:500 and 1:50, respectively). The 4 immunoblots were developed simultaneously by ECL (Amersham Pharmacia Biotech) onto the same piece of x-ray film to avoid differences in exposure time. Following β -dystroglycan detection, blots were stripped (65°C for 45 minutes in 62.5 mM Tris-HCl, pH 6.75, 2% SDS, 100 mM mercaptoethanol) blocked as above and reprobed with anti-phospho tyrosine antisera and detection by ECL as described above. Epitope mapping of β -dystroglycan monoclonal antibody MANDAG2 on 'SPOTS' membranes (see below) was performed as a normal western blot detection as described above. Following incubation with MANDAG2 antibody and washing blots were developed by ECL.

'SPOTS' membrane synthesis

The 'SPOTS' technique of peptide synthesis on derivatised cellulose

membrane was performed as described (Blankenmeyer-Menge et al., 1980; Frank and Doring, 1988; Kramer et al., 1993). All reagents and equipment, including amino acids, derivatised membranes, incubation trays and software ('SPOTS', release 1.0), were purchased from Cambridge research Biochemicals and Genosys Biotechnologies, Inc.

Hybridisation of 'SPOTS' membranes with ³²P-labeled utrophin GST fusion protein

A utrophin WW-EF-ZZ-GST fusion protein was purified from bacterial lysates on glutathione beads and phosphorylated in situ using the catalytic subunit of cAMP-dependent protein kinase as described previously (Rentschler et al., 1999). SPOTS membranes were blocked as above and then probed with the ³²P-labeled utrophin GST-fusion protein.

RESULTS

Treatment of tissue culture cells with phosphatase inhibitors such as peroxyvanadate or okadaic acid results in an apparent increase in phosphorylation levels, enabling the visualisation of transient phosphorylation events. Western blotting of HeLa cell extracts treated with peroxyvanadate, with antibodies to phosphotyrosine, showed a dramatic increase in the levels of tyrosine phosphorylation up to one hour with a slight decline in phospho-tyrosine levels up to six hours. With prolonged exposure to peroxyvanadate cells began to round up and detach from the substratum (data not shown). Western blotting of HeLa cell extracts with antibodies against β -dystroglycan

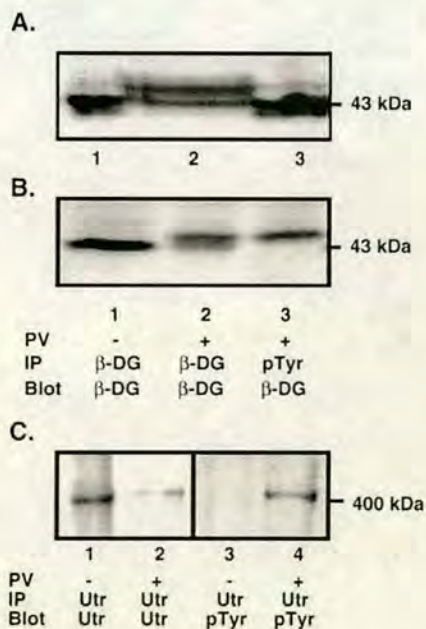


Fig. 1. Tyrosine phosphorylation of β -dystroglycan and utrophin in HeLa cells. (A) Western blot of: untreated confluent HeLa cells lane 1; peroxyvanadate treated HeLa cells lane 2; calyculin A treated HeLa cells lane 3; detected with β -dystroglycan antibody MANDAG2. (B,C) Western blots of RIPA extracts of HeLa cells with (+) or without (-) peroxyvanadate (PV) treatment, immunoprecipitated (IP) and detected (Blot) with the indicated antisera; β -DG, MANDAG2 against β -dystroglycan; pTyr, PY20 against phospho tyrosine; Utr, RAB5 against utrophin. Numbers represent approximate molecular mass in kDa.

revealed a pronounced retardation in electrophoretic mobility in extracts treated with peroxyvanadate (Fig. 1A; lane 1 and 2) consistent with β -dystroglycan being phosphorylated. Calyculin A, an inhibitor of type 1 and 2A serine/threonine phosphatases (Ishihara et al., 1989), added to HeLa cells over the same period however, did not result in any marked difference in electrophoretic mobility (Fig. 1A, lane 3). To confirm that the change in electrophoretic mobility was indeed due to tyrosine phosphorylation and not simply the activation of a serine/threonine kinase by a tyrosine kinase, we immunoprecipitated with antisera against β -dystroglycan or phospho-tyrosine and western blotted with β -dystroglycan antisera. As shown in Fig. 1B, β -dystroglycan antisera immunoprecipitated the authentic 43 kDa β -dystroglycan band and the electrophoretically retarded band (Fig. 1B, lanes 1, 2). Furthermore the phospho-tyrosine immunoprecipitate was recognised by β -dystroglycan antisera in the peroxyvanadate treated cell extracts (Fig. 1B, lane 3). Conversely western blotting of β -dystroglycan immunoprecipitates with anti-

phospho-tyrosine antibodies also detected β -dystroglycan (see Fig. 2B). Taken together, these data demonstrate unequivocally that the electrophoretically retarded β -dystroglycan is tyrosine phosphorylated. Similar results were obtained when utrophin immunoprecipitates were western blotted with antisera against either utrophin or phospho-tyrosine (Fig. 1C). In the presence of peroxyvanadate the electrophoretic mobility of utrophin was retarded and the electrophoretically retarded band was specifically recognised by antibodies to phospho-tyrosine. This demonstrates, that like β -dystroglycan, treatment of HeLa cells with peroxyvanadate leads to the phosphorylation of utrophin on tyrosine residues. Direct treatment of RIPA cell extracts with peroxyvanadate did not alter the electrophoretic mobility of β -dystroglycan or utrophin, indicating that the observed mobility shifts were not due to direct modification of thiols in β -dystroglycan and utrophin (Mikalsen and Kaalhus, 1998), data not shown. Similar results for both β -dystroglycan and utrophin were obtained with a number of cultured cell types including; fibroblast, REF52; myoblast, C2C12; epithelial, COS-7; and primary umbilical vein endothelial cells (data not shown) indicating that this is a widespread regulatory event.

Peroxyvanadate treatment of cells can cause artefactual phosphorylation of proteins due to inappropriate or uncontrolled activation of signalling cascades. Peroxyvanadate, however, has proven immensely useful for 'trapping' transient tyrosine phosphorylation events. To determine if the tyrosine phosphorylation of β -dystroglycan that we had observed was as a result of a physiological stimulus and not merely an artefact of the pharmacological dose of peroxyvanadate, we examined the effect of peroxyvanadate treatment on adherent and suspended HeLa cells. Fig. 2A shows a distinct electrophoretic mobility shift in response to cell adhesion and peroxyvanadate. Adhesion alone in the absence of peroxyvanadate caused a slight electrophoretic mobility shift by comparison with suspended cells (Fig. 2A, lanes 1, 2), addition of peroxyvanadate caused a further electrophoretic mobility shift in both suspended and adherent cells (Fig. 2A, lanes 3, 4). Notably the ability of the monoclonal antibody MANDAG2 to recognise β -dystroglycan in western blots was reduced with the increase in tyrosine phosphorylation, suggesting that the phosphorylated tyrosine is within the last 20 amino acids of β -dystroglycan against which the antibody was raised (compare also lanes 1 and 2 in Fig. 1B). Two-dimensional gel and western blotting analysis of β -dystroglycan immunoprecipitated from adherent or suspended HeLa cells in the presence or absence of peroxyvanadate, are shown in Fig. 2B. Western blot detection with anti-phospho tyrosine antisera reveals an increasing number of charged species as a result of both cell adhesion and addition of peroxyvanadate, suggesting a possible hierarchy of tyrosine phosphorylation leading to the adhesion-dependent phosphorylation of two tyrosine residues (Fig. 2B, upper panel). There is a complete absence of a phospho-tyrosine signal in the suspended peroxyvanadate treated cells. One clear spot of tyrosine phosphorylated β -dystroglycan is present in suspended cells treated with peroxyvanadate and in untreated adherent cells, whereas in adherent cells treated with peroxyvanadate we see at least two incompletely resolved spots giving the appearance of an ellipse. The pattern of β -dystroglycan spots in the lower panel recapitulates those of the upper panel (where present) and shows that approximately

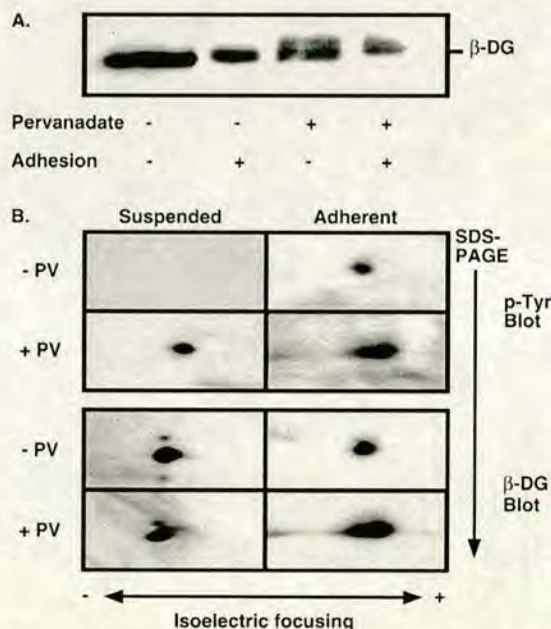


Fig. 2. Adhesion dependence of β -dystroglycan tyrosine phosphorylation. (A) Effect of peroxyvanadate and/or cell adhesion on the SDS-PAGE electrophoretic mobility of β -dystroglycan. HeLa cells were maintained in suspension or adherent for 1 hour in the presence or absence of peroxyvanadate as indicated. Adhesion and peroxyvanadate treatment resulted in a decrease in electrophoretic mobility as determined by western blotting of total cell extracts with MANDAG2 antisera against β -dystroglycan. (B) Tyrosine phosphorylation of β -dystroglycan in response to cell adhesion and peroxyvanadate. Two-dimensional gel analysis of β -dystroglycan immunoprecipitated from adherent or suspended HeLa cells in the presence or absence of peroxyvanadate (PV). Western blots were probed with antisera against β -dystroglycan (β -DG) and detected by ECL (lower panel), stripped, and reprobed with anti-phospho-tyrosine (p-Tyr) and detected by ECL (upper panel). In suspended cells in the absence of peroxyvanadate there is no detectable tyrosine phosphorylation. Adhesion or peroxyvanadate treatment results in one clear spot of tyrosine phosphorylated β -dystroglycan whereas in adherent cells in the presence of peroxyvanadate there are two closely migrating spots that appear as an ellipse.

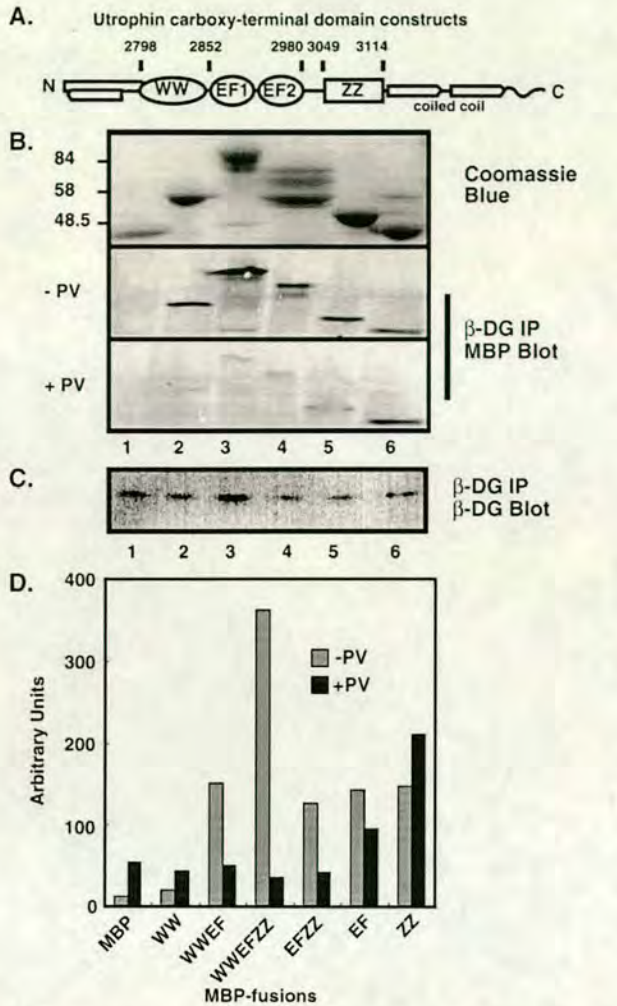


Fig. 3. MBP-utrophin fusion constructs, binding to β -dystroglycan and effect of tyrosine phosphorylation. The maltose binding protein (MBP) fusion constructs are shown schematically in A, with delimiting amino acid numbers corresponding to human utrophin above. (B) Top panel, Coomassie blue stained gel of MBP-fusions to the carboxy terminus of utrophin, relative position of molecular mass markers are shown on the left. Western blots against utrophin-MBP-fusion proteins immunoprecipitated with β -dystroglycan antisera. MBP-fusions were added to RIPA control extracts of HeLa cells, middle panel (-PV) or following treatment with peroxyvanadate for 1 hour, lower panel (+PV). Lanes in all three panels are: 1, MBP-WW; 2, MBP-WWEF; 3, MBP-WWEFZZ; 4, MBP-EFZZ; 5, MBP-EF; 6, MBP-ZZ. (C) Loading control for the experiment shown in B middle panel, blots were stripped and reprobed with β -dystroglycan monoclonal MANDAG2. (D) Quantification of the blots shown in B by densitometric scanning of enhanced chemi-fluorescence developed blots, including control immunoprecipitates with MBP alone.

equal amounts of β -dystroglycan were present in the original samples subjected to two-dimensional electrophoresis. These data therefore support the notion that β -dystroglycan is phosphorylated on tyrosine in response to cell adhesion.

In order to identify the β -dystroglycan binding site on utrophin, MBP-fusion proteins to various regions of the cysteine rich region of utrophin (Fig. 3A,B, upper) were used as affinity probes for β -dystroglycan in extracts of HeLa cells.

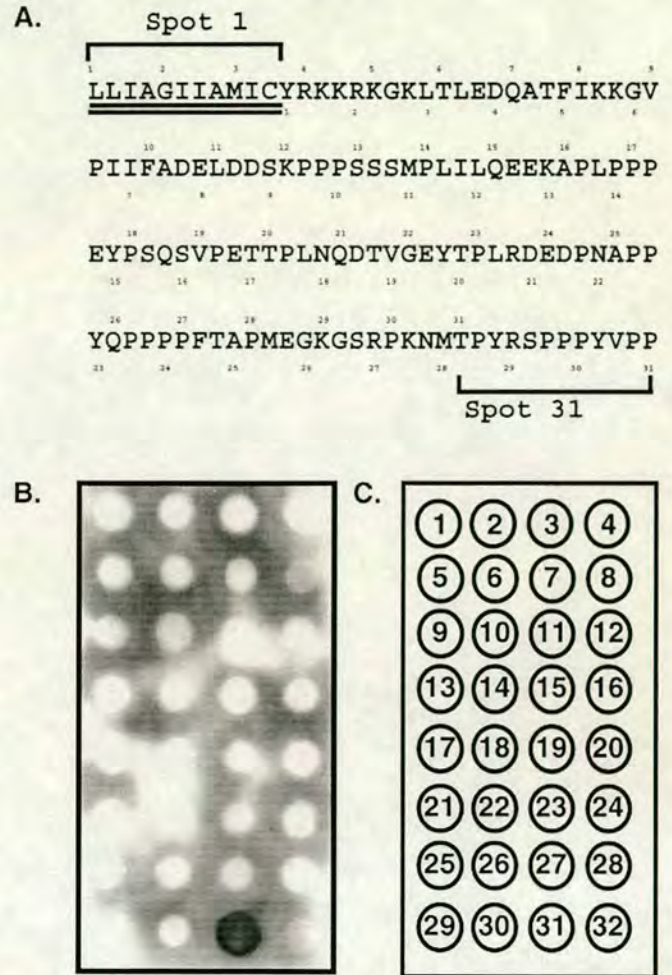


Fig. 4. Identification of β -dystroglycan peptides which interact with monoclonal MANDAG2. (A) Scheme for the synthesis of peptides on the 'SPOTS' membrane. Twelve amino acid long peptides representing part of the transmembrane region and the entire cytoplasmic domain of human β -dystroglycan (amino acids 764-895) were synthesised on derivatised 'SPOTS' membranes with an offset of four amino acids. The transmembrane region of β -dystroglycan is indicated by a double underline. Numbers above the β -dystroglycan sequence denote the first amino acid of the peptide found on the corresponding spot whilst numbers below the sequence denote the last amino acid of the peptide on the corresponding spot. Brackets identify the sequences of spots 1 and 31. Spot 32 is a negative control with no peptide synthesised. (B) 'SPOTS' membrane described as above probed with monoclonal antiserum MANDAG2 and detected by ECL. The antiserum recognised spot 31 only. (C) Numbered circles identify the location of each spot on the membrane.

Following immunoprecipitation with antibodies MANDAG2 and 43DAG1/8D5 against β -dystroglycan, MBP-utrophin fusion proteins co-immunoprecipitated with β -dystroglycan were detected by western blotting with anti-MBP antisera (Fig. 3B, middle). All MBP-fusion constructs were co-immunoprecipitated with β -dystroglycan with the exception of MBP-WW. Densitometric quantification of the MBP-fusions detected, revealed that almost twice as much MBP-WWEFZZ was bound as any other fusion, indicating that the complete cysteine-rich region of utrophin is the most effective in binding

β -dystroglycan under these conditions. Loading controls for β -dystroglycan (Fig. 3C) indicate that approximately equal amounts of β -dystroglycan were immunoprecipitated in each case. Fusions containing WVEF or EFZZ, as well as EF or ZZ alone, all bound equally well but not WW alone, suggesting that the presence of the EF and ZZ domains together were necessary for the interaction between WW and its presumed target motif on β -dystroglycan. Interactions between MBP-fusions containing the utrophin EF hand region and β -dystroglycan took place in the effective absence of Ca^{2+} and Mg^{2+} (presence of mM EGTA and EDTA). This would suggest that if the EF hands in utrophin are functional in terms of calcium binding, and this is doubtful (Winder et al., 1995a, 1997), then the presence of divalent cations is not required for binding to β -dystroglycan under these conditions.

Treatment of tissue culture cells with peroxyvanadate, but not calyculin A, resulted in the adhesion-dependent phosphorylation of β -dystroglycan and utrophin on tyrosine residues. We therefore repeated the experiments to identify the domains needed for utrophin binding to β -dystroglycan in extracts from HeLa cells that had previously been treated with peroxyvanadate. As can be seen in Fig. 3B (lower panel) the prior phosphorylation of β -dystroglycan on tyrosine almost completely abolished the ability of utrophin fusions to be co-immunoprecipitated with β -dystroglycan, with the exception of MBP-ZZ. As shown in Fig. 3D, the binding of the complete cysteine-rich region, domains WW, EF and ZZ, was completely inhibited by tyrosine phosphorylation of β -dystroglycan following treatment with peroxyvanadate as compared to MBP alone. There was no evidence from western blots of MBP-fusions undergoing mobility shifts, to suggest that any of the MBP-fusions themselves became phosphorylated during the course of the experiments, suggesting that the effect of peroxyvanadate was directly on β -dystroglycan within cells. Furthermore, despite the apparent inability of the β -dystroglycan monoclonal MANDAG2 to recognise tyrosine phosphorylated β -dystroglycan on western blots it was still able to recognise tyrosine

phosphorylated β -dystroglycan in solution. This is apparent from the ability of MANDAG2 to immunoprecipitate tyrosine phosphorylated β -dystroglycan which was detected on two dimensional gels with anti-phospho-tyrosine antisera (Fig. 2B) and to immunoprecipitate the ZZ domain fusion with tyrosine phosphorylated β -dystroglycan (Fig. 3B, lower panel).

As shown in Fig. 1B (lanes 1 and 2) and Fig. 2A, the monoclonal antibody MANDAG2 was less efficient at recognising tyrosine phosphorylated β -dystroglycan on western blots, though this did not appear to affect its ability to immunoprecipitate β -dystroglycan from cell extracts as indicated above. We therefore epitope mapped the MANDAG2 antibody using a series of 'SPOTs' membranes covering the entire cytoplasmic domain of β -dystroglycan (Rentschler et al., 1999). As shown in Fig. 4, MANDAG2 recognised a single peptide, spot 31, corresponding to the ultimate 12 amino acids of β -dystroglycan (TPYRSPPPYVPP). MANDAG2 was originally raised against a peptide corresponding to the ultimate 15 amino acids of β -dystroglycan (Helliwell et al., 1994). More detailed analysis of the epitope by systematic mutation of the peptide recognised in Fig. 4B revealed that the essential amino acids in the epitope were PxYVP in the ultimate 5 amino acids of β -dystroglycan (Fig. 5, Table 1). Furthermore as suggested by the relative inability of MANDAG2 to recognise tyrosine phosphorylated β -dystroglycan (Figs 1, 2), MANDAG2 was unable to recognise any peptide containing a phospho-tyrosine at the second last position (Y₈₉₂), but was unaffected by phosphorylation of tyrosine, serine or threonine alone in any of the other positions in the last 15 amino acids,

Fig. 5. Mutagenesis of a C-terminal peptide of β -dystroglycan to determine the epitope for monoclonal MANDAG2. (A) Fifteen amino acid long peptides representing the wild type C terminus of β -dystroglycan (K₈₈₁NMTPYRSPPPYVPP₈₉₅) and sequential single amino acid substitutions of the wild type peptide were synthesised on three 'SPOTs' membranes (see Table 1 for the scheme of synthesis and summary of results). The membranes were probed with MANDAG2 and detected by ECL to identify which amino acids in the C terminus of β -dystroglycan were part of the MANDAG2 epitope. Positive reacting spots appear with a dark halo around due to the short exposure times used and the density of peptide within the spot itself reducing the luminescence emission. The result, summarised in Table 2, indicates an epitope of PxYVP, since P₈₉₀, Y₈₉₂, V₈₉₃ and P₈₉₄ are sensitive to almost any substitution. (B) Numbered circles identify the location of each spot on the membrane.

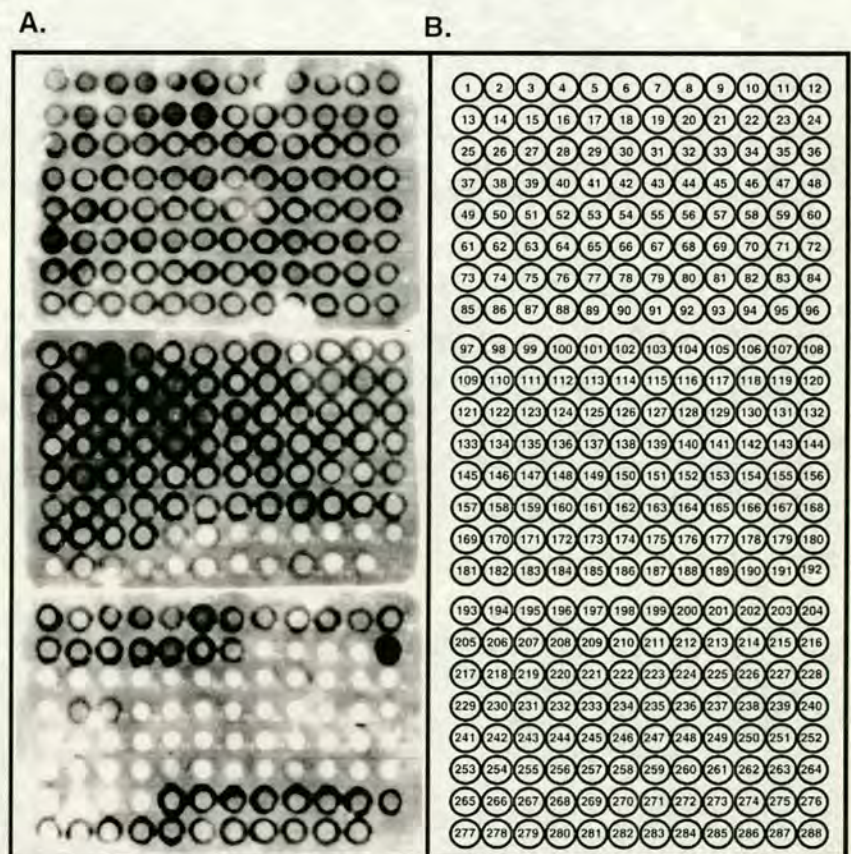


Table 1. Single amino acid substitutions within the β -dystroglycan carboxy-terminal peptide that significantly reduce binding of MANDAG2

	K	N	M	T	P	Y	R	S	P	P	P	Y	V	P	P
A	2	21	40	59	78	97	116	135	154	173	193	212	231	250	269
C	3	22	41	60	79	98	117	136	155	174	194	213	232	251	270
D	4	23	42	61	80	99	118	137	156	175	195	214	233	252	271
E	5	24	43	62	81	100	119	138	157	176	196	215	234	253	272
F	6	25	44	63	82	101	120	139	158	177	197	216	235	254	273
G	7	26	45	64	83	102	121	140	159	178	198	217	236	255	274
H	8	27	46	65	84	103	122	141	160	179	199	218	237	256	275
I	9	28	47	66	85	104	123	142	161	180	200	219	238	257	276
K	x	29	48	67	86	105	124	143	162	181	201	220	239	258	277
L	10	30	49	68	87	106	125	144	163	182	202	221	240	259	278
M	11	31	x	69	88	107	126	145	164	183	203	222	241	260	279
N	12	x	50	70	89	108	127	146	165	184	204	223	242	261	280
P	13	32	51	71	x	109	128	147	x	x	x	224	243	x	x
Q	14	33	52	72	90	110	129	148	166	185	205	225	244	262	281
R	15	34	53	73	91	111	x	149	167	186	206	226	245	263	282
S	16	35	54	74	92	112	130	x	168	187	207	227	246	264	283
T	17	36	55	x	93	113	131	150	169	188	208	228	247	265	284
V	18	37	56	75	94	114	132	151	170	189	209	229	x	266	285
W	19	38	57	76	95	115	133	152	171	190	210	230	248	267	286
Y	20	39	58	77	96	x	134	153	172	191	211	x	249	268	287

Summary of the results shown in Fig. 5. Each amino acid of the wild-type peptide was sequentially replaced with the other 19 amino acids, and binding of the β -dystroglycan monoclonal MANDAG2 was compared to that of the wild-type peptide. The uppermost row represents the amino acid positions of the wild-type β -dystroglycan peptide. The leftmost column represents all the replacement amino acids. The intersections correspond to the spot numbers where substitutions occurred. Substitutions which significantly reduced binding are indicated by shading. 'x' denotes the amino acid of the wild-type peptide. Spot 192 and 288 are control spots where no peptide was synthesised.

(Fig. 6A). MANDAG2 is sensitive to phosphorylation of tyrosine in the epitope PxYVP, which in part overlaps the WW domain binding motif in β -dystroglycan PPxY (Rentschler et al., 1999) and is therefore an effective reporter for tyrosine phosphorylation of the β -dystroglycan WW domain binding motif. From these data and those in Figs 1 and 2, it is possible to deduce that adhesion-dependent tyrosine phosphorylation of β -dystroglycan on its ultimate tyrosine within the WW domain binding motif is able to regulate the binding to utrophin. In order to test this more

directly we probed the same SPOTs membranes used in Figs 4 and 6 with a 32 P-labelled GST-fusion protein comprising the WWFZZ region of utrophin. As demonstrated previously for the dystrophin WWFZZ region (Jung et al., 1995; Rentschler et al., 1999) utrophin also bound almost exclusively to a peptide corresponding to the last 12 amino acids of β -dystroglycan (Fig. 7A). On longer exposure of the SPOTS membrane, weaker interactions with other peptides are apparent, e.g. peptides 2,3,4,6,7,29,30 and binding of utrophin to peptides occurred equally well in the presence or

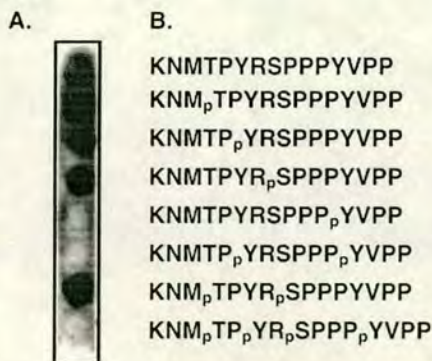


Fig. 6. Effect of phosphorylation on the binding of MANDAG2 to a C-terminal β -dystroglycan peptide. (A) A peptide equivalent to that described in Fig. 5A was synthesised containing the phospho-amino acids pY, pT and pS at each possible position in the peptide, either singly or in multiples. The membrane was probed with monoclonal MANDAG2 as described above. Only peptides containing a pY at Y₈₉₂ were not recognised by MANDAG2. (B) Actual sequence of the corresponding peptide spots in A.

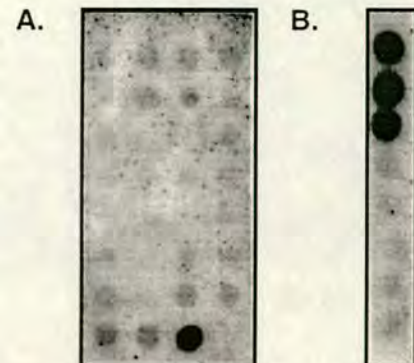


Fig. 7. Identification of β -dystroglycan peptides which interact with GST-utrophin WWFZZ and the effect of β -dystroglycan phosphorylation. (A) 'SPOTs' membrane as described in Fig. 4 was probed with 32 P-labelled GST-WWFZZ. This utrophin WWFZZ fusion protein bound almost exclusively to spot 31. (B) 'SPOTs' membrane as described in Fig. 6 was probed with 32 P-labelled GST-WWFZZ. The binding of the utrophin WWFZZ fusion protein was prevented by phosphorylation of either S₈₈₈ or Y₈₉₂.

absence of calcium (data not shown). Furthermore phosphorylation of the tyrosine residue in the WW domain binding motif PPxY was able to completely inhibit the binding of utrophin (Fig. 7B). Phosphorylation of the serine in the n-1 position adjacent to the PPxY motif was also able to regulate binding, as has been demonstrated for other WW domains binding to their cognate ligands (A. Korosi, A. Chang and M. Sudol, unpublished observations). This confirms the hypothesis that adhesion-dependent tyrosine phosphorylation of β -dystroglycan within the WW domain binding motif is able to regulate the WW domain-mediated interaction between utrophin and β -dystroglycan. This is the first demonstration of a physiologically relevant tyrosine phosphorylation of a WW domain ligand and has parallels with the tyrosine phosphorylation of SH3 domain ligands regulating SH3-mediated interactions.

DISCUSSION

Numerous studies have described serine/threonine phosphorylation of dystrophin *in vivo*, *in vitro* or by endogenous co-purifying kinases, reviewed in (Michalak et al., 1996; Winder et al., 1997), although few studies have identified functional consequences of these phosphorylation events. Dystrophin has been shown to be tyrosine phosphorylated in the postsynaptic membrane of *Torpedo* electric organ (Wagner and Haganir, 1994). Two-dimensional gel electrophoretic analysis of the dystrophin glycoprotein complex suggests phosphorylation of dystrophin, β -dystroglycan and possibly other components (Yamamoto et al., 1993). More recently, Yoshida and colleagues (1998) demonstrated adhesion-dependent tyrosine phosphorylation of α - and γ -sarcoglycan but not β -sarcoglycan or β -dystroglycan in L6 myoblasts. Tyrosine phosphorylation of utrophin and β -dystroglycan has not previously been described.

The role of β -dystroglycan in attachment to the extracellular matrix and regulation of utrophin binding to β -dystroglycan by tyrosine phosphorylation may be involved in cellular processes that require remodelling of cell-substratum interactions and cytoskeletal connections associated with them. β -dystroglycan and utrophin are located at sites of cell-cell and cell substratum contact (Belkin and Burridge, 1995a,b; Belkin and Smallheiser, 1996; James et al., 1996; Khurana et al., 1995). The adhesion-dependent tyrosine phosphorylation of β -dystroglycan occurred whether HeLa cells were plated onto fibronectin or laminin coated substrates, suggesting that the kinase responsible for phosphorylating β -dystroglycan was not specifically activated by the binding of α -dystroglycan to laminin (M. James and S. J. Winder, unpublished observations), but was a more general adhesion signal potentially involving integrin engagement. The phosphorylation of β -dystroglycan on tyrosine did still occur in focal adhesion kinase null cells (a generous gift from Dr D. Ilic, San Francisco) indicating that this kinase was not required for β -dystroglycan phosphorylation (S. J. Winder, unpublished observation). The adhesion-dependent phosphorylation of β -dystroglycan by as yet unidentified tyrosine kinases and in response to cell adhesion, leads to its release from utrophin and the underlying cytoskeleton. This may be required for processes such as cell adhesion, migration, proliferation and

differentiation. Furthermore the identification of tyrosine phosphorylation on β -dystroglycan from C2C12 myotubes (J. L. Ilesley and S. J. Winder, unpublished observations) suggests a role for tyrosine phosphorylation in regulating β -dystroglycan-dystrophin interactions. Such regulation may have implications in the pathogenesis and treatment of Duchenne muscular dystrophy. The recent identification of α -dystroglycan as the cellular receptor for *Mycobacterium leprae* (Rambukkana et al., 1998) and for arenaviruses such as Lassa fever virus (Cao et al., 1998), also raises the possibility that infection by these organisms may exert part of their pathogenic effect by disruption of signalling events associated with β -dystroglycan phosphorylation.

The modular protein domains of the dystrophin and utrophin cysteine-rich region show an extremely high degree of sequence identity, see Winder et al. (1997) for review. Studies *in vitro* translated β -dystroglycan and dystrophin (Jung et al., 1995), showed that high affinity binding of dystrophin carboxy terminus GST-fusion proteins to β -dystroglycan required the presence of the complete cysteine-rich domain (shown later to comprise WW, EF and ZZ domains) and that fusions comprising WW-EF bound weakly and WW alone not at all. However, no other dystrophin GST-fusion constructs; EF, ZZ, or EF-ZZ were found to bind under the conditions of these *in vitro* assays. These apparently conflicting results may reflect differences in the precise limits of the fusion proteins used in this study, the use of MBP as tags in recombinant proteins, and also that we used tissue cell extracts as a source of β -dystroglycan rather than *in vitro* translated protein. Additionally, more recent studies with dystrophin GST-fusion constructs containing well demarcated modular domains suggest that there is weak interaction between the EF hand region and also a contribution from the ZZ domain of dystrophin to β -dystroglycan binding (Rentschler et al., 1999). β -Dystroglycan from cellular sources may also be complexed with other proteins, such as the adapter protein Grb2 (Yang et al., 1995), or other as yet unidentified proteins, that facilitate binding of EF and ZZ domains to β -dystroglycan (Rentschler et al., 1999). The possibility remains that there are fundamental differences between the carboxy-terminal domains of utrophin and dystrophin with respect to the interaction with β -dystroglycan. This seems unlikely, however, as utrophin has been shown to effectively replace dystrophin in mouse models of muscular dystrophy (Tinsley et al., 1996).

Tyrosine phosphorylation of β -dystroglycan prevented the binding of all utrophin MBP-fusions with the exception of MBP-ZZ, suggesting that ZZ is involved in an interaction somehow different to the WW and EF domains, possibly with a different region of β -dystroglycan, or even with another protein associated with β -dystroglycan such as Grb2 (Yang et al., 1995). It is largely assumed that dystrophin and utrophin WW domains associate with β -dystroglycan via the WW domain binding motif PPxY (Chen and Sudol, 1995) located in the extreme carboxy terminus of β -dystroglycan (see Fig. 8). A recent thorough detailed analysis of the precise requirements for the dystrophin WW domain binding to β -dystroglycan has revealed that this is indeed the case (Rentschler et al., 1999). As shown here, the presence of both the WW domain and ZZ domain are required for the high affinity interaction between utrophin and β -dystroglycan, though the precise target sequence for the ZZ domain on β -

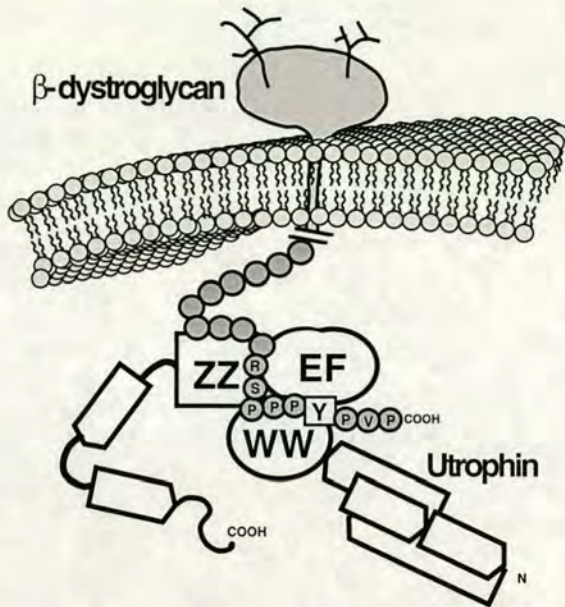


Fig. 8. Schematic organisation of β -dystroglycan and utrophin carboxy terminus showing potential interactions between the WW, EF and ZZ domains of utrophin and the carboxy terminus of the cytoplasmic domain of β -dystroglycan. EF represents the two EF hands in the cysteine-rich region of utrophin and ZZ the ZZ domain zinc finger. The 12 carboxy-terminal amino acids of β -dystroglycan, including the putative WW domain binding motif PPPY are shown as individual dark shaded circles with the phosphotyrosine that regulates utrophin binding, as a lighter shaded square.

dystroglycan has yet to be determined. The cytoplasmic domain of β -dystroglycan contains 5 tyrosine residues, none of which is in a known consensus for tyrosine phosphorylation. One of these tyrosines however, is within the WW binding motif PPxY in the carboxy-terminal 15 amino acids of β -dystroglycan (residues 888-892; see Fig. 8), which have been shown to be essential for dystrophin binding (Rentschler et al., 1999). There is also a second potential WW binding motif at residues 817-822 but this does not appear to be involved in dystrophin binding to β -dystroglycan (Rentschler et al., 1999). Two more tyrosines are within putative PxxP SH3 binding motifs (Ren et al., 1993) and therefore in potential sites to regulate the binding of SH3 containing proteins, such as Grb2 (Yang et al., 1995) and others, that may facilitate interactions between the carboxy terminus of utrophin and β -dystroglycan.

Phosphorylation of the Pin1 and Nedd4 WW domain ligands on serine or threonine, is a prerequisite for WW domain binding (Lu et al., 1999) see Table 2. It has also been clearly demonstrated in vitro, that tyrosine phosphorylation of the Yes-associated protein WW domain ligand, in the sequence PPxY, completely abolishes WW domain binding (Chen et al., 1997). Furthermore, we have demonstrated a physiological, adhesion-dependent, tyrosine phosphorylation of a WW domain ligand, and that this phosphorylation is able to regulate binding to the utrophin WW domain. Phosphorylation of WW domain binding motifs on tyrosine demonstrates that the WW domain motif PPxY can be regulated in an analogous way to tyrosine-containing SH3 motifs (Px[Y]P), see Table 2. This suggests a greater role for the WW domain, not only as a protein-protein interaction motif, but as a target and mediator of tyrosine

Table 2. Role of phosphorylation in regulation of SH and WW domain interactions

Domain	Ligand	Phosphorylation obligatory	Regulated by (de)phosphorylation
SH2	pYxx ψ *	Yes	(Yes)
SH3	PxxP	No	Yes \ddagger
WWI	PPLP	No	No
WWII	PPxY	No	Yes \S
WWIII	pS/TP \P	Yes	(Yes)

A brief list of consensus motifs for interaction with the indicated SH or WW domain. Domain, represents the domain class, SH2, SH3 or WW domain types I, II and III. Ligand, represents the defined consensus sequence for binding to the respective ligand. Phosphorylation obligatory, defines whether phosphorylation of a core residue is required for the interaction with the respective domain. Regulated by (de)phosphorylation, indicates where dephosphorylation (shown in parentheses) may act as a regulatory step or whether phosphorylation of the core residue itself leads directly to regulation of binding. *, ψ represents any hydrophobic residue. \ddagger , regulation occurs by phosphorylation of a tyrosine within the PxxP motif. \S , when the tyrosine is phosphorylated. \P , this represents the common residues identified between Pin1 and Cdc25 (Lu et al., 1999) and does not represent a true experimentally defined consensus.

kinase regulated signalling. Furthermore, this raises the possibility for SH2 domain mediated interaction with the phosphorylated WW domain ligand (Sudol, 1996b).

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REFERENCES

- Belkin, A. M. and Burridge, K. (1995a). Association of aciculin with dystrophin and utrophin. *J. Biol. Chem.* **270**, 6328-6337.
- Belkin, A. M. and Burridge, K. (1995b). Localization of utrophin and aciculin at sites of cell-matrix and cell-cell adhesion in cultured cells. *Exp. Cell Res.* **221**, 132-140.
- Belkin, A. M. and Smallheiser, N. R. (1996). Localization of crinin (dystroglycan) at sites of cell-matrix and cell-cell contact: recruitment to focal adhesions is dependent upon extracellular ligands. *Cell Adhes. Commun.* **4**, 281-296.
- Blankenmeyer-Menge, B., Nimitz, M. and Frank, R. (1990). An efficient method for anchoring Fmoc-amino acids to hydroxyl-functionalised solid supports. *Tetrahedron Lett.* **31**, 1701-1704.
- Bonnemann, C. G., Modi, R., Noguchi, S., Mizuno, Y., Yoshida, M., Gussoni, E., McNally, E. M., Duggan, D. J., Angelini, C., Hoffman, E. P. et al. (1995). β -Sarcoglycan (A3b) mutations cause autosomal recessive muscular dystrophy with loss of the sarcoglycan complex. *Nature Genet.* **11**, 266-273.
- Burridge, K. and Chrzanoska-Wodnicka, M. (1996). Focal adhesions, contractility, and signaling. *Annu. Rev. Cell Dev. Biol.* **12**, 463-518.
- Cao, W., Henry, M. D., Borrow, P., Yamada, H., Elder, J. H., Ravkov, E. V., Nichol, S. T., Compans, R. W., Campbell, K. P. and Oldstone, M. B. A. (1998). Identification of α -dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus. *Science* **282**, 2079-2081.
- Chen, H. I. and Sudol, M. (1995). The WW domain of the Yes-associated protein binds a proline-rich ligand that differs from the consensus established for Src homology 3-binding modules. *Proc. Nat. Acad. Sci. USA* **92**, 7819-7823.
- Chen, H. I., Einbond, A., Kwak, S.-J., Linn, H., Koepf, E., Peterson, S., Kelly, J. W. and Sudol, M. (1997). Characterisation of the WW domain of human Yes-associated protein and its polyproline-containing ligands. *J. Biol. Chem.* **272**, 17070-17077.

- Durbeej, M., Larsson, E., Ibraghimov-Beskrovnaya, O., Roberds, S. L., Campbell, K. P. and Ekblom, P. (1995). Nonmuscle alpha-dystroglycan is involved in epithelial development. *J. Cell Biol.* **130**, 79-91.
- Ervasti, J. M., Ohlendieck, K., Kahl, S. D., Gaver, M. G. and Campbell, K. P. (1990). Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. *Nature* **345**, 315-319.
- Ervasti, J. M. and Campbell, K. P. (1993). A role for the dystrophin glycoprotein complex as a transmembrane linker between laminin and actin. *J. Cell Biol.* **122**, 809-823.
- Frank, R. and Doring, R. (1988). Simultaneous multiple peptide synthesis under continuous flow conditions on cellulose paper discs as segmental solid synthesis. *Tetrahedron* **44**, 6031-6040.
- Helliwell, T. R., NguenthiMan and Morris, G. E. (1994). Expression of the 43-kDa dystrophin associated glycoprotein in human neuromuscular disease. *Neuromusc Dis.* **4**, 101-113.
- Henry, M. D. and Campbell, K. P. (1998). A role for dystroglycan in basement membrane assembly. *Cell* **95**, 859-870.
- Ibraghimov-Beskrovnaya, O., Ervasti, J. M., Leveille, C. J., Slaughter, C. A., Sernett, N. W. and Campbell, K. P. (1992). Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature* **355**, 696-702.
- Ishihara, H., Martin, B. L., Brautigam, D. L., Karaki, H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimoto, K., Uemera, D. et al. (1989). Calyculin-A and okadaic acid - inhibitors of protein phosphatase activity. *Biochem. Biophys. Res. Commun.* **159**, 871-877.
- James, M., NguenthiMan, Wise, C. J., Jones, G. E. and Morris, G. E. (1996). Utrophin-dystroglycan complex in membranes of adherent cultured cells. *Cell Motil. Cytoskel.* **33**, 163-174.
- Jung, D., Yang, B., Meyer, J., Chamberlain, J. S. and Campbell, K. P. (1995). Identification and characterization of the dystrophin anchoring site on beta-dystroglycan. *J. Biol. Chem.* **270**, 27305-27310.
- Khurana, T. S., Kunkel, L. M., Frederickson, A. D., Carbonetto, S. and Watkins, S. C. (1995). Interaction of chromosome 6-encoded dystrophin related protein with the extracellular matrix. *J. Cell Sci.* **108**, 173-185.
- Koenig, M., Monaco, A. P. and Kunkel, L. M. (1988). The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell* **53**, 219-228.
- Kramer, A., Schuster, A., Reineke, U., Malin, R., Volkmer-Engert, R. and Schneider-Mergener, J. (1993). Simultaneous synthesis of peptide libraries on a single cellulose membrane support: examples for the identification of protein, metal and DNA binding peptide mixtures. *Peptide Res* **6**, 314-319.
- Laemmlli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Lim, L. E., Duclos, F., Broux, O., Bourg, N., Sunada, Y., Allamand, V., Meyer, J., Richard, I., Moomaw, C., Slaughter, C. et al. (1995). β -Sarcoglycan: characterization and role in limb-girdle muscular dystrophy linked to 4q12. *Nature Genet.* **11**, 257-265.
- Lu, P.-J., Zhou, X. Z., Shen, M. and Lu, K. P. (1999). Function of WW domains as phosphoserine- or phosphothreonine-binding modules. *Science* **283**, 1325-1328.
- Matsumura, K., Ervasti, J. M., Ohlendieck, K., Kahl, S. D. and Campbell, K. P. (1992). Association of dystrophin-related protein with dystrophin-associated proteins in Mdx mouse muscle. *Nature* **360**, 588-591.
- Matsumura, K., Nonaka, I., Tome, F. M. S., Arahata, K., Collin, H., Leturcq, F., Recan, D., Kaplan, J. C., Fardeau, M. and Campbell, K. P. (1993). Mild deficiency of dystrophin-associated proteins in becker muscular-dystrophy patients having in-frame deletions in the rod domain of dystrophin. *Am. J. Hum. Genet.* **53**, 409-416.
- Matsumura, K., Chiba, A., Yamada, H., Fukuta-Ohi, H., Fujita, S., Endo, T., Kobata, A., Anderson, L. V. B., Kanazawa, I., Campbell, K. P. et al. (1997). A role of dystroglycan in schwannoma cell adhesion to laminin. *J. Biol. Chem.* **272**, 13904-13910.
- Michalak, M., Fu, S. Y., Milner, R. E., Busaan, J. L. and Hance, J. E. (1996). Phosphorylation of the carboxyl-terminal region of dystrophin. *Biochem. Cell Biol.* **74**, 431-437.
- Mikalsen, S.-O. and Kaalhus, O. (1998). Properties of pervanadate and permolybdate: connexin 43, phosphatase inhibition, and thiol reactivity as model systems. *J. Biol. Chem.* **273**, 10036-10045.
- Ponting, C. P., Blake, D. J., Davies, K. E., Kendrick-Jones, J. and Winder, S. J. (1996). ZZ and TAZ: new putative zinc fingers in dystrophin, CBP, p300 and other proteins. *Trends Biochem. Sci.* **21**, 11-13.
- Rambukkana, A., Yamada, H., Zanazzi, G., Mathus, T., Salzer, J. L., Yurchenko, P. D., Campbell, K. P. and Fischetti, V. A. (1998). Role of α -dystroglycan as a Schwann cell receptor for *Mycobacterium leprae*. *Science* **282**, 2076-2079.
- Ren, R. B., Mayer, B. J., Cicchetti, P. and Baltimore, D. (1993). Identification of a 10-amino acid proline-rich SH3 binding site. *Science* **259**, 1157-1161.
- Renshaw, M. W., Ren, X.-D. and Schwartz, M. A. (1997). Growth factor activation of MAP kinase requires cell adhesion. *EMBO J.* **16**, 5592-5599.
- Rentschler, S., Linn, H., Deininger, K., Bedford, M. T., Espanel, X. and Sudol, M. (1999). The WW domain of dystrophin requires EF-hands region to interact with β -dystroglycan. *Biol. Chem.* **380**, 431-442.
- Rosa, G., Ceccarini, M., Cavaldesi, M., Zini, M. and Petrucci, T. C. (1996). Localisation of the dystrophin binding site at the carboxyl terminus of β -dystroglycan. *Biochem. Biophys. Res. Commun.* **223**, 272-277.
- Rost, B., Sander, C. and Schneider, R. (1994). PHD - An automatic mail server for protein secondary structure prediction. *Computer Appl. Biosci.* **10**, 53-60.
- Schultz, J., Milpetz, F., Bork, P. and Ponting, C. P. (1998). SMART, a simple modular architecture research tool: Identification of signaling domains. *Proc. Nat. Acad. Sci. USA* **95**, 5857-5864.
- Sudol, M. (1996a). Structure and function of the WW domain. *Prog. Biophys. Mol. Biol.* **65**, 113-132.
- Sudol, M. (1996b). The WW module competes with the SH3 domain? *Trends Biochem. Sci.* **21**, 161-163.
- Suzuki, A., Yoshida, M., Hayashi, K., Mizuno, Y., Hagiwara, Y. and Ozawa, E. (1994). Molecular organisation at the glycoprotein-complex-binding site of dystrophin: three dystrophin-associated proteins bind directly to the carboxy-terminal portion of dystrophin. *Eur. J. Biochem.* **220**, 283-292.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997). The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res.* **25**, 4876-4882.
- Tinsley, J. M., Blake, D. J., Roche, A., Fairbrother, U., Riss, J., Byth, B. C., Knight, A. E., Kendrick-Jones, J., Suthers, G. K., Love, D. R. et al. (1992). Primary structure of dystrophin-related protein. *Nature* **360**, 591-593.
- Tinsley, J. M., Potter, A. C., Phelps, S. R., Fisher, R., Trickett, J. I. and Davies, K. E. (1996). Amelioration of the dystrophic phenotype of *mdx* mice using a truncated utrophin transgene. *Nature* **384**, 349-353.
- Tufty, R. M. and Kretsinger, R. H. (1975). Troponin and parvalbumin calcium binding regions predicted in myosin light chain and T4 lysozyme. *Science* **187**, 167-169.
- Wagner, K. R. and Haganir, R. L. (1994). Tyrosine and serine phosphorylation of dystrophin and the 58-kDa protein in the postsynaptic membrane of *Torpedo* electric organ. *J. Neurochem.* **62**, 1947-1952.
- Williamson, R. A., Henry, M. D., Daniels, K. J., Hrstka, R. F., Lee, J. C., Sunada, Y., Ibraghimov-Beskrovnaya, O. and Campbell, K. P. (1997). Dystroglycan is essential for early embryonic development: disruption of Reichert's membrane in *Dag1*-null mice. *Hum. Mol. Genet.* **6**, 831-841.
- Winder, S. J. (1997). The membrane-cytoskeleton interface: the role of dystrophin and utrophin. *J. Muscle Res. Cell Motil.* **18**, 617-629.
- Winder, S. J. and Kendrick-Jones, J. (1995). Protein production in 3 different expression vectors from a single PCR product. *Anal. Biochem.* **231**, 271-273.
- Winder, S. J., Gibson, T. J. and Kendrick-Jones, J. (1995a). Dystrophin and utrophin: the missing links! *FEBS Lett.* **369**, 27-33.
- Winder, S. J., Hemmings, L., Maciver, S. K., Bolton, S. J., Tinsley, J. M., Davies, K. E., Critchley, D. R. and Kendrick-Jones, J. (1995b). Utrophin actin binding domain: analysis of actin binding and cellular targeting. *J. Cell Sci.* **108**, 63-71.
- Winder, S. J., Knight, A. E. and Kendrick-Jones, J. (1997). Protein structure. In *Dystrophin: Gene, Protein and Cell Biology* (ed. S. C. Brown and J. A. Lucy), pp. 27-55. Cambridge: Cambridge University Press.
- Yamamoto, H., Hagiwara, Y., Mizuno, Y., Yoshida, M. and Ozawa, E. (1993). Heterogeneity of dystrophin-associated proteins. *J. Biochem.* **114**, 132-139.
- Yang, B., Jung, D., Motto, D., Meyer, J., Koretzky, G. and Campbell, K. P. (1995). SH3 domain-mediated interaction of dystroglycan and Grb2. *J. Biol. Chem.* **270**, 11711-11714.
- Yoshida, T., Pan, Y., Hanada, H., Iwata, Y. and Shigekawa, M. (1998). Bidirectional signaling between sarcoglycans and the integrin adhesion system in cultured L6 myoblasts. *J. Biol. Chem.* **273**, 1583-1590.