

**Neoplastic expression and cellular functions  
of proteolysis inducing factor / dermcidin**

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## **Declaration**

I declare that I have composed this thesis, that the work described is my own and that it has not been submitted for any other degree or professional qualification.

Some work was performed in collaboration -

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## Abstract

Proteolysis inducing factor (PIF) is a pro-cachectic glycopeptide purified from the urines of mice inoculated with the MAC16 tumour and from the urines of weight losing patients with pancreatic carcinoma. It arises from the dermcidin gene which produces 2 other peptides, Y-P30, an unglycosylated neuronal survival factor with homology to the PIF peptide core and DCD-1, an antibiotic peptide secreted by eccrine sweat glands which has no homology with PIF. We sought to investigate PIF / dermcidin expression in cell lines and pancreatic carcinoma tissue, the role of proteolysis inducing factor as a growth and survival factor in tumour cells and the influence on these effects of the structural features known to be important to the induction of cachexia by PIF and the functions of Y-P30 and dermcidin.

*In vitro* expression was assessed by PCR, Western blotting, *in vitro* translation and immunocytochemistry. *In vivo* expression was assessed using laser capture microdissection, PCR, real-time PCR, Western blotting, immunoprecipitation and the analysis of urine by mass spectrometry. Growth and survival functions were assessed in the HuH7 cell line with immunocytochemistry and flow cytometry. Cells were treated with a synthetic PIF peptide or stably transfected with PIF / dermcidin expression vectors. Site-directed mutagenesis of these vectors was used to assess glycosylation and the role of different sequences within the polypeptide.

Dermcidin was expressed by pancreatic carcinoma cell lines and primary human hepatocytes but not by the HuH7 cell line. In HuH7 cells induced expression promoted cell growth and improved survival following oxidative stress. The YP-30 / PIF core peptide sequence was sufficient to induce cell growth. Survival promotion

did not require glycosylation but was prevented by mutagenesis of the asparagine residues of the PIF core peptide. Analysis of mRNA expression suggested dermcidin was expressed in a low number of pancreatic carcinomas. Non-specific antibody binding prompted the development of mass spectrometry for detection of PIF in urine samples of patients with these tumours. This did not reveal PIF but did demonstrate other proteomic changes.

We have demonstrated growth and survival functions of PIF / dermcidin which may be relevant in a range of physiological and pathological processes including cachexia and neoplasia. The PIF core peptide sequence appears to be important for these effects but its glycosylation does not appear to be required. However, differential glycosylation may account for PIF's cachectic affects and the difficulty in its immunological detection. Further investigation using techniques such as mass spectrometry may cast further light on the structure of PIF and its relationship to disease processes.

**Neoplastic expression and cellular functions of  
proteolysis inducing factor / dermcidin**

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## Summary

The work presented in this thesis explores the cellular functions of proteolysis inducing factor (PIF) / dermcidin. The structural features of PIF / dermcidin suggested to be important for these functions have also been investigated.

The PIF molecule is a highly glycosylated, pro-cachectic polypeptide which arises from the dermcidin gene. Dermcidin also produces Y-P30, an unglycosylated neuronal survival peptide of which the unglycosylated PIF core peptide forms a part and DCD-1, a C-terminus peptide with antibiotic properties which does not overlap with the sequence of the PIF core peptide. We examined the expression pattern of PIF / dermcidin mRNA, its effects on cells known to respond to the cachectic effects of glycosylated PIF and the regions of the PIF / dermcidin sequence which may be important for these functions. We also attempted to relate these findings to the *in vivo* situation by examining PIF / dermcidin expression in tumours resected from patients with pancreatic carcinoma.

Several pancreatic carcinoma cell lines, a hepatic carcinoma cell line and primary cultures of adult and fetal liver cells were screened for PIF / dermcidin expression. Expression was observed in all of the pancreatic carcinoma cell lines but not in the HuH7 hepatic carcinoma cell line (Chapter 3). The latter cell line was therefore selected as a model in which to study the effects PIF / dermcidin expression. This was achieved by the transfection of HuH7 cells with the full-length PIF / dermcidin cDNA in a mammalian overexpression vector (Chapter 4). PIF / dermcidin produced from this vector contained a signal peptide and was targetted to the secretory pathway in HuH7 cells. Site-directed mutagenesis of the expression vector was employed to remove potential N-glycosylation sites, introduce premature

STOP codons and alter the domain predicted to be critical for PIF / dermcidin to function as a phosphatase.

In order to study the effect of PIF / dermcidin expression on survival transfected cells were subjected to oxidative stress (Chapter 5). This resulted in cell necrosis. Cells transfected with PIF / dermcidin exhibited improved survival in comparison with untransfected cells. This was abrogated by mutation of both potential N-glycosylation sites in combination but not individually. However, PIF / dermcidin glycosylation was not demonstrable in these cells or using an *in vitro* translation system. Both of the potentially glycosylated asparagine residues lie within the phosphatase domain, raising the possibility that this may be of relevance to the survival benefits.

The effect of PIF / dermcidin on cell proliferation was explored. Transfected cells were assayed for cell cycling by bromodeoxyuridine staining (Chapter 6). Cells transfected with native PIF demonstrated a significant increase in cell proliferation which was not inhibited by the introduction of a STOP codon 5' to the DCD-1 peptide sequence. In contrast with this, the use of a STOP codon to prevent translation of the PIF core peptide prevented PIF / dermcidin -induced cell proliferation. A similar effect was observed on mutation of what analysis of homologous phosphatases suggests is the most highly conserved residue in the PIF / dermcidin phosphatase domain. These results were shown to be relevant at the peptide level by the induction of proliferation by a synthetic peptide corresponding to the PIF core peptide. Thus, PIF / dermcidin appears to have effects on both cell proliferation and survival. Our results also suggest that the same peptide sequence

may be responsible, raising the possibility that the same signalling mechanism may exert both effects.

The survival and proliferation benefits of PIF / dermcidin suggest that expression may be of benefit to cells within the abnormal microenvironment of a tumour. We aimed to investigate this by relating the production of urinary PIF, believed to be a marker of cachexia, to the expression of PIF / dermcidin in tumours of the pancreas. Urine samples and tissue specimens were obtained from patients undergoing surgery for pancreatic carcinoma. Unfortunately, we were unable to detect urinary PIF by immunological methods and testing revealed non-specific reactions of anti-PIF antibodies. We subsequently developed MALDI-TOF mass spectrometry as an alternative means of urinary analysis. This sensitive technique was similarly unable to detect urinary PIF, but did demonstrate the presence of MEKK-3 and APC-binding protein EB1, proteins not present in normal urine. Further development of this technique may reveal important clues as to the pathogenesis of pancreatic carcinoma and cachexia.

Our analysis of tumour PIF expression was similarly limited by the lack of a reliable immunological method of detection. However, only one of the 19 tumour samples demonstrated expression of PIF / dermcidin mRNA. This is consistent with the lack of urinary PIF on assessment by mass spectrometry and suggests that expression may be of low frequency in these tumours. In contrast, all three of the pancreatic carcinoma cell lines which we screened demonstrated PIF / dermcidin expression. One explanation for this difference is that an influence of the *in vitro* environment is capable of inducing PIF expression.

PIF / dermcidin expression therefore appears to confer survival and proliferation benefits on cells which may be relevant in a subset of pancreatic carcinomas. It is feasible that these effects may result in the positive selection of expressing cells, thereby contributing to the development of cachexia. In the liver, these effects may render PIF / dermcidin relevant as a pre-conditioning agent.

The effects of PIF / dermcidin may be mediated separately or in concert. In favour of the latter, the phosphatase domain appears to be important in both survival and proliferation. Future studies will aim to further investigate this mechanism and to resolve the difficulties surrounding *in vivo* assessment of PIF / dermcidin expression.

# 1. Introduction

## Proteolysis inducing factor / dermcidin

### Identification of proteolysis inducing factor / dermcidin

Proteolysis inducing factor is a novel pro-cachectic glycopeptide which was first definitively isolated by affinity chromatography of antigenic material from the MAC16 murine adenocarcinoma (Todorov et al. 1996a). This tumour was known to induce cachexia via the secretion of circulatory catabolic factors (Bibby et al. 1987, Beck and Tisdale 1987) and the monoclonal antibody used to purify PIF had been developed by the fusion of myeloma cells with splenocytes from MAC 16-bearing mice demonstrating delayed cachexia (McDevitt et al. 1995). PIF eluted as two species of molecular mass 69kD and 24kD (Todorov et al. 1996a). On re-injection into mice the 24kD species induced rapid weight loss which could be prevented by prior administration of the monoclonal antibody (Todorov et al. 1996a). *In vitro*, the 24kD species was capable of inducing proteolysis in isolated gastrocnemius muscle leading it to be termed 'proteolysis inducing factor' (PIF)(Todorov et al. 1996a). PIF was subsequently identified in the urines of weight-losing patients with carcinomas of the pancreas, lung, ovary and breast, but not in non-weight losing patients with the same tumours or in weight-losing patients with non-malignant disease (Todorov et al. 1996a, Cariuk et al. 1997).

Amino acid sequencing of the 24kD species of PIF from the MAC 16 tumour revealed a 20 amino acid N-terminus sequence. An identical 14 amino acid sequence was obtained from 24kD PIF obtained from the urine of a weight-losing patient with pancreatic carcinoma (Todorov et al. 1996a). The sequence showed no homology to

any known cytokine or eukaryotic protein sequence, but 11 of the 13 N-terminus amino acids did show homology with a streptococcal preabsorbing antigen involved in the pathogenesis of post-streptococcal glomerulonephritis (Yoshizawa et al. 1992). At this point it was not possible to identify PIF's parent gene using the available techniques of Southern and Northern blotting due to the high degree of degeneracy of the codons encoding the terminal seven amino acids.

### **Structure and homologues of proteolysis inducing factor / dermcidin**

Structural analysis of MAC16-derived PIF demonstrated the presence of carbohydrate in the 24kD species, with N- and O-deglycosylation suggesting it consisted of a short core peptide with one O-linked sulphated oligosaccharide of molecular mass 6kD and one N-linked sulphated oligosaccharide of molecular mass 10kD (figure 1.1) (Todorov et al. 1996a, Todorov et al. 1997). The molecular weight of the core peptide was initially placed at 2kD in correspondence with the predicted weight of the sequenced N-terminus peptide (1.9kD) (Todorov et al. 1996a). However, more detailed structural analyses suggested it to be closer to 4kD (Todorov et al. 1997, Todorov et al. 1999), suggesting a longer core peptide of approximately 30 amino acids. The deglycosylated peptides did not induce gastrocnemius muscle proteolysis, suggesting that glycosylation is essential for PIF's pro-cachectic effects (Todorov et al. 1997). Deglycosylation with peptide *N*-glycosidase F or *O*-glycosidase also prevented detection of PIF with the monoclonal antibody. Similar results were obtained with human PIF purified from the melanoma cell line G361 (Todorov et al. 1999).

Shortly before the latter study, in 1997 and 1998, two patents describing the human cachexia associated protein (HCAP) gene encoding PIF and its putative peptide products were published (Genbank accession number AR053250, US patent 5834192). However, it was with the identification of PIF homologues that the cDNA and genomic sequences first entered the literature. Initially, a 30 amino acid peptide which showed a high degree of homology with the 20 sequenced amino acids of the PIF core was purified from the media of oxidatively-stressed neuronal cells (figure 1.1) (Cunningham et al. 1998). This peptide was named Y-P30. The cDNA encoding Y-P30 was subsequently identified in a search of the 'LifeSeq Gold' database of Incyte genomics, the authors of the 1997 PIF patent (Cunningham et al. 2002). The translated sequence of this cDNA (Genbank accession number AY044239) showed alignment with 18 of the 20 residues of the murine PIF core peptide sequence and 29 of 30 residues of Y-P30. However, the first group to publish this cDNA sequence had in fact independently identified it as a transcript differentially expressed by melanomas and melanocytic naevi (Hipfel et al. 2000). The parental gene was named dermcidin (DCD) following the discovery that it encoded a 47 amino acid peptide, termed DCD-1, secreted by eccrine sweat glands (Schitteck et al. 2001). Although DCD-1 showed no homology to either PIF or Y-P30, translation of DCD suggested that they arose from the same transcript. The gene encoding DCD was mapped to chromosome 12q13 and consisted of 5 exons and 4 introns (Schitteck et al. 2001).

Signal peptide      Y-P30 (extent of sequenced PIF core)

MRFM<sup>S</sup>TL<sup>S</sup>LL<sup>S</sup>FL<sup>S</sup>TALAGALVCA<sup>S</sup>YDPEAA<sup>S</sup>APG<sup>S</sup>SG<sup>N</sup>NP<sup>N</sup>CHEASAAQ<sup>S</sup>KEN<sup>S</sup>AGEDP

Propeptide      DCD-1 Peptide

GLARQAPKPRKQRSS<sup>S</sup>LLEKGLDGAKKAVGGLGKLGKDAVEDLESVGKGA

VHDVKDVLDSVL

**Figure 1.1.** Predicted peptide products of PIF / dermcidin.

S – Potential O-glycosylation site

N – Potential N-glycosylation site

It is possible to construct a map of the nascent polypeptide by assimilating the data on the sequences of the PIF, Y-P30 and DCD-1 peptides (figure 1.1). The presence of an N-terminus signal peptide was first suggested on the discovery of dermcidin (Schitteck et al. 2001) but its production has yet to be confirmed experimentally. Certainly, the sequence is that of a signal peptide which would be expected to target the polypeptide to the secretory pathway (<http://www.cbs.dtu.dk/sevices/SignalP>). It has been suggested that proteolytic processing may account for the production of the other peptides, but this has also to be confirmed and may potentially occur either intracellularly or extracellularly (Schitteck et al. 2001).

## Function of proteolysis inducing factor / dermcidin

Subsequent studies of PIF, Y-P30 and DCD-1 have provided further insight into the function of the dermcidin gene products. Following the initial discovery of the proteolytic effects of PIF on skeletal muscle (Todorov et al. 1996a, Todorov et al. 1996b, Todorov et al. 1997), an *in vitro* assay was developed using C<sub>2</sub>C<sub>12</sub> murine myoblast <sup>3</sup>H-phenylalanine uptake and release to assay protein synthesis and degradation (Smith et al. 1999). This confirmed a stimulatory effect of PIF on protein degradation and also an ability to inhibit protein synthesis. Interestingly, the effect on degradation but not synthesis was inhibited by treatment with the polyunsaturated fatty acid, eicosapentanoic acid (EPA), suggesting that these effects are mediated by different mechanisms. It was subsequently demonstrated that degradation involved expression of 20S proteasome subunits and downregulation of IκB, which was attenuated by EPA (Whitehouse and Tisdale 2003) and potentially mediated by phospholipase A<sub>2</sub> and phospholipase C-induced arachidonic acid release leading to mitosis activating protein kinase activation (Smith and Tisdale 2003).

The influence of PIF on hepatic protein metabolism was investigated using cultures of primary hepatocytes and the hepatoma cell line HepG2 (Watchorn et al. 2001). Here, it was again found that PIF had *in vitro* effects consistent with a role as a cachectic factor, activating the transcription factors NF-κB and STAT3 and subsequently stimulating IL-8, IL-6 and CRP production and inhibiting transferrin production. PIF was subsequently found to induce the expression of NF-κB, IL-6, IL-8, ICAM-1 and VCAM and the shedding of syndecans in the liver endothelial cell line SK-HEP-1 and human umbilical vein endothelial cells but not in pulmonary artery endothelial cells (Watchorn et al. 2002). These results confirmed the

specificity of PIF's effects, demonstrating differential effects on endothelial cell subtypes. In addition, the roles of ICAM-1, VCAM and syndecans in development, inflammation, tissue repair and host defence (Collins et al. 1995, Attar et al. 1999, Park et al. 1999, Zimmermann and David 1999) suggested for the first time that the glycosylated PIF peptide may have a role outwith the cachectic process.

Data on DCD-1 indicate that its primary function may be as an antibiotic peptide. This was initially suggested by its size and location at the COOH terminus of the protein, which are consistent with those of proteolytically-processed antibiotic peptides (Bals 2000), and confirmed by the finding of a high degree of antimicrobial activity against *e. coli*, *s. aureus*, *e. faecalis* and *c. albicans* (Schitteck et al. 2001). Of note, Y-P30 did not have any effect on the same organisms.

The first function of Y-P30 to be identified was that of a survival factor for neuronal cells (Cunningham et al. 1998). Synthetic Y-P30 increased the survival of neuronal cells on treatment with H<sub>2</sub>O<sub>2</sub> *in vitro* and was capable of sustaining the cortical neurons of rats following surgical injury *in vivo* (Cunningham et al. 1998). It was suggested that the *in vitro* effect may be due to cation-dependent phosphatase activity of the peptide, which contains a motif similar to the catalytic region of calcineurin-type phosphatases and this was subsequently confirmed (Zhuo et al. 1994, Mertz et al. 1997, Cunningham et al. 2002). The protective effect of Y-P30 was also found to be associated with binding to calreticulin (Cunningham et al. 2000). Transfection of the full-length dermcidin cDNA (termed DSEP for diffusible survival evasion peptide) into mouse and human neuronal cells resulted in increased resistance to H<sub>2</sub>O<sub>2</sub> and *trans*-retinoic acid (Cunningham et al. 2002), and it is feasible that this effect may be mediated via calreticulin given its ability to downregulate

transcription of retinoic acid-responsive genes (Dedhar et al. 1994, Desai et al. 1996, Shago et al. 1997). More recently, it has been suggested that the survival function of Y-P30 may be important in its action as an oncogene in breast cancer (Porter et al. 2003). This hypothesis is based on the finding that DCD is overexpressed in ~10% of breast cancers and promotes the growth and survival of breast cancer cells *in vitro*. However, these studies were based on the overexpression of the full length DCD cDNA and the peptide responsible for oncogenesis has not been definitively identified. The fact that synthetic Y-P30 peptide has survival benefits in neuronal cells (Cunningham et al. 1998) supports its promotion of tumour cell survival, but there is as yet no evidence to suggest it induces cell growth.

Recently, data has been published which raises important questions about the role of PIF in cancer cachexia. Using PCR and real-time PCR, Monitto et al (2004) demonstrated DCD expression in a subset of breast tumours but not in the MAC16 murine adenocarcinoma cell line or in human pancreas. Protein expression was also not detected using an antibody raised to amino acids 44-62 of the polypeptide. Furthermore, although a secreted protein was produced on transfection of several murine and human cell lines with the full-length DCD cDNA in an overexpression vector, no glycosylation was apparent. Xenografting of overexpressing tumours into nude mice also did not result in cachexia. These results are consistent with the lack of a consensus N-glycosylation sequence in the PIF sequence, although tumours are known to demonstrate abnormal glycosylation patterns (Kannagi et al. 2004). The glycosylation of PIF may therefore be critical in determining its biological activity although the mechanisms by which it is glycosylated remain unknown.

## **Protein glycosylation**

Glycosylation is the process by which glycoproteins are created through the covalent addition of sugars to proteins. The majority of soluble and membrane-bound proteins destined for the Golgi apparatus, lysosomes, cell membrane or extracellular space are glycosylated.

### **Mechanisms of glycosylation**

Glycosylation occurs both co- and post-translationally. Most proteins synthesised in the rough endoplasmic reticulum (ER) are initially co-translationally glycosylated by the addition of an N-linked oligosaccharide. This reaction is catalyzed by oligosaccharyl transferase, a membrane bound ER enzyme with its active site on the luminal side of the ER membrane (Alberts et al. 2002a). Oligosaccharyl transferase is specific and acts preferentially on asparagine residues contained in the consensus sequence Asn-X-Ser / Thr, where X is any amino acid other than proline (Lau et al. 1983, Welply et al. 1983). It has been proposed that the importance of the Ser / Thr residues lies in their ability to act as donors in hydrogen bond formation with oligosaccharyl transferase, a function which reduced cysteine may also fulfill creating the atypical glycosylation site Asn-X-Cys (Sato et al. 2000, Spiro 2002). As the asparagine residue of the nascent polypeptide chain enters the ER, oligosaccharyl transferase adds a preformed precursor oligosaccharide consisting of N-acetylglucosamine, mannose and glucose and containing a total of 14 sugars (Hubbard and Robbins 1980, Olden et al. 1980). Three glucose residues and one mannose residue are then removed by glucosidase and mannosidase while the glycoprotein remains in the rough ER (Bischoff and Kornfeld 1983, Esmon et al. 1984). If the protein is folded correctly at this stage, it is able to leave the ER. If,

however, it is incompletely folded then ER glucosyl transferase is able to access the remaining sugar moieties and add a single terminal glucose residue. This results in the binding of the chaperones calnexin and calreticulin and the retention of the protein in the ER (Kearse et al. 1994, Spiro et al. 1996). Co-translational glycosylation therefore serves to prevent the export of incompletely folded glycoproteins from the ER and provides the core carbohydrate for N-glycosylation. Further glycosylation takes place post-translationally in the Golgi apparatus.

### **N-glycosylation**

Modification of N-linked oligosaccharide in the Golgi apparatus results in 2 broad classes of N-linked carbohydrates, the complex oligosaccharides and the high-mannose oligosaccharides (Alberts et al. 2002b). Complex oligosaccharides are generated by a combination of trimming of the original oligosaccharide added in the ER and the addition of further sugar residues. In contrast, high-mannose oligosaccharides have no further sugars added and represent the remains of the original N-linked oligosaccharide following trimming.

The addition of further sugars to complex oligosaccharides is catalyzed by a group of enzymes known as the glycosyltransferases. Over one hundred glycosyltransferases have been identified and the enzymes are classified according to the type of sugar they transfer (eg. galactosyltransferases, sialyltransferases) (Beyer et al. 1981, Sadler 1984). Although these enzymes otherwise share little homology, they are anchored to the membrane of the Golgi apparatus by signal-anchor domains which orientate their catalytic domains within the lumen (Wickner and Lodish 1985, Paulson and Colley 1989). Cleavage of the signal anchor has been reported

(Lammers and Jamieson 1989) and soluble forms of glycosyltransferases have been purified from milk, serum and other body fluids (Beyer et al. 1981, Sadler 1984). Glycosyltransferases may therefore also be active extracellularly, although their primary site of action is the Golgi apparatus.

Whether a given oligosaccharide is processed to a complex or a high-mannose oligosaccharide depends largely on its position in the protein. If it is accessible to glycosyltransferases it is likely to be processed and if not, it is likely to remain in the high-mannose form. The precise structure of the carbohydrate chain synthesized is dependent on several factors, including protein conformation, substrate availability and the expression of glycosyltransferases within the cell (Smith and Baenziger 1988, Paulson and Colley 1989, Yarema and Bertozzi 2001). Subsequently, N-glycosylation is often incomplete or variable, creating multiple glycoforms of proteins.

### **O-glycosylation**

O-glycosylation describes the process by which carbohydrate is added to the hydroxyl groups of the serine or threonine residues of selected proteins. In common with the extension of N-linked oligosaccharide chains, this is catalyzed by a series of glycosyltransferase enzymes (Clausen and Bennett 1996, Ten Hagen et al. 2001) which are active in the cis-Golgi (Roth et al. 1994, Hirschberg et al. 1998). In contrast with N-glycosylation however, no specific consensus sequence for O-glycosylation has been identified and it has been suggested that this may be due to the fact that multiple O-glycosyltransferases may act directly on the protein sequence whereas a single oligosaccharyl transferase is involved in initial N-glycosylation in the ER (Spiro 2002).

The degree of O-glycosylation may vary from just a few to 10 or more sugar residues (Alberts et al. 2002b). Heavy O-glycosylation is observed in proteoglycan core proteins (Ten Hagen et al. 2001), where carbohydrate side chains are made up of repeating disaccharide units and which subsequently polymerise forming glycosaminoglycans (Alberts et al. 2002b). Subcellular processing and secretion of O-glycosylated proteins is similar to that of N-glycosylated proteins, with many proteoglycans targeted to the extracellular matrix or mucus secretions.

## **Biological importance of glycosylation**

### **Functions of glycosylation**

Glycosylation represents a mechanism by which proteins are modified by non-genomic influences, specifically the glycosyltransferase and sugar milieu of the cell. The glycoproteins expressed by a cell may therefore be said to reflect its genomic, proteomic and metabolic status and as described above, a complex series of pathways have evolved to synthesize oligosaccharide side-chains. Glycosylation would subsequently be expected to fulfill important biological functions. However, many of these functions remain uncharacterised.

N-linked oligosaccharides are found in all eukaryotes but not in prokaryotes (Spiro 2002). This may reflect their role in ensuring correct protein folding and suggests that they may fulfill functions relevant to higher organisms.

Due to the limited flexibility of carbohydrate side-chains, even a small oligosaccharide protrudes from the surface of a protein and can limit the approach of other molecules. For example, glycosylation may render a glycoprotein more

resistant to protease digestion (van Berkel et al. 1995, Remacle et al. 2006) or mediate interactions with other proteins. Selectins are a family of cell adhesion molecules found on leukocytes which recognise and bind mammalian oligosaccharides bearing fucose and sialic acid such as the sialyl Le<sup>X</sup> and sialyl Le<sup>A</sup> antigens, which are located mainly on the surface of endothelial cells (Varki 1994). Glycosylation is also important in interactions between extracellular proteins. Many coagulation and fibrinolytic factors are glycosylated (Spiro 2002) and their carbohydrate side-chains have been implicated in control of secretion, activity and plasma clearance (Rudd et al. 1995, Millar and Brown 2006).

Interactions involving carbohydrate moieties may result in signal transduction. For example, selectin-mediated cell adhesion stimulates leukocyte involvement in thrombosis, inflammation and leukocyte trafficking (Varki 1994). The degree of glycosylation has been demonstrated to directly influence signalling from the receptor Notch. This transmembrane protein is O-glycosylated by the addition of a single fucose to certain serine, threonine and hydroxylysine residues (Moloney et al. 2000). In some cell types an N-acetylglucosamine residue is then added by a separate glycosyltransferase, sensitizing the receptor to activating stimuli (Bruckner et al. 2000). This glycosylation is important in temporally regulating Notch signalling during development. Glycosylation is also important in modifying the effects of cytokines. It has the potential to enhance or inhibit their multimerisation, binding to other structures (including extracellular matrix molecules and parasites) and active effects (Tanaka et al. 1993, Lucas et al. 1994, Opdenakker et al. 1995). N-glycosylation has been demonstrated to decrease cytokine activity and receptor binding (Cebon et al. 1990, Opdenakker et al. 1995). As noted above,

glycosylation may be incomplete or variable, and this may result in cytokines isoforms of differing activity competing for receptor binding (Opdenakker et al. 1995).

### **Glycosylation in disease**

Altered glycosylation has been described in many disease states. In certain rare genetic conditions primary defects in glycosylation exist and here it is clear that the disease phenotype is secondary to altered glycosylation (Freeze and Westphal 2001, Schachter 2001). However, in inflammation and neoplasia it is not clear whether altered glycosylation is a cause or effect of the disease process.

In inflammation, there is increased hepatic production of the acute phase proteins  $\alpha$ 1 acid glycoprotein and  $\alpha$ 1 antitrypsin and a concomitant increase in glycosyltransferase expression is observed (Kim et al. 1972, Lammers and Jamieson 1989). Hepatic glycosyltransferase expression has also been demonstrated to increase in response to steroids (van et al. 1986, Wang et al. 1989) and it has been suggested that subsequent changes in coagulation factor and complement glycosylation may contribute to the inflammatory process.

In neoplasia, aberrant glycosylation has been reported in essentially all types of experimental and human cancers and many glycosylated epitopes form tumour-associated antigens (Hakomori 2002). Initially, this may result from oncogenic transformation and a growing amount of evidence suggests that glycosylation may subsequently influence tumour progression. Expression of the Neu3 sialidase has been demonstrated to increase production of lactosylceramide, resulting in the inhibition of apoptosis through increase *Bcl-2* and decreased caspase expression (Kakugawa et al. 2002). The ganglioside GM3 acts as a substrate in the formation of

lactosylceramide and a decrease in its concentration may result in a decrease in GM3 - CD9 / CD82 complexes (Ono et al. 1999), which are antimetastatic in colorectal and bladder cancer cell lines (Ono et al. 2001, Satoh et al. 2001). GM3 also inhibits epidermal growth factor (EGF) receptor tyrosine kinase activity, and a decrease in this action may also result in increased cell motility (Bremer et al. 1986).

Other glycosylation pathways contribute to tumour cell invasion and metastasis. Increased N-linked oligosaccharide branching is commonly seen in malignancy, enhanced by expression of GnT-V and inhibited by GnT-III (Schachter 1986). GnT-V is upregulated by the oncogene family Ets (Kang et al. 1996, Buckhaults et al. 1997), resulting in increased E-cadherin side-chain branching and subsequently decreased cell to cell adhesion and a prometastatic effect (Yoshimura et al. 1996). The sialyl Le<sup>X</sup> antigen has also been implicated in metastasis, with lung adenocarcinoma cells expressing long-chain sialyl Le<sup>X</sup> showing higher metastatic potential in nude mice (Inufusa et al. 1991). Colorectal cancer cell lines transfected with long-chain sialyl Le<sup>X</sup> also show preferential adhesion to hepatocytes (Ota et al. 2000). Significantly, both sialyl Le<sup>X</sup> and sialyl Le<sup>A</sup> expression have been associated with decreased postoperative survival (Nakamori et al. 1993, Kannagi 1997).

It is therefore clear that glycosylation of PIF / dermcidin has the potential to alter almost any aspect of its function. In particular, it may feasibly affect whether PIF / dermcidin induces cachexia or functions as a growth or survival factor.

## Growth Factors

A tumour has been defined as 'an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues, and persists in the same excessive manner after cessation of the stimuli which evoked the change' (Willis 1934). This definition lead to the hypothesis that tumour cells are autonomous in terms of exogenous growth control due to the autocrine action of growth factors (Sporn and Roberts 1985, Weinberg 1985). The first autocrine growth factor to be implicated in carcinogenesis was platelet-derived growth factor (PDGF). The B-chain of PDGF is encoded by the oncogene *v-sis*, the activation of which results in PDGF overproduction (Doolittle et al. 1983, Waterfield et al. 1983). Growth factors may also result in tumour cell proliferation due to the overexpression of cellular receptors, for example the EGF receptor encoded by the oncogene *c-Erb* (Downward et al. 1984), or by excessive amplification of the signal induced by receptor activation, for example by *ras* (Gilman 1984).

Through the activation of oncogenic pathways, growth factors may influence many aspects of cancer biology. As noted above, K-ras has been implicated in pancreatic cancer progression and chemoresistance (DiGiuseppe et al. 1994b, Ng et al. 2000). Angiogenesis may be promoted by overproduction of VEGF (Luo et al. 2001), which also acts as a tumour mitogen. Smad4 mutations may also prevent the negative effect of TGF- $\beta$  on tumour growth (Korc 1998).

It is therefore clear that growth factors have unique physiological functions in addition to inducing tumour cell proliferation. Indeed, almost no molecule is solely expressed by tumour cells but rather is likely to result in the selection of tumour cells if its expression is beneficial, either through its primary physiological function or a

secondary effect. Consistent with this diversity of function, a range of molecules originally identified through their physiological functions have subsequently been demonstrated to be growth factors for tumours. These include cytokines, chemokines, hormones and extracellular matrix molecules. In pancreatic cancer, IL-8 has proliferative and pro-angiogenic effects (Shi et al. 1999), gastrin induces cell growth and tumorigenesis (Harris et al. 2004) and fibronectin and collagen I and IV increase proliferation and chemoresistance (Miyamoto et al. 2004).

As would be expected from this diversity of physiological functions, growth factors show great structural diversity. Indeed, their only common features appear to be those essential to their extracellular function and interaction with receptors, namely the correct degree of hydrophobicity and having the necessary signal peptides to target them to the secretory pathway.

As our knowledge of cancer biology has expanded, it has emerged that carcinogenesis is influenced by abnormal control of cell death as well as abnormal regulation of proliferation. Indeed, the proliferation of cells with genetic abnormalities should perhaps prompt reconsideration of the definition of cancer given above as it suggests that there must be a defect in cell death which allows these cells to survive.

## Survival Factors

The influence of cell death on oncogenesis rose to prominence with the finding that the product of the wild-type p53 antioncogene induces apoptosis whereas the product of mutant p53 does not (Yonish-Rouach et al. 1991). Other oncogenes were subsequently found to promote cell survival, including ras, which acts via mitosis activating protein kinase (Bonni et al. 1999, Scheid et al. 1999) and src, which acts via the STAT family of transcription factors (Shen et al. 2001 Wang et al. 2000, Shen et al. 2001). Consistent with their activation of these signalling pathways, many growth factors have also been found to have pro-survival effects. For example, PDGF and EGF have been shown to rescue cells from apoptosis through their action on the ras pathway (Miyamoto and Fox 2000) and insulin-like growth factor decreases the apoptosis of pancreatic carcinoma cells in response to radiotherapy and chemotherapy (Min et al. 2003). The joint effects of these factors on growth and survival may be mediated through the cell cycle. It is at cell cycle checkpoints that apoptosis is triggered in cells with DNA abnormalities (Wu and Levine 1994, Nelson and Kastan 1994). Failure of this process has been suggested to be a key event in oncogenesis which may reflect either the actions of oncogenes, for example c-myc (Wyllie 1993), or the survival effects of the growth factor milieu (Wyllie 2002).

In addition to protecting cells from intracellular stimuli of apoptosis, tumour survival factors exist which protect cells from the insults of the hostile tumour microenvironment. The immune response to cancer involves cell-mediated cytotoxicity mediated by ligands of the TNF superfamily. Of these, TNF- $\alpha$ , FasL and TRAIL are most frequently involved in apoptotic signalling (Ashkenazi and Dixit 1999). The actions of these molecules are mediated by a system of soluble and

membrane bound receptors. These include the decoy receptors (DcR) 1 and 2, which are membrane bound (Mongkolsapaya et al. 1998, gli-Esposti et al. 1997), and DcR3 and osteoprotegerin, which are secreted proteins (Pitti et al. 1998, Simonet et al. 1997). DcR3 has been demonstrated to block the apoptotic signalling of Fas ligand and subsequently function as a survival factor in pancreatic, lung and colonic carcinoma cells (Tsuji et al. 2003, Pitti et al. 1998).

Another stimulus of cell death to which cancer cells are exposed is oxidative stress. Evidence from recent studies suggests that cancer cells are subject to increased oxidative stress associated with oncogenic transformation, changes in metabolism and increased generation of reactive oxygen intermediaries (ROIs) (Toyokuni et al. 1995, Hileman et al. 2001, Kang and Hamasaki 2003, Behrend et al. 2003). The oncogenes *c-myc* and *ras* have been demonstrated to induce the generation of reactive oxygen species (Vafa et al. 2002, Hlavata et al. 2003). Mitochondrial metabolism is also altered in cancer cells in that the generation of superoxide may be increased, although the mechanism of generation is unchanged (Konstantinov et al. 1987).

The increased ROI production in cancer cells may result in cellular damage including the promotion of mutations and genomic instability. This may result in cell death. Several mechanisms have evolved to protect cells from this damage and there is evidence to suggest that some of these may be altered in cancer. For example, expression of the antioxidant enzymes superoxide dismutase and glutathione-S-transferase may be increased in tumour cells (Janssen et al. 1999, Kanbagli et al. 2000), although this may partly be a feedback response to the increased levels of oxidative stress (Storey 1996) which are often inadequately controlled by these

enzymes (Pelicano et al. 2004). Extracellular stimuli may also influence the cellular response to oxidative stress and the use of such agents to pre-condition cells to oxidative stress has been extensively studied in the field of organ transplantation. Agents with protective, pre-conditioning effects include oxidative stress itself ('ischaemic preconditioning'), steroids and low levels of cytokines such as TNF $\alpha$  (Chiappa et al. 2004, Teoh et al. 2004). These agents subsequently have the potential to function as survival factors in neoplasia, in which they are known to be aberrantly regulated (Aderka et al. 1991, Knapp et al. 1991, Pelicano et al. 2004). Finally, a low level of oxidative stress has been recognised to transduce signals into cells via the activation of tyrosine kinases including *src* and the subsequent stimulation of MAP kinases, phospholipase C- $\gamma$  and phosphoinositol-3-kinase (Kamata and Hirata 1999, Otani 2004). In tumour cells these mediators may stimulate cellular proliferation and motility (Burdon 1995, Dorward et al. 1997, Schimmel and Bauer 2002, Behrend et al. 2003). The abnormal oxidative stress and oncogenic signalling seen in malignancy may subsequently influence the balance of redox signalling in favour of survival and proliferation. Oxidative stress can also be seen to provide a link between these two important processes of tumour progression. In addition, cells deprived of growth factors or subjected to radiotherapy or chemotherapy are also subjected to oxidative stress (Lieberthal et al. 1998, Pandey et al. 2003, Renschler 2004), suggesting that cells able to survive under conditions of oxidative stress may also be resistant to these agents

There are therefore several potential mechanisms by which Y-P30 may improve the survival of tumour cells (Y79 retinoblastoma cells) subjected to

oxidative stress (Cunningham et al. 1998). This may select for expressing cells and raises the possibility that pre-existing PIF / DCD expression could predispose to malignant progression, increased invasion, angiogenesis and an increased risk of metastasis formation (Hockel and Vaupel 2001).

## **Pancreatic cancer**

Pancreatic ductal adenocarcinoma is the fourth leading cause of cancer death in the Western world, accounting for 2% of all cancers and 5% of all cancer deaths in the United States and 2.3% of all cancers in Scotland (Jemal et al. 2004, ISD at [www.isdscotland.org](http://www.isdscotland.org)). Its aggressive behaviour and anatomical site commonly result in a late presentation, often when local invasion or distant metastasis have already occurred. The disease is subsequently almost universally fatal (Greenlee et al. 2000) with an overall 5 year survival rate of <5% (Kuvshinoff and Bryer 2000, ISD at [www.isdscotland.org](http://www.isdscotland.org)).

In localized pancreatic cancer surgery offers the only hope of cure. However, less than 20% of tumours are resectable by the time of diagnosis and even in patients with resectable cancers the 5 year survival rate is less than 25% in the best centres (Neoptolemos and Kerr 1995, Yeo et al. 1995). Indicators of both early disease and metastasis have subsequently been studied in order that patients suitable for surgery may be identified earlier and that those with incurable disease may be spared unnecessary operations (Hruban et al. 2000b). Studies of intraductal lesions with cell proliferation and atypia suggested that some of these lesions may progress to infiltrating carcinomas and these lesions have been named pancreatic intraepithelial neoplasia (PanIN) (Brat et al. 1998, Hruban et al. 1999). Several oncogenes have been found to be activated in PanIN, including K-ras, HER-2/neu, p16, p53, DPC4 and BRCA2 (DiGiuseppe et al. 1994b, Yamano et al. 2000, Day et al. 1996, Wilentz et al. 2000, Wilentz et al. 1998, Moskaluk et al. 1997, DiGiuseppe et al. 1994a, Goggins et al. 2000) which may have effects on tumour growth. A progression model for the development of malignancy and invasiveness has subsequently been

suggested (Hruban et al. 2000a). Although the relative difficulty of biopsying the pancreas renders direct screening of lesions for abnormal expression of these genes impractical, they may potentially prove useful as therapeutic targets or circulating markers.

For the majority of patients with unresectable pancreatic cancer there remain few treatment options. Trials of many radiotherapy and chemotherapy regimes as primary treatments for pancreatic cancer showed little benefit and significant toxicity (Thomas 1996, Schnall and Macdonald 1996). This resulted in the reservation of these therapies for those patients who have already undergone surgical resection (Neoptolemos and Kerr 1995). More recently however, gemcitabine has been demonstrated to have a marginal survival benefit over conventional fluorouracil chemotherapy (median survival 5.6 vs 4.4 months, 18% vs 2% 1 year survival) (Burris, III et al. 1997). Nevertheless, the management of these patients remains mainly palliative. The investigation of both the mechanisms of resistance to chemotherapy and the decline in quality and length of life in patients with irresectable pancreatic cancer therefore seems important.

Several mechanisms have been implicated in the resistance of pancreatic cancer to chemotherapy. The majority of cytotoxic agents act by inducing apoptosis in cells undergoing cycling or protein synthesis. It has subsequently been proposed that those cancer cells resistant to chemotherapy have low rates of mitosis and protein synthesis (Stein et al. 2004). A low expression of protein synthesis genes has been demonstrated in pancreatic cancer and has been shown to correlate with poor 5 year survival (Stein et al. 2004). In contrast, the expression of cell adhesion, cytoskeletal and ECM genes was found to be positively correlated with a poor 5 year

survival (Stein et al. 2004). Expression of these genes may favour survival by decreasing anoikis through strong interactions with the tumour stroma (Plath et al. 2000). Stromal cells have also been demonstrated to stimulate chemoresistance in pancreatic tumour cells by producing nitric oxide leading to the activation of the NF- $\kappa$ B pathway in tumour cells and subsequent IL-1 $\beta$  secretion (Muerkoster et al. 2004). Upstream mediators in this pathway may include K-ras and AKT, which have also been implicated in chemoresistance (Ng et al. 2000, Fahy et al. 2003, Fahy et al. 2004). Several other genes have also been implicated in pancreatic cancer chemoresistance including p53, cyclin D1, CEA-CAM 6, MDR I and src (Nio et al. 1998, Kornmann et al. 1998, Duxbury et al. 2004a, Lage and Dietel 2002, Duxbury et al. 2004b).

It can therefore be seen that molecules affecting tumour growth and survival appear to play an important role in pancreatic carcinogenesis and it is subsequently feasible that PIF / dermcidin may contribute to this process. This is supported by previous studies demonstrating that PIF appears to play a role in the development of cachexia in pancreatic cancer (Todorov et al. 1996a, Wigmore et al. 2000). In mediating cachexia PIF / dermcidin may act in concert with a range of other mediators to stimulate complex metabolic changes which may, in turn, influence the expression and function of PIF / dermcidin.

## Cancer cachexia

### Definition and importance of cachexia

The word 'cachexia' originates from the Greek words 'kakos', meaning bad, and 'hexis', meaning condition. It has been defined as a clinical syndrome of anorexia, early satiety, changes in taste perception, weight-loss, weakness, anaemia and oedema (Fearon 1992). Cachexia is classically associated with certain types of cancer, but may also be seen in other conditions including chronic heart failure, chronic inflammatory diseases and infective conditions such as the acquired immune deficiency syndrome (Anker and Sharma 2002, Kotler 2000, Rall and Roubenoff 2004). In cancer, it has been estimated that up to 80% of patients will develop cachexia before death (Dewys et al. 1980). It is commonest in those with pancreatic or gastric tumours, approximately one third of whom have substantial ( $\geq 10\%$ ) weight loss at the moment of diagnosis and is of higher frequency in patients with solid tumours, with the exception of breast cancer and sarcomas (Dewys et al. 1980, Bozzetti et al. 1982).

Duration of survival and quality of life are frequently drastically affected by cachexia. It has been estimated that cachexia accounts for 22% of deaths in cancer (Warren 1935). The rate of complications from surgery, radiotherapy and chemotherapy are increased (Studley 1936, Engelman et al. 1999, Curtis et al. 1995, Piquet et al. 2002, Dewys et al. 1980). Patients also suffer from the process itself, with weakness, chronic nausea and anorexia all contributing to a decrease in the quality of life in what may be a few short months until death (Stone et al. 2000, Vigano et al. 2004). This inevitably instigates psychological distress in the patient and their family, compounding that caused by the diagnosis of cancer (Padilla 1986).

## **Mechanisms of cachexia**

### **Anorexia**

Patients with cancer cachexia frequently have a decreased nutritional intake which may be due to a variety of factors. Some of these may be specific to the site of the tumour, for example, gastrointestinal obstruction, nausea and constipation in tumours of the gastrointestinal tract. In pancreatic cancer malabsorption may also occur (Perez et al. 1983) as the tumour results in loss of pancreatic exocrine function. However, even when these problems are adequately treated an adequate oral intake may remain difficult to establish due to effects including changes in taste, poor appetite and early satiety (Fearon 1992). These effects do not appear to be specific to the site of the tumour, and may also be seen in many patients with inflammatory and infective illnesses. As such, they may represent an influence of either the tumour itself or the host response to disease on the drive to eat.

In simple starvation patients undergo a physiological response whereby energy expenditure is reduced, protein is conserved and fat becomes the primary energy source through the generation of fatty acids and ketone bodies (Grande et al. 1958, Leibel et al. 1995). It has been suggested that in cancer cachexia this response is altered or absent, resulting in an increased energy expenditure and continued protein loss (Brennan 1977). This may also affect the pattern of tissue loss observed in cancer cachexia, in which visceral protein is conserved at the expense of muscle protein (Heymsfield and McManus 1985, Fearon and Preston 1990).

Many mediators contribute to both the metabolic response to starvation and the drive to eat including hypothalamic control, inflammatory cytokines and the leptin system. The potential roles of these mediators in cachexia are discussed below.

## **Hypermetabolism**

Studies of resting energy expenditure in cancer patients have shown a variation from less than 60% to more than 150% of predicted values (Bozzetti et al. 1980, MacFie et al. 1982, Knox et al. 1983, Dempsey et al. 1984, Hansell et al. 1986a, Hyltander et al. 1991, Fredrix et al. 1991). This may be influenced by tumour type, as patients with cancers more frequently associated with cachexia (including pancreas and lung) more commonly have an elevated resting energy expenditure (Fredrix et al. 1991, Staal-van den Brekel AJ et al. 1994, Falconer et al. 1994) and course of disease, as studies of carcinosarcoma-bearing rats have suggested that the animals go through an initial hypermetabolic phase then become briefly normometabolic prior to passing into a preterminal hypometabolic phase (Zylicz et al. 1990). Unchecked hypermetabolism would inevitably deplete body reserves and it has been suggested that there is a compensatory fall in physical activity, maintaining a constant total energy expenditure in spite of the increase in resting energy expenditure. This decrease in activity may be reflected in a reduction in the quality of life as the disease progresses, until eventually decompensation occurs and tissue depletion contributes to cachexia-related mortality. Clearly, it is likely that the change from hypermetabolism to hypometabolism reflects the influence of more than one process on the net metabolic rate.

## **Substrate metabolism**

The metabolism of many major nutrients including glucose, fat and protein has been shown to be altered in cancer cachexia.

Many cachectic patients exhibit relative glucose intolerance and insulin resistance (Rohdenburg et al. 1919, Waterhouse 1974, Holroyde et al. 1975, Lundholm et al. 1978, Chlebowski et al. 1982, Holroyde et al. 1984, Eden et al. 1984, Shaw and Wolfe 1987, Copeland et al. 1987). This results in an increased rate of glucose production and recycling via lactate which may have an energy cost of up to 260kcal per day (Eden et al. 1984). The subsequent rise in plasma glucose concentration may benefit solid tumours, which gain most of their energy from the anaerobic metabolism of glucose (WARBURG 1956, Zu and Guppy 2004). Certainly, alterations in glucose metabolism may become more pronounced with the progress of the disease (Chlebowski et al. 1982, Shaw and Wolfe 1987).

The primary alteration in fat metabolism in cancer cachexia is an increase in the fat oxidation rate (Hansell et al. 1986b). This results in a pronounced loss of adipose tissue (Heymsfield and McManus 1985, Fearon and Preston 1990), which appears to be due to a decrease in lipogenesis rather than an increase in lipolysis (Jeevanandam et al. 1986). This has been ascribed partly to reduced levels of lipoprotein lipase in weight-losing cancer patients (Vlassara et al. 1986). However, new evidence is emerging to suggest that other factors, such as the lipid-mobilizing factor, zinc alpha-2 glycoprotein (ZAG) may be involved (Russell et al. 2004, Bing et al. 2004). Notably, ZAG induces lipolysis, and confirmation of its involvement in human cachexia may prompt reassessment of the role of lipolysis in adipose tissue loss.

Protein metabolism is markedly affected in cancer cachexia. When compared with starved normal individuals and weight-losing non-cancer patients, patients with cancer cachexia have been shown to have an increased whole body protein turnover (Carmichael et al. 1980, Jeevanandam et al. 1984, Fearon et al. 1988, Melville et al. 1990). The differences between these groups support the theory that either the tumour itself or a host response to the cancer-bearing state may affect protein metabolism, mirroring theories of anorexia induction (see above). In animal studies, it has been suggested that tumour cells may use circulating albumin as a source of albumin for growth (Stehle et al. 1997). In humans, the relative size of tumours is much smaller and it is therefore less likely that their requirement for nutrients has a large effect on whole body protein turnover. However, a quantitative effect on protein catabolism does appear to exist, as turnover has been shown to increase with the progression of disease (Carmichael et al. 1980). Alterations in host metabolism include a change in the balance of liver export proteins with increased fibrinogen synthesis rates (Fearon et al. 1998, Preston et al. 1998). Overall, the increase in protein cycling has been estimated to have an energy cost of around 100kcal per day (Fearon et al. 1988). The net effect of this cycling is a urinary nitrogen loss and a negative nitrogen balance (Argiles et al. 1999). In mice this is maximal at low levels of weight loss and halts at higher levels, consistent with the passage of rats through hypermetabolic and normometabolic states prior to pre-terminal hypometabolism (Beck and Tisdale 1989, Zyllicz et al. 1990).

The different tissues of the body are affected by the increase in protein turnover in a highly variable manner. In general, visceral protein is conserved whereas skeletal muscle is commonly lost. This may result from either decreased

protein synthesis or increased protein degradation. In humans, the former has been demonstrated in cancer patients (Dworzak et al. 1998, Emery et al. 1984a). Animal studies have suggested that this may be secondary to a decrease in RNA synthesis (Emery et al. 1984b). In contrast, there is no direct evidence that protein breakdown is involved in human cachexia, although this is difficult to measure *in vivo*. However, in rat and mouse systems it has been suggested that the ATP-dependent ubiquitin-proteasome pathway is involved in proteolysis (Llovera et al. 1994, Llovera et al. 1995, Baracos et al. 1995, Lecker et al. 1999, Lorite et al. 2001). The calcium dependent and lysosomal pathways do not appear to be involved. On balance, it seems likely that confirmatory *in vivo* studies will show a role for proteolysis in human cachexia.

In the liver, export protein synthesis is deranged and in specific, there appears to be a loss of feedback control. With regard to albumin, production remains unchanged despite a drop in the circulating concentration of albumin (Fearon et al. 1998, Preston et al. 1998). In addition, as noted above fibrinogen synthesis increases and this occurs in the presence of elevated plasma fibrinogen levels.

### **Inflammatory and acute phase protein responses**

There is an increasing body of evidence to suggest that the induction of a systemic inflammatory response plays an important role in cancer cachexia. Leukocytosis has been associated with cachexia in human squamous cell carcinomas (Kato et al. 1999, Yoneda et al. 1991) and may be induced by tumour necrosis factor (TNF) and granulocyte-colony stimulating factor (G-CSF), cytokines common to

both inflammation and cachexia (Kato et al. 1999, Tracey et al. 1988). The subsequent increase in bone marrow activity will have an inevitable energy cost, although the importance of this in cancer cachexia has yet to be investigated.

There is a substantial amount of data to implicate the acute phase protein response in the pathogenesis of cachexia. The acute phase protein response represents a change in the pattern of serum proteins synthesized by the liver and is reflected in the changes in hepatic protein metabolism described above. It is characterised by an increase in the serum concentrations of positive acute phase proteins such as C-reactive protein and fibrinogen and a decrease in the concentrations of negative acute phase proteins such as albumin. It has been demonstrated to occur in a significant proportion of patients with pancreatic, lung, renal and oesophageal tumours (Falconer et al. 1994, Staal-van den Brekel AJ et al. 1995, Blay et al. 1992, Wayman et al. 1997). Furthermore, an acute phase response has been linked to weight loss in melanoma, lung cancer and pancreatic cancer (Staal-van den Brekel AJ et al. 1995, Wigmore et al. 1997c, Scott et al. 1996, Harvie et al. 1998). In pancreatic cancer, the response has been shown to increase with disease progression (Falconer et al. 1994) and this may be of clinical importance, as it has also been shown to be associated with a decreased duration of survival (Falconer et al. 1995). This is also the case in patients with renal and colorectal tumours (Blay et al. 1992, Nielsen et al. 2000). In patients with gastrointestinal tumours an acute phase protein response has been shown to be associated with a reduced quality of life. The presence of an acute phase protein response in cancer therefore seems to be closely linked to cachexia and has many of the same effects on clinical outcome.

Several effects of the acute phase protein response may stimulate cachexia. Firstly, it contributes to the increased protein cycling of cachexia described above. This may be compounded by the other mechanisms acting to induce cachexia. For instance, anorexia will result in a decreased protein intake resulting in an increase in muscle protein breakdown to provide amino acids for acute phase protein synthesis. This in turn may be further exaggerated as there is an imbalance between the amino acid composition of skeletal muscle and acute phase proteins (Reeds et al. 1994). Secondly, the acute phase response may induce weight loss through effects of the positive acute phase proteins as mediators of inflammation. CRP promotes monocyte extravasation and activation both by increasing the expression of ICAM-1, VCAM and E-selectin by endothelial cells and by a direct effect (Pasceri et al. 2000, Mortensen et al. 1976). In doing so, it increases the production of IL-1, TNF and IL-6, all of which are mediators of cachexia (see below) (Ballou and Lozanski 1992). Thirdly, the acute phase protein response may promote tumour growth and progression. It has been suggested that the altered energy substrate metabolism associated with the acute phase response may result in an increase in available nutrients for tumour cells (Eden et al. 1984, Holroyde et al. 1975). ICAM-1 expression by endothelial cells has also been shown to increase tumour cell adherence, increasing the chance of successful metastasis (Burrows et al. 1991). Increased tumour burden is associated with an increased cachectic response (Falconer et al. 1995, Carmichael et al. 1980), which could potentially be mediated via all of the mechanisms described above.

## **Antitumour therapy**

Many anticancer therapies have the unfortunate side-effect of contributing to cachexia. Surgery, particularly on the gastrointestinal tract, may decrease oral intake in the short term, stimulates the metabolic response to injury and has the potential to result in complications with long term effects on gastrointestinal function, for example adhesions or decreased bowel length resulting in the short bowel syndrome. Chemotherapy also has well recognised anorexigenic and emetic effects and affects the cells of the gastrointestinal tract resulting in the development of diarrhoea and food intolerance (Osoba et al. 1994, Parnes et al. 1994). Radiotherapy has also been demonstrated to cause weight loss (Munshi et al. 2003). These therapies can therefore be seen to induce some of the mechanisms of cachexia described above. The induction of common mediators may account for this effect.

## **Mediators of cachexia**

In seeking to explain how the mechanisms of cachexia are induced several theories have been developed. Initially, it was suggested that a tumour constitutes a new, metabolically active organ and could therefore induce cachexia through its metabolic demands. However, in contrast with animal tumours, human tumours are proportionally small and the presence and severity of cachexia often correlates poorly with their size. In addition, it cannot easily explain the complex effects on appetite and substrate metabolism. It was therefore proposed that circulating mediators may be partly responsible for cachexia. This theory was supported by parabiotic studies in rats in which cachexia is induced in both the tumour-bearing animal and the non-tumour bearing animal sharing its circulation. A wide variety of

factors with an influence on the mechanisms of cachexia have subsequently been identified.

## **Cytokines**

Several pro-inflammatory cytokines, including tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin-1 (IL-1), interleukin-6 (IL-6), interferon  $\gamma$  (IFN $\gamma$ ) and ciliary neurotrophic factor (CNTF) have been implicated in the development of cachexia. These molecules show variation in the tumours they have been identified in, their *in vivo* and *in vitro* mechanisms of action and in whether they are capable of inducing cachexia on inoculation into experimental animals or require the presence of tumour cells.

TNF $\alpha$  was the first cytokine identified as having a pro-cachectic effect and in this capacity was originally termed 'cachectin' (Beutler et al. 1985a, Beutler et al. 1985b). Administration of TNF $\alpha$  leads to weight loss, anorexia, increased levels of glucagon and cortisol, decreased levels of insulin and insulin resistance, protein and fat breakdown and an acute phase protein response in animals and humans (Tracey et al. 1988, Mahony and Tisdale 1988, Moldawer et al. 1988, Charters and Grimble 1989, Goodman 1991, Llovera et al. 1993, Llovera et al. 1997 (all animal refs), Warren et al. 1987, Selby et al. 1987, Starnes, Jr. et al. 1988, Michie et al. 1988 (human refs)). However, on repeated administration tolerance develops and food intake and body weight return to normal (Socher et al. 1988a). It is also rare to detect circulating TNF $\alpha$  in weight-losing patients with cancer (Socher et al. 1988b) and while anti-TNF $\alpha$  antibodies have been shown to increase food intake in a rodent model of cachexia (Gelin et al. 1991, Smith and Kluger 1993) they do not abrogate weight loss. These findings suggest that TNF $\alpha$  has a more subtle effect than first

thought. Circulating TNF $\alpha$  levels have been shown to exhibit circadian changes, with increased levels being found in cancer patients at 3am (Muc-Wierzgon et al. 1996). Soluble TNF $\alpha$  receptors may also influence the response to TNF $\alpha$ , which stimulates soluble TNF $\alpha$  receptor release masking its own presence and resulting in a correlation between circulating TNF $\alpha$  receptors and disease stage (Aderka et al. 1991). The effects of TNF $\alpha$  may similarly be affected by the co-secretion of other mediators, as transfection of TNF $\alpha$  into Chinese hamster ovary (CHO) cells results in the ability to induce cachexia on their implantation into nude mice (Oliff et al. 1987) yet TNF $\alpha$  is incapable of inducing protein breakdown on *in vitro* incubation with rat skeletal muscle (Goodman 1991).

Similar characteristics to those of TNF $\alpha$  have been attributed to IFN $\gamma$ , which also has pro-cachectic effects which may be blocked by anti-IFN $\gamma$  antibodies and which may be induced by transfection into CHO cells and yet are not observed on administration of IFN $\gamma$  alone (Matthys et al. 1991).

A more direct cachectic effect may be exerted by IL-6. Elevated levels of circulating IL-6 have been shown to be associated with weight loss and an acute phase protein response in pancreatic, lymphoma, lung and colorectal cancer patients (Fearon et al. 1991, Kurzrock et al. 1993, Scott et al. 1996, Wigmore et al. 1997a). Administration of IL-6 induces weight loss, increased energy expenditure and increased cortisol and glucagon concentrations in humans (Stouthard et al. 1996). Furthermore, treatment with anti-IL6 antibodies has been shown to suppress cachexia induced by a murine colonic adenocarcinoma (Strassmann et al. 1992). This may potentially be mediated through the acute phase protein response, which IL-6 has been demonstrated to induce (Morrone et al. 1988, Castell et al. 1990). However,

different tumour cell clones producing similar amounts of IL-6 can produce widely varying degrees of weight loss (Soda et al. 1995), suggesting that IL-6 acts in conjunction with other factors. Certainly, IL-6 is not able to induce proteolysis of rat skeletal muscle directly (Garcia-Martinez et al. 1994). However, transfection studies have demonstrated that tumour cells overexpressing IL-6 induce weight loss without increasing IL-1 or TNF levels, suggesting that these are not the mediators involved (Ohe et al. 1993). Indeed, the pro-cachectic effects of IL-1 may result from its stimulation of IL-6 secretion (Okusawa et al. 1988, Moldawer et al. 1988, Hellerstein et al. 1989, Ballmer et al. 1991, Yasumoto et al. 1995) and anti-IL-1 antibodies are capable of reducing both IL-6 production and weight loss in tumour bearing animals (Gelin et al. 1991, Oldenburg et al. 1993). IL-6 production and the acute phase response are also attenuated by anti-IL-1 antibodies in an inflammatory model (Gershenwald et al. 1990).

It is therefore apparent that cytokines work in combination and may require the presence of tumour cells to induce cachexia. Other molecules which are produced by or in response to tumour cells may also act as cofactors in the development of cachexia.

## **Neurotransmitters**

In common with other physiological processes, the regulation of food intake is influenced by conscious, subconscious and autonomic input from the central nervous system. Autonomic control is mediated by the hypothalamus, in which neuropeptide Y functions as an appetite stimulating neurotransmitter and serotonin and corticotrophin-releasing factor (CRF) function as appetite suppressants (Kalra et al. 1991, Heinrichs et al. 1993). In cancer patients, increased levels of tryptophan, the

precursor of serotonin, have been found and correlate with reduced food intake (Cangiano et al. 1994). Dysregulation of hypothalamic appetite control in cancer is also suggested by findings in tumour-bearing rats, in which decreased neuropeptide Y and increased CRF levels have been found (Inui 1999). Receptor downregulation may also play a role, as in a similar model, hypothalamic injection of neuropeptide Y failed to stimulate the appetite of tumour-bearing rats (Chance et al. 1996). In addition, as with the effects of cytokines neuropetidergic appetite control may be influenced by the levels of other mediators, in particular the hormone leptin.

### **Hormones**

Dysregulation of secretion of the stress hormones adrenaline, glucagon, hydrocortisone and cortisol may contribute to cancer cachexia. Administration of these hormones produces features of cachexia including glucose intolerance, protein loss, an acute phase protein response and energy expenditure (Bessey et al. 1984, Watters et al. 1986). In humans with cancer associated weight loss increases in cortisol and glucagon levels have been demonstrated (Schaur et al. 1979, Burt et al. 1983, Knapp et al. 1991). In tumour-bearing animals, large differences in hormone levels may also be observed, although the pattern of response appears to vary depending on which cell line is implanted (Besedovsky et al. 1985). Cortisol and glucagon may also modify the acute phase response (Baumann and Gauldie 1994). The latter two characteristics echo those described above for IFN $\gamma$  and IL-6, which may also influence cortisol levels (Spath-Schwalbe et al. 1989, Steensberg et al. 2003).

Anabolic hormones may also be dysregulated in cancer cachexia. The secretion of insulin in response to a rise in glucose has been shown to be decreased in

a group of patients with colorectal cancer (Holroyde et al. 1984). In pancreatic cancer insulin resistance has also been described, and appears to be due to an influence of the tumour on normal pancreatic tissue production of islet amyloid polypeptide (amylin) which reduces insulin sensitivity and glycogen synthesis (Schwartz et al. 1978, Gullo et al. 1993, Permert et al. 1994, Ding et al. 1998). In itself, amylin is also capable of inducing anorexia and weight loss in rats (Arnelo et al. 1996). Insulin also affects the acute phase protein response, stimulating albumin production and decreasing fibrinogen synthesis (De et al. 1993). However, it has been suggested that the anabolic effect of insulin on skeletal muscle is not affected in cancer (Newman et al. 1991) and it is increasingly recognised that it is changes in the cortisol:insulin ratio which may result in the catabolism of peripheral tissues (Fearon et al. 1998).

Other hormones may also play a role in cancer cachexia. For example, leptin is produced by fat, suppresses appetite and increases energy expenditure (Zhang et al. 1994). Leptin levels are elevated in some models of inflammation and may be influenced by IL-1 $\beta$  and TNF $\alpha$  (Grunfeld et al. 1996, Sarraf et al. 1997, Zumbach et al. 1997, Faggioni et al. 1998). However, studies have demonstrated that leptin levels are altered in only some patients with cancer cachexia and it has been suggested that levels are not abnormal in weight-losing patients with pancreatic cancer (Simons et al. 1997, Brown et al. 2001, Dulger et al. 2004, Tessitore et al. 2004). This evidence suggests that hormones such as this could potentially act as co-factors in the development of cachexia. Like cytokines, hormones do not appear to act alone in this process.

## **Prostaglandins**

Prostaglandins may be involved in cachexia both as humoral mediators and in signal transduction (Strelkov et al. 1989, Tessitore et al. 1993, Smith and Tisdale 2003). Circulating levels of prostaglandin E<sub>2</sub> and have been found to be elevated in a rat model of cachexia and rat hepatoma cells have been demonstrated to induce muscle protein loss by the production of this prostaglandin (Strelkov et al. 1989, Tessitore et al. 1993). In signal transduction, prostaglandin generation appears to be a major step in the initiation of skeletal muscle proteolysis (Thompson and Palmer 1998). Prostaglandins are also involved in the mediation of the effects of some pro-cachectic cytokines such as TNF $\alpha$  and IL-1 (Okusawa et al. 1988, Hellerstein et al. 1989, Uehara et al. 1989, Rothwell 1992, Kozak et al. 1997). Inhibitors of prostaglandin synthesis are capable of preventing the catabolic effect of serum from mice with cancer cachexia (Smith and Tisdale 1993) and muscle proteolysis in tumour-bearing rats (Strelkov et al. 1989).

## **Tumour-derived mediators**

With the establishment of animal models of cancer cachexia it became apparent that the serum of tumour-bearing animals was itself capable of inducing cachexia in normal animals. The subsequent screening of sera for fractions with cachectic activity lead to the isolation of cachexia-associated proteins which were previously unidentified and which were therefore labelled as tumour-specific cachectic mediators. These include lipid mobilizing factor (LMF) and proteolysis inducing factor (PIF). Lipid mobilising factor was originally described in a murine tumour model and has subsequently been found in the urine of weight-losing cancer patients (Hirai et al. 1997, Hirai et al. 1998). It is capable of inducing triglyceride

hydrolysis in adipose tissue through a cyclic AMP-mediated process by interaction with a  $\beta$ 3-adrenoreceptor (Sanders and Tisdale 2004, Russell et al. 2004). Recently however, production of LMF has also been shown to take place in normal white adipose tissue and to be upregulated by the presence of cachexia-inducing tumours in mice (Bing et al. 2004). Furthermore, sequencing of LMF showed it to be identical to zinc  $\alpha$ 2 glycoprotein (ZAG). Interestingly, ZAG is known to have a role as an acute phase protein.

As noted above, proteolysis inducing factor was also originally isolated from a murine cachexia-inducing tumour and was subsequently identified in the urines of weight losing patients with cancer but not weight losing patients with other pathologies (Todorov et al. 1996a, Todorov et al. 1996b). Its production may subsequently be a cause of the cachectic process, consistent with its induction of muscle proteolysis and alterations in hepatic gene expression (Todorov et al. 1997, Watchorn et al. 2001), a result of positive effects on tumour cell growth and survival or the result of induction by another mediator of cachexia.

## Summary and aims

Proteolysis inducing factor is a unique molecule with defined *in vitro* metabolic functions which are relevant to cancer cachexia. Its structure, expression pattern and function as a growth and survival factor are less well characterised. A more complete understanding of these features has the potential to increase our understanding of tumour progression and survival and how these processes may be linked with the development of cachexia.

The work presented in this thesis seeks to explore -

1. The *in vitro* and *in vivo* expression patterns of proteolysis inducing factor.

Specifically, the expression of PIF mRNA by cell lines and pancreatic carcinoma tissue and the relationship of expression to nutritional status and survival (chapters 3, 7 and 8).

2. The role of proteolysis inducing factor as a growth and survival factor in tumour cells.

In particular, the effects of *in vitro* PIF expression and synthetic PIF peptides on cellular, immunological and molecular biological markers of proliferation and apoptosis (chapters 5 and 6).

3. The influence of the structural features of PIF known to be involved in the induction of cachexia and the importance of the PIF homologues Y-P30 and dermcidin in the role of PIF as a growth and survival factor.

In specific, the effect of removing potentially glycosylated amino acids and preventing the production of specific peptide products on the *in vitro* system used to assess the role of PIF as a growth and survival factor.

## **2. Materials and methods**

### **Cell culture**

#### **Cell lines**

The HuH7, Mia PaCa2, CF-PAC and Panc1 cell lines were obtained from the European Collection of Cell Cultures (ECACC, Porton Down, UK).

#### **Culture technique**

All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM-Gibco-BRL, Paisley, UK) supplemented with 10% fetal calf serum (FCS) (Gibco). Medium was supplemented with 50 units/ml penicillin, 50µg/ml streptomycin and 2mmol glutamine (all from Gibco). Cells were grown in 25cm<sup>2</sup> or 75cm<sup>2</sup> flasks (Corning BV, Schipol-Rijk, the Netherlands).

For passage or use in experiments, cells were harvested from flasks by removal of all medium and incubation with 1ml of 0.25% trypsin / 0.02% EDTA (Gibco) per cm<sup>2</sup> for 30 seconds. On detachment cells were re-suspended in 10ml of medium, transferred to a fresh universal and pelleted by centrifugation at 1200g for 5 minutes prior to resuspension in 1ml of medium.

Viable cells were counted using the trypan blue exclusion method. 20µl cell suspension was added to 20µl trypan blue and 20µl of the resultant mix used to fill both sides of a multiwell counting chamber. Cells were then counted under the light microscope.

For standard passage cells were seeded at a concentration of  $1.33 \times 10^5$ /ml in 15ml medium (75cm<sup>2</sup> flasks) or 5ml medium (25cm<sup>2</sup> flasks). For use in experiments cells were seeded at the concentrations described in the individual chapters below.

## **Transfection**

The HuH7 cell line was transfected with PIF and PIF mutants in the mammalian expression vector pcDNA3.1+ using FuGENE-6 (Roche applied science, Lewes, UK). Prior to transfection the optimum concentration of the selective antibiotic, Geneticin (Invitrogen, Paisley, UK) was determined. This was performed by seeding  $1 \times 10^5$  cells per well in 6 well plates and incubating overnight prior to the addition of 0, 200, 400, 600, 800 or 1000µg/ml Geneticin. Following approximately 1 week in culture, wells containing 600µg/ml or more showed 100% cell death. Wells containing 400µg/ml or less contained some surviving cells. The optimum concentration was taken as the minimum required to kill 100% of cells, ie. 600µg/ml.

For transfection experiments,  $1 \times 10^5$  cells per well were seeded in 6 well plates and incubated overnight. Transfection complexes were then prepared in 0.5ml microcentrifuge tubes by the dilution of 3µl FuGENE-6 in 100µl serum-free DMEM and the addition of 2µg plasmid DNA. The complexes were mixed gently and incubated for 45 minutes at room temperature. They were then added dropwise to the cells seeded in 6 well plates. Cells were incubated overnight at 37°C prior to the addition of 600µg/ml Geneticin. From this point all culture media was supplemented with 600µg/ml Geneticin. Following 1 week in culture all wells contained only a few surviving cells. At 2 weeks these had begun to expand into colonies. Cells were then split into 25cm<sup>2</sup> flasks and grown until ~90% confluent. Standard culture and passage techniques were then used.

## **Liquid nitrogen storage**

For long term storage, aliquots of cells were stored in liquid nitrogen. Following washing and counting,  $\sim 5 \times 10^6$  cells were pelleted and resuspended in sterile dimethylsulfoxide (Sigma) plus 10% FCS. Samples were labelled and frozen overnight at  $-70^\circ\text{C}$  prior to transfer to liquid nitrogen. To re-establish culture, samples were thawed under running warm water then transferred into a universal using a pastette. 10ml of DMEM plus 10%FCS was then added slowly, trickling the medium down the side of the universal. A further 10ml medium was added rapidly. Cells were pelleted and all medium removed prior to resuspension in 5ml medium and seeding in a  $25\text{cm}^2$  flask.

## **Induction of oxidative stress**

In selected experiments HuH7 cells were subjected to oxidative stress by use of the enzyme glucose oxidase (Sigma, Dorset, UK). Cells were seeded at the desired concentration and allowed to settle overnight. Glucose oxidase was then added to the culture medium directly. Initial experiments were performed to optimize the concentration and duration of exposure. These suggested that a 2 hour exposure to 75mU/ml glucose oxidase was sufficient to kill approximately 50% of cells. This level of death facilitates the investigation of treatments which either increase or decrease cell death and this exposure was therefore adopted as the standard treatment. Following treatment culture medium was removed and cells were recovered for 4 hour in normal medium prior to analysis.

## **Synthetic PIF peptide treatment**

In selected experiments cell were treated with a synthetic PIF peptide (Albachem) custom synthesised to contain the 30 amino acids of the PIF core. The peptide had the sequence YDPEAASAPGSGNPCHEASAAQKENAGEDP and was of greater than 95% purity. Stock peptide was made up at a concentration of 20mg/ml in PBS. For use in experiments peptide was diluted to the desired concentration in PBS.

## **RNA Techniques**

### **RNA preparation**

#### **Trizol method**

RNA from cultured cells was prepared according to the Trizol method (Life Technologies, Paisley, UK). Certified RNase-free pipette tips and microcentrifuge tubes were used at all times. Culture medium was aspirated from adherent cells (HuH7, Mia PaCa2, CF-PAC and Panc1 cell lines) and cells lysed directly by the addition of 1ml Trizol reagent per cm<sup>2</sup> of culture flask area and lifting with a cell scraper. 1.25ml aliquots of each sample were then transferred to 1.5ml microcentrifuge tubes and incubated for 5 minutes at 21°C. Phase separation was then carried out by addition of 0.2ml chloroform per 1ml Trizol (ie. 0.25ml per 1.25ml aliquot of sample), vigorous shaking for 15 seconds to mix and centrifugation at 12000g for 15 minutes at 4°C. The aqueous phase was transferred to a fresh microcentrifuge tube. RNA was then precipitated by incubation of supernatants for

10 minutes at 21°C with 0.5ml isopropanol per 1ml Trizol used followed by centrifugation at 12000g for 10 minutes at 4°C. Supernatant was removed from the resultant RNA pellets which were then washed in 1ml of 75% ethanol (Sigma) per 1ml Trizol used by brief vortexing and centrifugation at 7500G for 5 minutes at 4°C. The ethanol was removed and the RNA pellet dissolved in 50µl of RNase-free water (Promega). Samples which had been aliquoted into separate microcentrifuge tubes following Trizol treatment were reunited by sequential suspension in the same 50µl of water. RNA was dissolved by incubation for 10 minutes at 55°C.

### **Paraffin block RNA isolation protocol**

RNA was prepared from laser-capture microdissection (LCMD) samples with a Paraffin-block RNA isolation kit (Ambion, Huntingdon, UK) using a modification of the manufacturer's protocol. Laser capture microdissection caps with adherent cells were placed on 0.5ml RNase-free microcentrifuge tubes containing 100µl proteinase K digestion buffer and 5µl proteinase K. The tubes were then inverted and incubated in a hybridisation oven for 90 minutes at 45°C with regular vortexing. Samples were then transferred to 1.5ml RNase-free microcentrifuge tubes and 600µl RNA extraction buffer was added to each. Tubes were then vortexed 5 times for 5 seconds each time and incubated at room temperature for 5 minutes. 700µl acid phenol:chloroform was added to each tube and samples were again vortexed 5 times for 5 seconds each time. Tubes were then spun at 13 000rpm for 5 minutes at room temperature and the aqueous upper phase transferred to a fresh microcentrifuge tube. Glycogen was then added to a final concentration of 250µg/ml and RNA was precipitated with 1 volume of isopropanol. Samples were mixed thoroughly and

incubated at  $-20^{\circ}\text{C}$  for at least 30 minutes (usually overnight) then spun at 13 000rpm for 15 minutes at  $4^{\circ}\text{C}$ . Supernatant was carefully decanted from the resultant RNA / glycogen pellet which was then washed by the addition of  $500\mu\text{l}$  75% ethanol, vortexing briefly and spinning at 13 000rpm for 5 minutes at  $4^{\circ}\text{C}$ . Ethanol was then carefully pipetted off and pellets air dried then resuspended in  $10\mu\text{l}$  nuclease-free water (Promega). RNA samples not immediately used in experiments were stored at  $-70^{\circ}\text{C}$ .

### **RNEasy RNA preparation protocol**

RNA was prepared from frozen tissue samples using rotor-stator homogenisation and an RNEasy Mini kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Tissue samples of approximately  $3\text{mm}^3$  (30-35mg) were cut from frozen specimens of frozen normal and malignant pancreatic tissue which had been mounted in OCT (see below). Samples were immediately placed in  $600\mu\text{l}$  buffer RLT in 5ml bijoux and homogenised with a rotor-stator for 30 seconds at full speed or until completely homogenised. Samples were then transferred to RNase-free 1.5ml microcentrifuge tubes and centrifuged for 3 minutes at 13 000rpm. Supernatants were transferred into fresh 1.5ml microcentrifuge tubes and 1 volume ( $600\mu\text{l}$ ) of 70% ethanol was added and mixed by pipetting.  $700\mu\text{l}$  of sample was then applied to an RNEasy mini column placed in a 2ml collection tube and centrifuged for 15 seconds at 8 000g. Flow-through was discarded. RNA was then subjected to either on-column DNase digestion (see below) or an additional wash by the application of  $700\mu\text{l}$  buffer RW1 and centrifugation for 15 seconds at 8 000g. Flow through was discarded and the columns transferred to new collection tubes.  $500\mu\text{l}$

buffer RPE was then added, columns centrifuged for 15 seconds at 8 000g and flow through again discarded. This step was repeated once and tubes were then spun again for 1 minute at 13 000rpm to eliminate any possible buffer RPE carryover. Columns were then transferred to fresh RNase-free 1.5ml microcentrifuge tubes and RNA eluted by the application of 30µl RNase-free water and centrifugation for 1 minute at 8 000g. To increase RNA concentration, the eluate was re-applied to the column and the final centrifugation step repeated.

## **DNase I treatment**

### **Treatment of RNA in solution**

RNA in RNase-free water prepared using the Trizol method or the paraffin block RNA isolation kit was DNase I treated using RQ1 DNase (Promega) according to the manufacturer's instructions. 10µl reactions were set up containing 8µl RNA, 1µl RQ1 DNase buffer and 1µl RQ1 DNase. These samples were then incubated for 30 minutes at 37°C. 1µl stop solution was then added and samples incubated for 10 minutes at 65°C.

### **On-column digestion of RNEasy-prepared RNA**

RNA prepared using the RNEasy Mini kit was DNase I treated using an on-column RNase-free DNase set (Qiagen) according to the manufacturer's instructions. Following application of RNA samples to RNEasy mini columns (see above), 350µl buffer RW1 was pipetted onto the columns and samples centrifuged

for 15 seconds at 8 000g. Flow through was discarded. 10 $\mu$ l of DNase I stock solution was then diluted in 70 $\mu$ l buffer RDD and applied to each column for 15 minutes at room temperature. 350 $\mu$ l buffer RW1 was then added and columns were centrifuged for 15 seconds at 8 000g. Flow through was discarded and the RNEasy RNA preparation protocol was continued as described above.

## **Assessment of RNA quality**

### **Spectrophotometry**

The concentration of RNA samples was assessed by spectrophotometry using a Thermo spectronic Biomate 5 spectrophotometer (Thermo electron corporation, West Palm Beach, FL, USA) and Vision 32 V1.25 analysis software. 1 $\mu$ l of sample was diluted 1 in 50 in distilled water. 50 $\mu$ l of distilled water only was used to blank the spectrophotometer. RNA concentration was calculated from the ratio of absorbance at 260nm to absorbance at 280nm.

### **Agilent capillary electrophoresis**

For use in real-time PCR experiments, RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent technologies, South Queensferry, UK) with either an RNA 6000 Nano or Pico LabChip Kits according to the manufacturer's instructions. All reagents were stored at 4°C and equilibrated to room temperature for 30 minutes prior to use. For Nano chips, gel was first prepared by centrifugation of 550 $\mu$ l RNA 6000 Nano gel matrix in a spin filter for 10 minutes at 1 500g. 65 $\mu$ l aliquots of gel were then placed in 0.5ml microcentrifuge tubes and used within 4

weeks. The gel-dye mix was prepared by vortexing the Nano dye concentrate for 10 seconds, centrifuging briefly and then adding 1 $\mu$ l of dye concentrate to a 65 $\mu$ l aliquot of gel. This mix was then vortexed, centrifuged at 13 000g for 10 minutes at room temperature and pipetted as indicated into a new RNA chip held in the chip priming station. The station was then closed and the plunger depressed until it was held by the clip. After 30 seconds, the clip was removed and a further 9 $\mu$ l of gel-dye mix pipetted into the indicated wells. 5 $\mu$ l of RNA 6000 marker was then added to the ladder well and all sample wells. 1 $\mu$ l of RNA 6000 ladder was added to the ladder well. 1 $\mu$ l of sample was then added to each sample well and the chip was vortexed for 1 minute at 2400rpm prior to analysis on the bioanalyzer.

For Pico chips, the gel matrix and gel-dye mix were prepared using RNA 6000 Pico gel matrix and dye concentrate and loaded into the RNA 6000 Pico chip as described above for Nano chips. 9 $\mu$ l of RNA 6000 Pico conditioning solution was then pipetted into the well marked CS and 5 $\mu$ l of RNA 6000 Pico marker pipetted into all sample wells and the ladder well. 1 $\mu$ l of ladder solution was then added to the ladder well and 1 $\mu$ l of sample added to each sample well. The chip was then vortexed for 1 minute at 2400rpm and analyzed on the bioanalyzer

### **Exclusion of genomic DNA contamination**

Following DNase I treatment, samples were screened for remaining genomic DNA by PCR for the housekeeping gene, cytochrome B. Standard 25 $\mu$ l reactions containing 1 $\mu$ l DNase-treated RNA were made up as described below. All samples were cycled as described for 35 cycles with an annealing temperature of 56°C. Positive and negative controls of purified genomic DNA and nuclease-free water

were used in every case. Samples were run on 1.4% agarose gels, stained with ethidium bromide and visualized under UV illumination.

### **Reverse transcription**

Reverse transcription was performed using AMV reverse transcriptase (Promega) according to the manufacturer's instructions. A reaction mixture sufficient to provide 20.5µl per RNA sample was prepared. This consisted of 8µl 25mM MgCl<sub>2</sub>, 4µl reverse transcription 10x buffer, 4µl dNTP mixture, 2µl oligo(dT)<sub>15</sub> primer, 1.5µl AMV reverse transcriptase (high concentration) and 1µl recombinant RNasin ribonuclease inhibitor. 20µl of this mix was then added to 20µl of sample RNA and mixed by centrifugation. Samples were then incubated in a thermal cycler (PHC-3, Techne) for 1 hour at 42°C followed by 5 minutes at 99°C.

## **Polymerase chain reaction**

### **Reaction constituents**

Polymerase chain reaction (PCR) was performed using a modification of the Promega PCR amplification protocol. A reaction mixture was prepared containing 0.75µl 25mM MgCl<sub>2</sub>, 2.5µl taq poly 10x buffer and 2.5µl dNTPs (all from Promega) per sample. This mixture was then aliquoted into thin-walled 0.75ml PCR microcentrifuge tubes at a volume of 5.75µl per tube. 2.5µl of both forward and reverse primer solutions at a concentration of 10mM were then added to each tube followed by 1µl of target cDNA. Positive and negative controls for the gene of

interest, consisting of purified cDNA or plasmid and nuclease-free water respectively, were used in every case. Finally, 1 $\mu$ l of taq polymerase diluted 1 in 5 in nuclease-free water was added to each tube and the total volume made up to 25 $\mu$ l with nuclease-free water. Samples were mixed by centrifugation at 4000rpm for 10 seconds.

### **Thermal cycling**

Samples were run on a thermal cycler (PHC-3, Techne). For most primers a standard program was used. This consisted of 94°C for 5 minutes, 72°C for 5 minutes then 35 cycles of 94°C for 20 seconds, 55°C for 20 seconds followed by 72°C for 30 seconds.

### **Agarose gel electrophoresis**

Following thermal cycling 15 $\mu$ l of each sample was run on an agarose gel. Gels were prepared by adding 1.4g of electrophoresis-grade agarose (Seakem agarose, Flowgen, Ashby de la Zouch, UK) in 100ml 1xTAE buffer and dissolving by microwaving for two 30 second bursts. Gels were cooled to below 65°C and 5 $\mu$ l ethidium bromide (Sigma) added prior to pouring into levelled gel reservoirs and the insertion of 10 or 20 well combs. One sample of 5 $\mu$ l of molecular weight marker was run on every gel. 1 $\mu$ l loading dye (Promega) was added to samples and markers and mixed by centrifugation at 13 000rpm for 30 seconds. Combs were removed and gels submersed in 1xTAE buffer. Samples were then pipetted into the wells of the gel and

run at 70V for 45 minutes, or until the dye front neared the end of the gel. Gels were removed from gel tanks and visualized under UV illumination.

### **Real-time PCR**

Real-time PCR was performed using an ABI Prism thermal cycler (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. DNase treated RNA samples were assessed for quality and reverse transcribed as described above. 25 $\mu$ l real-time PCR reactions were then set up containing 12.5 $\mu$ l master mix, 1.25 $\mu$ l anti-18S ribosomal RNA primer-probe mix, 7 $\mu$ l anti-PIF primer-probe mix (90 $\mu$ l forward primer, 90 $\mu$ l reverse primer, 300 $\mu$ l TAMRA probe and 1520 $\mu$ l nuclease-free water in 2ml)(table)(all from Applied Biosystems), 1.75 $\mu$ l nuclease-free water (Promega) and 2.5 $\mu$ l of sample cDNA. All samples were run in duplicate and positive (MiaPaCa2 cDNA) and negative (nuclease-free water) controls were run on each plate. Samples were then pipetted into the wells of a 96-well real-time PCR plate (Applied Biosystems) and the wells sealed with the supplied strips of caps. The plate was centrifuged at 500rpm for 5 minutes then inserted into the thermal cycler. VIC was selected as internal standard, FAM as unknown and sample volume as 25 $\mu$ l. Plates were then run on a standard 40 cycle program. Results were analysed by calculating the ratio of mean cycle count value for PIF positivity to the mean cycle count for 18S positivity for each sample and comparing this with the ratio calculated for the positive control. Samples requiring more than 23 cycles before threshold for VIC positivity was reached or more than 36 cycles before threshold for FAM positivity was reached were discarded.

## **Protein purification**

### **Preparation of cell lysates**

For preparation of protein lysates, cells were seeded in 25cm<sup>2</sup> flasks and grown to 90% confluency prior to protein preparation using radio-immunoprecipitation assay (RIPA) buffer (1xPBS, 1% Nonidet P-40 (BDH Chemicals, Poole, UK), 0.5% sodium deoxycholate and 0.1% SDS). One protease inhibitor tablet (Roche) was added to each 10ml of RIPA prior to use. Medium was removed and cells washed with sterile PBS. 500µl RIPA buffer was then added to each well and cells scraped thoroughly from the flask bottom. Lysates were then transferred to 1.5ml microcentrifuge tubes and passed several times through a 21G needle. Tubes were then centrifuged at 10 000g for 10 minutes at 4°C. The resultant protein supernatant was transferred to a fresh microcentrifuge tube for protein assay and pellets were discarded.

### **Protein assay**

Protein assays were performed according to the method of Lowry (1951) using a Dc protein quantification kit (Biorad). A/ reagent was first prepared by the addition of 5µl solution S to 3ml solution A and dissolving by incubation at 37°C. All samples were prepared and assayed in a 96-well plate. A standard curve was prepared by performing 4 serial 1 in 2 dilutions of 20µl of the 1.45mg/ml protein standard supplied in 20µl RIPA buffer. Samples were diluted 1 in 5 by the addition of 4µl sample to 16µl RIPA buffer. 5µl per well of standards and samples was then pipetted into the assay wells of the 96-well plate in triplicate. 25µl A/ and 200µl

reagent B were then added to each well and plates were shaken for 15 minutes at room temperature. Protein concentrations were determined from absorbance at 750 nm using a Dynex Technologies MRX-II plate reader and Assayzap interpretation software.

## **Western blotting**

### **NuPAGE gel electrophoresis and transfer**

In selected experiments protein samples were run on 10-well 4-12% pre-cast NuPAGE Bis-Tris gels using an XCell SureLock mini-cell and XCell II blot module (Invitrogen) according to the manufacturer's protocol. Gels were carefully removed from packaging and mounted in the XCell SureLock mini-cell. 1xMOPS buffer was then added to both the anode and cathode compartments making sure to cover the tops of sample wells. 20 $\mu$ g of each protein sample (approximately 10 $\mu$ l) was then diluted with sufficient 5x Laemmli sample buffer (LSB) to give a final concentration of 1x LSB (approximately 2.5 $\mu$ l). Samples were then incubated for 5 minutes at 95°C. The gel was then loaded with 5 $\mu$ l of Multimark molecular weight markers (Invitrogen) in the first lane and the full sample volumes in subsequent lanes. Gels were run at 100V for 1 hour or until the dye front neared the end of the gel.

Protein was transferred from NuPAGE gels to PVDF membrane (Invitrogen) by wet blotting in the XCell SureLock mini-cell. Gels were carefully removed from gel plates and wells trimmed off. Two double layers of Whatman paper and a sheet of PVDF membrane were then cut to size and soaked in 1x transfer buffer plus 20% methanol for 5 minutes at room temperature. Two transfer pads were also soaked. A sandwich was then constructed consisting of the cathode plate, a blotting pad, a

double layer of Whatman paper, the gel, the PVDF membrane, a double layer of Whatman paper, a further blotting pad and the anode plate. This sandwich was then inserted vertically into the gel tank, secured with the tension wedge and topped-up with transfer buffer plus 20% methanol. Electrophoretic transfer was then performed for 1 hour at 30V.

### **Polyacrylamide gel electrophoresis and transfer**

In selected experiments, protein samples were run on hand-cast polyacrylamide gels using a Protean II mini-cell and power pack (Biorad). 12.5% separating gels were prepared by mixing 3.12ml distilled water, 2.5ml 1.5M Tris (pH8.8), 100 $\mu$ l 10% SDS and 4.17ml acrylamide / Bis 30% stock (Biorad). Polymerisation was initiated by the addition of 10 $\mu$ l teramethylethylenediamine (TEMED) and 100 $\mu$ l 10% ammonium persulfate (AMPS) (both from Sigma) and the gel poured between two glass gel plates, one short and one long, held in the pouring rack, to approximately 1.5cm below the top of the short plate. The top of the gel was covered with isobutanol (Sigma) to obtain a level, meniscus-free surface and the gel was allowed to set. 4% loading gels were then prepared by mixing 5.98ml distilled water, 2.5ml 1.5M Tris (pH8.8), 100 $\mu$ l 10% SDS and 1.3ml acrylamide / Bis 30% stock (Biorad) and polymerizing with 20 $\mu$ l TEMED and 100 $\mu$ l 10% AMPS. Isobutanol was washed from the separating gel with distilled water and the loading gel poured on to the top of the short plate. A comb with the desired number of wells (usually 10) was then inserted and the loading gel was allowed to set.

To run, gels were placed in running racks in the gel tank and cathode and anode compartments filled with 1x running buffer (). 10 $\mu$ g of sample per well was

run on polyacrylamide gels. All samples were pre-incubated with LSB and loaded together with molecular weight markers as described above for NuPAGE gels. Gels were run at 100V for 1 hour or until the dye front neared the end of the gel.

Protein was transferred from polyacrylamide gels to nitrocellulose membrane by semi-dry blotting using an LKB Bromma 2117 Multiphor II electrophoresis unit. Gels were carefully removed from gel plates, the loading wells trimmed off and 4 sheets of blotting paper (Amersham) and one sheet of nitrocellulose (Biorad) cut to the size of the remaining gel. A sandwich was then constructed containing, from bottom up, a double layer of blotting paper soaked in transfer buffer (1x stock of 5.86g glycine, 11.625g Tris, 0.75g SDS and 400ml methanol made up to 2l with distilled water), the nitrocellulose, also soaked in transfer buffer, the gel and a further double layer of blotting paper soaked in transfer buffer. The cathode and anode plates of the blotter were wet with transfer buffer and the sandwich placed between them. Electrophoretic transfer was then performed for 1 hour at 100V.

### **Staining procedure**

Following transfer, both PVDF and nitrocellulose membranes were stained by the same method. Membranes were removed from transfer sandwiches, placed in a pyrex dish and stained with Ponceau red (Sigma) for 1 minute with rocking. They were then washed with 1xTBS to clarify lanes and molecular weight markers, which were marked with a pencil. Membranes were then blocked with 5% Marvel in TBS for 1 hour with rocking. Blocking solution was discarded and the primary antibody, diluted appropriately in 10ml blocking solution, was applied to the membranes for 1 hour at room temperature (or overnight at 4°C) with rocking. Membranes were then

washed 3 times, for 5 minutes each time, with 0.05% Tween 20 (Sigma) in 1xTBS.

The secondary antibody, diluted appropriately in blocking solution, was then applied for 1 hour at room temperature with rocking. Membranes were washed 3 times, twice as before in 0.05% Tween 20 in 1xTBS and once for 5 minutes in 1xTBS.

Western blots were visualized with an Enhanced Chemiluminescence (ECL) kit (Amersham Biosciences, Chalfont St Giles, UK) according to the manufacturer's instructions. Membranes were first applied to a piece of card wrapped in Saran wrap and taken to the dark room. 1ml each of the 2 ECL reagents was then mixed in a bijoux tube and applied to the membranes for 1 minute. This solution was then carefully blotted off and the mounting card was taped to a film cassette. A further piece of Saran wrap was used to cover the membranes and, under safe lighting, a piece of film was inserted into the cassette. Exposure was routinely performed for 15, 30, 60 and 300 seconds and film developed using an Amersham Hyperprocessor automated developer.

## **Immunoprecipitation**

Immunoprecipitation was performed using the mouse monoclonal anti-glycosylated PIF antibody and anti- pan-mouse Ig-labelled magnetic beads (Dynabeads – Dynal Biotech, Bromborough, UK) according to the manufacturer's instructions. 100 $\mu$ l of beads were first pipetted into a 1ml round-bottomed screw-cap tube and washed 3 times by applying 1ml of PBS plus cations (Sigma), inverting to mix and using a magnet to hold beads at the base of the tube while removing the PBS. 500 $\mu$ l of antibody and 500 $\mu$ l PBS plus cations was added and tubes were

incubated for 30 minutes at 4°C with rotation. Following a further 3 washes with PBS, 50µg protein was added and tubes were incubated for 2 hours at 4°C with rotation. Beads were then washed 5 times as before and transferred to a clean 0.5ml microcentrifuge tube. 30µl of 1xLSB was then added to each tube and antibody-antigen complexes were released by incubation at 95°C for 5 minutes. Samples were run on pre-cast NuPAGE tris-tricine gels as described above. Gels were then stained with Gelcode (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. First, gels were transferred to pyrex dishes and washed with deionized water 3 times for 15 minutes with rocking. Gels were then stained by incubation with 20ml Gelcode Blue Stain reagent for 1 hour with rocking. Gels were destained overnight with deionized water and visualized directly.

## **Plasmid techniques**

### **Plasmids**

The mammalian expression vectors pcDNA3.1+ and pcDNA3.1+V5-His (Invitrogen) were used. Both contain a multiple cloning site (MCS) under the control of a constitutive CMV promoter (figure 2.1). The pcDNA3.1+V5-His vector has an additional 3' V5-His tag. pcDNA3.1+ vector with PIF directionally cloned into the MCS using the EcoR-I and BamH-I restriction enzymes was created from pBluescript PIF (kind gift of Ian Waddell) by James A. Ross. This plasmid, termed pcDNA3.1+PIF, contained the full length PIF cDNA with a 5' GCCGCC Kozak vertebrate translation initiation sequence (Kozak 1987).

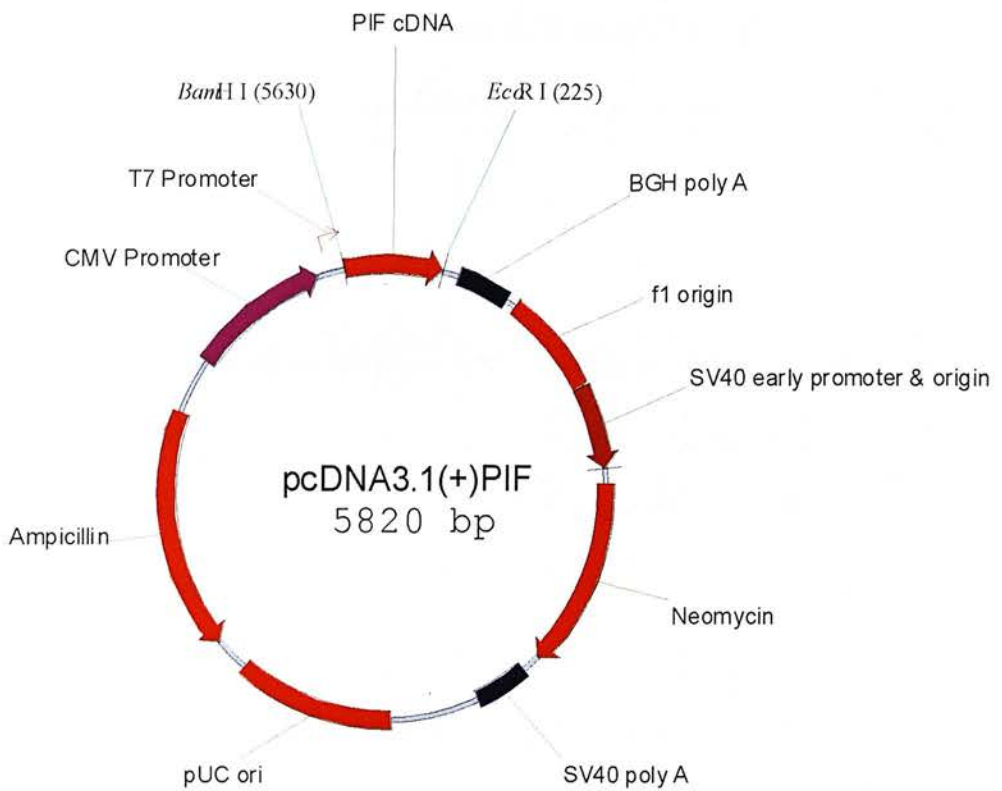


Figure 2.1. pcDNA3.1(+)-PIF showing the full-length PIF / dermcidin DNA cloned into the multiple cloning site.

### Transformation

For routine plasmid growth, vectors were transformed into ‘One shot’ TOP10 chemically competent *e. coli* (Invitrogen) using a modification of the manufacturer’s protocol. Cells were stored at  $-70^{\circ}\text{C}$  and defrosted on ice immediately prior to use.  $1\mu\text{g}$  purified plasmid (approximately  $1\mu\text{l}$ ) was then added to a minimum of  $16.67\mu\text{l}$  (1/3 vial) *e. coli* and mixed gently prior to incubation on ice for 5 minutes. Cells were then heat shocked for 30 seconds at  $42^{\circ}\text{C}$  in a water bath and returned immediately to ice.  $250\mu\text{l}$  of room temperature SOC medium was added and the samples shaken horizontally at 200rpm for 1 hour at  $37^{\circ}\text{C}$ .  $50\mu\text{l}$  of neat, 1 in 10 and 1 in 100

dilutions of each sample were then plated on Luria-Bertrani (LB)(Sigma) agar plates containing 50µg/ml ampicillin (Sigma) and incubated overnight at 37°C.

### **Analysis of colonies**

Selected colonies from LB plates were analysed by PCR. Standard PCR reactions containing a forward primer to the T7 promoter region and the 4730 PIF reverse primer were prepared. Each colony was then harvested with the stick end of a microbiological loop and inoculated into the PCR reaction. Colonies were simultaneously inoculated into 3ml LB mini-broths containing 50µg/ml ampicillin. PCR reactions were cycled and run on agarose gels as standard. Mini-broths were incubated overnight at 37°C with shaking at 220rpm. Mini-broths which showed growth and corresponded to PCR reactions that produced the correct size of band for the insert were selected for plasmid preparation and large-scale culture.

### **Plasmid mini-prep**

Plasmid DNA was prepared from 3ml *e.coli* broths using alkaline lysis. 1.5ml of broth was placed in a 1.5ml sterile microcentrifuge tube and centrifuged at 13 000rpm for 2 minutes at 4°C. Supernatant was removed and the pellet resuspended in 100µl Tris-EDTA (TE) buffer (pH 8, 10mmol Tris-HCl, 1mmol EDTA) (Sigma). 200µl of fresh NaOH / SDS solution (20µl 10M NaOH, 100µl 10% SDS and 880µl sterile water in 1ml), 150µl potassium acetate solution (60ml of 5M potassium acetate, 11.5ml glacial acetic acid and 28.5ml sterile water in 100ml) and 400µl phenol/chloroform were then added. Samples were vortexed well and spun at 13

000rpm for 5 minutes at 4°C. The upper phase was transferred into a new microcentrifuge tube. 200µl of isopropanol (Sigma) was added and mixed by inversion prior to spinning at 13 000rpm for 10 minutes at 4°C. Supernatant was removed and the pellet washed with 100µl cold 70% ethanol by mixing and centrifugation at 13 000rpm for 2 minutes. The ethanol was then removed and the pellet air-dried prior to redissolving in 20µl of TE buffer containing RNase (Gentra Systems, Flowgen, Ashby de la Zouch, UK)(1µl of 10mg/ml RNase per 100µl TE). Samples were finally dissolved by incubation at 37°C for 5 minutes. The molecular weight of purified plasmids was assessed by running 1µl of mini-prep on a standard agarose gel. The integrity of the insert sequence was determined by direct sequencing.

### **Large scale culture and maxi-prep**

For use in experiments plasmids were grown in 250ml LB broths and purified using an Endofree plasmid maxi kit (Qiagen) according to the manufacturer's instructions. 250ml broths were prepared by the dilution of 1ml mini-broth containing *e.coli* with the desired plasmid into 249ml of LB medium containing 50µg/ml ampicillin. These maxi-broths were cultured overnight at 37°C with shaking at 220rpm. Bacteria were then pelleted by centrifugation at 6000g in an ultracentrifuge fitted with a Sorvall GSA rotor for 15 minutes at 4°C. The supernatants were discarded and pellets lysed by resuspension in 10ml buffer P1. 10ml of buffer P2 was added and samples were mixed thoroughly by inverting 6 times then incubated for 5 minutes at room temperature. 10ml of buffer P3 was then

added to the lysate and samples mixed by inverting 6 times. The lysate was then poured into the barrel of a prepared Qiafilter cartridge and incubated at room temperature for 10 minutes. The plunger was inserted into the cartridge and samples were filtered into 50ml tubes. 2.5ml of buffer ER was added, mixed by inverting the tube 10 times and samples were incubated for 30 minutes on ice. During this incubation, a Qiagen-tip 500 was equilibrated by adding 10ml buffer QBT and allowing the tip to empty by gravity flow. The filtered lysate was then applied to the tip and allowed to flow through. The tip was then washed twice with 30ml of buffer QC and plasmid DNA eluted into a 40ml round-bottomed ultracentrifuge tube with 15ml buffer QN. DNA was then precipitated by adding 10.5ml isopropanol (Sigma), mixing and centrifuging at 15 000g for 30 minutes at 4°C. The supernatant was carefully decanted and the resultant DNA pellet washed by vortexing with 5ml 70% ethanol in diethylpyrocarbonate (DEPC) treated water and centrifuging at 15 000g for 10 minutes at 4°C. The ethanol was then removed and the pellet air-dried before redissolution in 500µl nuclease-free water (Promega).

DNA concentration was assessed by spectrophotometry. Quality was controlled by isopropanol precipitation and agarose gel electrophoresis of samples removed from the initial filtered lysate, the Qiagen-tip filtrate, the buffer QC washes and the DNA eluate.

### **Directional cloning**

For experiments to analyse the subcellular localisation of PIF the full-length cDNA was excised from pcDNA3.1+PIF and directionally cloned into pcDNA3.1+V5-His. First, 1µg of pcDNA3.1+PIF was subjected to restriction

digestion with EcoRI and BamHI (Promega). This was performed in a 20µl reaction containing 2µl 10x restriction enzyme reaction buffer appropriate to the enzymes used (Multicore buffer, Promega), 1µl EcoRI, 1µl BamHI, 1µg pcDNA3.1+PIF (approximately 1µl) and nuclease-free water to make up to 20µl. Reactions were then incubated overnight at 37°C and run on an agarose gel as described above.

Simultaneous double EcoRI / BamHI digestion of the empty pcDNA3.1+V5His vector was performed. In order to ensure the PIF insert was in frame with the V5His epitope on insertion, pcDNA3.1+V5His vector A was used. Both digest reactions were then run on an agarose gel and bands corresponding to the 418bp PIF excision fragment and the 5479bp pcDNA3.1+V5HisA digestion product excised under UV illumination using a clean scalpel, placed in 1.5ml microcentrifuge tubes and weighed.

DNA was extracted from agarose gel slices using a Wizard SV gel and PCR clean-up kit (Promega) according to the manufacturer's instructions. 10µl membrane binding solution per 10mg gel slice was added and samples vortexed and incubated at 50-65°C until the agarose was completely dissolved. An SV minicolumn was then inserted into a collection tube, the dissolved gel mixture applied and the column incubated for 1 minute at room temperature prior to centrifugation for 1 minute at 10 000g. Flow through was discarded and the column reinserted into the collection tube. 700µl membrane wash solution, to which 95% ethanol had been added as indicated, was then applied and columns centrifuged for 1 minute at 10 000g. Flow through was discarded and the column washed again by the addition of 500µl membrane wash solution and centrifugation for 5 minutes at 10 000g. Minicolumns were then placed in clean 1.5ml microcentrifuge tubes and 30µl nuclease-free water applied for 1

minute at room temperature. DNA was then eluted by centrifugation for 1 minute at 10 000g and quantified by spectrophotometry.

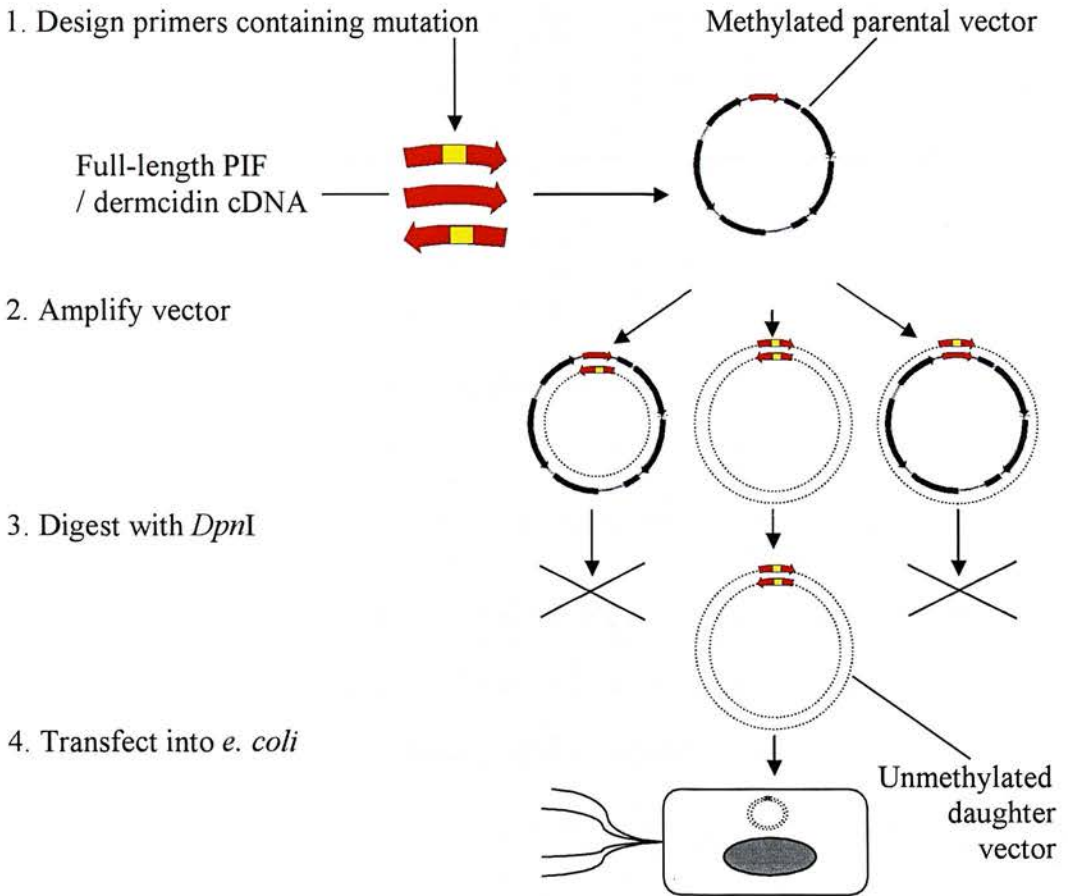
Ligation reactions were performed using T4 DNA ligase (Promega) according to the manufacturer's instructions. Three reactions containing 1:3, 1:1 and 3:1 molar ratios of vector to insert were used. Ligation mix was prepared using 1µl 10x ligation buffer, 0.2µl T4 DNA ligase and 4.8µl nuclease-free water per reaction. 2µl of vector DNA and 2µl of insert DNA, diluted to give the desired molar ratios using 10ng insert DNA, were then added to 6µl reaction mix to give a total reaction volume of 10µl. Samples were then incubated overnight at 15°C in a thermal cycler (Techne) and the full 10µl used to transform TOP10 *e.coli* as described above. Following mini-prep, the orientation and sequence of the PIF insert was confirmed using direct sequencing and plasmids grown and prepared for use in experiments by maxi-prep as described above.

### **Site-directed mutagenesis**

Site-directed mutagenesis was performed using a Quikchange II site-directed mutagenesis kit according to the manufacturer's instructions (figure 2.2) (Stratagene, Amsterdam, the Netherlands). Forward and reverse mutagenic primers containing the desired mutation in a central location were designed to meet the recommendations of the kit (25-45bp length,  $T_m \geq 78^\circ\text{C}$ , 10-15bp flanking mutation and GC content  $\geq 40\%$ ) and purchased from a commercial supplier (TAGN, Newcastle, UK).

Sample reactions containing 5µl 10x reaction buffer, 10ng pcDNA3.1+PIF plasmid template, 125ng forward primer, 125ng reverse primer, 1µl dNTP mix and

nuclease-free water to a final volume of 50µl were made up in 0.5ml thin-walled microcentrifuge tubes. 1µl *PfuUltra* DNA polymerase was then added. Samples were cycled in an Express thermal cycler (Thermo-Hybaidd, Middlesex, UK) for one cycle of 30 seconds at 95°C and sixteen cycles of 95°C for 30 seconds, 55°C for 1 minute and 68°C for 7 minutes. Samples were then cooled on ice for 2 minutes prior to digestion with 1µl *DpnI* at 37°C for 1 hour.



**Figure 2.2.** Site directed mutagenesis procedure.

Only the unmethylated, mutated daughter vector is saved from *DpnI* digestion and subsequently able to transform *e. coli*.

Samples were transformed into XL1-Blue supercompetent *e.coli*. 50µl aliquots of supercompetent cells were transferred into pre-chilled Falcon 2059 tubes (BD Biosciences, Oxford, UK). 4µl of sample was then added and the transformation reaction mixed gently and incubated for 30 minutes on ice. Reactions were then heat pulsed for 45 seconds at 42°C and placed on ice for 2 minutes. 0.5ml of NZY+ broth (Gibco), preheated to 42°C, was added and reactions were incubated at 37°C for 1 hour with shaking at 240rpm. 100µl cells were then plated on LB agar plates containing 50µg/ml ampicillin (Sigma) and incubated at 37°C for 16 hours. Colonies were analysed by PCR and inoculated into 3ml LB broths containing 50µg/ml ampicillin. Plasmids were prepared from broths grown from colonies containing the correct band on PCR by standard mini-prep technique and the sequence of the mutation was confirmed by direct sequencing. The plasmids containing the desired mutations were then used to transform TOP10 *e.coli*, grown in large scale culture and purified by maxi-prep and as described above.

### ***In vitro* translation**

*In vitro* translation was performed by coupled transcription and translation using T7 RNA polymerase and rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions. Radioisotopes used were <sup>35</sup>S methionine (10mCi/ml), <sup>14</sup>C leucine (0.5mCi/ml) and <sup>35</sup>S cysteine (10mCi/ml) (ICN Pharmaceuticals Ltd, Basingstoke, UK). Reagents were rapidly thawed from storage at -70°C by hand-warming and placed on ice. Reactions were then set up containing 12.5µl rabbit reticulocyte lysate, 0.5µl TNT buffer, 0.5µl RNAsin (Promega), 0.5µl T7 RNA polymerase, 2µl radiolabelled amino acid, 0.5µl amino acids minus the radiolabel

used, 1 µg plasmid DNA (approximately 1 µl) and nuclease-free water to 25 µl. In selected reactions 1.2 µl canine pancreatic microsomal membranes (CPMM) (Promega) were also used. Samples were incubated for 45 minutes at 30°C. 7.5 µl of sample was then removed, mixed with 7.5 µl 2x Laemmli sample buffer and incubated for 5 minutes at 95°C. Samples were spun at 13 000rpm for 2 minutes and supernatants removed from the precipitated haemin to fresh microcentrifuge tubes.

Samples were run on 10-well, pre-cast 10-20% tris-tricine gels (Biorad, Hemel Hempstead, UK) using Protean II mini-electrophoresis equipment (Biorad). Gels were carefully removed from packaging, inserted into the mounting rack and a reservoir for the anode buffer was created with a second gel (if required) or a buffer dam. Sufficient cathode buffer (100mM Tris / 100mM Tricine / 0.1% SDS) to cover the wells was then poured into the reservoir. The anode buffer (200mM Tris, pH 8.9) was poured into the exterior of the gel tank to cover the electrode. 5 µl of Multimark molecular weight markers (Invitrogen) were loaded into the first lane of each gel. 15 µl of sample per well was loaded. Gels were run at 70V for 2 hours or until the dye front neared the end of the gel. Gels were then removed from the gel tank and mounting plates and placed in a pyrex dish.

Gels were visualized by fluorography and autoradiography. All steps were performed at room temperature with rocking. Gels were stained for 15 minutes with Coomassie blue then destained with destain (40% methanol, 10% glacial acetic acid, 50% distilled water) for 30 minutes. Dehydration was then performed by 3, hour long incubations in dimethyl sulfoxide (DMSO). A fluor was added to gels by incubation for 1 hour 22% 2, 5-diphenyl oxazole (PPO)(Fisons, Manchester, UK) in DMSO. Gels were then rehydrated by incubation in distilled water for 3 hours with 3 changes

of water over this period. They were then removed from the pyrex dish and sandwiched between a double layer of blotting paper soaked in distilled water and Saran wrap prior to drying with heat on a vacuum drier for 1 hour. Sandwiches were then taken to the dark room, taped to a film cassette and a piece of GRI AX film (Genetic Research Instrumentation, Braintree, UK) applied. Cassettes were transferred to the  $-70^{\circ}\text{C}$  freezer. Exposure times varied from 16 hours to several weeks. Film was developed using an Amersham Hyperprocessor developing machine.

### **MTT assays**

For the assessment of cell survival by methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma) assay cells were plated in 96 well plates (Gibco) at  $1.5 \times 10^4$  cells per well and cultured overnight prior to treatment with glucose oxidase. Each sample was plated in sextuplicate. Following recovery,  $10\mu\text{l}$  of  $5\text{mg/ml}$  MTT was added to each well and plates were incubated for 4 hours at  $37^{\circ}\text{C}$ .  $100\mu\text{l}$  per well of 10%SDS, pH 3.0 was then added and plates were incubated overnight at  $37^{\circ}\text{C}$  prior to reading at 570nm on a Dynex Technologies MRX-II plate reader.

The coefficient of variation over the range of absorbances studied was  $<1.3\%$ .

### **Flow cytometry**

For use flow cytometry experiments HuH7 cells were seeded in 6 well plates at  $1 \times 10^6$  per well and allowed to settle overnight. Following glucose oxidase treatment cells and their culture medium were harvested using trypsin / EDTA then stained and analysed as described below.

## **Annexin V staining**

Annexin V / propidium iodide (PI) flow cytometry was performed using a Bender Medsystems kit according to the manufacturer's instructions (Bender Medsystems, Vienna, Austria). Following harvesting, cells were pelleted and washed twice in annexin binding buffer by resuspension and centrifugation for 5 minutes at 450g. Cells were then resuspended in annexin binding buffer at a concentration of  $2 \times 10^6$ /ml and split into 2 groups. The first group was not treated with annexin V and served as a negative control. 195 $\mu$ l aliquots of the second group were pipetted into flow cytometry tubes and 5 $\mu$ l annexin V-FITC was added. Tubes were vortexed and incubated for 10 minutes, at room temperature, in the dark. Cells were then washed and resuspended in 190 $\mu$ l annexin binding buffer. 10 $\mu$ l of propidium iodide solution was added to tubes from both groups of cells immediately prior to flow cytometric analysis.

The coefficient of variation for the assay over the range measured was 27%.

## **BOB78 staining**

Flow cytometry using the BOB78 antibody, which binds a surface marker of apoptotic cells, was performed as previously described (Hart et al. 2000). Following harvesting, cells were pelleted and washed twice in PBS by resuspension and centrifugation for 5 minutes at 450g. Cells were then resuspended in PBS at a concentration of  $1 \times 10^6$ /ml and split into two groups, one of  $2-5 \times 10^6$  and one of  $6-8 \times 10^6$ . These samples were then blocked by incubation with 20% normal rabbit

serum (Diagnostics Scotland, Carlisle, Scotland) in PBS for 20 minutes at room temperature. The first group of cells was treated with 5µl of negative control mouse IgM (DakoCytomation, Ely, UK) at 1 in 100 in PBS. The second group was treated with 5µl of BOB78 antibody at 1 in 100 in PBS. Both groups were incubated for 30 minutes at room temperature and washed by the addition of 1ml PBS/1% BSA and centrifugation for 5 minutes at 450g. Cells were then resuspended in 50µl PBS/1% BSA and 50µl of 1% sheep anti-mouse IgG FITC conjugate (Dako) in PBS was added to each tube. Cells were then incubated for 30 minutes at room temperature and washed as before in 1ml PBS/1% BSA. Negative control cells were analysed immediately. 5µl of propidium iodide was added to BOB78-treated cells before analysis.

The coefficient of variation for the assays over the range measured was 26%.

### **Anti-cleaved caspase 3 staining**

Cleaved caspase 3 flow cytometry was performed using an anti-cleaved caspase-3 (Asp175) (5A1) rabbit monoclonal antibody (Cell Signaling, Hitchin, UK) and control rabbit IgG antibody (Dako) according to the manufacturer's instructions. Following harvesting, cells were pelleted, resuspended in 700µl PBS and fixed by the addition of 300µl formaldehyde and incubation for 10 minutes at 37°C. Cells were then chilled on ice for one minute. Cells were permeabilized by the addition of 9ml ice-cold 100% methanol and vortexing prior to incubation for 30 minutes on ice.

For staining,  $1 \times 10^6$  cells per tube were aliquoted into assay tubes. 3ml PBS/1%BSA was then added to each tube and cells rinsed by centrifugation for 5 minutes at 450g. This step was repeated once then cells were blocked by resuspension in 100µl 20% normal goat serum (Dako) in PBS and incubation for 20

minutes at room temperature. 2µl of anti-cleaved caspase 3 antibody or control rabbit IgG antibody were then added and tubes were incubated for 30 minutes at room temperature. Cells were then rinsed, again by the addition of 3ml PBS/1%BSA and centrifugation for 5 minutes at 450g. Cells were then resuspended in 50µl PBS/1% BSA and 50µl of goat anti-rabbit IgG FITC conjugate (Dako) at 1 in 100 in PBS was added to each tube. Following a 30 minute incubation at room temperature and a further wash in 1ml PBS/1% BSA cells were analysed.

### **Flow cytometry analysis**

Samples were analysed using an Epics XL-MCL flow cytometer (Beckman Coulter Inc., Miami, USA) and System II acquisition software. Post-acquisition analysis was performed using Win-MDI 2.8 software (Scripps Research Institute, La Jolla, USA).

All cells were processed according to forward- and side-scatter. The coefficient of variation for assays over the range studied was 16%.

## **Immunocytochemistry**

### **Anti-V5-His immunocytochemistry**

For V5-His visualisation cells were treated with 5µM monensin (Sigma) and visualised using mouse monoclonal anti-V5 antibody (Invitrogen) and a rabbit anti-mouse FITC-labelled secondary (Dako). Cells were seeded in 8-well chambered slides at 10 000 cells per well and allowed to settle overnight. Monensin was then

added at a concentration of 5 $\mu$ M and cells incubated for 12 hours. Medium and chambers were then removed and the slides washed by immersion in PBS for 5 minutes. A greasepen was used to demarcate wells. Cells were then fixed by immersion in 3% paraformaldehyde in PBS for 15 minutes at room temperature. Following a further wash in PBS, permeabilization was performed by the addition of 50 $\mu$ l per well 0.2% Triton X-100 in PBS for 5 minutes. Slides were then washed and blocked with 50 $\mu$ l per well 20% normal rabbit serum (Dako) in PBS for 30 minutes at room temperature. 50 $\mu$ l per well of anti-V5 antibody at 1 in 200 in blocking buffer was then added and slides were incubated for 1 hour at room temperature. Following a further wash, cells were incubated with 50 $\mu$ l per well of rabbit anti-mouse FITC-labelled secondary at 1 in 100 in PBS for 30 minutes at room temperature then washed again. Cells were then stained with 50 $\mu$ l Hoescht 33342 (at 10 $\mu$ g/ml in distilled water) for 5 minutes at room temperature, washed, mounted in fluorescent mounting medium (Dako) and visualized by immunofluorescent microscopy.

## **Cytospins**

For assessment of cell death with annexin V, BOB78 and anti-cleaved caspase 3, cells were seeded at  $2 \times 10^4$  cells per well in 8-well chambered slides and allowed to settle overnight prior to glucose oxidase treatment. Dead cells were then harvested from the medium and cytospun onto positively charged slides at 300rpm for 3 minutes using a Shandon Cytospin 2 centrifuge.

### **Annexin V immunocytochemistry**

Annexin V staining was performed by incubation of cytopins for 5 minutes at room temperature in 50 $\mu$ l of annexin binding buffer containing 1.25 $\mu$ l annexin V and 1.25 $\mu$ l propidium iodide (Bender Medsystems). Slides were then washed by immersion in PBS for 5 minutes and nuclei stained by incubation with 50 $\mu$ l Hoescht 33342 (at 10 $\mu$ g/ml in distilled water) for 5 minutes at room temperature. Following a further wash in PBS, cytopins were washed, mounted in fluorescent mounting medium (Dako) and visualized by immunofluorescent microscopy.

### **BOB78 immunocytochemistry**

BOB78 staining of cytopins was performed by blocking for 1 hour at room temperature with 50 $\mu$ l of 20% normal rabbit serum (Dako) in PBS then staining for 1 hour at room temperature with BOB78 diluted 1/100 in PBS. Slides were then washed by immersion in PBS for 5 minutes and incubated at room temperature for 30 minutes with 50 $\mu$ l of secondary rabbit anti-mouse FITC conjugated immunoglobulins (Dako) diluted 1/100 in PBS. Following another wash in PBS, cells were fixed by the addition of 50 $\mu$ l 3% paraformaldehyde for 5 minutes then washed again. Cytopins were then stained with 50 $\mu$ l Hoescht 33342 (at 10 $\mu$ g/ml in distilled water) for 5 minutes at room temperature, washed, mounted in fluorescent mounting medium (Dako) and visualized by immunofluorescent microscopy.

### **Anti-cleaved caspase 3 immunocytochemistry**

Anti-cleaved caspase 3 immunocytochemistry was performed using an anti-cleaved caspase-3 (Asp175) (5A1) rabbit monoclonal antibody (Cell Signaling, Hitchin, UK) according to the manufacturer's instructions. Cytospins were first fixed by the addition of 50µl 3% paraformaldehyde for 10 minutes at room temperature then washed 3 times by immersion in tris-buffered saline (TBS) for 5 minutes. Permeabilization was then performed by the addition of 50µl 0.2% Triton X-100 (Sigma) for 5 minutes at room temperature. Slides were washed in TBS as before then blocked by incubation with 50µl 20% normal goat serum (Dako) in TBS for 1 hour at room temperature. 50µl of anti-cleaved caspase 3 antibody at 1 in 100 in 1%BSA in TBS was then added to cytopins and incubated overnight at 4°C. Slides were then washed in TBS as before and incubated with 50µl of secondary goat anti-rabbit IgG FITC conjugate (Dako) at 1 in 100 in 1%BSA in TBS. Following a further 3 washes in TBS, cytopins were stained with 50µl Hoescht 33342 (at 10µg/ml in distilled water) for 5 minutes at room temperature, washed again, mounted in fluorescent mounting medium (Dako) and visualized by immunofluorescent microscopy.

### **Immunofluorescent microscopy**

All slides were visualized with a Leica DMIRB fluorescent microscope (Leica, Wetzlar, Germany).

## **Bromodeoxyuridine immunocytochemistry**

### **BrDU labelling and immunostaining**

In experiments investigating cell proliferation, cells were labelled with Cell Proliferation Labelling Reagent, an aqueous solution of 5-bromo-2'-deoxyuridine and 5-fluoro-2'-deoxyuridine (BrDU) (Amersham) and visualized by immunocytochemistry using a monoclonal rat anti-BrDU antibody (Oxford Biotechnology, Kidlington, UK). Cells were seeded in 8-well chambered slides (Nunc, VWR International, Poole, UK) and cultured overnight under standard conditions. In experiments in which the synthetic PIF peptide was used, this was added at the desired concentration and cells cultured for a further 24 hours (see cell culture section above). BrDU was then added directly to culture medium at a dilution of 1 in 1000 and cells incubated for a further hour. Culture chambers and medium were then removed and slides were washed briefly by immersion in PBS prior to fixation in 80% ethanol overnight at 4°C. Slides were then washed in PBS for 10 minutes and incubated in 5M HCl for 45 minutes at room temperature. Two further 5 minute PBS washes were performed and the chambers on the slides were demarcated using a greasepen. Slides were blocked by incubation in 50µl per well of 20% normal goat serum (Dako) in PBS plus 0.05% Tween-20 for 10 minutes at room temperature. 50µl of primary antibody at a concentration of 1 in 100 in blocking solution was then added to each well and slides were incubated for 1 hour at room temperature. Slides were then washed in PBS 3 times, for 5 minutes each time. 50µl of goat anti-rat peroxidase-linked secondary antibody (Santa Cruz Biotechnology, Heidelberg, Germany) at a concentration of 1 in 300 in blocking solution was then

added to each well and slides were incubated for 30 minutes at room temperature prior to a further 3 washes in PBS.

### **DAB peroxidase staining and visualization**

Slides were stained using a liquid DAB+ substrate-chromogen system (Dako) and haematoxylin counterstaining and visualized by light microscopy. 1 drop of liquid chromogen solution was added to 1 ml of substrate solution and mixed. 50 $\mu$ l of this mixture was then added to each well and slides were placed on a piece of white paper and observed for the development of brown colour. Once this was seen, slides were placed in a rack and washed by immersion in distilled water.

Counterstaining with haematoxylin was then performed. A plastic box placed under the cold tap and continually replenished with running water was used for all wash steps. Slides were first immersed in haematoxylin for 1-2 minutes then dipped several times in the bath of running tap water. They were then dipped in 1% acid-alcohol several times, washed again and dipped in Scott's tap water for 1 minute to blue. Following a further rinse in tap water, slides were dehydrated by incubation for 5 minutes each in 50, 70, 80, 90 and 100% ethanol then 2 changes of xylene and mounted with a coverslip using DPX. Cell counts were performed using a light microscope and graticule to count of  $\geq 500$  total BrDU positive and negative cells per well.

The coefficient of variation for the assay over the range measured was 11% in experiments using transfected cells and 7% in experiments using untransfected cells.

# Patients

## **Ethical permission**

Full ethical permission from the Local Research and Ethics Committee (LREC) for Lothian was obtained prior to the recruitment of any patients.

## **Recruitment**

All patients were recruited from the hepatobiliary unit of the Royal Infirmary of Edinburgh. Inclusion criteria were suspected pancreatic neoplasia and age over 18. Exclusion criteria were pre-existing cachexia, active infection and non-pancreatic neoplasia. Informed consent was obtained 24 hours prior to surgery.

## **Data collection**

Data was collected by patient interview and case-note review. All data was recorded on a standardised proforma (appendix A). Nutritional data collected consisted of weight, serum albumin, serum uric acid and Karnofsky index (appendix B) (Karnofsky et al. 1948). Prognostic information consisted of patient age, co-morbidity, smoking, serum CRP and post-operatively, histology and staging.

## **Sample collection**

Samples of urine and normal and malignant pancreatic tissues were collected. Urine samples were collected pre-operatively by mid-stream urine collection and stored in sterile containers at -20°C until the time of analysis. Tissue samples of

approximately 1 cm<sup>3</sup> were harvested from surgical resection specimens (Whipple's) or core biopsies with the guidance of a consultant pathologist, snap frozen in sterile containers using liquid nitrogen and stored at -70°C until the time of analysis.

## **Laser capture microdissection**

### **Mounting and sectioning of tissue**

Whole tissue samples were mounted in Tissue-Tek(R) OCT™ (Sakura Finetek Europe BV, The Netherlands). 8µm frozen sections were then cut using a Leitz 1720 cryostat. (Leitz, Germany), and 3 sections per sample were mounted on positively charged slides. Excess OCT was then removed by rinsing in distilled water and slides stained by submersion for 15-20 seconds in 1% aqueous eosin and washed again. Slides were then dehydrated in ascending concentrations of alcohol for 1 minute each and cleared in 2 changes of clean xylene for 2 minutes each as described above under BrDU immunocytochemistry. Slides were finally air-dried and stored in an airtight container prior to analysis.

### **Cell capture**

Cells were captured using Capsure adhesive-coated caps and a Pixcell II laser capture microscope (Arcturus) according to the manufacturer's instructions. Under the guidance of a consultant pathologist, slides were reviewed under the laser capture microscope. A cap was then inserted into the microscope, placed in contact with the tissue section and the laser used to capture the entire tumour cell population onto the cap. This procedure was repeated using a fresh cap to capture normal cells from the

slide. RNA was then prepared from both samples using a paraffin block RNA isolation kit as described above.

## **Immunohistochemistry**

Immunohistochemistry was performed on sections of normal and tumour tissues using mouse monoclonal anti-glycosylated PIF antibody and rabbit polyclonal anti-PIF-peptide antiserum and a biotin-amplified peroxidase protocol. Frozen sections were cut as described above under laser capture microdissection. Endogenous peroxidase activity was then blocked by incubation in 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 10 minutes at room temperature. Slides were washed in distilled water and transferred to TBS for 5 minutes. They were then wet with distilled water and placed in a Sequenza slide rack (Ted Pella Inc, Redding, CA, USA), in which all subsequent steps were performed. 2-3 drops of avidin (Vector Laboratories, Peterborough, UK) were then applied to each slide for 15 minutes. Slides were washed with TBS and 2-3 drops of biotin applied for 15 minutes. A further TBS wash was performed then slides were blocked by the addition of 100µl per slide of either 10% normal rabbit serum (Dako) in TBS for experiments using the mouse monoclonal anti-glycosylated PIF primary or 10% normal swine serum (Dako) in TBS for experiments using the rabbit polyclonal anti-PIF-peptide primary. After a 30 minute incubation, 100µl per slide of the primary antibody diluted to the desired concentration in the appropriate blocking solution was added and slides were incubated for 1 hour at room temperature. A 10 minute wash with TBS was then performed. 100µl per slide of secondary antibody was then added. For experiments using the mouse monoclonal anti-glycosylated PIF primary, a rabbit anti-mouse

biotin-linked secondary (Dako) was used. For experiments using the rabbit polyclonal anti-PIF-peptide primary, a swine anti-rabbit biotin-linked secondary (Dako) was used. Both secondary antibodies were diluted 1 in 300 in the appropriate blocking solution. Following a 30 minute incubation at room temperature, the secondary was washed off with TBS for 10 minutes. Three drops of ABCComplex/HRP solution prepared as per the manufacturer's instructions (Dako)(1 drop reagent A and 1 drop reagent B in 5ml TBS, pH 7.6) were then added to each slide and incubated at room temperature for 30 minutes prior to washing with TBS for 10 minutes. Slides were then developed using a liquid DAB+ substrate-chromogen system (Dako), haematoxylin counterstained and mounted as described above for BrDU immunocytochemistry. All slides were reviewed by a consultant pathologist.

## **Urine concentration**

### **Filter centrifugation**

Urine samples were concentrated using Amicon Ultra 5000 molecular weight cut-off centrifugal filter columns (Millipore) according to the manufacturer's instructions. Urine samples were defrosted on ice and 20ml aliquoted into 40ml round-bottomed ultracentrifuge tubes. Sediment was then removed by ultracentrifugation for 20 minutes at 16 000g and transfer of only the supernatants to Amicon columns. The columns were then spun for 30 minutes at 4 000g and the urine not filtered through the column (~200µl) was transferred to a clean 1.5ml microcentrifuge tube.

## **Protein assay**

Prior to use in experiments, the protein concentration in concentrated samples was assayed using a Protein Micro-Assay kit (Biorad), based on the method of Bradford (1976), according to a modification of the manufacturer's instructions. All samples and standards were prepared and assayed in a 96-well plate (Costar, Cole-Parmer, London, UK). A standard curve was prepared by performing 4 serial 1 in 2 dilutions of 160 $\mu$ l of the 1.45mg/ml protein standard supplied in distilled water. 40 $\mu$ l of each sample was then diluted 1 in 5 in distilled water. Standards and samples were then pipetted into the left-hand, assay wells of the plate in duplicate, adding 80 $\mu$ l per well. 20 $\mu$ l per well of dye reagent concentrate was then added and the plate placed on the shaker for 15 minutes. The optical density (OD) at 590nm was then read using a Dynex Technologies MRX-II plate reader and Assayzap interpretation software.

## **MALDI-TOF Mass spectrometry**

### **Intact protein analysis**

Urine samples were analysed for intact proteins by matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry using a Voyager-DE STR mass spectrometer (Applied Biosystems) under the guidance of the staff at the Wellcome Institute for Cell Biology, King's Buildings, University of Edinburgh. Samples were diluted to a concentration of 1 $\mu$ g/ $\mu$ l in distilled water. 0.5 $\mu$ l per well of each sample was then loaded onto a MALDI plate and 0.5 $\mu$ l of matrix added immediately. For determination of whole mass spectra, a sinapinic acid

(3,5 dimethyl 4-hydroxycinnamic acid) matrix was used. For analysis of peptide products of tryptic digests, an alpha-cyano cinnamic acid matrix was used. Plates were then inserted into the mass spectrometer and samples ionised by targeting with the laser.

## **Peptide analysis**

### **One dimensional gel electrophoresis**

For determination of specific urinary constituents, urinary proteins were first separated using 1-dimensional gel electrophoresis. This was performed using 10-well 4-12% pre-cast NuPAGE Bis-Tris gels (Invitrogen) as described above for Western blotting to run 20µg per well of total urinary protein. Gels were run at 100V until the dye front neared the end of the gel. Gels were then stained with Gelcode (Pierce) as described above for immunoprecipitation. Bands of specific molecular weights were excised using a clean scalpel for each band and placed in clean 1.5ml microcentrifuge tubes.

### **Tryptic digest**

The protein constituents of specific molecular-weight bands were determined by tryptic digest and MALDI-TOF mass spectrometry of the resultant peptides. Each band was first incubated twice in 300µl of 200mM ammonium bicarbonate in 50% acetonitrile (ABC/ACN) for 30 minutes at 30°C. The gel pieces were then incubated in 300µl of 20mM DTT in ABC/CAN for 1 hour at 30°C and washed 3 times with 300µl ABC/ACN. Cysteines were then alkylated by treatment with 100µl of freshly-

made 50mM iodoacetamide (IAA) in ABC/ACN for 20 minutes in the dark. Gel pieces were then washed 3 times with 500 $\mu$ l ABC/ACN and cut into 2x1mm pieces with a clean scalpel blade. These samples were then centrifuged for 2 minutes at 13 000rpm and covered in acetonitrile until they turned white. Acetonitrile was then removed and the gel air-dried. Tryptic digest was then performed by adding 1 $\mu$ l trypsin in 110 $\mu$ l of 50mM ammonium bicarbonate and incubating overnight at 32°C. Samples were then analysed by MALDI-TOF mass spectrometry as described above, using 2 internal control tryptic peptide peaks of 842.5 and 2211.5 Daltons. Sample peaks were de-isotoped from these controls and the parental proteins identified by screening of the resultant mass spectra using the MIS-FIT database.

### **Statistical analysis**

Statistical analysis was carried out using Microsoft Excel. All experiments comparing the means of 2 samples were analysed using the Student's *t* test.

Data are presented as the mean with error bars representing the standard error unless otherwise stated.

Results were considered to be statistically significant with a P value of <0.05.

## Results

### 3. *In vitro* expression of proteolysis inducing factor

#### Summary

It is clear that PIF / dermcidin has diverse biological functions. However, little is known of the contexts in which they may be important. Previous studies have suggested that PIF / dermcidin expression may be limited to only a few tissues, but the expression of PIF / dermcidin *in vitro* or at different stages of development remains unknown. This chapter describes the screening of cell lines and cultured primary cells at different stages of differentiation and maturity for PIF / dermcidin mRNA expression.

A variety of cell lines demonstrated PIF / dermcidin expression. Notably, the pancreatic carcinoma cell lines MiaPaCa2, Panc1 and CF-PAC consistently showed strong expression whereas the cell lines HuH7, THP1 and MCF7, which are derived from tumours not normally associated with cachexia, did not express PIF / dermcidin.

To assess PIF / dermcidin expression at different stages of differentiation and maturity, mRNA derived from primary cultures of fetal liver cells and adult hepatocytes was analysed. Fetal liver cells did not express PIF / dermcidin, in contrast with adult hepatocytes. However, fetal cells selected for the stem cell markers CD34 and CD90 did demonstrate PIF / dermcidin expression. In each case cells were derived from a single organ and confirmation of these results in other livers will be required.

To investigate co-translational processing of PIF / dermcidin, an *in vitro* translation system was used to produce radiolabelled polypeptide from the full length cDNA in the pcDNA3.1+ expression vector. This resulted in a nascent polypeptide of 11kD. An additional, 2.5kD product was formed on the addition of canine pancreatic microsomal membranes. This is consistent with computer modelling and suggests the production of a signal peptide which may target PIF / dermcidin to the secretory pathway. Subcellular localization was then examined by cloning the PIF / dermcidin cDNA into the pcDNA3.1+ V5 His vector and transiently transfecting HuH7 cells. Anti-V5 His immunocytochemistry demonstrated staining only after treatment with monensin, an inhibitor of intracellular protein transport.

These results suggest that PIF / dermcidin expression is more widespread than previous studies have suggested. In cell lines, this could represent an effect of transformation or of prolonged culture. It is certainly feasible that expression may be induced, as non-expressing HuH7 cells are capable of producing PIF / dermcidin from an expression vector. This might explain why we found adult hepatocytes to express PIF / dermcidin whereas previous studies have suggested that the liver does not produce PIF / dermcidin mRNA (Schitteck et al. 2001). In cultures of primary fetal hepatocytes PIF / dermcidin expression may reflect a low degree of differentiation.

## Introduction

The PIF peptide was originally isolated from lysates of the MAC16 tumour using a monoclonal antibody developed to a cachexia-inducing factor present in the serum of mice (McDevitt et al. 1995, Todorov et al. 1996a, Todorov et al. 1996b). Using this antibody, it was subsequently possible to detect PIF in the urines of human patients with pancreatic, hepatic, colonic, ovarian, breast and lung tumours by Western blotting (Cariuk et al. 1997), although whether PIF was a product of tumour cells or another cell type was not investigated. More recently, immunohistochemical studies using the same antibody have suggested that PIF may arise directly from the tissue of gastrointestinal tumours, specifically colorectal, pancreatic, gastric and bile duct carcinomas (Cabal-Manzano et al. 2001). Further weight has been added to this theory by studies on prostatic carcinoma which demonstrated PIF / dermcidin expression by tumour cells using *in situ* hybridization (Wang et al. 2003). Recent studies have demonstrated that PIF may be produced by stromal cells as well as tumour cells (Deans et al. 2006).

In non-malignant cells, studies on dermcidin have suggested that expression may be more restricted. In specific, RT-PCR analysis demonstrated expression in skin, melanocytic naevi and melanoma tissues but not in 16 other human adult or fetal tissues (Schitteck et al. 2001). These results were supported by the findings of Porter et al. (2003), who analysed 75 tissues by dot-blot expression array and found DCD expression only in the pons of the brain. Interestingly, both Schitteck and Porter also suggested that PIF / dermcidin expression was similarly restricted in tumours, Schitteck reporting no expression in several tumour tissues and cell lines and Porter finding that just 2 of 64 pancreatic carcinomas were positive on

immunohistochemical tissue array. However, the significance of these findings is unclear as Schitteck did not report which tissues or cells were negative and Porter used an antibody to amino acids 53-64 of the nascent polypeptide, which may subsequently fail to recognize the proteolytically-processed and potentially glycosylated PIF core peptide. Therefore, although expression of PIF / dermcidin in normal tissues appears limited, whether it is similarly restricted in tumour tissues and cell lines remains unclear.

During development, it has been suggested that PIF may play a role in the formation of skeletal muscle and liver tissues (Watchorn et al. 2001). Support for this arises from the observation that there is a peak in PIF expression at E8.5 in the mouse embryo (I. Waddell, unpublished observation). This coincides with the period of maximal skeletal muscle and liver morphogenesis. PIF has been demonstrated to have effects on cellular function and gene transcription in these tissues (Watchorn et al. 2001, Whitehouse and Tisdale 2003). In liver cells this includes induction of IL-6, IL-8 and ICAM expression and syndecan 1 and 2 shedding (Watchorn et al. 2002). The roles of these molecules in cell adhesion and proliferation (Collins et al. 1995, Zimmermann and David 1999) therefore suggest that PIF may be involved in hepatocyte differentiation.

The functions of PIF / dermcidin as a cancer cachectic factor, neuronal survival factor and antibiotic peptide suggest that it is likely to be a secretory product. The dermcidin product DCD-1 is certainly secreted, and was originally isolated from sweat by high-performance liquid chromatography (Schitteck et al. 2001). More recently, four different forms of dermcidin have been discovered (Flad et al. 2002) and it has been suggested that these may be produced by proteolytic

processing either intracellularly or following secretion. Certain features of the expression pattern of DCD-1 in normal skin also suggests that it is a secretory product, with *in situ* hybridization, immunohistochemistry and immunofluorescence demonstrating expression in only eccrine sweat glands (Schitteck et al. 2001). Immunoelectronmicroscopy further demonstrated that DCD-1 is localized to the Golgi complex and secretory granules in eccrine cells. In addition, the presence of a NH-terminus signal peptide sequence is consistent with targeting of the PIF / dermcidin polypeptide to the secretory pathway (Nielsen et al. 1997).

In order to address the differences in reported PIF / dermcidin expression and to establish a background pattern of expression and a suitable cell line in which to study the functional effects of PIF / dermcidin expression we used RT-PCR to screen a range of cell types for PIF / dermcidin expression. We further aimed to confirm both the production of a signal peptide using an *in vitro* translation system and the targeting of the nascent polypeptide to the secretory pathway in the cell line selected to study functional effects using a V5-His labelled PIF / dermcidin mammalian expression vector.

## Materials and methods

### RNA preparation

RNA was prepared from cell cultures using Trizol (Life Technologies) (cf. p.49). RNA derived from primary hepatocytes cultures which had been treated with cyclosporin A, heat-shocked or left untreated was the kind gift of S. McNally. RNA derived from fetal liver cells was the kind gift of N. Masson. All samples were DNase I treated prior to reverse transcription (cf. p.52).

### Reverse transcription

Samples were reverse-transcribed in 25µl reactions using AMV reverse transcriptase and random primers (Promega) (cf. p.55).

### PCR

Standard 25µl reactions with 1µl sample and 1µl *taq* polymerase were used (cf. p.55). All DNase I treated RNA samples were checked for genomic DNA contamination by amplification with primers to the housekeeping gene, cytochrome B. Primer sequences were -

F - GGTTCTCTGGGAGGGAGTGC

R - GACAACACAGTAAGAACCAGG

Primers used for PIF / dermcidin PCR were -

F - CTCGGATCCGCCCATGAGGTTTCATGACTCTCC

R - CAGAATTCCTGGGTATCATTCTCAGCT

Primers were purchased from TAGN. All reactions underwent 35 cycles with a 56°C annealing temperature.

## **Cell culture**

HuH7, CF-PAC, Mia PaCa2 and Panc1 cells were cultured at 37°C in a 5% CO<sub>2</sub> in air atmosphere in DMEM plus 10% fetal calf serum, L-glutamine, penicillin and streptomycin (cf. p.46).

## **Vectors**

For *in vitro* translation experiments the pcDNA3.1+PIF vector was used. For transient transfection experiments the full length PIF / dermcidin insert in this vector was directionally cloned into the pcDNA3.1+V5-His vector (Invitrogen) (cf. p.67).

## ***In vitro* translation**

*In vitro* translation was performed using a rabbit reticulocyte system (Promega) according to the manufacturer's instructions (cf. p.71). Radioisotopes used were <sup>35</sup>S methionine, <sup>14</sup>C leucine and <sup>35</sup>S cysteine (ICN). In selected experiments reactions were supplemented with 1.2µl canine pancreatic microsomal membranes (Promega). Samples were run on 10-20% tris-tricine gels (Biorad) and visualized by fluorography.

## **Transfection and cell culture**

Plasmids were transiently transfected into the HuH7 cell line (ECACC) using Fugene (Roche Applied Science) according to the manufacturer's instructions (cf. p.47). Cells were cultured for 24 hours under standard conditions in the presence of 600µg/ml G418 (Sigma). For use in immunocytochemistry experiments cells were seeded in 8-well chambered slides at a concentration of 10 000 cells per well. After 24 hours cells were treated with 5µM monensin for 12 hours before fixation.

## **Immunocytochemistry**

Following permeabilization cells were probed with anti-V5 His FITC conjugated antibody (Invitrogen) and visualized by fluorescence microscopy (cf. p.76). Nuclei were counterstained using Hoescht 33342.

## Results

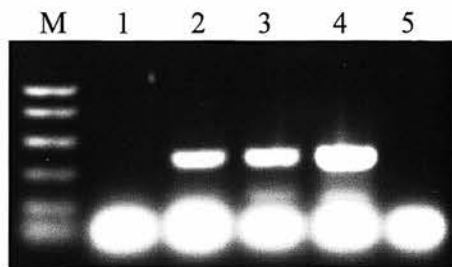
### Determination of RNA and cDNA integrity

All mRNA samples were assayed for the presence of contamination with genomic DNA by PCR amplification with primers to cytochrome B. When a band for the cytochrome B gene was observed, indicating genomic contamination, a smaller sample of RNA was retreated with DNase I and reamplified. Elimination of genomic contamination was achievable in all cases.

cDNA integrity was assessed by PCR with primers to cytochrome B. This resulted in products of the correct size without exception.

### Expression of PIF / dermcidin in cell lines

PCR of cDNA derived from the HuH7 cell line did not demonstrate PIF / dermcidin expression (figure 3.1). In contrast, the cDNAs from the pancreatic carcinoma cell lines MiaPaCa2, CF-PAC and Panc1 all produced strong bands on PIF / dermcidin PCR (figure 3.1). This result was confirmed in triplicate.

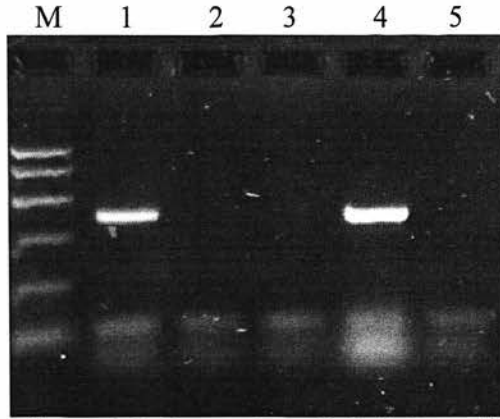


**Figure 3.1.** Expression of PIF / dermcidin by cell lines.

1. HuH7
2. Mia PaCa2
3. CF-PAC
4. Panc1
5. Blank (no cDNA)

### Expression of PIF / dermcidin in primary hepatocytes

cDNA from one preparation of unstimulated primary hepatocytes was positive for PIF / dermcidin expression (figure 3.2). In contrast, cDNA from hepatocytes treated with cyclosporin A or heat-shocked was negative for PIF / dermcidin. A repeat PCR confirmed these results.

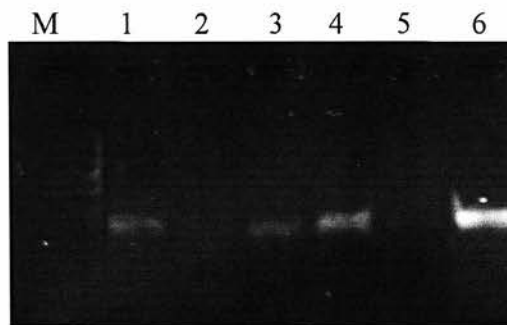


**Figure 3.2.** Expression of PIF / dermcidin by cultured primary adult human hepatocytes

1. Untreated
2. Heat shock treated
3. Cyclosporin treated
4. Positive control (CF-PAC cDNA)
5. Negative control (no cDNA)

### Expression of PIF / dermcidin by fetal liver cells

PCR of cDNA prepared from a mixed population of cells derived from a single fetal liver did not demonstrate PIF / dermcidin amplification (figure 3.3). In contrast, PCR of cells from single livers which had been sorted on the basis of either CD34 or CD90 expression was positive for PIF / dermcidin expression. This effect was independent of length of time in culture, which was 13 days for unsorted cells, 2 days for CD34+ cells and 41 days for CD90+ cells. A repeat PCR confirmed these results.

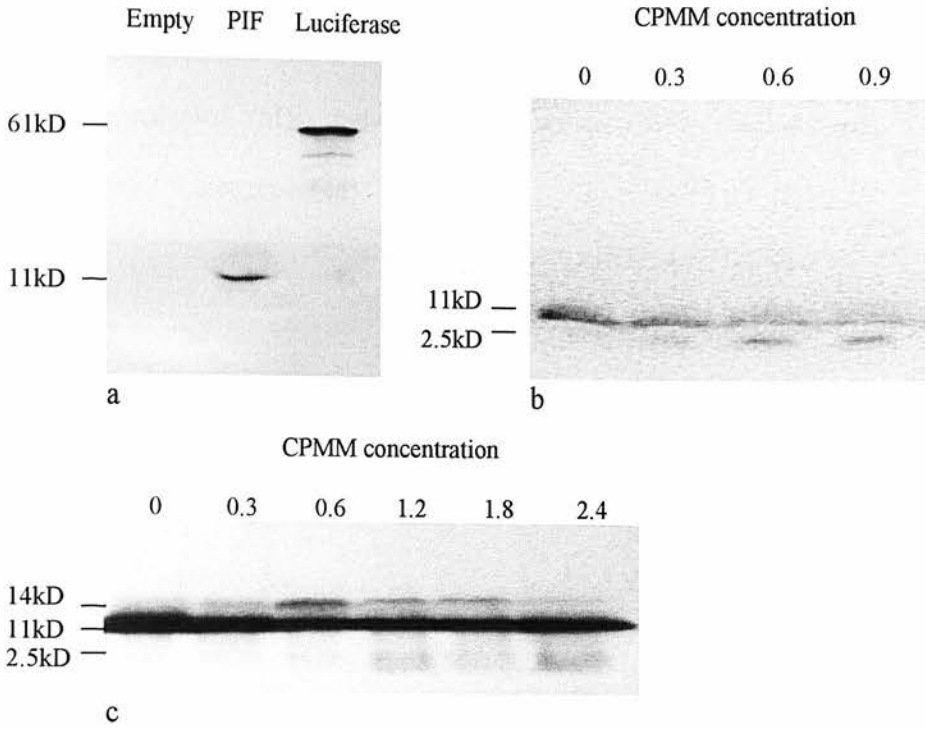


**Figure 3.3.** Expression of PIF / dermcidin by fetal liver cells

1. Primary adult hepatocytes
2. Unsorted fetal liver cells
3. CD34+ fetal liver cells
4. CD90+ fetal liver cells
5. Negative control (no cDNA)
6. Positive control (CF-PAC cDNA)

## **Production of a signal peptide**

Translation of pcDNA3.1+PIF in the presence of  $^{35}\text{S}$  methionine gave an unprocessed product of 11kDa (figure 3.4a, repeated twice). Co-translational processing, in the presence of canine pancreatic microsomal membranes, produced a cleavage product of approximately 2.5 kDa (figure 3.4b, repeated twice). As the larger fragment of PIF / dermcidin does not contain any methionine residues following cleavage, its visualisation must either reflect incomplete cleavage of signal peptide or a stage between translation and cleavage. Therefore, to assess processing after this stage reactions were performed in the presence of  $^{35}\text{S}$  cysteine, as cysteine residues are present in both the signal peptide and larger fragment. In this experiment an additional band of approximate molecular weight 14kDa was apparent in all reactions to which canine pancreatic microsomal membranes had been added (figure 3.4c, not repeated). No fragment of larger molecular weight could be identified.

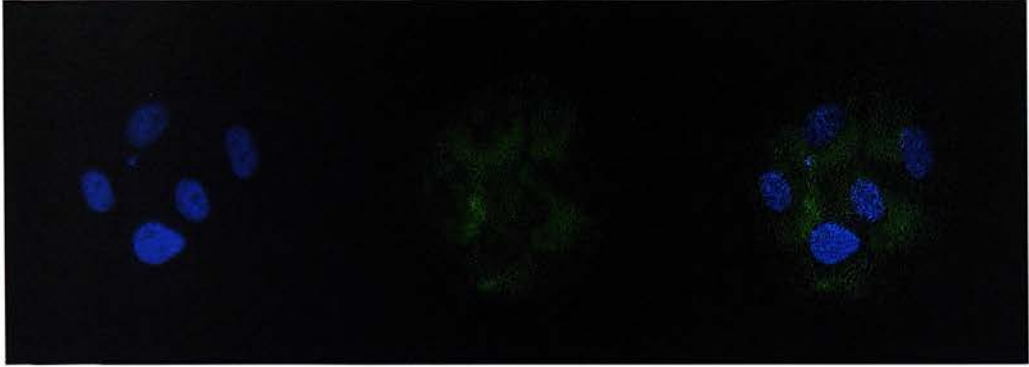


**Figure 3.4.** *In vitro* translation of pcDNA3.1(+)/PIF.

- a.  $^{35}\text{S}$  methionine label, no canine pancreatic microsomal membranes (CPMM)
- b.  $^{35}\text{S}$  methionine label with CPMM ( $\mu\text{l}$  per reaction)
- c.  $^{35}\text{S}$  cysteine label with CPMM

### Secretion of PIF / dermcidin by HuH7 cells

HuH7 cells which had been transiently transfected with the pcDNA+V5His PIF vector demonstrated V5His immunofluorescence only after treatment with monensin (figure 3.5, not repeated).



DAPI

FITC

Merge

**Figure 3.5.** Immunocytochemistry of V5His labelled PIF / dermcidin in monensin treated HuH7 cells (FITC labelled secondary antibody).

## Discussion

The finding that primary cultures of human hepatocytes express PIF / dermcidin was surprising given the previous studies on dermcidin expression (Schitteck et al. 2001, Porter et al. 2003). However, as noted above PIF / dermcidin expression may have been under-represented in Porter's study due to the use of an antibody which may not recognise the PIF core peptide. Schitteck used commercially available multiple tissue cDNA arrays to screen for PIF / dermcidin expression, and would be expected to gain high quality results, but it is not clear from the manufacturer's information whether all tissues derived from one individual. If this were the case, it is feasible that individual differences in PIF / dermcidin expression may have affected the results. A further explanation for the differences may be that PIF / dermcidin expression is somehow influenced by the processing or culture of hepatocytes. This procedure involves the harvesting of liver tissue removed during partial hepatectomy, usually for metastatic cancer, disintegration of the tissue architecture by collagenase perfusion and culture on type I collagen coated plates (Wigmore et al. 1997b). The cells are therefore subjected to a range of stresses and stimuli not encountered physiologically and any or all of these may induce PIF / dermcidin expression. Confirmatory studies of PIF / dermcidin expression in unprocessed liver tissue would help to address this area.

In contrast with adult hepatocytes, PIF / dermcidin expression was not detected in whole preparations of fetal liver cells. This is consistent with the dermcidin studies. However, it is difficult to comment on the contrast between this result and our finding that adult hepatocytes express PIF / dermcidin. Firstly, as noted above there are caveats regarding potential induction of expression in adult

hepatocytes. Secondly, it is not possible to cannulate the vessels of the fetal liver and the preparation subsequently differs from the adult in using tissue homogenization rather than collagenase perfusion. Fetal liver preparations therefore initially contain a high number of blood cells, although these are believed to disappear rapidly in culture (Malhi et al. 2002).

When cDNA was prepared from fetal liver cells selected for the markers CD34 and CD90, PIF / dermcidin expression was demonstrated. The expression could not be ascribed to length of time in culture, as both CD34 and CD90 positive cells were cultured for less time than unsorted preparations prior to RNA preparation. It is feasible that the process of sorting or of expansion from a small population could induce expression, although previous studies have demonstrated little qualitative change in fetal liver cells over prolonged culture periods (Malhi et al. 2002). PIF / dermcidin expression may therefore potentially mark cells which have expanded from small populations of CD34 and CD90 positive cells.

The production of a 2.5kD band on addition of canine pancreatic microsomal membranes to the *in vitro* translation system is highly suggestive of signal peptide production. The other potential proteolytic products of PIF, Y-P30 and DCD-1, are too large to produce a band of this size and their formation would be more likely to require the action of a specific protease. These results are consistent with those of computer modelling, which rely on sophisticated methods of neural mapping to identify signal peptides and the site of their cleavage from nascent polypeptides (Nielsen et al. 1997). Signal peptides control the entry of virtually all proteins to the secretory pathway, both in eukaryotes and prokaryotes (Gierasch 1989, von and Manoil 1990, Rapoport 1992). Similar targeting of PIF / dermcidin is therefore likely

and is consistent with our visualisation of V5 His-tagged PIF / dermcidin in HuH7 cell only after monensin treatment and also with the localisation of DCD-1 to the secretory granules of dark mucous cells in eccrine sweat glands (Schitteck et al. 2001). It therefore appears that these malignant cells, which do not endogenously express PIF / dermcidin, can produce the polypeptide from our expression vector.

The following chapters will examine the links between PIF / dermcidin mRNA expression, the production of active forms of PIF and its functional effects.

## 4. Generation of PIF / dermcidin mutants

### Summary

Previous studies suggest that post-translational modification may have important effects on PIF function. Deglycosylation of the core peptide at N- and O-glycosylated amino acid residues has been shown to remove its ability to induce muscle proteolysis (Todorov et al. 1997). Proteolytic processing may also be important in generating the DCD-1 and PIF core peptides from the nascent polypeptide chain (Cunningham et al. 1998, Flad et al. 2002).

To investigate the effect of these processes we utilized computer modelling to identify sites in the PIF / dermcidin amino acid sequence which may be subject to post-translational modification. We then employed site-directed mutagenesis to alter these residues and stably transfected PIF / dermcidin into HuH7 hepatic carcinoma cells.

Cleavage of the signal peptide which may target PIF / dermcidin to the secretory pathway was predicted by Signal P. A search for N-glycosylation sites did not reveal any typical consensus sequences but found one atypical sequence at N32. O-glycosylation was not predicted at any of the 8 serine and threonine residues.

Site-directed mutagenesis was performed on the full-length PIF / dermcidin cDNA in the pcDNA3.1+PIF vector using a Quikchange II kit. To study N-glycosylation, the two codons coding for asparagine residues were converted to code for glutamines both singly and in combination. To generate mutants coding only for the PIF core peptide, STOP codons were introduced immediately 5' to the sequences coding for the signal peptide and the PIF core peptide (Y-P30). All mutations were

confirmed by direct sequencing prior to large scale culture and purification using an Endofree plasmid maxi kit.

On stable transfection into HuH7 cells, analysis of PIF / dermcidin expression by PCR, Western blotting and immunoprecipitation suggested that endogenous expression was induced as a side-effect of prolonged culture. However, Western blotting for neomycin phosphotransferase demonstrated expression only in transfected cells suggesting that PIF / dermcidin was also overexpressed from the vector.

## Introduction

By assessing the available structural data on PIF and its homologues, Y-P30 and dermcidin, it is possible to construct a model of the parental polypeptide. When originally purified from the MAC16 tumour, glycosylated PIF was found to have an amino-terminal sequence of YDPEAASAPGSGDPSHEASA (Todorov et al. 1996a). Two years later, the sequence of the Y-P30 peptide, which had been purified from the supernatants of oxidatively stressed neuronal cells, was found to share close homology with that of PIF and extended the carboxyl terminus sequence by a further 9 amino acids (figure 1.1)(Cunningham et al. 1998). This was followed by the identification of the cDNA for Y-P30 (Cunningham et al. 2002). With a coding sequence of 330bp, this had a predicted peptide product of 110 amino acids and encoded the PIF core peptide and Y-P30 from amino acids 20 to 50, immediately after the signal peptide. Another product of the cDNA came to light with the discovery of dermcidin (Schitteck et al. 2001). This study isolated a 46 amino acid peptide termed DCD-1 from human sweat, the sequence of which was 100% homologous with the predicted 3' translation product of the Y-P30 cDNA. The cDNA for dermcidin was also isolated and showed 100% homology with that of Y-P30. Independently, our collaborators had also identified the cDNA for PIF / dermcidin by screening a cDNA database for sequences which when translated, produced peptides with homology to the known amino terminus sequence of PIF (Waddell et al., unpublished observation).

These studies therefore confirm that PIF, Y-P30 and DCD-1 arise from the same gene, dermcidin. In the polypeptide produced by this gene the signal peptide is sequentially followed by the PIF core / Y-P30, a pro-peptide component and the

DCD-1 peptide (figure 1.1). In order that these separate peptides can be produced, it therefore seems likely that proteolytic processing of the nascent polypeptide must occur. The identification of four additional forms of DCD-1 in sweat further supports this (Flad et al. 2002). In determining which peptide is produced, proteolytic processing may subsequently control the function PIF / dermcidin serves in any particular cell.

Other features of PIF / dermcidin's structure may also have important effects on its function. The original description of PIF determined its sequence from two species of molecular weight 24kD and 69 kD isolated by affinity chromatography, the latter of which also contained albumin and which were both found to contain carbohydrate (Todorov et al. 1996a). Subsequent analyses suggested that PIF is both N- and O- glycosylated, with one N-linked carbohydrate chain of 10kD and one O-linked chain of 6kD attached to the central peptide core of 4kD (Todorov et al. 1997, Todorov et al. 1999). Removal of these carbohydrate chains with peptide-N-glycosidase F and O-glycosidase also removed the ability of PIF to induce proteolysis in isolated mouse gastrocnemius muscle (Todorov et al. 1997). In the case of Y-P30, it has also been suggested that phosphatase activity may be involved in the peptide's effect (Cunningham et al. 1998). The central part of the Y-P30 sequence shares homology with the calcineurin phosphatase domain and the peptide has been demonstrated to have phosphatase activity *in vitro* (Cunningham et al. 1998).

In order to investigate the relationship between these structural characteristics and the function of PIF / dermcidin we aimed to alter crucial codons in the PIF

cDNA using site-directed mutagenesis and transfect these mutants into the HuH7 cell line to facilitate functional studies.

## Materials and methods

### Computer searches

N- and O-glycosylation were modelled using Net N-Glyc (<http://www.cbs.dtu.dk/services/NetNGlyc/>) and Net O-Glyc (<http://www.cbs.dtu.dk/services/NetOGlyc/>) respectively. Cleavage of the signal peptide was modelled using Signal P (<http://www.cbs.dtu.dk/services/SignalP/>).

### Site-directed mutagenesis

Site-directed mutagenesis was performed on the full-length PIF / dermcidin cDNA in pcDNA3.1+PIF using a Quikchange II kit (Stratagene). The modification of the manufacturer's protocol described in materials and methods (p.69) was used in every case.

### Primers

Primers used were all designed using the Quikchange primer design tool (Stratagene, <http://labtools.stratagene.com/>) to optimize  $T_m$  to approximately 78°C.

Primer sequences were –

Mutcleav F	GGTCTGTGCCTATATTCCAGAGGCCGCTCTGC
Mutcleav R	GCAGAGGCCGCCTCTGGAATATAGGCACAGACC
N32Q F	CCAGGATCGGGGCAGCCTTGCCATGAAGC
N32Q R	GCTTCATGGCAAGGCTGCCCCGATCCTGG

N44Q F	CAGCAGCTCAAAAGGAACAGGCAGGTGAAGA CCCAG
N44Q R	CTGGGTCTTCACCTGCCTGTTCTTTTGAGCTGCTG
H35N F	CGGGGAACCCTTGCAATGAAGCATCAGCAGC
H35N R	GCTGCTGATGCTTCATTGCAAGGGTTCCCCG
ProP STOP F	TGCAGGTGAAGACCCATAGTTAGCCAGACAGGCAC
ProP STOP R	GTGCCTGTCTGGCTAACTATGGGTCTTCACCTGCA
Y-P30 STOP F	CTGGTCTGTGCCTAGGATCCAGAGGCC
Y-P30 STOP R	GGCCTCTGGATCCTAGGCACAGACCAG

### **Plasmid culture and preparation**

Following mutagenesis cycling plasmids were transformed into XL-1 Blue supercompetent e.coli which were cultured overnight in 3ml LB broths at 37°C with agitation (cf. p.64). Plasmids were then prepared by alkaline lysis of 1ml of broth (cf. p.65) and mutations confirmed by direct sequencing. The remaining broth was then added to 250ml of LB broth and cultured overnight at 37°C with agitation (cf. p.66). Plasmid DNA for use in experiments was then prepared with an Endofree plasmid maxi kit (Qiagen) according to the manufacturer's instructions (cf. p.66).

### **DNA sequencing**

All plasmid sequencing was performed by Nicola Wrobel of the MRC centre for cardiovascular research, Centre for Inflammation Research, University of Edinburgh.

## Transfection and cell culture

HuH7 cells were stably transfected with empty, native PIF / dermcidin and mutant PIF / dermcidin vectors as described in materials and methods (p.47).

Following selection, cells were cultured under standard conditions in the presence of 600µg/ml G418.

## RT-PCR

RNA was prepared from transfected cells using Trizol and DNase I treated as described in materials and methods (pp.49 and 52). Samples were reverse-transcribed in standard 25µl reactions using AMV reverse transcriptase and random primers (Promega) (cf. p.55). PCR was then performed in standard 25µl reactions with 1µl sample and 1µl *taq* polymerase (cf. p.55). All DNase I treated RNA samples were also checked for genomic DNA contamination by amplification with primers to the housekeeping gene, cytochrome B. Primer sequences were -

F - GGTTCTCTGGGAGGGAGTGC

R - GACAACACAGTAAGAACCAGG

Primers used for PIF / dermcidin PCR were -

F - CTCGGATCCGCCGCCATGAGGTTTCATGACTCTCC

R - CAGAATTCCTGGGTATCATTCTCAGCT

All reactions underwent 35 cycles with a 56°C annealing temperature.

## **Western blotting**

Protein was prepared from transfected HuH7 cells using RIPA buffer and quantified using the Bradford method as described in materials and methods (p.58). Western blotting was performed as described in materials and methods (p.59) using 10µg of sample per well.

For PIF / dermcidin blotting, 4-12% pre-cast NuPAGE Bis-Tris gels in MOPS buffer (Invitrogen) were used. The primary antibody was polyclonal rabbit antisera raised to a synthetic peptide corresponding to the sequence of PIF / dermcidin with the highest predicted antigenicity (sequence HEASAAQKENAGEDPC) (Sigma) at 1 in 1000 in blocking solution. A goat anti-rabbit peroxidase linked secondary antibody (Santa Cruz) at 1 in 5000 in blocking solution and an ECL kit (Amersham biosciences) were used for visualization.

For neomycin phosphotransferase blotting, standard 12.5% polyacrylamide gels were used and protein transferred to nitrocellulose. The primary antibody was anti-neomycin phosphotransferase (Upstate) at 1µg/ml in blocking solution. Blots were visualized using secondary goat anti-mouse peroxidase linked antibody (Santa Cruz) at 1 in 5000 in blocking solution and an ECL kit (Amersham).

## **Immunoprecipitation**

Immunoprecipitation was performed using Dynabeads as described in materials and methods (p.62). The antibody used was mouse monoclonal anti-PIF antibody (McDevitt et al. 1995). Samples were run on a 4-12% pre-cast NuPAGE Bis-Tris gel in MOPS buffer (Invitrogen). Gels were stained with Gelcode (Pierce) and destained in deionized water.

## Results

### Computer modelling of PIF / dermcidin

Net-O-Glyc did not reveal any amino acids reaching the threshold for O-glycosylation. There were no typical consensus N-glycosylation sites in the PIF / dermcidin polypeptide. However, a search with Net-N-Glyc for atypical glycosylation sites revealed a consensus sequence at N32 with a potential of 0.77.

Signal P predicted cleavage of a signal peptide between amino acids 16 and 17. This peptide would be expected to target PIF / dermcidin to the secretory pathway.

### Generation of PIF / dermcidin mutants

Studies of MAC-16 and urine-derived PIF suggest heavy glycosylation at both asparagines 32 and asparagines 44 and at least one serine / threonine residue (Todorov et al. 1997). This contrasts with the results of computer searches. The calcineurin-like phosphatase domain has been suggested to lie at amino acids 12 to 17 of Y-P30, ie. amino acids 31 to 38 of the full length polypeptide (Cunningham et al. 1998). Mutants were therefore generated to convert both N residues to glutamines both singly and in combination, to truncate the core peptide by moving the point at which the signal peptide is cleaved to lengthen the signal peptide, truncate the polypeptide chain through the introduction of STOP codons at the start of the core peptide and propeptide and to replace the most conserved and functionally important residue of the calcineurin-like domain (Rusnak and Mertz 2000), H35, to asparagine.

5 $\mu$ l of each mutagenesis reaction was run on a confirmatory gel following *Dpn-I* digestion. This demonstrated bands corresponding to linear and supercoiled plasmid as expected. Transformation into supercompetent XL-1 Blue *e. coli* resulted in 10 to 100 colonies. This was below the expected efficiency of the Quikchange kit. The majority of colonies grew when transferred to 3ml minibroths. When plasmids were prepared by miniprep and analysed by direct sequencing the majority were also found to contain the desired mutation. No replication errors were noted.

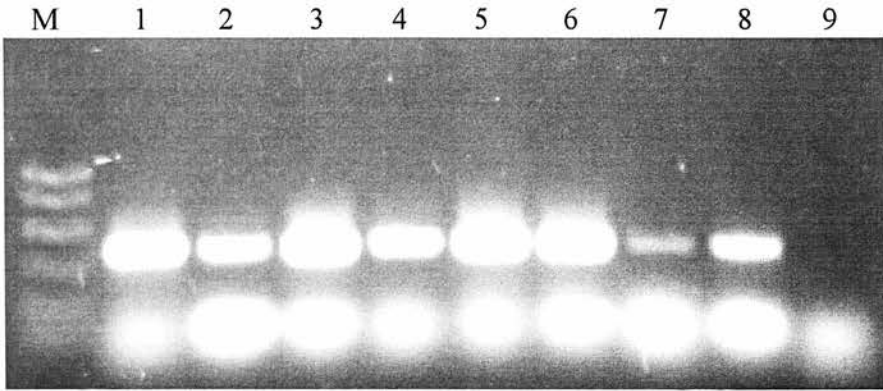
### **Large scale culture**

Innoculation of minibroths into 250ml maxibroths resulted in satisfactory growth in each case. Preparation of plasmids with the Endofree kit produced approximately 500 $\mu$ g of purified plasmid which was dissolved in 500 $\mu$ l of endotoxin-free TE buffer giving a final concentration of 1 $\mu$ g/ $\mu$ l. Isopropanol precipitated samples from each stage of the preparation process were run on agarose gels as a quality control and revealed satisfactory preparation in each case.

### **Confirmation of PIF / dermcidin expression in transfected cells**

PIF / dermcidin PCR of cDNA from cells transfected with PIF / dermcidin-containing vectors demonstrated PIF / dermcidin expression in every case (figure 4.1). However, expression was also demonstrated in cells transfected with empty pcDNA3.1(+). The PCR was repeated 3 times with the same result. This result was validated by sequencing of the empty vector, confirming that it was free from PIF / dermcidin contamination and by repeating the whole transfection experiment with

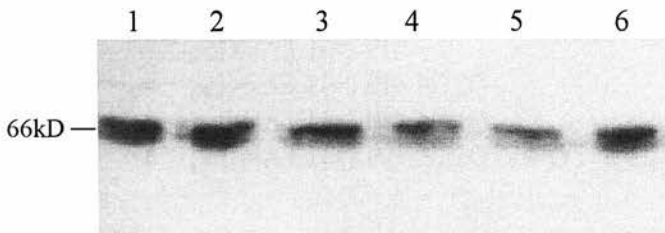
fresh HuH7 cells, excluding the possibility of inadvertent mixing of cells during passage. Following prolonged passage, PIF / dermcidin expression also became evident in untransfected HuH7 cells (figure 4.1). PCR of the DNase-treated RNA from which the cDNAs were prepared did not produce bands for PIF / dermcidin, suggesting that contamination with genomic DNA containing the integrated PIF cDNA had not occurred (experiment repeated once).



**Figure 4.1.** PIF / dermcidin PCR of cDNA derived from cells transfected with pcDNA3.1+ containing the PIF / dermcidin cDNA

1. Empty pcDNA3.1(+) transfected cells
2. pcDNA3.1(+) PIF transfected cells
3. pcDNA3.1(+) Mutcleav transfected cells
4. pcDNA3.1(+) N32Q transfected cells
5. pcDNA3.1(+) N44Q transfected cells
6. pcDNA3.1(+) N32Q N44Q transfected cells
7. Untransfected HuH7 cells
8. Positive control (CF-PAC cDNA)
9. Negative control (no cDNA)

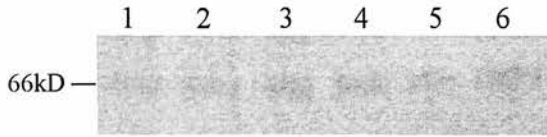
To confirm the production of PIF / dermcidin protein Western blotting of transfected cell lysates was performed. Probing with rabbit polyclonal antisera to the PIF / dermcidin peptide revealed bands of molecular weight 66kD in all cell lysates (figure 4.2, experiment repeated twice, transfection also repeated). Again, these bands were present in untransfected HuH7 cells and cells transfected with the empty pcDNA3.1(+) vector. In cells containing the mutant PIF / dermcidin vectors pcDNA3.1(+)PIF N32Q, pcDNA3.1(+)PIF N44Q and pcDNA3.1(+)PIF N32QN44Q, which would not be expected to produce N-glycosylated PIF, no difference in molecular weight was apparent.



**Figure 4.2.** Western blot of transfected HuH7 cell lysates using polyclonal anti-PIF / dermcidin antibody. Transfected vector -

1. None (untransfected)
2. Empty pcDNA3.1(+)
3. pcDNA3.1(+) PIF
4. pcDNA3.1(+) PIF N32Q
5. pcDNA3.1(+) PIF N44Q
6. pcDNA3.1(+) PIF N32Q N44Q

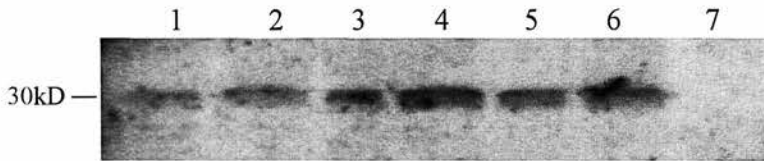
To exclude non-detection of glycosylated PIF due to the antibody being directed to the PIF core peptide, immunoprecipitation using the mouse monoclonal antibody demonstrated to bind to glycosylated PIF (Todorov et al. 1997) was undertaken. Again, bands of weight 66kD were detected in each cell lysate (figure 4.3, experiment repeated once).



**Figure 4.3.** Immunoprecipitation of transfected HuH7 cell lysates using mouse monoclonal anti-glycosylated PIF antibody. Transfected vector –

1. None (untransfected)
2. Empty pcDNA3.1(+)
3. pcDNA3.1(+) PIF
4. pcDNA3.1(+) PIF N32Q
5. pcDNA3.1(+) PIF N44Q
6. pcDNA3.1(+) PIF N32Q N44Q

We hypothesized that these results could represent either non-specific binding of either antibody or, as suggested by the results of PCR of HuH7 cells cultured for prolonged periods (figure 4.1), the induction of PIF expression by the culture environment. In pcDNA3.1+ the CMV promoter drives expression of both the antibiotic resistance gene, neomycin phosphotransferase and the insert, in this case the PIF / dermcidin cDNA. Therefore, in order to confirm function of the CMV promoter in transfected HuH7 cells we performed Western blotting for neomycin phosphotransferase. This demonstrated expression of the 30kD protein neomycin phosphotransferase II in all cells transfected with pcDNA3.1+, including those transfected with the empty vector, but not in untransfected cells (figure 4.4, not repeated).



**Figure 4.4.** Anti-neomycin phosphotransferase Western of PIF / dermcidin-transfected cells

1. Empty pcDNA3.1(+) transfected cells
2. pcDNA3.1(+) PIF transfected cells
3. pcDNA3.1(+) Mutcleav transfected cells
4. pcDNA3.1(+) N32Q transfected cells
5. pcDNA3.1(+) N44Q transfected cells
6. pcDNA3.1(+) N32Q N44Q transfected cells
7. Untransfected HuH7 cells

## Discussion

We have successfully used the technique of site-directed mutagenesis to alter codons of the PIF / dermcidin cDNA. The mutations introduced were carefully designed both to facilitate this process and to create amino acid substitutions which would affect the structure of the polypeptide. In the case of N-glycosylation, substitution of asparagine with glutamine is a widely accepted technique to prevent carbohydrate addition (Choi et al. 2003, Li et al. 2004b, Wujek et al. 2004). Similarly, the use of STOP codons is an established and effective method of sequentially truncating proteins (Revilla et al. 1999, Rebrin et al. 2001). Finally, in the case of the calcineurin-like phosphatase domain of the PIF core peptide, the histidine at position 35 corresponds to the most highly conserved amino acid in calcineurin itself, in which substitution with asparagine results in a significant reduction in phosphatase activity (Mertz et al. 1997). All of these mutations would therefore be expected to have significant effects on the functions of PIF. However, confirming their production from the pcDNA3.1+ vector in the HuH7 cell system did not prove straightforward.

When assessed by RT-PCR, HuH7 cells did not appear to express PIF / dermcidin (cf. chapter 3). We were therefore surprised to find the presence of PIF / dermcidin cDNA following prolonged passage or transfection with empty pcDNA3.1+. The stronger bands seen following transfection further suggest that an additional stimulus may be acting on the transfected cells. In the case of prolonged passage, several factors might influence expression. Firstly, repeated oxidative stress may induce PIF / dermcidin and is the only factor known to stimulate PIF / dermcidin expression (Cunningham et al. 1998). It would be expected to affect cells

in culture at several stages, for example when nutrients are depleted pre-passage or during the process of trypsinisation. The mechanism of induction is unclear, but examination of the putative PIF / dermcidin promoter reveals ample scope for transcriptional regulation. We identified this region from the human genome mapping project and found 127 binding sites for 27 different transcription factors in the 2000 base pairs immediately upstream of the transcriptional start site. These included binding sites for c-Jun, which is known to be induced by oxidative stress via a Raf1-dependent pathway (Chakraborti and Chakraborti 1998) and whose activity is redox regulated (Hwang et al. 2004).

Transcriptional regulation may also be affected by cell senescence, a second factor which may contribute to PIF / dermcidin expression following prolonged passage. Senescence is known to affect the transcriptional activity of E2F-1 and p53 (Dimri et al. 1994, Tsou et al. 1996, Itahana et al. 2001), both of which have predicted binding sites in the PIF / dermcidin promoter. Alternatively, the effect of senescence on cell signalling, including alterations in cellular redox status, could potentially affect PIF / dermcidin expression (Esposito et al. 2004).

In the case of transfected cells, PIF / dermcidin expression could also be induced by either antibiotic selection or the presence of the plasmid. The antibiotic used, G418, acts by inhibiting protein synthesis and is inactivated by the plasmid-encoded enzyme neomycin phosphotransferase. However, it is feasible that either of these molecules or their product may have additional effects on gene expression, although there are no descriptions of such an effect in the literature. Similarly, the production of proteins from the plasmid or the process of integration with the genome could also potentially influence gene expression.

The induction of endogenous PIF / dermcidin is therefore certainly feasible and as we had extensively validated this experimentally, we sought to confirm production of mutant PIF / dermcidin from the transfected vector. Unfortunately, Western blotting for PIF / dermcidin demonstrated bands of uniform molecular weight in all transfectants including those transfected with N-glycosylation mutant PIF / dermcidin. Of these, the double mutant would certainly not be expected to carry any N-linked carbohydrate and would therefore be predicted to be 10kD lighter than native, glycosylated PIF (Todorov et al. 1997). As the rabbit-polyclonal anti-PIF / dermcidin antibody used was developed to an unglycosylated peptide fragment of PIF / dermcidin it may not bind to glycosylated PIF. However, this possibility was excluded by the detection of the same molecular weight bands by immunoprecipitation using the anti-glycosylated PIF mouse monoclonal antibody (McDevitt et al. 1995). The weight of this band, at 66kD, is suggestive that if the antibodies are indeed detecting PIF they are detecting the albumin-bound form (Todorov et al. 1996a). Albumin is known to be secreted by HuH7 cells (Nakabayashi et al. 1982) and its association with endogenous PIF as well as mutant, vector-derived PIF could mask the small differences in molecular weight the mutations would be expected to produce.

In order to confirm the function of the CMV promoter, the component of the pcDNA3.1+ vector that drives expression in mammalian cells and to avoid the problems of immunological detection of native and mutant PIF / dermcidin, we examined the production of neomycin phosphotransferase in HuH7 cells. As expected, this revealed expression in only transfected cells. This confirms successful transfection of empty, native PIF / dermcidin and mutant vectors and demonstrates

that the CMV promoter functions in HuH7 cells. This suggests that the native and mutant PIF / dermcidin inserts in pcDNA3.1+, under the control of the same promoter, are also likely to be expressed. In light of this, the fact that the problems with immunological detection of PIF / dermcidin could also occur in any other cell system used for transfection and the possibility of assessing vector expression by *in vitro* translation, it was decided to proceed with functional studies of transfected HuH7 cells ensuring that every experiment was appropriately controlled and that any difference detected could subsequently only be attributed to overexpression of the transfected sequence from the CMV promoter.

## 5. Analysis of the survival-promoting effect of proteolysis inducing factor

### Summary

Proteolysis inducing factor shares the same peptide core sequence as the Y-P30 survival peptide but differs in being heavily N-glycosylated. In this chapter we describe our investigation of whether PIF functions as a survival factor in non-neuronal cells and the influence of mutating the potentially glycosylated asparagine residues on this effect. These aims were achieved by analyzing the survival of HuH7 cells, stably transfected with either native or mutant PIF / dermcidin vectors, following the induction of oxidative stress.

Oxidative stress was induced by treatment with glucose oxidase. Survival was assessed by MTT assay and examination of annexin V, BOB78 and anti-cleaved caspase 3 staining by flow cytometry and immunocytochemistry. The molecular weights of plasmid peptides products were additionally assessed by *in vitro* translation.

On the induction of oxidative stress PIF / dermcidin was observed to increase survival by 26% when assessed by MTT assay (not significant) and 42% when assessed by forward- and side-scatter on flow cytometry ( $P < 0.01$ ). The mode of death was necrosis, as assessed by annexin V, BOB78 and cleaved caspase 3 staining. Mutation of both asparagine residues in combination removed the protective effect of PIF / dermcidin but mutation of either N32Q or N44Q singly had no significant effect. *In vitro* translation did not reveal any difference in the molecular weights of PIF / dermcidin produced from native or mutant vectors.

These results demonstrate that PIF / dermcidin is capable of functioning as a survival factor in non-neuronal, transformed cells. This effect does not appear to require the N-glycosylation essential for muscle proteolysis but is inhibited by mutation of asparagine residues, potentially through an influence on the calcineurin-like phosphatase domain.

## Introduction

The first clue to the potential physiological function of the PIF peptide core came with the discovery of Y-P30 (Cunningham et al. 1998). This 30 amino acid peptide was purified from the medium of Y79 human retinoblastoma and the HN33.1 mouse hippocampal cell lines following their exposure to hydrogen peroxide ( $H_2O_2$ ). N-terminus sequencing of Y-P30 revealed a high degree of homology with PIF, as discussed in chapter 1. However, Y-P30 did not appear to be glycosylated and was capable of protecting cells from oxidative stress. Specifically, when either purified or synthetic, full-length Y-P30 were applied to differentiated HN33.1 cells (ie. multipolar cells with cell processes), both survival and differentiation following treatment with  $H_2O_2$  were improved (Cunningham et al. 1998). This finding was supported by *in vivo* work, demonstrating a reduction in pyramidal neuron loss and microglial infiltration in lesions of rat cerebral cortices treated with Y-P30 (Cunningham et al. 1998). More recently, the identification of the cDNA encoding Y-P30 facilitated transfection of HN33.1 cells, producing Y-P30 overexpressing cells (Cunningham et al. 2002). These similarly demonstrated improved survival and differentiation following stress with  $H_2O_2$ , incubation with activated macrophages and xenografting into the brains of immunocompetent rats, as assessed by microscopy and counting (Cunningham et al. 2002).

The function of Y-P30 as a survival factor therefore appears to confer a selective advantage on overexpressing neuronal cells both *in vitro* and *in vivo*. If this function were also to be active in tumour cells, it could help to explain the development of cachexia as cells would be selected for PIF expression. The metabolism and microenvironment of a tumour frequently results in the exposure of

cells to high levels of oxidative stress. (Spitz et al. 2000, Das 2002, Raghunand et al. 2003). This may induce death in susceptible cells and lead to genomic instability and drug resistance in those that survive, resulting in the positive selection and progression of resistant cells (Pelicano et al. 2004). A survival benefit of PIF / dermcidin expression may therefore have significant implications.

We subsequently aimed to determine whether PIF / dermcidin expression might contribute to the survival of tumour cells under conditions of oxidative stress. Furthermore, we sought to investigate whether there was a direct link between the function of PIF / dermcidin as a survival factor and its induction of muscle proteolysis, for which N-glycosylation is essential (Todorov et al. 1997). These aims were addressed by studying the survival of HuH7 cells transfected with native and mutant PIF / dermcidin-containing vectors under conditions of oxidative stress.

## **Materials and methods**

### **Induction of oxidative stress**

Cells were plated in 96 well plates (Gibco) at  $1.5 \times 10^4$  per well, 6 well plates (Gibco) at  $1 \times 10^6$  per well or 8-well chambered slides at  $2 \times 10^4$  per well and cultured overnight prior to the induction of oxidative stress by the addition of 75mU/ml glucose oxidase (Sigma) for 2 hours (cf. pp.46 and 48). Medium was then replaced and cells incubated under standard conditions for 4 hours.

### **MTT assays**

The survival of cells in 96 well plates was assessed by MTT assay (cf. p.73). 100µl of 5mg/ml MTT was added to each well and incubated for 4 hours at 37°C. 100µl per well of 10%SDS, pH 3.0 was then added and plates were incubated overnight at 37°C prior to reading at 570nm on an Assayzap plate reader.

### **Flow Cytometry**

Cells grown in 6 well plates were analysed by flow cytometry (cf. p.73). Following glucose oxidase treatment cells and their culture medium were harvested, pelleted and washed twice by resuspension in PBS and centrifugation at 1500xG for 5 minutes. Annexin V / PI flow cytometry was performed using a Bender Medsystems kit according to the manufacturer's instructions (Bender Medsystems). Cleaved caspase 3 flow cytometry was performed using the cleaved caspase-3 (Asp175) (5A1) rabbit monoclonal antibody (Cell Signaling) according to the

manufacturer's instructions. Flow cytometry using the BOB78 antibody at 1 in 100 and control antibody, also of IgM isotype (Sigma), was performed as described previously (Hart et al. 2000). The secondary antibody used was sheep anti-mouse IgG FITC (Dako, Ely, UK). Samples were analysed using a Coulter Epics XL flow cytometer.

### **Immunocytochemistry**

For immunocytochemistry experiments (cf. p.76) cells cultured in 8-well chambered slides were used. For the assessment of cell death with annexin V, BOB78 and anti-cleaved caspase 3, cells were treated with glucose oxidase and dead cells harvested from the medium. These were then cytospun onto positively charged slides at 300rpm for 3 minutes.

Annexin V staining was performed by incubation of cytospins for 5 minutes at room temperature in 50 $\mu$ l of annexin binding buffer containing 1.25 $\mu$ l annexin V and 1.25 $\mu$ l propidium iodide (Bender Medsystems).

BOB78 staining was performed by blocking for 1 hour at room temperature in 20% rabbit serum then staining for 1 hour at room temperature with BOB78 diluted 1/100 in PBS. Slides were washed in PBS then incubated at room temperature for 30 minutes with secondary rabbit anti mouse FITC conjugated immunoglobulins (Dako) diluted 1/100 in PBS. Cells were fixed in 3% paraformaldehyde for 5 minutes and mounted in fluorescent mounting medium (Dako) prior to visualisation on a Leica DMIRB fluorescent microscope (Wetzlar, Germany).

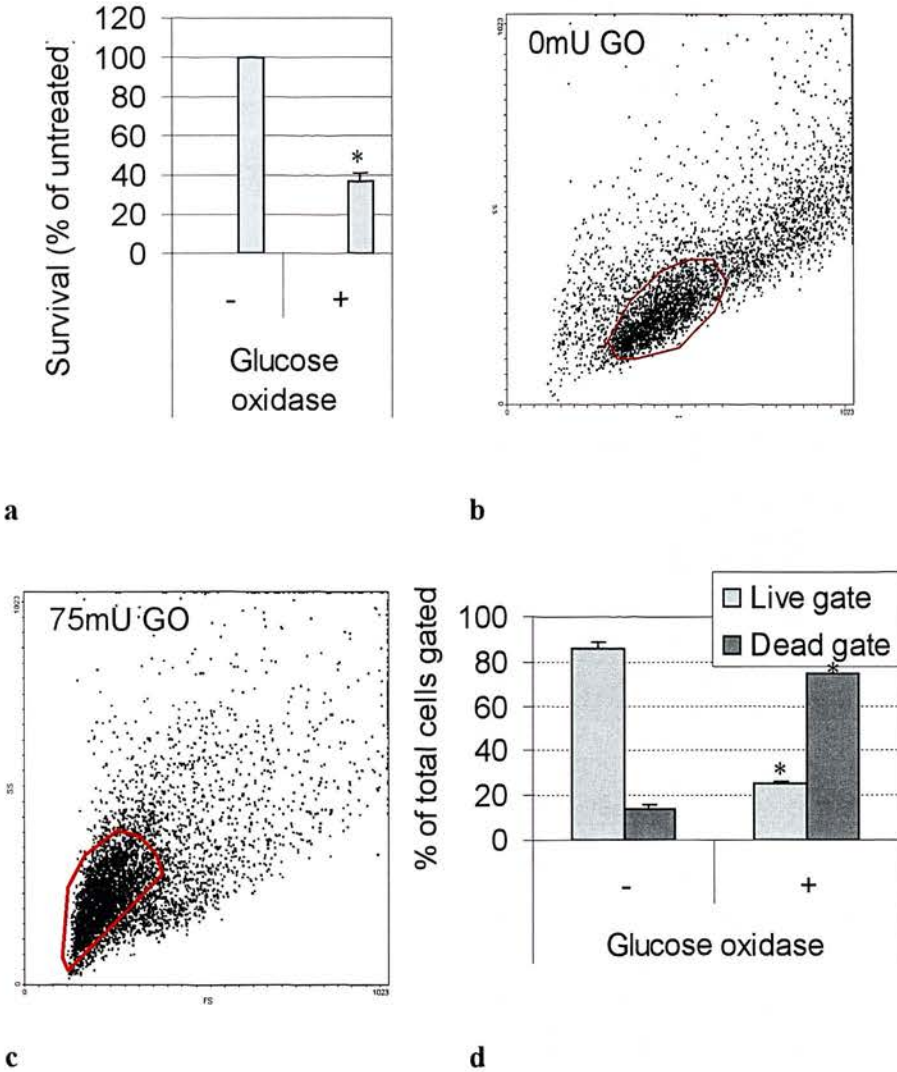
### ***In vitro* Translation**

*In vitro* translation was performed using a rabbit reticulocyte system (Promega) according to the manufacturer's instructions (cf. p.71). Samples were labelled with  $^{35}\text{S}$  cysteine (ICN). In selected experiments reactions were supplemented with 1.2 $\mu\text{l}$  canine pancreatic microsomal membranes (Promega). Samples were run on 10-20% tris-tricine gels (Biorad) and visualized by fluorography.

# Results

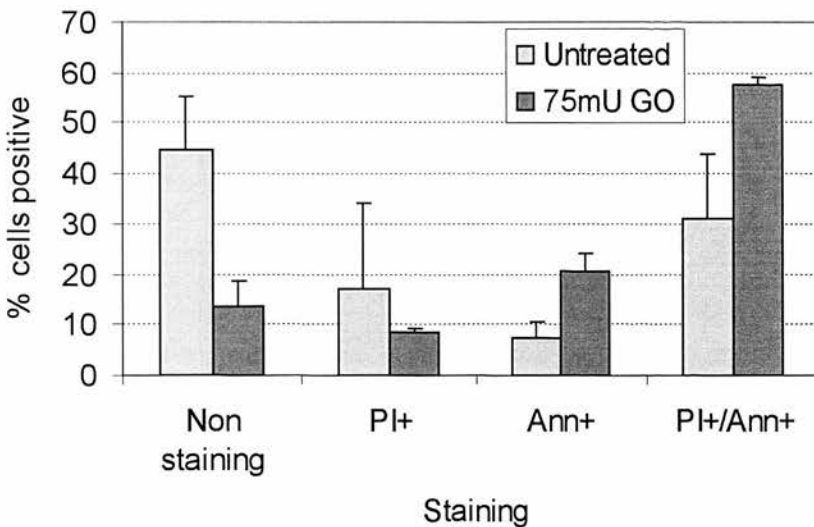
## Analysis of HuH7 cell response to oxidative stress with glucose oxidase

In order to assess the effect of glucose oxidase empty vector-transfected cells were analysed. Treatment for 2 hours with 75mU/ml glucose oxidase resulted in a decrease in survival of 66% on MTT assay (figure 5.1a). This was significant (Student's *t*,  $P < 0.02$ , 3 repeats).



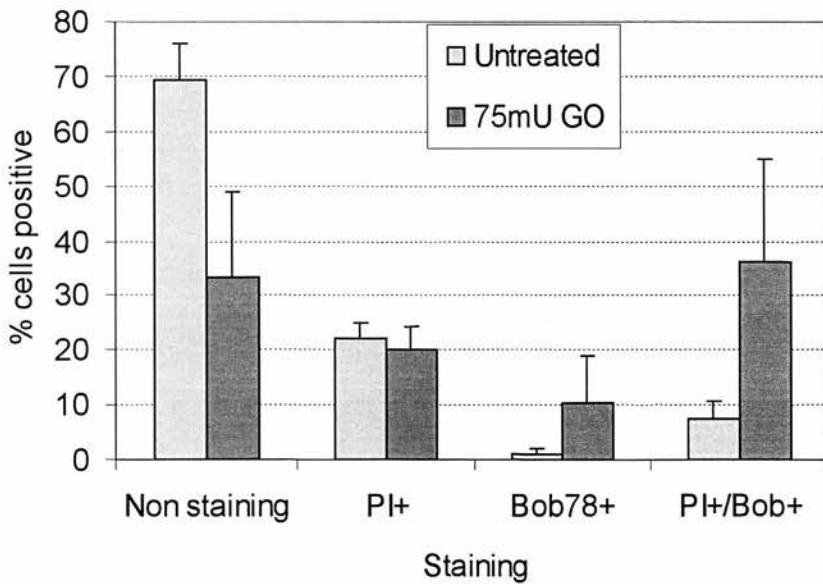
**Figure 5.1.** Effect of glucose oxidase on sham transfected HuH7 cells. **a.** MTT assay. **b.** Forward vs side-scatter, untreated and **c.** glucose oxidase treated. **d.** Quantification of live and dead gates.

On flow cytometry, glucose oxidase treatment resulted in a characteristic change in the forward- and side-scatter of cells suggesting an increase in cell death (figure 5.1b, c). 82% of untreated cells were gated as live and 18% as dead (figure 5.1d). Glucose oxidase treatment decreased the live gate to 29%, a 65% reduction (figure 5.1d). There was a commensurate increase in the dead gate to 71%. This effect was significant (Student's *t* test,  $P < 0.05$ , 3 repeats). Analysis of the annexin V staining patterns of dead-gated cells revealed that untreated, 7% stained with annexin only, 17% with PI only, 31% with both and 45% with neither (figure 5.2). When treated with glucose oxidase, 20% stained with annexin only, 8% with PI only, 58% with both and 14% with neither. These differences were not significant despite 3 repeats of the experiment.

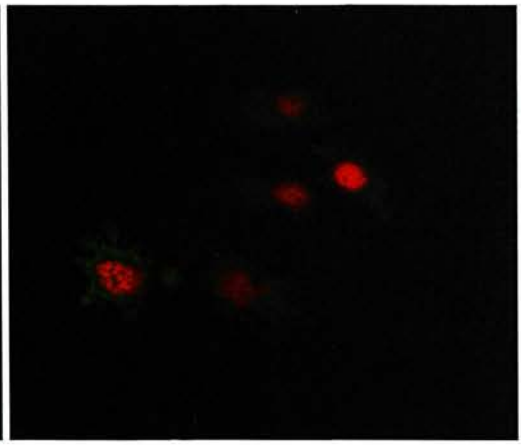
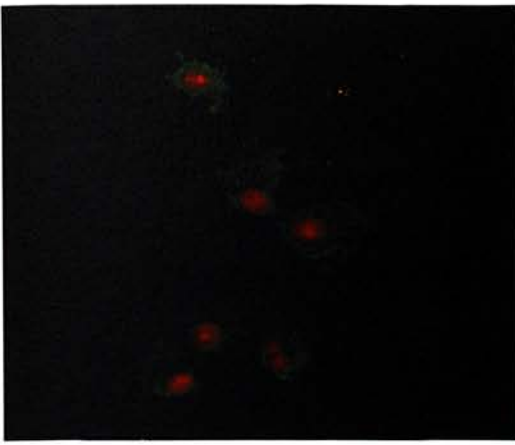


**Figure 5.2.** Effect of glucose oxidase treatment on annexin / propidium iodide staining of sham transfected HuH7 cells.

On staining of dead-gated cells with the BOB78 antibody, a surface marker of apoptosis (Hart et al. 2000), 1% stained with BOB78 only in the untreated sample, 22% with PI only, 7% with both and 70% with neither (figure 5.3). On glucose oxidase treatment, 10% stained with BOB78 only, 20% with PI only, 36% with both and 33% with neither. These differences were not significant despite 3 repeats of the experiment. Dead-gated HuH7 cells did not stain for cleaved caspase 3. These data suggested that glucose oxidase induced necrosis of HuH7 cells. This was supported by the results of immunocytochemistry with both annexin V and BOB78 (figure 5.4a, b) in which cells appeared predominantly necrotic both in morphology and staining pattern.



**Figure 5.3.** Effect of glucose oxidase treatment on BOB78 / propidium iodide staining of sham transfected HuH7 cells



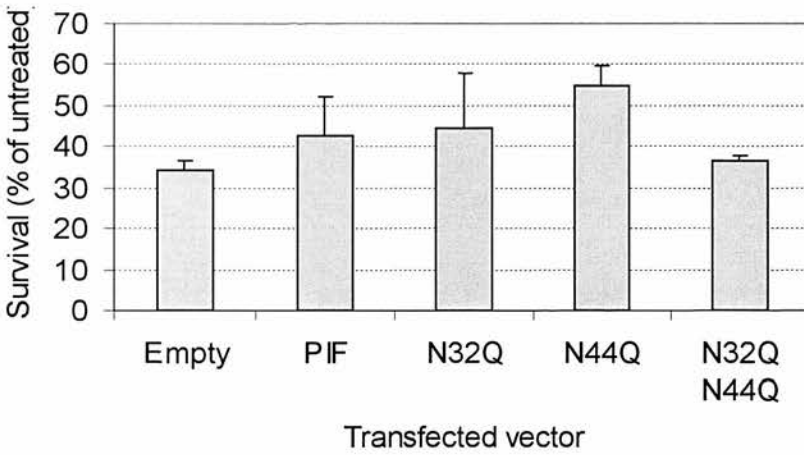
**a**

**b**

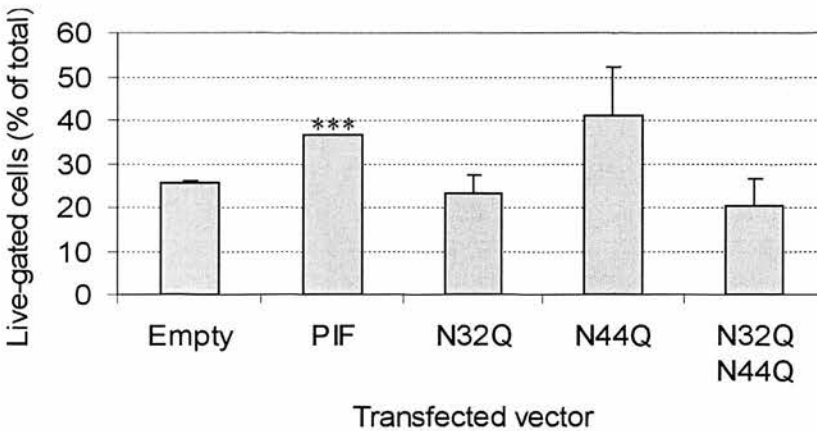
**Figure 5.4.** Effect of glucose oxidase on cell morphology and staining patterns. **a.** Annexin V / propidium iodide. **b.** BOB78 / propidium iodide.

## Effect of PIF / dermcidin on the response of HuH7 cells to oxidative stress

Following glucose oxidase treatment the mean survival of HuH7 cells as assessed by MTT assay was increased from 34% to 43% by transfection of the PIF / dermcidin-containing vector, a survival benefit of 26% (figure 5.5a). However, despite 5 repeats of the experiment this difference did not reach significance. On flow cytometry, forward- and side-scatter gating demonstrated an increase in live-gated cells from 26% to 37% following PIF / dermcidin transfection, a survival benefit of 42% (figure 5.5b). This was significant (Student's *t*,  $P < 0.01$ , 3 repeats).



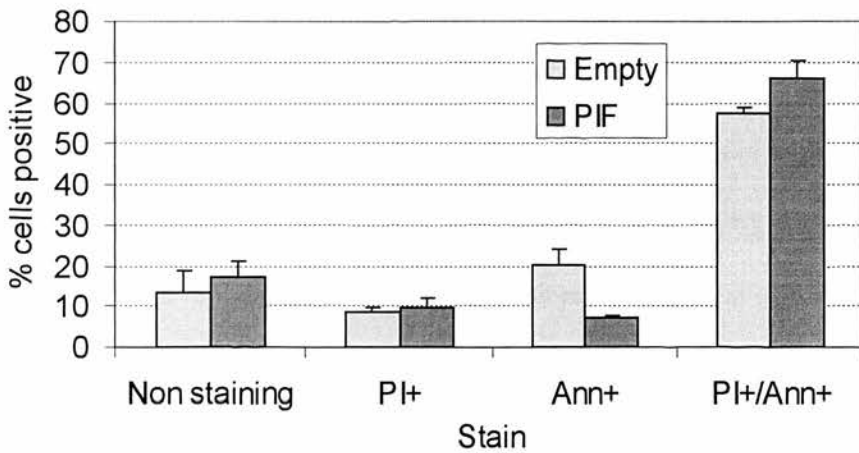
**a**



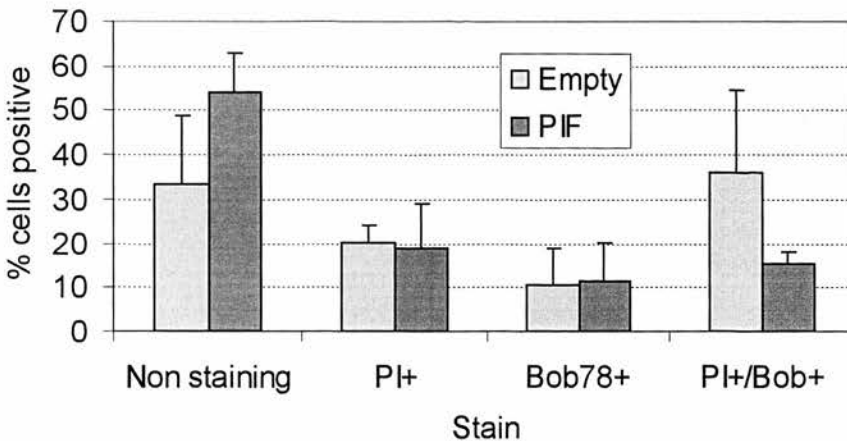
**b**

**Figure 5.5.** Effect of PIF / dermcidin transfection on survival following glucose oxidase treatment. **a.** MTT assay. **b.** Flow cytometry, forward and side scatter. \*\*\* $P < 0.01$ .

Analysis of the dead-gated cells revealed a largely unchanged pattern of annexin V staining following PIF / dermcidin transfection without glucose oxidase treatment. However, on glucose oxidase treatment, PIF / dermcidin transfected cells demonstrated a further decrease in the small proportion of dead cells staining with annexin only in favour of double annexin / PI staining (figure 5.6a), although this was not significant (3 repeats). BOB78 staining was also affected, with a decrease in total (BOB78 alone or double BOB78 / PI) staining (figure 5.6b). No staining of HuH7 cells was observed with anti-cleaved caspase 3 antibody.



a

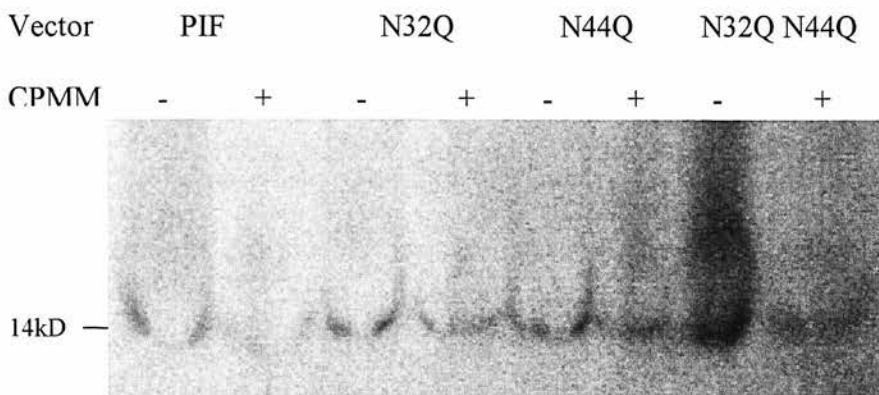


b

**Figure 5.6.** Effect of PIF / dermcidin transfection on flow cytometric staining patterns following glucose oxidase treatment. **a.** Annexin V / propidium iodide. **b.** BOB78 / propidium iodide.

## Effect of site-directed mutagenesis of asparagine residues

As previous studies have reported heavy N-glycosylation of the PIF peptide (Todorov et al. 1997), we substituted glutamine for each of the two asparagine residues in the PIF / dermcidin polypeptide sequence, both singly and in combination. *In vitro* translation of these mutant vectors with <sup>35</sup>S-cysteine resulted in products of identical molecular weight to wild-type PIF / dermcidin (figure 5.7). When transfected into HuH7 cells, the single mutations N32Q and N44Q conferred the same survival benefit as wild-type PIF / dermcidin as assessed by the MTT assay (figure 5.5a). However, on flow cytometry the N32Q mutation abrogated the effect on cell survival, whereas the N44Q mutation had no effect (figure 5.5b). Double mutation of N32 and N44 removed the ability of PIF / dermcidin to protect cells as assessed by both MTT assay and flow cytometry (figure 5.5a, b). Although these differences were replicated 3 times they did not reach statistical significance. No difference in the annexin V and BOB78 staining patterns between N32Q, N44Q and PIF / dermcidin or N32Q N44Q and sham transfectants was observed.



**Figure 5.7.** *In vitro* translation of PIF / dermcidin asparagine mutants.

## Discussion

Previous studies have shown that glucose oxidase induces necrosis in hepatic cells through the generation of a constant dose of H<sub>2</sub>O<sub>2</sub> and subsequently free oxygen radicals (Lemasters 1999). In HuH7 cells we also found that cell death occurred almost exclusively by necrosis. In particular, there was no activation of cleaved caspase 3, a mediator common to both the receptor-activated and intrinsic pathways of apoptosis which is known to be functional in HuH7 cells (Ashkenazi and Dixit 1998, Feng and Kaplowitz 2002). The low level of annexin staining in comparison with double annexin / PI staining on both flow cytometry and immunocytochemistry was also consistent with necrotic cell death. In addition, BOB78 staining showed a similar pattern of changes to annexin V. We have previously demonstrated that BOB78 antigen translocation to an intact plasma membrane is an indicator of apoptosis (Hart et al. 2000). Further repeats will help improve the reproducibility of annexin V and BOB78 flow cytometry.

In the glucose oxidase system, we demonstrated a highly significant survival benefit of PIF / dermcidin expression using forward and side scatter gated flow cytometry. On MTT assay a similar but non-significant effect was observed. Shifts from cell necrosis to apoptosis are known to occur in primary hepatocytes, where inhibition of caspases converts apoptotic to necrotic cell death in the presence of an overwhelming death stimulus from staurosporine (Feng and Kaplowitz 2002). However, this did not occur in the HuH7 cells, as cleaved caspase 3 was not activated. In addition, although not significant the main changes in annexin V staining PIF / dermcidin transfection were a fall in annexin-only and a rise in double annexin / PI staining, likely to be due to the increased proportional effect of live cells

falling into the dead gate. PIF / dermcidin therefore appears to improve survival by decreasing necrosis. The mechanisms by which this occurs remain to be determined.

We attempted to correlate the ability of PIF / dermcidin to promote survival with structural mutations designed to alter N-glycosylation. Previous studies have suggested heavy N-glycosylation of up to 10kD, possibly at one site in the 24kD moiety (Todorov et al. 1997). Treatment of PIF with peptide N-glycosidase F destroys its antigenic and cachexia-inducing properties (Todorov et al. 1997). However, when we eliminated either of the 2 N-glycosylation sites at N32 and N44 singly, no significant effect on the survival benefits of PIF / dermcidin expression was observed. In contrast, mutation of both sites in combination appeared to inhibit the survival-promoting effect of wild-type PIF / dermcidin. None of the mutations altered the molecular weight of PIF / dermcidin produced by *in vitro* translation. These results are interesting as they appear to suggest that in a cell free translation system, PIF / dermcidin is not substantially glycosylated at either N-residue. This is feasible as neither site contains a typical consensus N-glycosylation sequence, although N32 has characteristics of an atypical site (Welply et al. 1983, Sato et al. 2000). The loss of protection on mutation of both asparagine residues therefore suggests that either one of the sites must be glycosylated for this effect or that the double mutation interferes with function in another way, for example by structural modification which alters receptor binding. In light of the lack of consensus glycosylation sites or glycosylation in the *in vitro* translation system, the latter explanation seems more likely. In addition, there is no evidence that the Y-P30 / DSEP molecule is glycosylated (Cunningham et al. 1998, Cunningham et al. 2000). Interestingly, the phosphatase domain identified in Y-P30 as similar to the similar to

that of calcineurin lies between N32 and N44 (Cunningham et al. 1998, Cunningham et al. 2000). X-ray crystallography has shown that asparagine residues contribute to this domain in calcineurin (Rusnak and Mertz 2000). It is therefore feasible that loss of both N residues but not one alone is sufficient to impair such phosphatase activity. Further structural studies will be help to determine whether it is indeed this domain which is responsible for PIF / dermcidin's survival effect.

## 6. Analysis of the induction of cell proliferation by proteolysis inducing factor

### Summary

It has been suggested that DCD acts as a growth factor in breast cancer cells (Porter et al. 2003). In their study, Porter et al. stably transfected an alkaline phosphatase tagged CDC construct into the 21NT breast carcinoma cell line and observed an increase in cell numbers at 1,3,5 and 7 days following seeding. This raises several questions. Firstly, which peptide induces proliferation, DCD or PIF / Y-P30, secondly, can the increase in cell numbers be ascribed to an increase in cell cycling and thirdly, is proliferation also induced in other cell types and particularly in HuH7 cells, which do not express PIF / dermcidin endogenously.

To address these questions we utilized two approaches. Firstly, the proliferation of HuH7 cells stably transfected with wild-type and mutant PIF / dermcidin vectors was assessed. Secondly, a synthetic PIF peptide was used to treat HuH7 cells. Cell cycling was quantified by direct counting of cells labelled with bromodeoxyuridine (BrDU) and visualized by immunocytochemistry.

Cells stably transfected with wild-type PIF / dermcidin had a higher rate of proliferation than cells transfected with empty vector ( $P < 0.02$ ). This increase in proliferation was inhibited by mutation introducing a STOP codon immediately following the signal peptide sequence but not by mutation introducing a STOP codon immediately 3' to the sequence encoding the PIF peptide ( $P < 0.02$ ). Mutation of the residue corresponding to the most highly conserved amino acid in the calcineurin phosphatase domain, histidine 35, to asparagine also prevented PIF / dermcidin

induced cell proliferation. Treatment of cells with the synthetic PIF peptide resulted in significant HuH7 cell proliferation with a bell-shaped dose-response curve ( $P < 0.05$  at peak).

These results suggest that the PIF core peptide is sufficient to induce cell proliferation and that the phosphatase domain may be involved in mediating this effect. These findings confirm the potential of PIF to act as an oncogene, promoting the selection of clones with the capacity to induce cachexia.

## Introduction

The first direct evidence that PIF / dermcidin might stimulate cell proliferation came from an investigation into the effect of DSEP overexpression in neural cells (Cunningham et al. 2002). Here, it was found that overexpression decreased the ability of all-*trans*-retinoic acid to slow cell proliferation. Just under three times more overexpressing cells than sham transfectants were counted at 3 days post treatment. More recently, transfection of dermcidin (essentially the same cDNA as DSEP) has been demonstrated to increase cell numbers in breast cancer cell lines (Porter et al. 2003). In this study, increased numbers of 21NT breast cancer cells were observed at 5 and 7 days when stably transfected with a full-length DCD-containing construct as opposed to the empty vector. In both the DSEP and DCD studies the full length cDNA was used, and it is therefore not clear whether it is the Y-P30, DCD-1 or another peptide that induces proliferation. Both studies also demonstrated an improvement in cell survival following oxidative stress, but the increase in numbers remains more likely to be accounted for by increased proliferation than by decreased death as the cells were not stressed in proliferation assays.

Several features of PIF / dermcidin's structure and the signalling pathways it induces are consistent with a proliferative effect. Structural analysis of the PIF polypeptide has demonstrated cleavage of a signal peptide and targeting to the secretory pathway (chapter 3). An extracellular action is consistent with a role as a growth factor and therefore an oncogene (Porter et al. 2003). How this may be mediated is unclear. The central sequence of the PIF core peptide has homology with the phosphatase of calcineurin (Cunningham et al. 1998), but although calcineurin

may result in T-cell activation (Bommireddy et al. 2003), it may inhibit proliferation in neoplastic cells via cdk-4 dephosphorylation (Baksh et al. 2000). However, PIF / dermcidin may have different targets to those calcineurin and it is feasible that dephosphorylation of these targets may result in cell proliferation. There is evidence that calreticulin and low- and high-affinity receptors in 21NT cells, breast cancer tissue and human brain all interact with PIF / dermcidin (Cunningham et al. 2000, Porter et al. 2003). It has also been suggested that there exists a membrane receptor for PIF on skeletal muscle (Smith and Tisdale 2003, Waddell et al, unpublished observation). It subsequently appears feasible that different receptors may exist for glycosylated and unglycosylated PIF. It is likely that at least one of these receptors has significant functional effects as several signal transduction pathways have been shown to respond to PIF.

Perhaps the PIF-induced pathway with the most significant proliferative effects is that of NF- $\kappa$ B. PIF has been demonstrated to induce activation of NF- $\kappa$ B in hepatic cells (Watchorn et al. 2001). NF- $\kappa$ B induces proliferation in a variety of cell types including pancreatic carcinoma and hepatic cells (Kirillova et al. 1999, Liptay et al. 2003, Li et al. 2004a, Yao et al. 2004). In addition, the STAT3 transcription factor is also known to be activated by PIF treatment. STAT3 has also been demonstrated to be involved in the proliferation of pancreatic carcinoma and hepatic cells (Kirillova et al. 1999, Kountouras et al. 2001, Scholz et al. 2003). Upstream of transcriptional regulation, PIF has been shown to induce signalling in skeletal muscle via the release of arachidonic acid and its conversion to 15(*S*)-hydroxyeicosatetranoic acid (15(*S*)-HETE). 15(*S*)-HETE has been demonstrated to induce the proliferation of leukaemia cells (Postoak et al. 1990). In skeletal muscle, it

has been proposed that 15(S)-HETE leads to activation of the ubiquitin-proteasome pathway and this may also have pro-proliferative effects in pancreatic cancer (Shah et al. 2001). It can therefore be seen that many of the signal transduction pathways putatively activated by PIF / dermcidin have the potential to induce cell proliferation.

In this chapter we describe our investigation of two aspects of PIF / dermcidin's effect on cell proliferation. Firstly, we have aimed to confirm definitively that cell cycling was responsible for the increases in cell numbers found in other studies. In order to achieve this, we utilized bromodeoxyuridine staining. This technique labels newly formed DNA allowing cells in S-phase of the cell cycle to be identified with an anti-BrDU antibody on immunocytochemistry.

Secondly, we sought to determine the portion of the PIF / dermcidin polypeptide responsible for the induction of cell proliferation. Two approaches were used, the synthesis of a peptide containing the full-length PIF core / Y-P30 sequence and the analysis of the proliferative effects of truncation mutants generated by the introduction of STOP codons into the PIF / dermcidin cDNA sequence. Mutants containing the asparagine to glutamine substitutions N32Q, N44Q and N32QN44Q and the histidine to asparagine substitution H35N were also examined to investigate the potential effects of loss of N-glycosylation and phosphatase activity on proliferation.

## **Materials and methods**

### **PIF / dermcidin mutants**

PIF / dermcidin mutants were created by site-directed mutagenesis of the pcDNA3.1+PIF vector and transfected into HuH7 cells as described in materials and methods (cf. pp.69 & 47). The vectors used in experiments were empty vector (pcDNA3.1+), native pcDNA3.1+PIF, pcDNA3.1+PIF ProP STOP, pcDNA3.1+PIF Y-P30 STOP, pcDNA3.1+PIF H35N, pcDNA3.1+PIF N32Q, pcDNA3.1+PIF N44Q and pcDNA3.1+PIF N32QN44Q.

### **Synthetic PIF peptide**

A 30 amino acid peptide incorporating the full length PIF core / Y-P30 sequence was designed (cf. p.49). The full sequence was – YDPEAASAPGSGNPCHEASAAQKENAGEDP. Custom synthesized peptide of >95% purity was purchased from Albachem.

### **Cell culture**

The HuH7 cell line was cultured under standard conditions as described in materials and methods (cf. p.46). For use in experiments, cells were seeded in 8-well chambered slides at a density of 10 000 per well and allowed to settle overnight. In experiments using the synthetic peptide, peptide was added at a range of concentrations and cells cultured under standard conditions for a further 24 hours prior to BrDU immunocytochemistry.

For experiments using the mutant PIF / dermcidin vectors cells were stably transfected as described above. Following seeding and overnight incubation BrDU immunocytochemistry was performed.

### **BrDU labelling and immunocytochemistry**

Following a 1 hour incubation in BrDU (Proliferating cell labelling reagent, Amersham) cells were fixed in 80% ethanol and subjected to standard BrDU immunocytochemistry as described in materials and methods (cf. p.79). Cells were then counted under direct vision using a graticule. Each sample was plated in 3 chambers and at least 500 cells per chamber were counted and marked as positive or negative. Experiments were performed in triplicate.

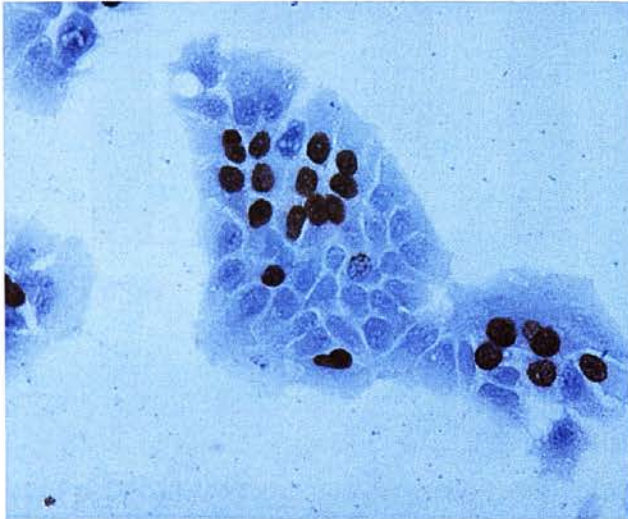
### **Assessment of transfectants**

In order to assess PIF / dermcidin expression in transfected cells, Western blots of cell lysates were performed as described in materials and methods (cf. p.59). The primary antibody used was polyclonal anti-PIF / dermcidin rabbit antisera (Sigma). Blots were visualized with a goat anti-rabbit peroxidase-linked secondary antibody (Santa Cruz) and an ECL kit (Amersham).

## Results

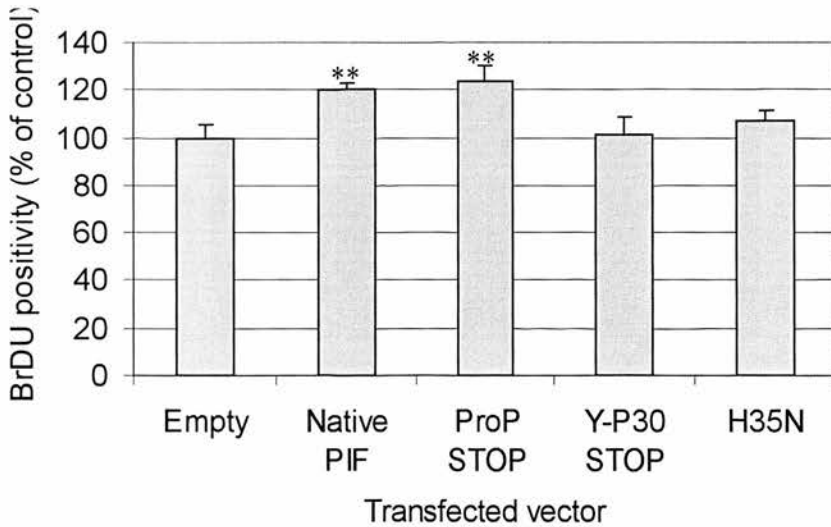
### Assessment of cell proliferation in transfected HuH7 cells

In experiments examining the effects of transfection of vectors containing native PIF / dermcidin, the truncation mutants ProP STOP and Y-P30 STOP or the H35N mutation, BrDU immunocytochemistry resulted in readily distinguishable positive and negative nuclear staining (figure 6.1).



**Figure 6.1.** BrDU staining of pcDNA3.1(+) transfected HuH7 cells.

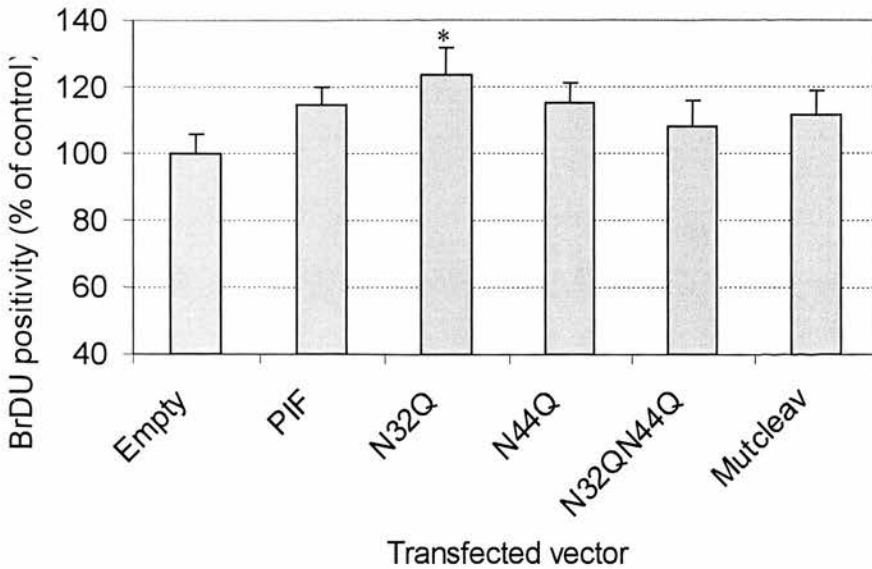
Stable transfection of the PIF / dermcidin-containing vector resulted in a 20% increase in cells positive for BrDU in comparison with transfection of the empty vector (figure 6.2). This was significant (Student's *t* test,  $P < 0.02$ , 8 repeats). The effect was abrogated by the H35N and Y-P30 STOP mutants but not by the ProP STOP mutant, transfection of which also resulted in a significant increase in cell numbers (figure 6.2) (Student's *t* test,  $P < 0.02$ , 8 repeats).



**Figure 6.2.** Effect of pcDNA3.1(+) insert on proportion of transfected HuH7 cells staining for BrDU, truncation mutants. \*\* $P < 0.02$ .

In experiments examining the effects of the asparagine mutations N32Q, N44Q and N32QN44Q and the Mutcleav mutation, no difference in cell morphology was observed on BrDU staining. In these experiments, cells transfected with the native PIF / dermcidin-containing vector demonstrated a 14% increase in proliferation over controls (figure 6.3). This was increased to 24% in cells

transfected with the N32Q mutant vector (significant at  $P < 0.05$ , Student's *t* test, 3 repeats). Cells transfected with the N44Q vector showed no significant difference in proliferation in comparison with those transfected with native PIF / dermcidin, demonstrating an increase in proliferation of 15% above control. The N32QN44Q and Mutcleav vectors had intermediate effects on cell proliferation with transfected cell demonstrating 8% and 11% increases above controls respectively.



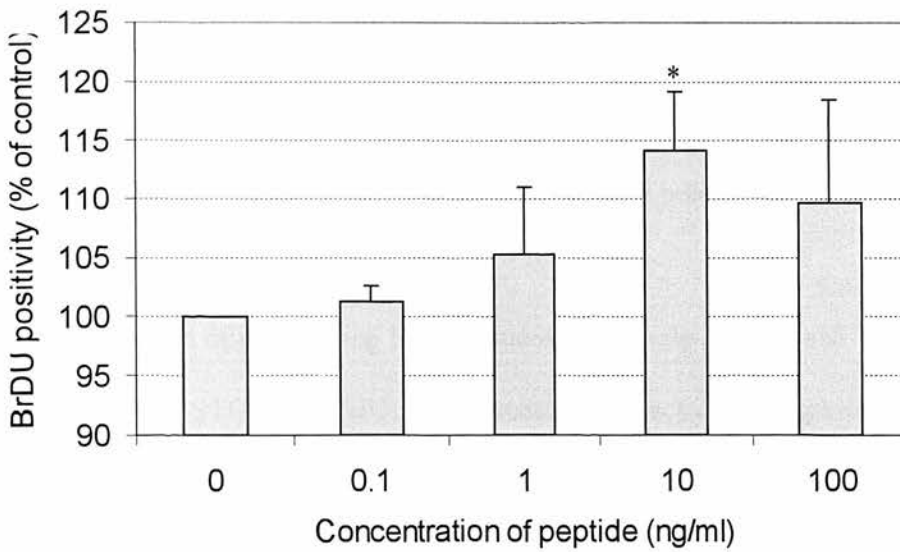
**Figure 6.3.** Effect of pcDNA3.1(+) insert on proportion of transfected HuH7 cells staining for BrDU, asparagine mutants and mutcleav. \* $P < 0.05$ .

### Assessment of PIF / dermcidin expression in transfectants

The assessment of PIF / dermcidin expression in cells transfected with the N32Q, N44Q, N32QN44Q and Mutcleav vectors has been described in chapter 4. Western blotting of lysates from cells transfected with the ProP STOP, Y-P30 STOP and H35N mutant PIF / dermcidin vectors similarly demonstrated bands at 66kD in all instances.

### Induction of cell proliferation by a synthetic PIF peptide

When HuH7 cells were treated with the synthetic PIF peptide there was no identifiable change in cell morphology. Cell counts for BrDU positivity revealed that the peptide increased cell proliferation (figure 6.4). The response had a bell-shaped dose-response curve and produced a maximum increase of 14% at a peptide concentration of 10ng/ml (significant at  $P < 0.05$ , Student's *t* test, 8 repeats)



**Figure 6.4.** Effect of HuH7 cell treatment with the PIF core peptide on BrDU positivity. \* $P < 0.05$ .

## Discussion

The results of transfection experiments suggest that PIF / dermcidin expression induces cell proliferation. At 20%, the magnitude of the increase is significant as this represents the increase in the proportion of cycling cells following a 24 hour incubation, which would therefore be expected to have an exponential effect on cell numbers over time. This is consistent with the previous studies on the proliferative effects of DSEP (Cunningham et al. 2002, Porter et al. 2003). Furthermore, the increased proportion of cells in S phase revealed by bromodeoxyuridine staining suggests that PIF / dermcidin may act by increasing entry to the cell cycle rather than decreasing apoptosis or inducing population expansion by another mechanism. Such an effect could potentially be mediated at the G<sub>1</sub>-S checkpoint.

The effect of introducing STOP codons prior to the DCD-1 and Y-P30 sequences (ProP STOP and Y-P30 STOP mutants respectively) suggests that the PIF core / Y-P30 sequence is required for the induction of cell proliferation. When the sequence 3' to Y-P30 including the propeptide and DCD-1 coding areas (ProP STOP) was knocked out there was little effect on PIF / dermcidin-induced proliferation but this was abrogated by further truncation, removing the Y-P30 sequence. As discussed above, the CMV promoter is active in HuH7 cells and the appropriate use of controls means that the only factor which should be able to account for the differences in proliferation observed is the difference in vector sequence introduced by site-directed mutagenesis. In addition, experiments using the synthetic PIF peptide demonstrate that the PIF core peptide is sufficient to induce proliferation. The bell-shaped dose-response curve is characteristic of biological

responses to several cytokines and growth factors including IFN- $\gamma$ , IL-6, IL-8, TNF- $\alpha$  and IL-1 (Talmadge et al. 1987, Utsunomiya et al. 1996, Ben-Baruch et al. 1997, Tsigos et al. 1999). Furthermore, it has been suggested that bell-shaped dose-response curves may result from the action of an agonist on two separate receptors (Ashby 1990). It is therefore intriguing to suppose that HuH7 cells may express both the high- and low-affinity PIF / dermcidin receptors previously reported (Porter et al. 2003), one of which may stimulate proliferation and one which may inhibit it. It is highly unlikely that the effects are due to any contaminant of the peptide as synthesis is performed from the constituent amino acids entirely by machine. This eliminates the potential for endotoxin or any other peptide product of PIF / dermcidin to induce the proliferation observed. It is hoped that further studies using the *in vitro* translation system will confirm that the STOP codons introduced indeed truncate the polypeptide, but due to the problems with anti-PIF antibodies (cf chapter 7) and the requirement for separate antibodies to detect the product of each mutant vector, one of which potentially produces only a ubiquitous signal peptide, it does not seem feasible to confirm the truncations in HuH7 cells.

The finding that PIF / dermcidin induced cell proliferation is prevented by the N32QN44Q and H35N mutations further supports the role of the PIF core in the induction of proliferation, as both of these mutations lie within it. These mutations also lie within the calcineurin phosphatase-like domain (Cunningham et al. 1998). Histidine is the most highly conserved amino acid across the calcineurin-like family of phosphatases (Rusnak and Mertz 2000) and its removal has been demonstrated to cause a significant decrease in enzyme activity (Zhuo et al. 1994, Mertz et al. 1997). It is therefore feasible that its replacement with asparagine in the H35N mutant

reduces phosphatase activity. The effect of the N32QN44Q mutant is less clear. As described above, this mutation was designed to prevent N-glycosylation of PIF, but we have been unable to demonstrate this in HuH7 cells. However, the single mutations N32Q and N44Q did not affect PIF / dermcidin-induced cell proliferation. In the calcineurin phosphatase domain, the role of asparagine residues has not been investigated, although they are a conserved part of the active phosphatase site (Rusnak and Mertz 2000). It is therefore feasible that mutation of both asparagines disrupts the phosphatase site but mutation of either alone is insufficient to do so. One way to test this hypothesis would be to extend the studies of para-nitrophenyl phosphate cleavage by Y-P30 performed by Cunningham et al. (1998). In particular, it would be possible to synthesize peptides containing the H35N and N32QN44Q mutations and compare their ability to induce proliferation with that of the native peptide. These studies will cast further light on the mechanism which allows PIF / dermcidin to act as an oncogene (Porter et al. 2003).

## 7. Investigation of urinary PIF expression and proteomic profiles

### Summary

In previous studies, detection of urinary PIF has been the gold standard for identification of PIF production and is the measure which has been correlated with weight loss. We therefore set out to investigate the link between the presence of urinary PIF and PIF production in patients with pancreatic carcinoma. However, during the course of our studies, other workers in our laboratory demonstrated that the anti-PIF antibody used in Western blotting of urines also recognizes CD59, a ubiquitous cellular marker of epithelial lineage. We therefore sought an alternative method of identifying urinary PIF. This led to the development of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry for urine analysis.

Nineteen patients undergoing surgery for pancreatic carcinoma were recruited and nutritional and prognostic information was collected. Urine and tissue samples were obtained immediately prior to and during surgery respectively. For mass spectrometry, urine was concentrated by a factor of 100 using an ultracentrifugation filter cell. Samples were analysed by matrix-assisted laser desorption-ionisation time-of-flight mass spectrometry using a Voyager-DE STR mass spectrometer. In order to identify specific urinary proteins, concentrated urines were run on polyacrylamide gels and bands excised and subjected to tryptic digest. Peaks obtained from these bands on mass spectrometry were compared with those of known and predicted tryptic digest products using the MIS-FIT database.

Full mass profiles of the urine samples revealed peaks at 23 500, 9 750 and 6 200 which were not present in urines from 4 non-matched healthy male volunteers

aged between 25 and 30 years. There was no significant link between the presence of any band and weight loss or duration of survival. When samples were run on polyacrylamide gels, protein bands did not correspond exactly to these peaks but were identifiable at 15, 23, 30 and 40kD. Tryptic digests of these bands identified in them mitogen-activated protein kinase kinase kinase 3,) and APC-binding protein EB1, which have not previously been reported in normal urine and immunoglobulin light chain, keratin,  $\alpha$ -1 microglobulin and mannose binding lectin-associated serine protease-2 related protein, which have previously been found in normal urine. PIF was not identified.

Mass spectrometry is a promising technique for the analysis of urine. The identification of cachexia-associated factors may in future provide a method of screening patients for early nutritional intervention. Further studies of the statistical significance of these factors and refinements in MALDI-TOF technique have the potential to yield great advances in this field.

## Introduction

Following the discovery of PIF, the importance of urinary PIF as a nutritional indicator in pancreatic cancer was one of the main areas addressed by researchers, establishing the clinical relevance of the molecule. As discussed in chapter 1, in humans PIF was first isolated from the urines of patients with carcinoma of the pancreas, in whom an association with weight loss was reported (Todorov et al. 1996a). The same study demonstrated PIF in the urines of patients with carcinomas of the lung, breast, ovary and one patient with T-cell lymphoma. Taken with the lack of PIF in the urines of non-weight losing pancreatic carcinoma patients or those of patients with weight loss due to major burns, multiple injuries or surgery-associated catabolism and sepsis, this suggested that the presence of urinary PIF was specific to the cachectic state in malignancy and was not restricted to a particular type of cancer.

Subsequent studies examined the nutritional status of PIF-positive patients with pancreatic carcinoma in more detail. Urinary PIF was demonstrated in 80% of patients with pancreatic carcinoma and this group was found to have a significantly greater total weight loss and rate of weight loss than patients whose urine did not contain PIF (Wigmore et al. 2000). In addition, the presence of PIF was independent of serum CRP concentration, the acute-phase response and survival (Wigmore et al. 2000), suggesting that it is not a by-product of the inflammatory response to malignancy, although it remains possible that PIF may be produced by the liver or another tissue as part of another response to malignancy.

In this study, we sought to determine urinary PIF levels in patients with pancreatic carcinoma as part of their nutritional assessment prior to the determination of tumour PIF production (cf. chapter 8). As described above, a monoclonal antibody

to a cachexia-inducing factor present in the serum of mice was originally used to purify PIF from the MAC16 tumour (McDevitt et al. 1995, Todorov et al. 1996a, Todorov et al. 1996b). Subsequently, the same antibody was used to purify PIF from the urines of weight-losing patients with pancreatic carcinoma (Cariuk et al. 1997) and Western blotting became the standard technique for PIF detection (Todorov et al. 1996a, Todorov et al. 1996b, Wigmore et al. 2000). The Western blotting technique used was straightforward, concentrating urines by ammonium sulphate precipitation then running 5µg protein per well on a 15% polyacrylamide gel prior to transfer to nitrocellulose and probing using the antibody described, which had been biotinylated (Wigmore et al. 2000). However, a high degree of urine concentration was required, and it was not possible to isolate PIF from plasma due to the higher overall and relative protein concentrations.

When workers in our laboratory attempted to identify PIF in urine samples which had previously been found to be positive a band of molecular weight 24kD could not be identified. Instead, a 21kD band was found. When excised and sequenced, this was identified as CD59, a ubiquitous cellular marker of epithelial lineage which is also N-glycosylated (Venneker and Asghar 1992). It was therefore concluded that although PIF may previously have been found using this antibody, in our hands it did not provide the necessary specificity, potentially due to a cross-reaction with a common carbohydrate antigen on CD59. We subsequently sought a new assay to meet several criteria. Firstly, it should be suitable for the analysis of urine in order to validate earlier studies. Secondly, it should provide definitive and reproducible protein identification. Thirdly, it should be fast, as a high number of

specimens were to be analysed. Having considered these criteria, we settled on the technique of mass spectrometry.

Mass spectrometry, and in specific, matrix-assisted laser desorption ionisation-time-of-flight (MALDI-TOF) mass spectrometry, is an enormously sensitive technique which allows the determination of the precise mass of a protein or of peptides derived from it by enzymatic or chemical cleavage. The latter provides a unique 'fingerprint' for each protein which can subsequently be identified by screening against databases in which the masses of all proteins and their predicted peptide fragments are held. In MALDI-TOF mass spectrometry this is achieved by mixing samples with an organic acid and drying them onto a metal slide. The samples are then targeted with a laser, resulting in their conversion to an ionized gas in which each protein or peptide contains at least one positive charge. They are then accelerated through an electric field towards a detector. The time taken to reach the detector is dependant on the mass and charge of the sample, allowing determination of the precise mass from ions with one positive charge. This technique is capable of identifying proteins from samples in the nanogram range with an error rate of  $<1$  in  $10^6$ .

Mass spectrometry has been used extensively in the field of proteomics, the attempt to characterise all of the proteins encoded in an organism's genome including their post-translational modifications. However, in each organism, different cells express different sets of proteins which may be influenced by the conditions the cell is subject to. Examination of these proteomes is facilitated by mass spectrometry and can provide a valuable insight into disease by identifying differences in the proteins expressed by normal and abnormal cells. In cancer, MALDI-TOF mass spectrometry

has been used to identify proteins which are differentially expressed by tumour cells in comparison with normal cells (Chen et al. 2004, Kreunin et al. 2004). These proteins have the potential to function as biomarkers which could be of great clinical benefit and to this end mass spectrometry for the detection of proteins in bodily fluids, including plasma and urine, has been investigated (Ostergaard et al. 1997, Vlahou et al. 2001, Koomen et al. 2004).

In urine, protein may be present in the absence of systemic or renal disease. MALDI-TOF mass spectrometry has identified 67 forms of 47 different proteins in normal urine (Thongboonkerd et al. 2002). In systemic disease it is generally increases in plasma protein loading which result in the presence of abnormal proteins in the urine (Nedelkov and Nelson 2001). Frequently found proteins include cystatin C,  $\beta$ -2 microglobulin, urinary protein 1 and retinol binding protein.

The techniques used to prepare urine for protein analysis are straightforward and allow for the processing of multiple samples simultaneously. We therefore sought to develop MALDI-TOF mass spectrometry to examine the urines of patients with pancreatic cancer for changes in the total protein profile and the presence of proteins, including PIF, which might reflect the molecular basis of cachexia.

## **Materials and methods**

### **Patients**

Patients undergoing surgery for suspected pancreatic carcinoma were recruited from the hepatobiliary unit of the department of surgery, Royal Infirmary of Edinburgh (cf. p.81). Full ethical permission was obtained from the Local Research and Ethics Committee (LREC) for Lothian. Informed consent was obtained from every patient (appendix C). Information pertaining to the diagnosis of malignancy, nutritional status and prognosis was collected as described in materials and methods and recorded on a standardised proforma (appendix A).

Control urine samples were obtained from four healthy male laboratory workers aged between 25 and 30 years.

### **Urine samples**

200ml pre-operative mid-stream urine (MSU) samples were obtained and stored at -20°C.

### **Concentration of urine**

For use in mass spectrometry experiments urine was concentrated using Amicon Ultra 5000 molecular weight cut-off centrifugal filter columns (Millipore) according to the manufacturer's instructions (cf. p.85). This resulted in the concentration of 20ml samples by a factor of around 100 giving a final volume of 200µl concentrated urine.

## **Protein assay**

The protein concentration in all samples was assayed using a Biorad protein assay as per the manufacturer's instructions (cf. p.58).

## **One-dimensional polyacrylamide gel electrophoresis**

In experiments to examine the identity of proteins of specific molecular weights 20µg per well of sample was run on a 4-12% pre-cast NuPAGE Bis-Tris gel in MOPS buffer (Invitrogen) (cf. p.59). Gels were stained with Gelcode (Pierce) and destained in deionized water. Bands were then excised with a clean scalpel.

## **Tryptic digest**

Bands from polyacrylamide gels were treated with ammonium bicarbonate and acetonitrile then subjected to tryptic digest as described in materials and methods (cf. p.86).

## **MALDI-TOF mass spectrometry**

For analysis in the mass spectrometer 0.5µl of sample (either tryptic digest or concentrated urine) was applied to each well of the MALDI plate and 0.5µl of matrix added (cf. p.86). The matrix used was  $\alpha$ -cyano cinnamic acid for tryptic digests and sinapinic acid for whole urine samples. The plate was then allowed to dry, loaded into the Voyager-DE<sup>TM</sup> STR mass spectrometer (Applied Biosystems) and analysed. Results of tryptic digests were compared with the known and predicted tryptic digest profiles of known proteins using the MIS-FIT database.

## Results

### Patient data

A total of 19 patients undergoing surgery for pancreatic carcinoma were recruited over an 18 month period from January 2002 to June 2004 (appendix D). Tissue and urine samples were obtained from every patient.

Assessment of nutritional status revealed that 16 patients had lost weight prior to diagnosis, 1 did not know if they had gained or lost weight and 2 patients' weights had remained stable. The mean percentage weight loss was 8.4% with a range of 0 to 18%. Three patients remained weight stable. Two of these patients were the only patients not to describe a decrease in oral intake. One patient described an increase in intake following the insertion of a biliary stent. All but one patient had experienced a decrease in performance status as assessed by the Karnofsky index. Serum albumin was determined in every case and was low in the 2 patients with the highest weight losses, 12 and 13.15kg. Serum urate was assayed in 14 patients and was normal in every case.

Prognostic information was collected in every case. At the end of the study, the mean duration of follow-up was 11 months. Eleven patients had developed recurrence or metastasis, 5 of whom had residual tumour or metastases at the time of initial surgery. Of the 19 patients recruited, 9 (47.4%) had died by the end of the study. The mean duration of survival of the deceased patients was 254 days post-diagnosis. Including patients who were still alive at the end of the study the overall duration of survival exceeded 323 days. Examination of routine histopathology reports revealed a variety of tumours consisting of 8 pancreatic adenocarcinomas, 4 cholangiocarcinomas, 2 ampullary tumours, 1 ampullary adenoma, 1 serous

microcystadenoma of pancreas, 1 tubulovillous adenoma of the ampulla, 1 metastatic fibrosarcoma and 1 duodenal carcinoma. Of the pancreatic adenocarcinomas, 2 were staged as pT3N0Mx, 4 as pT3N1Mx and 2 as M1. At the end of the study, none of the patients with pT3N0Mx tumours, 2 of the patients with pT3N1Mx tumours and 1 of the patients with M1 tumours had died. The other tumours were of various stages (figure). Six of the 19 patients were smokers at the time of surgery and of these, 2 (33.3%) had died by the end of the study. Serum CRP levels were determined in 15 patients and were elevated in 6, of whom 2 (33.3%) had died by the end of the study.

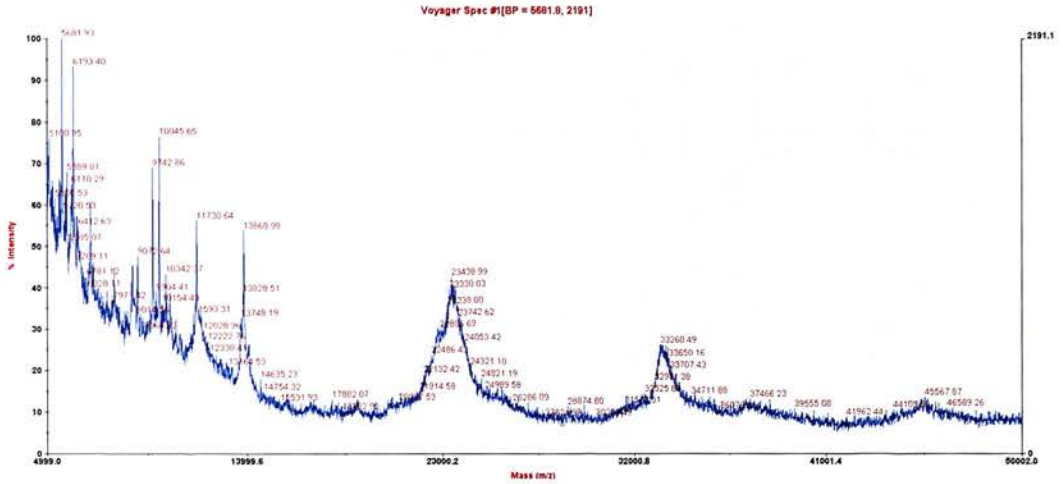
There was no statistically significant difference in the duration of survival of patients with a specific tumour type, patients with a tumour of a more advanced stage, patients who smoked or patients who had an elevated CRP.

### **Urinary whole mass spectra**

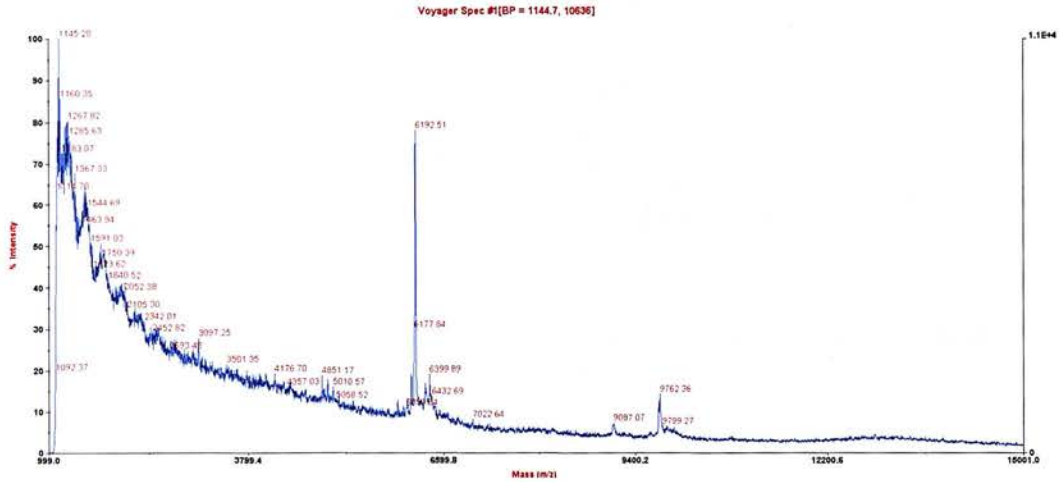
Urine samples from the nineteen patients recruited were concentrated and their protein content assayed. This revealed a range of concentrations from 102 $\mu$ g/ml to 4769 $\mu$ g/ml. Samples were diluted to give a concentration of approximately 100 $\mu$ g/ml then run on the mass spectrometer. Satisfactory spectra were obtained for 15 of 19 samples (figure 7.1a), 1 gave a spectrum of intermediate quality and 3 samples did not give satisfactory mass spectra despite the use of alternative matrices and serial dilutions.

Of the 16 samples analysed, 14 produced peaks at a mass of 6200, 13 produced peaks at a mass of 9100, 16 produced peaks a mass of 9750, 7 produced peaks a mass of 11730, 4 produced peaks a mass of 13800 and 15 produced peaks at a mass of 23500. Of these peaks, those at 6200, 9100 and 9750 were seen in the 4

samples of normal urine analysed (figure 7.1b). Those at 11730, 13800 and 23500 were not seen. There was no significant increase in the mean weight loss of patients with any specific band in their urine (Student's *t* test).



**a**

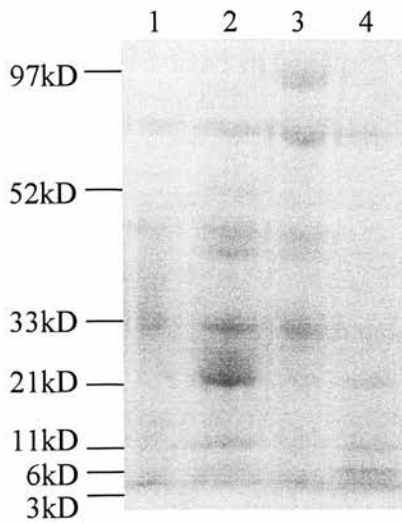


**b**

**Figure 7.1.** Whole mass spectra of concentrated urine samples from **a.** patient with pancreatic carcinoma and **b.** normal individual.

## One dimensional gel electrophoresis and tryptic digests

In order to further investigate the protein composition of the peaks identified on whole mass analysis and to try to identify PIF / dermcidin peptides, patients urine samples were separated by one-dimensional gel electrophoresis. This resulted in a characteristic banding pattern (figure 7.2), with bands at 6, 12, 21, 33, ~40, ~45, ~77 and 97 kD. These did not correspond to the molecular mass peaks identified by MALDI-TOF mass spectrometry.



**Figure 7.2.** One-dimensional gel electrophoresis of concentrated urine samples from 4 patients with pancreatic carcinoma.

As the molecular weight of glycosylated PIF is believed to be around 24kD and the predicted molecular weight of the full-length nascent polypeptide is 12kD, the bands of these molecular weights from 2 patients, one of whom was positive for PIF / dermcidin mRNA by real-time PCR (see following chapter) were excised and subjected to tryptic digest. MALDI-TOF mass spectrometry of the peptide products of these digests failed to detect any predicted peptide products of PIF / dermcidin. However, a range of other proteins were identified. In patient JG, who was positive for PIF / dermcidin expression on real-time PCR, the 15kD and 30kD bands were identified as immunoglobulin kappa light chain (molecular weight 23481 / 23512). The 40kD band contained keratin (type II cytoskeletal 1)(molecular weight 66067Da) and alpha-1-microglobulin/bikunin precursor (molecular weight 39000Da). The patient did not have an elevated serum urea or creatinine at the time of urine collection. In patient DB, the 23kD band contained mitogen-activated protein kinase kinase kinase 3 isoform 1 (molecular weight 74090), immunoglobulin kappa light chain (molecular weight 29506), APC-binding protein EB1 (molecular weight 24282) and mannose binding lectin-associated serine protease-2 related protein (molecular weight 19505). The 33kD band contained immunoglobulin kappa light chain (molecular weight 23512). Of these proteins, only MEKK3 and APC-binding protein EB1 have not previously been found in normal urine (Nedelkov and Nelson 2001, Thongboonkerd et al. 2002, Pisitkun et al. 2004).

## Discussion

The use of mass spectrometry in the investigation of urinary proteomes in cancer has previously been described for transitional and squamous cell carcinomas of the urinary tract (Ostergaard et al. 1997, Vlahou et al. 2001). Protein products of these tumours may directly enter the urine and are subsequently likely to be readily detectable. In contrast, although the urinary proteome in cancer cachexia has also been studied (Choudhary et al. 1999a, Choudhary et al. 1999c), the protein peaks observed on full mass spectra were not definitively identified. Our confirmation of protein identities using tryptic digest therefore represents the first definitive characterisation of alterations in the urinary proteome in non-urinary cancer.

Unfortunately, our attempts to correlate urinary PIF excretion with the nutritional and clinical status of patients with pancreatic carcinoma were unsuccessful, firstly as it was not possible to recruit sufficient patients in the available time and secondly due to problems with the immunological detection of PIF. The finding that the anti-glycosylated PIF antibody also binds CD59 was disappointing and presented a technical difficulty to our laboratory. This was addressed mainly by a co-worker, who carefully replicated the published urine blotting protocols. Advice and training from their authors was obtained but it was not possible to replicate their results. Indeed, in our hands the only urinary protein found to bind the antibody was CD59, the identity of which was confirmed beyond reasonable doubt by protein sequencing. This does not mean that PIF does not bind under other conditions. As noted above, the antibody was originally used to purify MAC16 tumour-derived PIF by affinity chromatography (Todorov et al. 1996a), and it was from PIF produced by this method that the core peptide sequence and the

pattern of glycosylation was determined. Unfortunately, none of the published studies describe confirmatory sequencing of urine-derived PIF.

MALDI-TOF mass spectrometry also failed to identify PIF in the urines we analysed. As noted above, this technique is capable of detecting tiny amounts of protein with great accuracy. However, communication with the authors of previous studies of glycosylated PIF has suggested that it is present at extremely low concentrations in urine, circa 0.5ng per ml (M. Tisdale, personal communication). We concentrated our urine samples approximately 100 fold, resulting in a PIF concentration of 50ng per ml in the 5 $\mu$ l placed on the mass spectrometry plate. This represents 25pg of PIF, equivalent to approximately 1 femtomole of the 24kD species. As this is the very limit of sensitivity of the mass spectrometer used it is possible that PIF was not detected in our samples as it was present at too low a concentration. Although further concentration would be possible it would not increase the low amount of PIF present in relation to other proteins. While this may be problematic in Western blotting, it may not be so for mass spectrometry and could prove worthwhile. Similarly, the low relative abundance of PIF in plasma might also prove less problematic for mass spectrometric analysis and plasma analysis could be revisited.

Post-translational modification and the method by which samples are prepared from urine may also affect the spectrum of proteins detected by mass spectrometry (Thongboonkerd et al. 2002). In particular, heavy glycosylation may render PIF highly adherent, potentially resulting retention on the vessels used in purification and can also impair the detection of peptides by MALDI-TOF. Heavy glycosylation may also have contributed to our failure to identify PIF in the gel

bands analysed as they were not stained with silver stain, which would be expected to demonstrate carbohydrate epitopes. The bands at 21kD and 11kD which were analysed were chosen as they lay close to the predicted weights of glycosylated and non-glycosylated PIF / dermcidin. The fact that carbohydrate ions are metastable in mass spectrometers and therefore subject to degradation prior to detection (Mortz et al. 1996) and that multiple glycoforms of PIF may exist (Wirth et al. 2002) may also have resulted in unexpected molecular weights, further hampering identification.

A combination of the above factors may potentially have resulted in the failure to detect urinary PIF. Urinary PIF has previously been reported to be present in up to 80% of urines from weight-losing patients with pancreatic carcinoma (Wigmore et al. 2000). Our cohort of patients did not differ significantly from those in these studies and it would seem unlikely that all 19 of our patients were non-PIF producers. The apparent absence of urinary PIF in our studies therefore appears to contrast directly with previous work (Todorov et al. 1996a, Wigmore et al. 2000). However, if urinary PIF is indeed not present in these patients' urines, this would be in keeping with the results of Monitto et al. (2004), who have demonstrated that PIF / dermcidin overexpressing xenografts do not induce cachexia. Furthermore, the previous study claiming to have detected urinary PIF by mass spectrometry did not confirm it as a constituent of the identified 24kD urinary peak (Choudhary et al. 1999b). Reconciling these studies and those describing the *in vitro* pro-cachectic effects of glycosylated PIF on myotubes and hepatocytes (Smith et al. 1999, Watchorn et al. 2001) will require further detailed evaluation of PIF glycosylation.

## 8. Assessment of PIF / dermcidin expression in pancreatic carcinoma tissue

### Summary

The source of PIF previous studies detected in the urine of weight-losing patients with pancreatic carcinoma has not been definitively determined. Antigenic cross-reactivity with PIF isolated from MAC-16 tumours suggests that tumour cells may produce PIF. However, screening of tissues for dermcidin mRNA demonstrated expression only in skin and brain (Schittek et al. 2001) and SAGE analysis has not demonstrated DSEP expression in pancreatic tumours (Porter et al. 2003). This chapter describes the investigation of whether pancreatic tumour cells produce PIF *in vivo*.

Samples of pancreatic tumours were obtained from specimens removed during surgery for pancreatic cancer. PIF / dermcidin expression was then assessed by analysis of mRNA and protein production. mRNA was prepared by laser capture microdissection and tissue homogenization. Following reverse transcription PIF / dermcidin expression was assessed by both standard and real-time PCR. Production of PIF peptide was assessed by immunohistochemistry.

cDNA produced from mRNA prepared by laser capture microdissection was positive for PIF / dermcidin by standard PCR in both tumour and normal samples. However, analysis of this mRNA by Agilent capillary electrophoresis suggested it was not of sufficient quality for quantitative PCR and it did not prove possible to improve yields through refinements in technique. mRNA derived from homogenized

tissues was of better quality. However, quantitative PCR identified significant PIF / dermcidin expression in only one sample and this experiment was not repeated.

Staining of frozen sections with the monoclonal antibody to glycosylated PIF did not reveal any positivity. Staining with rabbit polyclonal antiserum to the PIF peptide demonstrated staining of interstitial cells, peripheral nerves, and vascular smooth muscle cells, but not tumour cells. However, it was not possible to inhibit this staining with either the antigenic fragment of PIF to which the antisera was raised or the full-length Y-P30/PIF synthetic peptide.

The results of mRNA experiments conflict. On balance, the finding that mRNA isolated from homogenized tumour was of better quality and that quantitative PCR is more specific than standard PCR suggests that pancreatic tumours may not express PIF / dermcidin mRNA, although the development of better RNA purification and the analysis of larger numbers is required. The results of immunohistochemistry experiments appear to support the results of real-time PCR, as although staining was observed it was not inhibited by the antigenic peptide and may therefore be non-specific. The source of PIF previously identified in the urines of patients with pancreatic carcinoma therefore remains unclear.

## Introduction

The most comprehensive study of tumour PIF / dermcidin expression to date examined production in prostatic carcinoma (Wang et al. 2003). This study demonstrated the presence of PIF / dermcidin mRNA in prostatic carcinoma cell lines, primary prostatic carcinoma and osseous metastases and confirmed by *in situ* hybridisation that it was produced by only malignant epithelial cells. Interestingly, urinary PIF was demonstrated in 9 of 19 cachectic patients but 0 of 19 non-cachectic patients. However, the correlation between tumour expression of PIF / dermcidin mRNA and the presence of urinary PIF was not examined. Another study did investigate this relationship, assessing PIF expression by immunohistochemistry and the presence of urinary PIF by Western blotting in patients with gastrointestinal cancers (Cabal-Manzano et al. 2001). Of the 16 patients examined, 5 showed granular, cytoplasmic staining of tumour cells which was stronger in less differentiated tumours. All of these patients were also positive for urinary PIF, in contrast with patients negative for tumour PIF staining, who were all negative. The positive tumours arose from the pancreas, colon, liver, lung and oesophagus. These results strongly argue that tumour cells are the source of urinary PIF. However, the numbers examined were small and as discussed above, there is now evidence to suggest that the monoclonal mouse anti-glycosylated PIF antibody used recognises epitopes of other antigens including CD59 (cf. chapter 7). Indeed, another study obtained contrasting results with an antibody to the DCD-1 peptide. This gave only 2 positive results on immunohistochemical array examination of 64 pancreatic tumours (Porter et al. 2003). However, this antibody would not be expected to recognise the Y-P30 / PIF core peptide which may be present even in the absence of DCD-1 due to

proteolytic cleavage. Examination of PIF / dermcidin mRNA in either of these studies would have helped to resolve the apparent conflict between their findings, but this was not performed. In fact, PIF / dermcidin mRNA expression in gastrointestinal tissues has only once been assessed, and although expression was not found, only normal tissues and no tumours were examined (Schitteck et al. 2001).

We therefore sought to examine the production of PIF / dermcidin mRNA by pancreatic tumour cells and correlate this with PIF peptide production. Initially, it was also planned to correlate these findings with both the clinical status of patients and the presence of urinary PIF. However, as described in the previous chapter, the latter did not prove possible.

## **Materials and methods**

### **Tumour samples**

Tumour samples were collected at the time of surgery (cf. p.82). For patients undergoing Whipples resections, samples of tumour and normal tissue were removed from fresh surgical specimens under the guidance of a consultant pathologist. For patients undergoing palliative bypasses, core biopsies of tumour and normal tissue were taken intraoperatively by the operating surgeon. All samples were immediately snap frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  prior to analysis.

### **Laser capture microdissection**

Frozen sections were cut from tumour and normal tissue samples, mounted on charged slides and stained with eosin only. Histology was determined with the help of a consultant pathologist. Capsure laser capture microdissection caps and a Pixcell laser capture microscope (Arcturus) were used to generate separate samples of carcinoma and normal cells as per the manufacturer's instructions (cf. p.82).

### **RNA preparation**

RNA was prepared from laser-capture microdissected cells using a paraffin block RNA isolation kit (Ambion) with a modified protocol as described in materials and methods (cf. p.50).

For the preparation of RNA from intact tissue samples tissue a rotor-stator homogeniser was used for homogenisation. RNA was then extracted using an RNEasy kit (Qiagen) according to the manufacturer's instructions (cf. p.51).

All samples were DNase I treated prior to reverse transcription (cf. p.52). Samples prepared by laser capture microdissection were treated using Promega DNase I. Samples prepared by tissue homogenisation and RNEasy column purification were treated by on-column DNase digestion as described in materials and methods.

### **Assessment of RNA**

RNA concentrations were assessed by spectrophotometry. RNA quality in samples prepared by laser-capture microdissection and direct tissue homogenisation was assessed by Agilent chip capillary electrophoresis as described in materials and methods (p.53).

### **Reverse transcription**

Samples were reverse-transcribed in 25µl reactions using AMV reverse transcriptase and random primers (Promega) (cf. p.55).

### **PCR**

Standard 25µl reactions with 1µl sample and 1µl *taq* polymerase were used (cf. p.55). All DNase I treated RNA samples were checked for genomic DNA

contamination by amplification with primers to the housekeeping gene, cytochrome

B. Primer sequences were -

F - GGTTCTCTGGGAGGGAGTGC

R - GACAACACAGTAAGAACCAGG (TAGN)

Primers used for PIF / dermcidin PCR were -

F - CTCGGATCCGCCGCCATGAGGTTTCATGACTCTCC

R - CAGAATTCCTGGGTATCATTCTCAGCT (TAGN).

All reactions underwent 35 cycles with a 56°C annealing temperature.

### **Real-time PCR**

Real-time PCR was performed using an ABI Prism cycler (Applied Biosystems) and 25µl reaction volumes as described in materials and methods (p.57).

Samples were run in duplicate using a 18S ribosomal RNA VIC-labelled internal control and FAM-labelled PIF / dermcidin unknown. 18S primer and probe sequences were from Applied Biosystems (sequences copyright Applied Biosystems).

PIF / dermcidin primer and probe sequences were -

F - CAAAAGGAAAATGCAGGTGAAGA

R - CTCCGTCTAGGCCTTTTCCA

Probe - ACAGGCACCAAAGCCAAGGAAGCA

## **Immunohistochemistry**

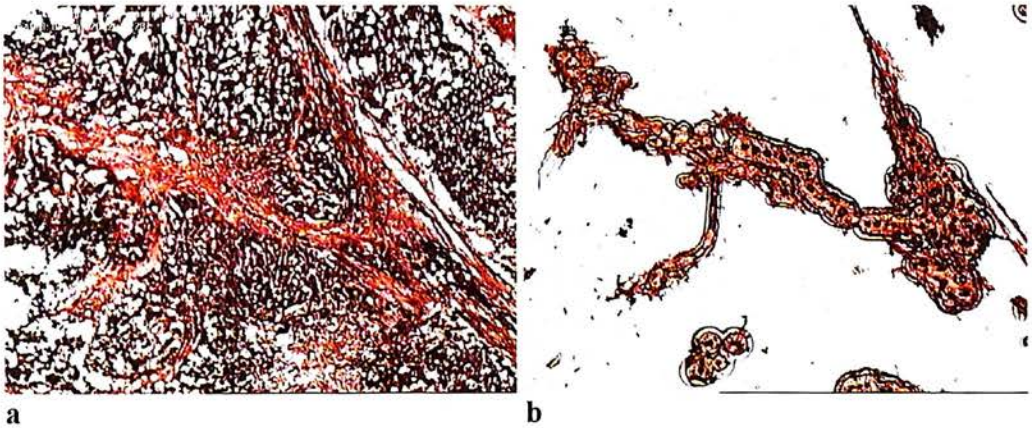
Biotin-amplified DAB immunohistochemistry was performed on frozen sections as described in materials and methods (p.83). The primary antibodies used were rabbit polyclonal anti-PIF peptide antiserum (Sigma) and mouse monoclonal anti-glycosylated PIF monoclonal antibody (cf. McDevitt et al. 1995) at concentrations of 1 in 1000 and 1 in 100 in blocking solution. The secondary antibodies used were swine anti-rabbit biotin-linked antibodies and rabbit anti-mouse biotin-linked antibodies respectively. Secondary antibodies were diluted 1 in 300 in blocking solution. All sections were counterstained with haematoxylin. Histology and staining patterns were assessed with the help of a consultant pathologist.

In peptide inhibition experiments, 10mg/ml of either the peptide used to raise the rabbit antiserum (Sigma) or the full-length synthetic PIF core peptide (Albachim) were added to the primary antibody prior to its application to tissue sections.

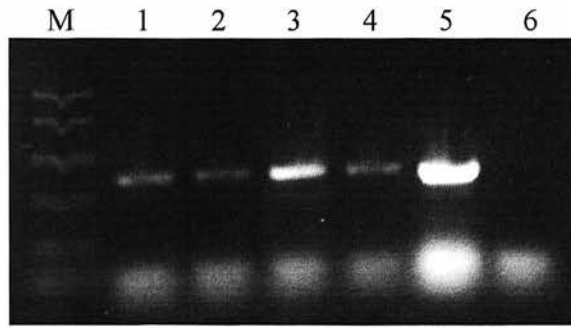
## Results

### Investigation of PIF / dermcidin mRNA expression in pancreatic tumour tissue

Using laser capture microdissection, it was possible to separate cells from areas of tumour and areas of normal pancreas (figure 8.1). PCR of cDNA derived from these samples was positive for PIF / dermcidin expression in every case (figure 8.2). However, despite our best efforts to ensure tissue was snap-frozen as quickly as possible and repeated attempts at RNA extraction, analysis of RNA concentration revealed extremely low amounts of RNA in LCMD samples. Analysis of RNA quality by Agilent chip capillary electrophoresis also revealed that RNA was of insufficient quality for real-time PCR experiments (figure 8.3). It was therefore decided to prepare RNA from gross, matched samples of normal and tumour tissue.

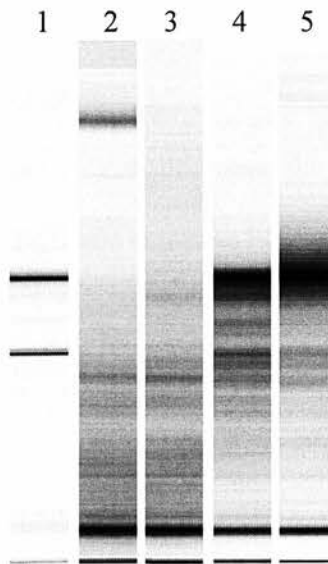


**Figure 8.1.** Laser capture microdissection. **a** Slide prior to capture. **b** Cap with adherent cells post capture.



**Figure 8.2.** PCR of LCMD-derived cDNA for PIF / dermcidin expression.

1. Patient 1, normal tissue
2. Patient 1, tumour tissue
3. Patient 2, normal tissue
4. Patient 2, tumour tissue
5. Positive control (CF-PAC cDNA)
6. Blank (no cDNA)



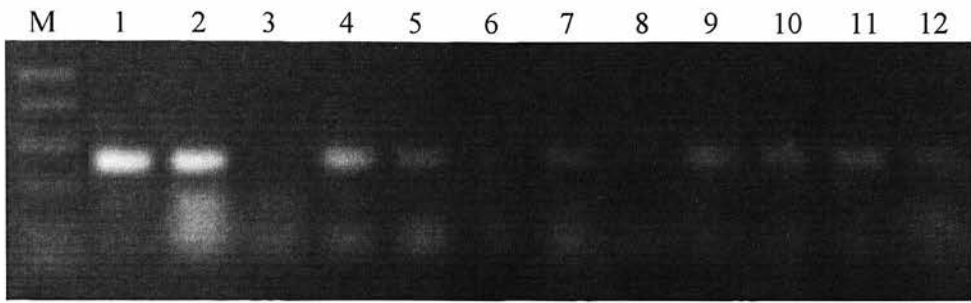
**Figure 8.3.** Agilent bioanalyzer virtual RNA gel.

1. HuH7-derived RNA showing strong 18S and 28S bands (positive control)
2. Patient 1, LCMD-derived RNA
3. Patient 2, LCMD-derived RNA
4. Patient 1, homogenization-derived RNA
5. Patient 2, homogenization-derived RNA

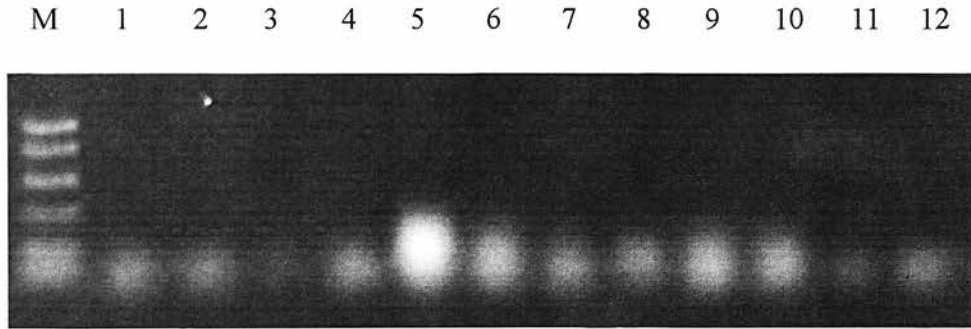
Preparation by tissue homogenisation yielded higher amounts of RNA.

Agilent analysis revealed that the quality of this RNA was significantly better than that obtained from LCMD (figure 8.3). Standard PCR of cDNA derived from these samples was positive for cytochrome B in the majority of cases (figure 8.4a). In contrast, PIF / dermcidin PCR was negative in every case (figure 8.4b). Real-time PCR of the same samples revealed a satisfactory level of VIC (28S ribosomal RNA) positivity (count >23) in only 11 of 28 samples. Only one of the samples positive for PIF / dermcidin expression on standard PCR, GT-normal, had a VIC count of >23. In only one of the VIC positive samples, JG-tumour, was the FAM count <36. This sample had a FAM : VIC ratio of 0.96. These results suggest an undetectable level of PIF / dermcidin expression in the 10 samples with FAM counts of >36, including those found positive for PIF / dermcidin on standard PCR, but a relatively high level of expression in the JG-tumour sample.

These results did not permit statistical analysis. The RNA extraction, reverse transcription, quality control PCR and real-time PCR procedures were not repeated.



**a**

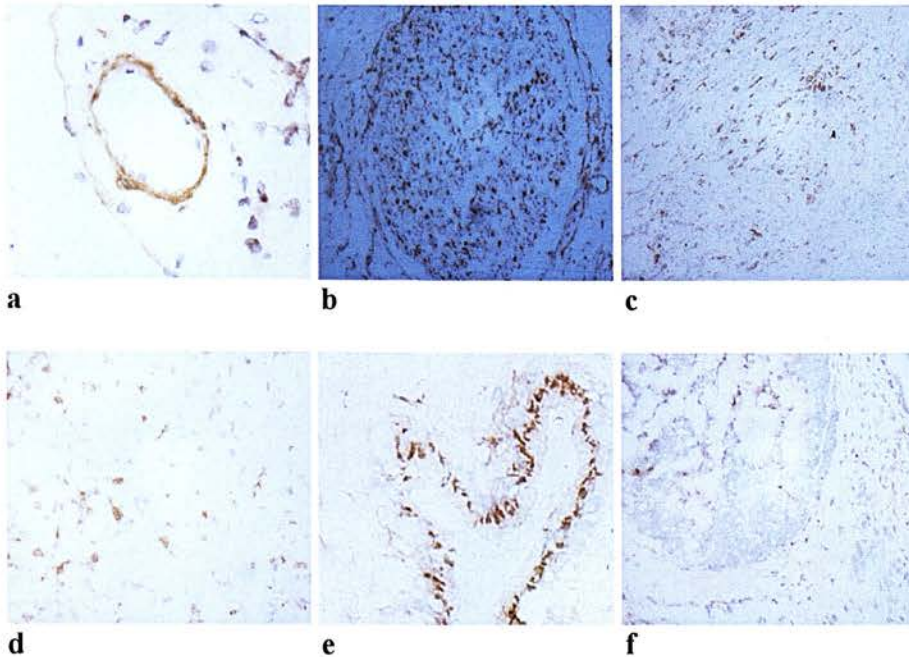


**b**

**Figure 8.4.** PCR of homogenization-derived cDNA from 12 samples of pancreatic tumour. **a.** Cytochrome B. **b.** PIF / dermcidin. Bands in the gel are short and do not correspond to the 358bp length of PIF / dermcidin PCR product and are therefore felt to represent primer artefact.

## Investigation of PIF peptide production in pancreatic tumour tissue

In order to determine whether the production of PIF peptide mirrored PIF / dermcidin mRNA expression we investigated tumour PIF production using immunohistochemistry. Staining of frozen sections with monoclonal anti-glycosylated PIF antibody revealed no specific staining at concentrations of neat, 1 in 100 or 1 in 1000. Staining of the same sections with the rabbit polyclonal anti-PIF peptide antiserum gave a characteristic pattern. Positivity was observed in smooth muscle cells of the walls of small blood vessels, nerve cells and interstitial cells (figure 8.5a-g). The latter had a fusiform appearance and were considered to be either myofibroblasts or possibly tissue macrophages or mast cells. Staining of some epithelial cells was also seen and was localised to what appeared to be secretory granules. However, no tumour epithelium was positive.



**Figure 8.5.** Staining pattern of pancreatic tumours stained with rabbit polyclonal anti-PIF peptide antiserum. **a.** Small blood vessel. **b.** Nerve. **c.** Interstitial cells. **d.** Fusiform cells. **e.** Epithelial cells. **f.** Pancreatic tumour tissue.

In order to test the specificity of the rabbit polyclonal antisera, immunohistochemistry was repeated on the same sections having pre-incubated the primary antibody with an excess of either the immunogenic peptide to which it was raised or the full-length synthetic PIF core peptide. No change in the staining pattern was observed. In light of these findings, no further immunohistochemistry was performed with this antiserum.

## Discussion

Our initial results with laser-capture microdissection prepared RNA suggested that PIF / dermcidin was expressed by both normal and neoplastic cells from samples of human pancreatic carcinoma. This would have suggested a much broader expression profile than has previously been described (Schitteck et al. 2001, Porter et al. 2003). However, doubt was cast on this by the fact that the better quality samples prepared by tissue homogenisation were not positive for PIF / dermcidin expression on PCR. These samples contained greater amounts of RNA than the LCMD samples and should have contained all cell types present in the LCMD samples. False negatives would therefore seem unlikely and the negative results of real-time PCR support this. We were also unable to confirm the LCMD sample results with real-time PCR as the RNA was found to be of insufficient quality. Therefore, in the absence of further time to improve the LCMD technique, we feel that tissue homogenisation provided more reliable results and conclude that in the majority of cases, PIF / dermcidin is expressed in neither normal nor neoplastic pancreatic tissue. This is consistent with the previous studies of expression (Schitteck et al. 2001, Porter et al. 2003).

In one case, JG, we observed a significant signal for PIF / dermcidin expression on real-time PCR. This sample was not positive on standard PCR, although this is a less sensitive assay. The tumour in this case was a ductal adenocarcinoma of pancreas, and that this may produce PIF / dermcidin is supported by the findings of Porter et al. (2003), who as noted above, found expression in 2 of 64 pancreatic adenocarcinomas. However, urine from the same patient was analysed by tryptic digest and mass spectrometry and PIF was not identified (cf. chapter 7)

although it cannot be stated categorically that PIF has not entered the urine due to the possibility that MALDI-TOF analysis may have failed to detect glycosylated PIF.

The initial results of immunohistochemistry with the rabbit polyclonal anti-PIF peptide antibody were of great interest, as they suggested expression by a variety of cells. Theoretically, this antisera should recognise only the most antigenic part of the PIF core peptide. This has no close homologues and the post-streptococcal glomerular antigen (Yoshizawa et al. 1992), to which it is related, would not be expected to be found in the patterns observed. Specific staining of other antigens is therefore unlikely to account for the widespread staining observed. However, as the antisera is polyclonal, it is feasible that antibodies to other antigens could produce positive staining. In order to exclude this, we attempted to block specific binding by incubation with the immunogenic peptide and the full-length PIF core peptide. The large excess of these peptides would be expected to bind all anti-PIF antibodies in the antisera, leaving those capable of non-specific binding and allowing the determination of PIF expression by analysing the differences between slides stained in their presence and absence. Unfortunately, no differences were observed, suggesting that the staining was non-specific. This is consistent with the results of both RNA analysis and urine mass spectrometry and supports the lack of PIF / dermcidin expression in the majority of pancreatic tumours. The lack of staining observed when the mouse monoclonal anti-PIF antibody was used is also consistent with these results. Despite the recognition of CD59 by this antibody, previous studies suggest that it is also potentially capable of detecting the glycosylated form of PIF. Glycosylated PIF would therefore also not appear to be produced by pancreatic tumours.

In order to confirm these negative results, careful analysis of positive controls for both antibodies will be required. In the case of the polyclonal antisera, as it was obtained from a reliable commercial source it would be hoped that some immunoreactivity to the inoculated peptide would be present. It may therefore be possible to detect binding by a simple shift in weight of peptide-bound antibody on a standard immunoblot. This could also be confirmed by performing tryptic digest and mass spectrometric analysis of immunoprecipitated, antibody-bound peptide. This technique could also potentially be used to identify any antigens binding to the antisera non-specifically. In the case of the mouse monoclonal antisera, a reliable source of glycosylated PIF would be required as a positive control. Unfortunately, such a source was not available to us at the time of this study.

Potentially, the staining pattern of slides from the tumour of patient JG, which was positive for PIF / dermcidin expression on real-time PCR, could also have demonstrated a positive staining pattern. However, when compared with other slides no difference could be detected. Staining was also not affected by preincubation of the antisera with the peptides. If PIF / dermcidin mRNA is indeed expressed by this tumour, it would therefore not seem to be converted into a peptide form which is detectable either by immunohistochemistry with the available antibodies or by mass spectrometric analysis of urine. This could potentially be due to the production of a low amount of peptide, which would be consistent with the results of PCR in which mRNA was undetectable by standard PCR and at the lower limit of real-time PCR detection. Further work on the reliable detection of PIF / dermcidin peptides will be required to confirm and extend these studies.

## 9. Discussion

This thesis has sought to investigate how PIF / dermcidin is involved in cell survival and growth and how these functions are related to the structural characteristics of the molecule. We also sought to relate our findings to the *in vivo* situation in the context of pancreatic carcinoma and cachexia. The results obtained provide insight into the potential physiological and pathological roles of PIF / dermcidin, with particular reference to neoplasia.

### **The importance of PIF / dermcidin to cell proliferation**

Our finding that PIF / dermcidin induces tumour cell proliferation confirms its oncogenic potential. Autocrine secretion of PIF / dermcidin would be expected to contribute to the autonomous growth of tumour cells. This has been suggested to occur by the amplification of the dermcidin gene locus in breast cancer (Porter et al. 2003), which may result in overproduction. Alternatively, as with other oncogene pathways involving secreted products, abnormalities in the receptor system or signalling pathways could contribute to tumour cell expansion (Downward et al. 1984). Despite the finding of putative PIF receptors by two groups (Porter et al. 2003, Smith and Tisdale 2003), as yet their identity is unknown. Cloning will facilitate the investigation of their role in neoplasia. Of the signalling pathways known to be stimulated by glycosylated PIF, there is some evidence that the proteasome pathway may be involved in the induction of cell proliferation. In pancreatic cancer, inhibition of the 26S proteasome has been found to inhibit proliferation, potentially via prevention of p21<sup>waf-1</sup> degradation (Shah et al. 2001).

This serves as a reminder that the ubiquitin-proteasome system is active not just in muscle tissue but in every cell type and could be activated by ligation of aberrantly expressed PIF / dermcidin receptors. Were the effect to be similar to the stimulatory effect of glycosylated PIF on murine myotube proteasomes this could contribute to the pro-proliferative effects we have observed (Lorite et al. 2001, Whitehouse and Tisdale 2003). The stimulation of 15(*S*)-HETE production seems less likely to stimulate proliferation, having been demonstrated to inhibit cell growth in colorectal and prostatic cancer cells (Shappell et al. 1999, Shappell et al. 2001, Chen et al. 2003).

### **The importance of PIF / dermcidin to cell survival**

We have demonstrated that PIF / dermcidin overexpression results in an increase in cell resistance to oxidative stress. This is in keeping with the results of previous studies on neuronal cells (Cunningham et al. 1998, Cunningham et al. 2002). We also confirmed that the mode of cell death in our system is necrosis, which is reduced by PIF / dermcidin expression. In neuronal cells oxidative stress similarly results in necrotic cell death which may be reduced by preconditioning, which results in the induction of several antioxidant enzymes (Arthur et al. 2004). Preconditioning by enzymatic induction is well described in hepatic cells and it is subsequently feasible that PIF / dermcidin may act in this manner in HuH7 cells. Alternatively, PIF / dermcidin expression may protect cells from oxidative stress by induction of the proteasome system (Smith and Tisdale 2003, Whitehouse and Tisdale 2003). Proteasome induction has been associated with improved survival of both neuronal cells and liver cells following oxidative stress (Lee et al. 2004,

Donohue, Jr. et al. 2004). PIF / dermcidin may therefore potentially work via either of these systems or by another, as yet unidentified mechanism. Intriguingly, low-level oxidative stress has been demonstrated to induce the proteasome system, and it is subsequently feasible that PIF / dermcidin may exert its effects indirectly, via the induction of oxidative stress. This could also help to explain the down regulation of PIF / dermcidin expression in adult hepatocytes by the preconditioning agents cyclosporin and heat-shock, but would require further investigation to be substantiated.

One final mechanism by which PIF / dermcidin may influence cell necrosis is via the calcineurin-like phosphatase domain. Cell necrosis is increasingly recognised as having elements of an ordered pathway which appears to include onset of the mitochondrial permeability transition (Lemasters et al. 1998, Lemasters 1999). This transition may be induced by oxidative stress and interestingly, may be blocked by cyclosporin A. Cyclosporin's inhibition of calcineurin (Rusnak and Mertz 2000) raises the possibility that this may be the point at which PIF / dermcidin acts on the necrotic pathway and that phosphatase activity may be involved. This would be consistent with our finding that mutation of both asparagines was required to inhibit PIF / dermcidin's survival effects. Similar results with cells transfected with the H35N PIF / dermcidin mutant would further support this.

An effect of the phosphatase domain on the anti-necrotic properties of PIF / dermcidin would suggest some overlap between the survival and proliferative effects of the molecule, as our results suggest that this domain is required for the proliferative action of PIF / dermcidin. In contrast with the proposed 'preconditioning' mechanisms of action, which suggest that PIF / dermcidin may act

as a modifier of the response to environmental stress rather than as an anti-apoptotic survival factor, this would be in favour of PIF / dermcidin acting primarily as a conventional growth factor, with secondary effects on cell survival via cell cycle modulation. In support of this, we have already described the pro-proliferative effect of transfection, and shown changes in the proportion of cycling cells. This may involve a range of factors such as c-myc and ras which have been demonstrated to inhibit apoptosis (Wyllie 1993, Bonni et al. 1999, Scheid et al. 1999). In particular, PIF has been demonstrated to induce STAT-3 activation in hepatic cells (Watchorn et al. 2001), and this transcription factor family is known to inhibit apoptosis (Shen et al. 2001 Wang et al. 2000, Shen et al. 2001). Investigation of the effects of classical inducers of apoptosis, for example staurosporine and camptothecin, on PIF / dermcidin overexpressing cells would help to determine if this influences apoptosis and subsequently confirm whether cell proliferation and survival are separable phenomena.

### **The importance of PIF / dermcidin expression in hepatic cells**

Our demonstration of PIF / dermcidin expression in untreated primary hepatocytes, CD34 positive fetal liver cells and CD90 positive fetal liver cells but not in primary hepatocytes treated with cyclosporin or heat-shocked, the HuH7 cells line or unsorted fetal liver cells suggests that hepatic cells are capable of regulating PIF expression in different stages of development and following different stimuli. As discussed above (cf. chapters 3 and 9), it is difficult to propose a single mechanism or stimulus to account for these differences. However, control will ultimately be mediated through the PIF / dermcidin promoter. We have begun to study the

sequence immediately 5' to the dermcidin gene and have discovered 127 putative binding sites for 21 different transcription factors using computer modelling. The identification of DNase I hypersensitive sites in fetal and adult hepatocytes subjected to different stimuli will help identify the transcription factors that regulate PIF / dermcidin expression.

If PIF / dermcidin expression by cultured primary hepatocytes reflects the *in vivo* situation, PIF may have a greater physiological role than previously proposed. Glycosylated PIF is known to activate the transcription factors NF- $\kappa$ B and STAT3 in primary hepatocytes and induces IL-6, IL-8 and C-reactive protein production while decreasing transferrin production (Watchorn et al. 2001). These effects are consistent with a pro-inflammatory action and may reflect aberrant glycosylation of PIF in patients with cachexia but would not be expected to be active in the normal liver. Alternatively it is possible that the *in vivo* effect of PIF is blocked by the action of another pathway whose influence is lost *in vitro*.

In our studies, it appears feasible that the ischaemia and oxidative stress to which hepatocytes are subjected during harvesting might induce PIF / dermcidin expression in primary cultures. This would be consistent with the inhibition of PIF / dermcidin expression by the preconditioning agents cyclosporin and heat-shock. The potential role of PIF / dermcidin in hepatic preconditioning is subsequently hard to determine, as it appears to protect cells from oxidative stress and yet its expression is inhibited by preconditioning agents. One possible explanation is that as discussed above, PIF / dermcidin does generate a low level of oxidative stress and that prevention of this stress is part of the protective effect of these preconditioning agents. Another is that by protecting cells from oxidative stress these agents prevent

the induction of PIF / dermcidin expression. This would imply that they act independently of PIF / dermcidin. Therefore, were PIF / dermcidin to have a direct effect on the mitochondrial permeability transition, this would not seem to be required for cyclosporin's inhibition of cell death via this pathway (see above). Finally, a form of feedback may be involved, with a decrease in phosphatase activity secondary to cyclosporin inhibition resulting in decreased positive feedback on PIF / dermcidin expression. In any case, PIF / dermcidin would appear to work via mechanisms independent of cyclosporin or heat shock. Our finding that it protects HuH7 cells from oxidative stress subsequently implies that it may prove valuable as an adjunct to these agents in hepatic pre-conditioning.

We found that PIF / dermcidin mRNA was not expressed by unsorted fetal liver cells but was produced by those selected for the markers CD34 and CD90. CD34 is a cell surface glycoprotein originally identified on cells from the immature human myeloid cell line KG-1 and subsequently demonstrated to be expressed on haemopoietic stem cells capable of differentiating into either biliary cells or hepatocytes (Civin et al. 1984, Theise et al. 2000, Lanza et al. 2001, Crosby et al. 2001). CD90 is also a haemopoietic stem cell marker, the expression of which correlates with liver-specific gene expression and which jointly stains hepatic oval cells with CD34 (Petersen et al. 1998, Okumoto et al. 2003). The co-expression of PIF / dermcidin with both of these cluster determinants therefore raises the possibility that it may be a marker of bi-potential stem cells. This would be in keeping with the effects of PIF on liver cell ICAM-1 and VCAM expression and syndecan shedding, which have lead to the suggestion of a role in development (Watchorn et al. 2001, Watchorn et al. 2002). To more firmly establish this role,

further studies of PIF protein expression and culture from limiting dilutions of potential stem cells will be required.

The targeting of PIF / dermcidin to the secretory pathway in HuH7 cells raises the question of why previous studies which have used immunological methods to detect glycosylated PIF have always required cell lysates, as opposed to supernatants, for success (Todorov et al. 1996a, Todorov et al. 1999). One explanation could be that PIF is processed differently in these cells and is retained in the cytosol. The antibody used in these studies appears to recognise a glycosylated epitope (Todorov et al. 1997), and glycosylation of the PIF core could be a signal for intracellular retention. However, this does not explain the finding that *in vivo*, studies using the same antibody have demonstrated PIF in urine and suggest that it may be found, bound to albumin, in serum (Todorov et al. 1996a, Wigmore et al. 2000). This may be explained were cell lysis to be required for PIF to enter the extracellular compartment. The alternative hypothesis, that the peptide is somehow secondarily glycosylated following secretion, does not seem likely given its identification in cell lysates. Further studies of the antigen specificity of the different antibodies and careful analysis of glycosylation in different cell types will be help to address these issues.

### **The importance of asparagine residues in the PIF / dermcidin polypeptide**

In our *in vitro* systems, PIF / dermcidin did not appear to be glycosylated. Use of the anti-glycosylated PIF monoclonal antibody, which also detected CD59, did not demonstrate any bands with the molecular weight of glycosylated PIF on

Western blotting or immunoprecipitation. Similarly, the use of rabbit polyclonal anti-PIF peptide demonstrated only products with molecular weights corresponding to aggregated or albumin-bound PIF. Furthermore, the asparagine residues of human PIF do not lie in classical consensus N-glycosylation sites. Despite the possibility that they may represent atypical or novel glycosylation sites, mutation of both residues to glutamines also did not affect the molecular weight of PIF detected by either Western blotting or *in vitro* translation. *In vivo*, we were also unable to detect glycosylated PIF in the urines of weight losing patients with pancreatic carcinoma by Western blotting and neither glycosylated nor unglycosylated PIF / dermcidin were detectable by MALDI-TOF mass spectrometry. These findings are in agreement with those of Monitto et al. (2004), who were unable to demonstrate PIF glycosylation in a range of cell types and did not find overexpressing cell lines to be pro-cachectic. Previous studies on Y-P30 have also suggested it is not glycosylated (Cunningham et al. 1998, Porter et al. 2003). These findings do not exclude the possibility that PIF glycosylation is detectable under different circumstances, such as in cachexia, for which glycosylation appears to be required.

The inhibition of PIF / dermcidin's survival benefits and proliferative effects by mutation of both asparagine residues suggests that in combination, they are important to this function. Whether this is due to an effect on the potential phosphatase domain or on receptor binding is not clear. Studies comparing the survival and proliferative effects of synthetic peptides containing asparagine to glutamine substitution with the wild type PIF core peptide and identification of the PIF receptor will help to clarify the role of these residues.

## **The importance of the PIF core peptide to PIF / dermcidin function**

Our results demonstrate that the PIF core peptide is sufficient to induce HuH7 cell proliferation. This is consistent with studies showing the proliferative effects of DSEP transfection, which implied but did not confirm that these were due to the Y-P30 portion of the molecule (Cunningham et al. 2002, Porter et al. 2003). Potentially therefore, proteolytic processing of the nascent PIF / dermcidin transcript as suggested by Schitteck et al. (2001) may result in the production of a signal peptide, a peptide with proliferative effects, an antibiotic peptide and an as yet uncharacterised 'pro-peptide'. Why and how these peptides with such different functions should arise from the same gene is unclear. In the hypothalamus, the pro-opiomelanocortin (POMC) polypeptide is cleaved to give rise to several hormones (Chretien and Seidah 1981). Different forms of POMC-derived peptides are secreted depending on the proteolysis and glycosylation of the full-length precursor and secretion of one form of POMC peptide may occur without the secretion of another (Chretien and Seidah 1981). It is feasible that the production of PIF / dermcidin peptides may similarly be separately regulated. This could explain the previously reported differences in PIF expression and glycosylation (Todorov et al. 1996a, Monitto et al. 2004). Alternatively, the functions of the PIF / dermcidin peptides may have evolved to act in concert, potentially in response to an as yet unidentified stimulus. For example, it is feasible that secretion of anti-bacterial and pro-survival / proliferation peptides might benefit cells under conditions of oxidative stress in bacterial infection (Liaudet et al. 2002). Identification of the mechanisms controlling PIF / dermcidin expression, confirmation that proteolytic processing occurs, identification of the

proteases responsible and determination of the destinations of the peptides produced will address these issues.

Several mechanisms may potentially account for the effect of the PIF core peptide. Firstly, this peptide contains the sequence with homology to the phosphatase domain of calcineurin and has previously been demonstrated to cleave p-nitrophenyl phosphate (Cunningham et al. 1998). Our results suggest that this may be important for the induction of cell proliferation and potentially also for protection from oxidative stress. However, as calcineurin has not been reported to stimulate cell proliferation it seems likely that the PIF phosphatase will act on different targets. As yet, these remain unknown. Secondly, the PIF core peptide may exert its effects through interactions with calreticulin. It has been suggested that the survival effect of Y-P30 involves binding to cell surface calreticulin, inducing its translocation to the cytoplasm and resulting in increased calcium binding and subsequent protection from reactive oxygen intermediaries (Cunningham et al. 2000). However, what effect this may have on proliferation in our system is difficult to determine, as calcium has previously been demonstrated to induce the proliferation of normal hepatocytes but not malignant cells (Swierenga et al. 1980). Thirdly, the PIF core peptide might feasibly act by interacting with one of the uncharacterised PIF receptors. Given the small size of the peptide, it is possible that the H35N and N32QN44Q mutations may interfere with core peptide binding one of these receptors or alternatively, to calreticulin or the phosphatase target. Investigation of the biological and phosphatase activity of mutation-containing synthetic peptides and their binding patterns will help elucidate which of these mechanisms are important in cell survival and proliferation.

## Characterisation of the urinary proteome and PIF excretion

Using mass spectrometry, we have demonstrated proteins in the urines of patients with pancreatic carcinoma which are not present in the urines of healthy individuals. Although we did not quantify these changes, they are unlikely to be of the correct order of magnitude to affect overall nitrogen balance as nephrotic syndrome is rare in patients with malignant disease (Lund et al. 1989) and the proteins found therefore potentially reflect other aspects of the neoplastic process. Although they may subsequently prove to be important tumour markers, as yet little is known of their function. This reflects the power of proteomic technology to identify proteins which may have been sequenced only as part of the human genome project or described in minimal detail in the literature. However, to identify their clinical relevance studies with sufficient numbers of patients to prove statistical significance will be required. Similarly, to identify their possible relevance as therapeutic targets, conventional scientific assessment of their biological function is necessary. These caveats are often levelled as criticisms of 'fishing expeditions' using genomic technology. However, changes in the overall patterns of protein expression may also act as tumour markers and could reflect biological processes of therapeutic importance. In non-urinary tract cancers changes in the urinary proteome may potentially be of increased importance as these tumours cannot directly secrete proteins into urine. Changes in the proteome may therefore reflect not only abnormalities in renal function but also the presence of abnormal proteins in the circulation. The urinary proteomes of patients with pancreatic carcinoma therefore provide a potential insight into the pathophysiology of cancer and cachexia and their relationship with renal function.

We identified two proteins in the urines of patients with pancreatic carcinoma which have not previously been reported in normal urine, mitogen-activated protein kinase kinase kinase 3 isoform 1 (MEKK3) and APC-binding protein EB1 (Nedelkov and Nelson 2001, Thongboonkerd et al. 2002, Pisitkun et al. 2004). EB-1 interacts directly with the tumour-suppressor APC, linking it to microtubules (Mimori-Kiyosue et al. 2000). MEKK3 has been demonstrated to inhibit apoptosis through NF- $\kappa$ B activation and is overexpressed in certain tumours (Samanta et al. 2004). It is therefore feasible that these proteins may influence progression or cachexia in pancreatic cancer. Studies of their expression by tumour cells and their effects on progression and malignancy will help to determine their roles.

The significance of the other proteins we identified in the urines assayed is difficult to determine. Mannose binding lectin-associated serine protease-2 related protein (MBP2-RP), immunoglobulin light chains, the alpha-1-microglobulin/bikunin precursor and keratin have all previously been detected in normal urine (Nedelkov and Nelson 2001, Thongboonkerd et al. 2002, Pisitkun et al. 2004). However, the passage of these proteins into urine may be altered by disease, particularly those affecting the kidneys and urinary tract (Ostergaard et al. 1997, Vlahou et al. 2001, Thongboonkerd 2004). In this respect,  $\alpha$ 1-microglobulin and bikunin have been investigated extensively as markers of renal function and it has been suggested that urinary  $\alpha$ 1-microglobulin may be a marker of asymptomatic renal tubular dysfunction (Yu et al. 1983). Both  $\alpha$ 1-microglobulin and bikunin are abundant serum glycoproteins (Vetr and Gebhard 1990) and it is therefore feasible that their presence in urine may be due to an effect of the neoplastic or cachectic processes on renal function. In favour of cachexia being the relevant pathology are

the findings that bikunin has negative effects on the malignancy of ovarian carcinoma and serum  $\alpha$ 1-microglobulin levels do not correlate with the presence of neoplasia (Takagi et al. 1980, Tanaka et al. 2004). Altered renal function may also potentially account for the presence of MBP2-RP, immunoglobulin light chains and keratin in urine. Certainly, urinary immunoglobulin excretion has been demonstrated to be elevated in prostatic and renal carcinoma and is thought to reflect glomerular damage (Hemmingsen and Skaarup 1977, Abdul and Hoosein 1995). In pancreatic carcinoma the production of immunoglobulins is also known to be both qualitatively different and greater in comparison with the normal pancreas (Goodale et al. 1979, Goodale, Jr. et al. 1981, Maacke et al. 2002). Ultimately, changes in urinary protein excretion may contribute to the negative nitrogen balance of cachexia and could potentially prove to be an important indicator of the cachectic process. Larger studies to investigate the significance of this process and the importance of individual urinary proteins appear warranted.

### **Implications of PIF / dermcidin - induced cell proliferation and survival for the development of cachexia**

Although the mechanisms by which cachexia develops are increasingly well understood, little is known of the reasons why tumours produce pro-cachectic substances. One theory is that production of cachectic factors may bestow a survival advantage on tumour cells. This would suggest that the metabolic changes of cachexia develop as a by-product of oversecretion of these molecules. However, it is feasible that this alteration in metabolism may also benefit the tumour, selecting for cells which are capable of survival in adverse conditions. In particular, levels of

oxidative stress are often high within tumours (Pelicano et al. 2004). Subsequently, PIF / dermcidin expression would again be expected to benefit cells, and a form of positive feedback loop could develop.

We found that the unglycosylated PIF core peptide is sufficient to induce cell proliferation and that the survival benefits of PIF / dermcidin expression do not require glycosylation. It has previously been suggested that unglycosylated PIF is unlikely to induce cachexia (Monitto et al. 2004) and it would therefore seem feasible that the stimulation of cell growth and survival are the primary functions of the unglycosylated molecule. In contrast, studies of glycosylated PIF suggest that this form is capable of inducing muscle proteolysis and alterations in hepatic gene expression (Todorov et al. 1996a, Watchorn et al. 2001) and that glycosylation is required for the action on muscle (Todorov et al. 1997). Glycosylation subsequently appears to be a key event which may potentially separate PIF / dermcidin's growth and survival promoting effects of from its pro-cachectic ones. Differential glycosylation may also help to explain the difficulties in the immunological detection of PIF. We did not find PIF / dermcidin to be glycosylated in either HuH7 cell or our *in vitro* translation system and unglycosylated PIF / dermcidin has previously been demonstrated in neuronal cells and several overexpressing cell lines (Cunningham et al. 1998, Monitto et al. 2004). In contrast, PIF purified by affinity-chromatography from the MAC16 cell line and from the urines of patients with pancreatic carcinoma appears to be glycosylated. Identification of the stage at which PIF becomes glycosylated may subsequently reveal cellular abnormalities contributing to cachexia. Tumours are known to exhibit abnormal patterns of glycosylation, which may change with progression (Kannagi et al. 2004). Interestingly, the PIF core

peptide lacks consensus sequences for N-glycosylation and would therefore not be expected to be a target of the principle N-glycosylating enzymes (Welply et al. 1983). Investigation of PIF glycosylation therefore has the potential to reveal novel cellular biochemistry as well as improve our understanding of PIF / dermcidin biology.

### **The importance of PIF / dermcidin in pancreatic carcinoma**

We have demonstrated that PIF / dermcidin is widely expressed in human pancreatic carcinoma cell lines. In addition, the HuH7 cell line did not exhibit PIF / dermcidin expression, suggesting an association between cell lines derived from cachexia-inducing tumours and PIF / dermcidin expression. However, it has previously been reported that *in vivo* only 2 of 64 pancreatic tumours demonstrated PIF / dermcidin expression (Porter et al. 2003), and this is consistent with our findings of a restrictive expression pattern both by immunological and PCR analysis of tumours and urinary analysis. Furthermore, glycosylation is required for the induction of muscle proteolysis (Todorov et al. 1997). Subsequently, a direct link between PIF / dermcidin expression *in vitro* and the induction of cachexia *in vivo* does not appear to exist, although the production of PIF / dermcidin mRNA would be necessary for any cachectic effect. The role of PIF may therefore be restricted to a subset of carcinomas. In these tumours, PIF may have the potential to influence cell growth and survival and subsequently progression. Examination of expressing tumours will help to identify characteristics associated with PIF / dermcidin expression, for example malignancy and cachexia. Furthermore, it may lead to the establishment of PIF / dermcidin's position in the PIN - carcinoma sequence. The low

frequency of expression observed suggests that it is unlikely to be a central molecule in this process, but could be important at later stages when the development of oxidative stress provides a selective pressure for expression. If so, it may be of relevance to the prognosis of these tumours. Oxidative stress is known to contribute to the malignancy of cancer (Pelicano et al. 2004). Furthermore, many cancer therapies function via the induction of oxidative stress (Renschler 2004) and tumour cell resistance to this may subsequently confer resistance to these agents. PIF / dermcidin expression may therefore potentially influence the poor clinical outcome in these patients through effects on tumour progression, resistance to therapy and development of cachexia.

Oxidative stress may also potentially account for the more widespread *in vitro* expression of PIF / dermcidin. In culture, cells must survive in an altered nutritional environment and with different oxygenation to *in vivo* conditions. This may result in higher levels of oxidative stress (Lluis et al. 2005). As yet, the only stimulus for PIF / dermcidin expression to be identified is oxidative stress (Cunningham et al. 1998) and this may therefore stimulate *in vitro* expression. Alternatively, another effect of the culture environment may influence expression via one of the many putative transcription factor binding sites in the PIF / dermcidin promoter. Elucidating the mechanisms controlling expression may aid in understanding the differences between *in vitro* and *in vivo* expression.

### **Limitations of the current studies**

There were some areas in which it was not possible to fully achieve our aims. We were technically unable to demonstrate PIF / dermcidin protein production

immunologically. *In vitro*, it was possible to confirm expression at the mRNA level although expression in HuH7 cells appeared to be variable and potentially inducible in prolonged culture necessitating careful validation of results. We were unable to detect PIF / dermcidin expression *in vivo*, possibly due to the difficulty of preparing high-quality RNA from pancreatic samples but also possibly reflecting the low level of expression reported by other authors (Porter et al. 2003). We were subsequently unable to fully relate our laboratory studies to the clinical situation or to resolve the conflicting evidence from previous studies regarding PIF expression. However, the technical difficulty we experienced does raise the possibility that the available anti-PIF antibodies, particularly the monoclonal anti-glycosylated PIF antibody, are not as reliable as previously thought and that previous work based on this antibody should perhaps be re-evaluated using new antibodies.

The transfection of mammalian overexpression vectors is a useful technique but may produce results which are not relevant to the physiological function of a gene. The HuH7 cell line has not been characterised for the expression of PIF / dermcidin receptors and it is subsequently not possible to say whether its response to overexpression is either typical or representative of the *in vivo* situation.

Overexpression vectors result in the unregulated production of large amounts of mRNA for the gene of interest and feedback or other methods of control of expression are subsequently not possible. Studies of gene function using this method are therefore usefully supported by studies using RNAi, transgenic animals and more traditional methods of promoter and expression analysis.

It was not possible to recruit sufficient patients with pancreatic carcinoma in the available time to draw statistically significant conclusions regarding their urinary

proteomic profiles. In addition, the need to develop this technology restricted the time available to fully analyse all patients' urine samples using mass spectrometry of tryptic digests. These factors leave several opportunities for the future study of this promising technique.

## Conclusion

Proteolysis inducing factor / dermcidin is a novel molecule with multiple functions in muscle proteolysis, hepatic metabolism, the innate immune system and cell survival and proliferation. These functions may be relevant in a range of physiological and pathological processes including cachexia and neoplasia. This thesis has described the effect of expression on cell survival and proliferation in a hepatic carcinoma cell line system. Within the molecule, an important structure for these effects appears to be the PIF core peptide, which may act through the calcineurin-like phosphatase domain or potentially via receptor interaction and effects on oxidative stress and the proteasome. This thesis also attempted to relate these findings to the role of PIF / dermcidin in pancreatic carcinoma and cachexia, although problems with the detection of glycosylated PIF by immunological methods prevented this aim from being fulfilled. In attempting to develop an alternative method of detecting urinary PIF / dermcidin MALDI-TOF mass spectrometry revealed changes in the urinary proteome of patients with pancreatic carcinoma. Further studies of the *in vivo* roles of these proteins and of PIF / dermcidin has the potential to further improve our understanding of tumorigenesis, cachexia and the cellular processes which underlie them.

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# Research Data Collection Sheet

## Patient Details

Name/Addressograph

Date

Sex

M / F

Age at diagnosis

Date of Diagnosis –

Duration of symptoms -

Co-morbidity/PMH -

Current Health (eg URTI) -

Smoker

Current Medications - NSAIDS  
- Steroids  
- Immunosuppressants  
- Others

ASA

Pre-illness stable weight

Pre-illness BMI

# Appendix A – Patient Data Collection Proforma

## Pathology Details

Tumour Position                      **Head / neck / body**

Vascular involvement                **No / portal vein / SMA / SMV**

Histology                                **Ductal adenocarcinoma/Other**

Differentiation                         **Poorly/moderately/well**

Other Prognostic Features    (eg Necrosis)

Staging Investigations - CT	T	N	M
	Tumour volume <input style="width: 150px; height: 25px;" type="text"/>		
- EUS	T	N	M
- Lap/LUS	T	N	M
- CXR			
- Other (eg peritoneal washings)			

Final Stage:			
	<b>Clinical</b>	<b>T</b>	<b>N</b>
			<b>M</b>
	<b>Pathological</b>	<b>T</b>	<b>N</b>
			<b>M</b>

# Appendix A – Patient Data Collection Proforma

## Investigations

	Premorbid	At Diagnosis	Clinic	Clinic
DATE				
CRP				
Albumin				
Uric acid				
Urinary PIF				
Blood for storage				
Height				
Weight				
BMI				
Karnofsky Index				
Estimated oral intake				

## Tissue Samples

Source procedure -

Ischaemic time - Whipples - time from transaction of neck to freezing =  
 - Other – time from specimen removal to freezing =

Skeletal muscle taken Y/N

# Appendix A – Patient Data Collection Proforma

## LCM

Site of cell collection (eg core, rim) –

## PCR

mRNA	PCR Level		
	Tumour cells	Stroma	Normal Pancreas
PIF			

## Outcome

Treatment undertaken:

**Surgery**      Yes/No

Operation –

Date –

Adjuvant Treatment –

Complications -

**Palliation**      Chemotherapy

Radiotherapy

Stenting (inc type)

Laser

Nothing

## Outcome

Date of Recurrence -

Date of death –

Cause of death -

## Appendix B – Karnofsky Index

Normal, no complaints	100
Able to carry out normal activities, minor signs of disease	90
Normal activity but with effort	80
Self-caring but unable to carry out normal activity or work	70
Requires occasional assistance but able to care for most needs	60
Requires considerable assistance and frequent medical care	50
Disabled, requires special care and assistance	40
Severely disabled, hospitalization indicated although death not imminent	30
Very sick, requires hospitalization. Active supportive treatment necessary	20
Moribund	10
Dead	0

## Appendix C – Patient Information Sheet and Consent Form

### Transcriptional Regulation of Proteolysis Inducing Factor in Pancreatic Carcinoma **A Study into Weight Loss in Patients with Pancreatic Cancer**

(Version 2)

*You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.*

Thank you for reading this

#### **What is the purpose of the study?**

Proteolysis inducing factor (PIF) is a molecule detected in the urine of patients with pancreatic tumours. Its presence is associated with increased weight loss. PIF is not found in the body normally, and we plan to confirm that it is produced by tumour cells and investigate why. We will do this by DNA analysis of the tumour and by analysis of your urine for PIF and other molecules which might cause weight loss or affect the behaviour of the tumour.

#### **Why have I been chosen?**

We plan to look at PIF production in tumours which have been removed surgically. You have been chosen as we believe you have this type of tumour and your planned treatment is surgery.

#### **Do I have to take part?**

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

#### **What will happen to me if I take part?**

Your treatment will not be altered by taking part in our study. For the experiments we are planning, we need a sample of your tumour which we will take at the time of surgery. If it is possible to remove the tumour we will take it to the pathologist who will take the sample for us. If it is not possible to remove the tumour your surgeon will take an additional biopsy sample (about the size of the head of a matchstick) for the study. We also need a urine sample before the operation, and access to the results of some of the blood tests which have been performed.

You should be aware that it will not be possible to return the samples of tumour or urine to you once they have been taken.

#### **What do I have to do?**

We would be grateful if you could pass 200ml (about 1 mug) of urine into the container provided. Otherwise, your treatment will continue unchanged.

#### **What is the drug or procedure being tested?**

We are not testing any drugs or procedures. You will receive treatment for your condition as normal.

#### **What are the side effects of any treatment received when taking part?**

Your treatment will proceed as normal and any side effects will be dealt with as normal.

#### **What are the possible disadvantages and risks of taking part?**

If we take an additional biopsy sample you will be exposed to the associated risks of bleeding, leakage from the pancreas and inflammation of the pancreas. If your surgeon is able to remove the tumour the only

## **Appendix C – Patient Information Sheet and Consent Form**

risk is that by removing a part of your tumour we could affect the information gained from it by the pathologists. To avoid this, all samples will be taken under their guidance.

### **What are the possible benefits of taking part?**

As your treatment will be unchanged, there is no direct benefit to you for taking part. However, you will be helping research into cancer, and specifically into the reasons why people with cancer lose weight.

### **What if new information becomes available?**

Sometimes during the course of a research project, new information becomes available about the subject being studied. If this happens your research doctor will tell you about it and discuss with you any changes in the way your samples will be used. If you decide to continue in the study you will be asked to sign an updated consent form.

Also, on receiving new information your research doctor may consider it necessary to withdraw you from the study. He / she will explain the reasons and arrange for your care to continue.

### **What happens when the research study stops?**

Your treatment and follow up will continue as normal.

### **What if something goes wrong?**

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

### **Will my taking part in this study be kept confidential?**

Yes. All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it.

If you agree, we will write to your GP and inform them of your involvement in the study.

### **What will happen to the results of the research study?**

We plan to continue the study until 2004. We will then analyze the results and hope to publish them in a medical journal. You will not be identified in any publication. If you would like a copy of the results, please contact the lead researcher around December 2004.

### **Who is organizing and funding the research?**

This research is supported by the University of Edinburgh Department of Surgery at the Royal Infirmary of Edinburgh.

### **Who has reviewed the study?**

The Local Research and Ethics Committee of the Royal Infirmary of Edinburgh.

### **Contact for further information**

If you would like to know more, please contact the lead researcher -

Mr. A. Lowrie

Tissue Injury and Repair Group

Centre for Inflammation Research (6<sup>th</sup> Floor)

University of Edinburgh Medical School

Teviot Place

Edinburgh

Tel – 0131 650 2985

**Thank you for taking part.**

**Appendix C – Patient Information Sheet and Consent Form**

**CONSENT FORM**

**Transcriptional Regulation of Proteolysis Inducing Factor in Pancreatic Cancer**

**A Study into Weight Loss in Patients with Pancreatic Cancer**

Version 2

Name of Researcher: Mr A. Lowrie, Tissue Injury and Repair Group, Centre for Inflammation Research (6<sup>th</sup> Floor), School of Clinical Sciences and Community Health, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG Tel – 0131 650 2879

Please initial box

- 1. I confirm that I have read and understand the information sheet dated \_\_\_\_\_ for the above study and have had the opportunity to ask questions.
- 2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.
- 3. I understand that samples of my body tissue, urine and blood will be kept and analysed for research purposes which will include analysis of DNA and the mix of proteins in my urine. I understand these samples will not be returned to me.
- 4. I consent to my GP being informed of my participation in this study
- 5. I agree to take part in the above study.

\_\_\_\_\_  
**Name of Patient**

\_\_\_\_\_  
**Date**

\_\_\_\_\_  
**Signature**

\_\_\_\_\_  
**Name of Person taking consent**

\_\_\_\_\_  
**Date**

\_\_\_\_\_  
**Signature**

(if different from researcher)

\_\_\_\_\_  
**Researcher**

\_\_\_\_\_  
**Date**

\_\_\_\_\_  
**Signature**

1 for patient; 1 for researcher; 1 to be kept with hospital notes

## Appendix D – Patient Details

Age	Sex	%weight loss	Pathology	Surgery	Survival (days)
63	M	11	PDA*	Resection	500
71	F	4	PDA	Resection	112
68	F	7	Serous microcystadenoma	Resection	>562
50	M	9	Liposarcoma	Resection	>536
51	F	12	PDA	Resection	>515
60	M	13	Duodenal carcinoma	Resection	171
47	F	5	Ampullary adenocarcinoma	Resection	>568
52	M	18	Cholangiocarcinoma	Resection	>447
80	F	10	Ampullary TVA	Resection	>471
45	M	11	Cholangiocarcinoma	Resection	267
65	F	0	Cholangiocarcinoma	Resection	341
55	F	0	PDA	Bypass	219
74	F	14	PDA	Resection	284
52	M	8	Cholangiocarcinoma	Resection	254
58	F	13	PDA	Bypass	139
77	F	3	PDA	Resection	>214
45	F	3	Ampullary adenocarcinoma	Resection	>171
60	F	0	Ampullary adenoma	Resection	>123
47	M	18	PDA	Resection	>243

\*PDA – Pancreatic ductal adenocarcinoma

## **Appendix F – Publications arising from work contained in this thesis**

Dermcidin expression in hepatic cells improves survival without N-glycosylation but requires asparagine residues.

**Lowrie A. G.,** Wigmore S. J., Wright D. J., Waddell I., Ross J. A.

British Journal of Cancer, 2006; Jun 5;94(11):1663-71.