

**Phosphoinositide 3-kinase Regulation of Anchorage-independent
Growth and Drug Resistance in Small Cell Lung Cancer Cells.**

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The work presented in this thesis was performed solely by the author, unless otherwise stated, under the supervision of Dr. Tariq Sethi and Prof. Edwin R. Chilvers, Respiratory Medicine Unit, Rayne Laboratory, University of Edinburgh.

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Abstract.

Human small cell lung cancer (SCLC) accounts for approximately 25% of all primary lung cancers. It is known to metastasise early and follow an aggressive clinical course. Despite initial sensitivity to chemotherapy, patients almost invariably relapse and become chemoresistant so that the 2 year survival rate is between 3 - 8%. Widespread metastasis and resistance to chemotherapy are the two main causes of these dismal survival figures in SCLC. This thesis focuses on the potential mechanisms involved.

Anchorage-independent proliferation is essential to enable tumour cells *in vivo* to survive and metastasise, and under *in vitro* conditions allows transformed cells to form colonies in semi-solid media. Phosphoinositide 3-kinase (PI 3-kinase) has been shown to critically involved in cell anchorage-independent growth and tumourigenicity. In this thesis it is shown that PI 3-kinase is constitutively activated in SCLC cell lines (H69, H345 and H510). Inhibition of PI 3-kinase activity using the selective inhibitors wortmannin and LY294002 markedly inhibited cell proliferation and stimulated apoptosis in liquid media. This inhibition of proliferation was shown to occur via both a ribosomal protein s6 kinase (p70^{s6k}) dependent and independent mechanism. Furthermore, PI 3-kinase inhibition reduced basal SCLC cell growth in agarose semi-solid media which could not be rescued by the addition of neuropeptides. This is the first description of a constitutively activated PI 3-kinase in any human cancer. It is proposed that this constitutive activity plays an important role in promoting growth, anchorage-independence and tumourigenicity in SCLC, and may account for the non-adherent phenotype and highly metastatic nature of this cancer.

Resistance to chemotherapy is a major problem in the treatment of SCLC. Adhesion to extracellular matrix (ECM) proteins can protect cells from undergoing detachment-induced apoptosis. SCLC cells *in vivo* are surrounded by a specialised micro-environment rich in ECM containing in part laminin, fibronectin and collagen

IV. It is shown that SCLC cells adhere to the ECM proteins *in vitro* via a β 1 integrin-dependent manner, enhancing tumourigenicity and conferring resistance to standard chemotherapeutic agents. This adhesion to ECM proteins increases SCLC cell growth and protects cells from the pro-apoptotic effects of the chemotherapy agents via a β 1-integrin-dependent mechanism requiring tyrosine kinase activation.

Thus ECM proteins *in vivo* may provide a signal resulting in resistance to chemotherapy, which accounts for the partial responses and the local recurrence of SCLC often seen in patients after primary chemotherapy.

These findings may begin to provide us with novel targets for the therapeutic intervention of SCLC.

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Abbreviations

AEBSF	4-(2-aminoethyl)benzenesulfonyl flouride
ATP	adenosine 5'-triphosphate
NH ₄ Cl	ammonium chloride
(NH ₄) ₂ PO ₄	ammonium phosphate
Bax	pro-apoptotic Bcl-2 homology protein
Bcl-2	B cell leukaemia oncogene 2
BSA	bovine serum albumin
CMF-PBS	calcium and magnesium free – phosphate buffered saline
CaCl ₂	calcium chloride
CO ₂	carbon dioxide
CHCl ₃	chloroform
CHO	Chinese hamster ovary cells
C IV	collagen IV
DNA	deoxyribonucleic acid
°C	degrees centigrade
DAG	diacyl glycerol
dH ₂ O	distilled water
DTT	dithiotheritol
DMEM	Dulbecco's modified Eagle's medium
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbant assay
ECM	extracellular matrix
ERK	extracellular signal-regulated protein kinase (ERK 1 = p ⁴⁴ MAPK, ERK 2 = p ⁴² MAPK)
FRAP	FKBP12 rapamyin associated protein

FKBP12	FK506 binding protein 12
FAK	focal adhesion kinase
FCS	foetal calf serum
FGF	fibroblast growth factor
Fn	fibronectin
FITC	fluorescein isothiocyanate
GST	glutathione-s transferase
GSK3	glycogen synthase kinase-3
Grb2	growth-factor-receptor-bound protein 2
GDP	guanosine di-phosphate
GTP	guanosine tri-phosphate
HPLC	high pressure liquid chromatography
HRP	horse radish peroxidase
HCl	hydrochloric acid
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
IgG	immunoglobulin G
IP ₃	inositol 1,4,5-trisphosphate
IGF-1	insulin-like growth factor 1
ILK	integrin-linked kinase
IL	interleukin
ICE	interleukin 1- β converting enzyme
Ln	laminin
LiCl	lithium chloride
LPA	lysophosphatidic acid
MgAc	magnesium acetate
MgCl ₂	magnesium chloride
mTOR	mammalian target of rapamycin
MEK	MAP/ERK kinase
MMP	matrix metaloproteinase
MeOH	methanol
M	methionine

min(s)	minute(s)
MAP kinase	mitogen activated protein kinase
MTT	(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue)
NGF	nerve growth factor
NaCl	sodium chloride
NaF	sodium fluoride
PMSF	phenylmethylsulfonyl fluoride
PI	phosphatidylinositol or phosphoinositol
PS	phosphatidylserine
PI3P	phosphatidylinositol (3) phosphate
PI4P	phosphatidylinositol (4) phosphate
PI(3,4,5)P ₃	phosphatidylinositol (3,4,5)-trisphosphate
PI(4,5)P ₂	phosphatidylinositol (4,5)-bisphosphate
PBS	phosphate buffered saline
PI 3-kinase	phosphoinositide 3-kinase
PLC	phospholipase C
PDK1	PI(3,4,5)P ₃ -dependent kinase 1
PDGF	platelet derived growth factor
PH	pleckstrin homology domain
PCR	polymerase chain reaction
PYK2	proline rich tyrosine kinase 2
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
Rho	ras homologous
Rac	ras-related C ₃ -botulinum toxin substrate
Ras	rat sarcoma virus
p70 ^{s6k}	ribosomal protein s6 kinase
src	rous sarcoma virus
Shc	SH2-domain-containing α 2-collagen related protein

SCLC	small cell lung cancer
$\text{Na}_4\text{P}_2\text{O}_7$	sodium pyrophosphate
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SOS	son of sevenless
SH2	src homology 2
SH3	src homology 3
TGF- β	transforming growth factor beta (β)
TCA	trichloroacetic acid
TLC	thin layer chromatography
TNF- α	tumour necrosis factor- α
Tyrphostin-25	3,4,5-tri-hydroxy-phenyl-methylene(-propanedinitrile)
Y	tyrosine
VEGF	vascular endothelial growth factor

CHAPTER 1

Introduction.

1.1 Lung Cancer.

The World Health Organisation estimates that there are approximately 6 million new patients diagnosed with a form of cancer each year. Two thirds of these people will then go on to die from cancer, making up 10% of the total world mortality rate. Lung cancer remains the most frequent cause of cancer death, and is therefore a major public health problem. Lung cancer is primarily caused by cigarette smoking (85 - 95% of all lung cancer cases). Other causes of lung cancer are the result of environmental and occupational factors such as asbestos, arsenic, mustard gas, polycyclic hydrocarbons and ionising radiation.

1.1.1 Classification.

1.1.1.1 Lung Cancer Classification.

Lung tumours are carcinomas originating from the respiratory epithelium and are classified by The World Health Organisation (WHO, 1982) on the basis of histology. The two main types of lung cancer are small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (includes squamous, large and adenocarcinoma).

A recent hypothesis by Mabry (1991) suggests that all types of lung cancer originate in the bronchial epithelium and are related via a common differentiation pathway. This hypothesis has been further demonstrated by the use of a non-tumourigenic cell

line derived from bronchial epithelium cells (BEAS-2B). These cells were transfected with the Simian virus (SV40) enabling them to grow continuously (Gruenert et al., 1988, Pfeifer et al., 1989, Bonfil et al., 1989). This lung cancer model then demonstrated that the overexpression or point mutation of various oncogenes caused the production of different histological tumour types (Mabry et al., 1991, Suda et al., 1987). Insertion of the viral *Harvey-ras* (*v-Ha-ras*) gene into a classical SCLC cell which already endogenously expressed amplified the *N-myc* gene, caused transition to the large cell cancer phenotype. Alterations in the morphological, biochemical and growth characteristics were noted (Falco et al., 1990, Mabry et al., 1988 and 1989). Amplification of the *c-myc* gene (known to be overexpressed in variant SCLC types (Little et al., 1983)), in a classical SCLC cell line caused alterations to the cell growth rates, an increase in cloning efficiency, but did not effect neuroendocrine enzyme levels (Johnson, 1986). These studies suggest that amplification of certain oncogenes may cause a shift of SCLC cell line characteristics from a classical to a variant or large cell phenotype.

Conversion from a classical to large cell phenotype might also be associated with cell resistance to chemotherapy in some clinical cases. However, other mechanisms are likely to be involved in this change, as only 10% of SCLC patients present with small / large cell mixtures. At autopsy, this rate increases moderately to 13 - 28% despite the majority of the tumours becoming chemoresistant (Sehested et al., 1986).

1.1.1.2 Small Cell Lung Cancer Classification.

SCLC is the most aggressive form of lung cancer. It occurs predominantly in people in their seventh or eighth decade of life, constituting 25% of all cases of bronchial carcinomas (Weiss, 1981 and 1984). SCLC is strongly associated in a dose-dependent manner with smoking and is rare in non-smokers.

Given that over 90% of patients with SCLC have either local advanced or systemic cancer at initial presentation, treatment by primary resection is rarely an option. This

is due to the fact that SCLC metastasises early, and has the fastest growth rates of the four main types of lung cancer.

The main characteristics of SCLC cells are that they appear as a dense cellular tumour of round monomorphic cells with an extremely high nuclear to cytoplasmic ratio. SCLC is classified as a neuroendocrine cancer. Neuroendocrine cells are defined as being able to produce and secrete polypeptide hormones or amines. Therefore, small dense core granules containing peptides and hormones are often seen in the cytoplasm of SCLC cells. The nuclear features of SCLC cells include the inconspicuous nuclei and uniformly dispersed fine chromatin. When processed for cytological examination, SCLC cells are seen to be approximately 2-3 times the size of mature lymphocytes (7 - 11 microns in diameter) and mitosis is particularly noticeable.

In a study where cells were removed from SCLC patients either during life or at post mortem, it was clear that heterogeneity existed within the SCLC general classification. This allows for further subclassification of SCLC into two types namely classic SCLC and variant SCLC. Classical SCLC cells account for approximately 70% of small cell tumours (Belper et al., 1987a and b and c, Carney et al., 1985a, Gazdar et al., 1980 and 1981 and 1985). Variant cell lines have a larger cell morphology which is less differentiated and more resistant to chemo- and radiotherapy (see Table 1.1 for major differences between cell types). It is accepted that variant cell lines derive not as a result of *in vitro* culture, but reflect an inherent property of the *in vivo* tumour from which they originated.

Table 1.1: Major Differences between Classical and Variant forms of Small Cell Lung Cancer Cells in Culture.

Feature	Classical (SCLC-C)	Variant (SCLC-V)
Growth Pattern	Tightly packed floating aggregates	Loose floating aggregates or monolayers
Histology of xenografts	Small cell carcinoma	Small / large cell carcinoma
Substrate adherence	Absent	Sometimes present
Doubling time	Long	Short
Colony-forming efficiency	Low	Relatively high
Activation of <i>c-myc</i>	Absent	Often present
Sensitivity to radiation	High	Low
Enzymes		
L-dopa decarboxylase	Present	Reduced or absent
Neuron-specific enolase	Present	Reduced
Creatine-kinase brain isoenzyme	Present	Present
Dense-core vesicles	Present	Usually absent
Secretory products		
Bombesin-like peptides	Present	Usually absent
Other peptides	Usually present	Usually absent

A feature of SCLC cells is their ability to secrete a large number of hormonal neuropeptides including gastrin releasing peptide, vasopressin, bombesin and neurotensin (Sorenson et al., 1981, Sausville et al., 1985, Goedert et al., 1984, Cuttitta et al., 1985, Belper et al., 1988. Reviewed Moody & Cuttitta, 1993). These neuropeptides have been shown to induce the mobilisation of intracellular calcium stores in SCLC cells (Heikkila et al., 1987, Woll & Rozengurt, 1989, Bunn et al., 1990 and 1992, Sethi & Rozengurt, 1991). However, the degree of responsiveness of the different SCLC cell lines to these neuropeptides varies (Bunn et al., 1992). This heterogeneity is thought to be due to the presence of different receptor types and numbers on the different SCLC cell lines.

The ability of these neuropeptides to promote SCLC clonal growth in semi-solid agar has previously been demonstrated (Sethi & Rozengurt, 1992, Carney et al., 1987, Weber et al., 1985). Sethi and Rozengurt (1992) were further able to correlate the increases in neuropeptide-stimulated clonal growth with the increases in calcium

mobilisation. It is therefore hypothesised that the aggressive growth of SCLC *in vivo* is driven by multiple autocrine and paracrine interactions involving these hormonal neuropeptides. The ability to survive without the requirement of exogenous growth factors (serum-independence) is a characteristic feature of the transformed phenotype, and is highlighted in SCLC cell lines *in vitro*. In liquid culture these cells are routinely grown in minimal media, i.e. in the presence of key growth factors/hormones only (e.g. insulin, selenium, transferrin and hydrocortisone) (Carney et al., 1985b, Bepler et al., 1988).

1.1.2 Hereditary Predisposition.

Most genetic alterations in lung tumours have arisen somatically. However, evidence is beginning to suggest that an inherited component may predispose people to lung cancer development. Given that only 10 - 15% of smokers (20⁺ cigarettes per day) actually develop lung cancer, it is hypothesised that host factors must alter a persons risk of developing lung cancer. Also the fact that non-smokers and relatives of smokers may also develop lung cancer (not taking into account passive smoking), suggests that people can be predisposed to lung cancer development.

A multi-stage model proposes that two or more genetic mutations are required to fully transform a cell. If one or more of these mutations was inherited, it may allow transformation to occur at an earlier stage in life. Increasing evidence has suggested that genetic alterations that lead to an increased risk in lung cancer formation are inherited in a mendelian manner (Sellers et al., 1994). However, with increased smoking and age, the relative contribution of this inheritance pattern decreases. A number of epidemiological studies have noted the existence of families where first-degree relatives have a 2-4 fold increased risk in developing lung cancer (Ooi et al., 1986) as well as an increased risk in developing other non-smoking related cancers (Lynch et al., 1986). It has been observed that relatives of retinoblastoma patients (Rb), who were carriers of a mutated Rb gene, were 15 times more likely to die from lung cancer than a person from the general population. This is further evidence

indicating that inherited predisposition can be a factor in the development of lung cancer (Saunders et al., 1989).

The reason why only 10 - 15% of smokers develop lung cancer may be explained by their ability to metabolise/inactivate and remove potential carcinogens from the body. The majority of carcinogenic factors are the metabolites of environmental carcinogens (Sims et al., 1974). Enzymes known as phase I enzymes, such as aryl hydrocarbon hydroxylase and epoxide hydrolase, activate potential compounds into mutagenic and carcinogenic diol-epoxides (Grover et al., 1976, Newbold & Brookes, 1976). Phase II enzymes such as glutathione-S-transferase and UDP-glucuronosyltransferase then cause the production of non-toxic water soluble metabolites (Pelkonen & Nebert, 1982). Theoretically, if patients were unable to metabolise (activate) the ingested carcinogens, they may not be so prone to developing cancer. Cigarette smoke has been shown to cause the induction of the phase I enzymes cytochrome-P450-dependent aryl hydrocarbon hydroxylase and epoxide hydrolase and the phase II enzyme UDP-glucuronyl transferase (Petruzzeli et al., 1988). In contrast significant decreases in the phase II enzyme glutathione-S-transferase were noted. These results suggest that cigarette smoking may be able to alter the balance between the activation and detoxifying enzymes in the body and this may modulate susceptibility to carcinogenic damage.

1.1.3 Genetic Pathology.

Development of lung cancer is a multistep process involving a number of histopathological, biological, molecular and epigenetic changes. These involve either activation of dominant proto-oncogenes or deactivation of recessive tumour suppresser oncogenes (anti-oncogenes). The progressive loss of genetic stability is known to cause cell transformation and cancer development.

Lung cancer formation models have been proposed in which a number of different stages are incorporated. Broadly these can be split into three main stages:

1) Exposure to an environmental carcinogen. The extent of injury can depend upon the genotype and metabolic phenotype of the cell, enabling the extent of injury to become more enhanced. The formation of DNA:benzo(a)pyrene adducts (from cigarette smoke) have been detected in people predisposed to lung cancer (Bohr et al., 1987). This suggests that upsets in the DNA-adduct excision repair mechanism may be crucial in the onset of lung cancer.

2) Proliferative Stage. In response to exposure, there is production of growth factors. This may either be due to initiation of new, or the enhancement of existing growth factors. Paracrine growth of cells then leads to focal cellular proliferation (normally benign). These tumours often consist of cells with identical morphological and phenotypical characteristics to the original cell, and are situated in the same tissue environment (i.e. as they are not anchorage-independent, no invasion or metastasising occurs, which is often associated with malignancy). This dysregulation of growth leading to benign hyperplasia has often been seen in animal models (Chang et al., 1989, Sandgren et al., 1990). Expression of different oncogenes that interrupt and cause activation of the growth stimulatory pathways in the absence of any ligand (due to mutation or overexpression) can also lead to cells bypassing the requirement of growth factors causing serum-independence.

3) Promotion and Induction. Involves several chromosome abnormalities (somatic or inherited) in recessive oncogenes, p53, and epigenetic changes such as the activation of several proto-oncogenes.

Over the last decade the understanding of lung cancer formation has greatly increased due to identification of some of the genetic abnormalities involved. Studies have shown the presence of a large number of acquired (somatic) mutations in lung cancer. These have been shown to take on many different forms ranging from numerical abnormalities (hypodiploidy, hyperdiploidy, tetradiploidy) to structural changes (translocations and deletions) (Whang-Peng, 1989). Some of these mutations are

present in one type of tumour only, and mutations of the 3p, 13q and 17p chromosomes have been found in the majority of SCLC cells (Yokota et al., 1987).

Cytogenetic analysis of SCLC reveals the presence of non-random deletions in chromosome 3 in greater than 90% of cases, with the shortest region being 3p14-23 (Whang-Peng et al., 1982, Falor et al., 1985, Buys et al., 1987). These deletions have been confirmed in both primary tumours and cell lines. Deletion of 3p has also been seen in NSCLC (with lesser frequency) (Weston et al., 1989), breast carcinomas and in renal carcinomas suggesting that it may play a more general role in the pathogenesis of cancer (Kok et al., 1987, Zbar et al., 1987). In Wilms' tumour (child's kidney tumour), a deletion in the short arm of chromosome 11 causes the loss of a tumour suppressor gene. Absence of genetic material in the same region has been noted in lung carcinomas (Yokota et al., 1987) and it seems likely that the same gene is involved.

1.1.3.1 Recessive Oncogenes.

Recessive oncogenes (tumour suppressor genes) are genes whose recessive inactivation or loss allows for dysregulation of cell growth. (Both alleles need to be inactivated).

Retinoblastoma Gene. Retinoblastoma is a childhood tumour in which both alleles of the Retinoblastoma (Rb) gene (13q14) are inactivated. The Rb protein is known to be a negative regulator of cell death. Studies of somatic genetic changes in SCLC noted that a number of mutations were found to be localised to the 13q chromosome. Harbour and colleagues (1988) found upon investigation, the absence of Rb gene mRNA expression in 60% of SCLC cell lines but only 10% of NSCLC cell lines and normal lung tissue. Further studies by a number of different groups have indicated the loss of the 13q allele in virtually all SCLC tumours (Mori, 1989, Yokota et al., 1987, Hensel, 1990). These results sharply contrast with a loss in only 32-55% of NSCLC tumours.

There have been studies performed concluding that survivors of hereditary Rb might be predisposed to lung cancer (Leonard et al., 1988). Sanders and colleagues (1989) also showed that carriers of the Rb1 mutation had a 15-fold increased risk in developing lung cancer (mainly small cell type). Once again these results indicate that a predisposition to lung cancer can be inherited.

p53. p53 is the most commonly mutated gene in human cancer (Hollstein et al., 1991). Loss of one allele at 17p13 (where p53 is known to reside (Isobe et al., 1986)) is common in lung tumours with a mutation (often in the DNA binding domain region) on the remaining allele. The most common mutation is the transversion of the nucleotides GC to TA (Harris & Hollstein, 1993). Carcinogens found in cigarette smoke can cause this transversion. Point mutations in p53 mRNA have been found in all types of primary tumour specimens (Takahashi et al., 1989). The highest percentage incidence of p53 mutations in lung cancers have been shown in SCLC patients (70%) followed by squamous (65%), large cell (60%) and adenocarcinoma (33%) patients (Greenblatt et al., 1994).

Abrogation of the p53 signalling pathway is the most common specific alteration in human neoplasia suggesting a central role for the protein in the progression of cancer (Lane, 1993). Wild type p53 is able to bind to DNA as a multiprotein homo-dimer/trimer. However, mutated p53 is able to bind to wild type p53 inactivating it in a dominant negative manner. Wild type p53 is a nuclear protein involved in the G1/S checkpoint of the cell cycle. Down regulation of this pathway leads to uncontrolled cell proliferation due to the loss of p53 regulated growth arrest. The inability of cells to arrest growth prevents DNA repair from occurring and enables DNA mutations to be passed on. This may ultimately lead to malignant transformation.

p53 was first shown to be involved in the apoptotic process of cells (section 1.2) in a temperature-sensitive mutant cell line (Yonish-Rouach et al., 1991). Increases in p53 levels can cause transactivation of the pro-apoptotic gene Bax (Miyashita et al., 1994, Selvakumaran et al., 1994) and repress the anti-apoptotic protein Bcl-2 (Shen & Shen, 1994). This causes an alteration in the balance of Bax : Bcl-2 protein levels within the cell ultimately leading to apoptosis (discussed in section 1.2). However, p53 activation has since been shown not to be required for all apoptotic mechanisms. Thymocytes from p53 null mice were resistant to apoptosis induced by chemically induced DNA breaks (whilst wild type were not). Mutated p53 could not however prevent glucocorticoid mediated apoptosis (Lowe et al., 1993, Clarke et al., 1993). Thus, there is more than one signalling pathway present in cells that leads to cell death via apoptosis.

1.1.3.2 Dominant Oncogenes.

Dominant acting oncogenes are genes whose altered expression contributes to the malignant phenotype. (Only one of the alleles needs to be activated)

Myc Family. This family is composed of six members, c-, L-, N-, P-, R- and B-*myc* (Cook et al., 1993). Among them, only three (c-, L- and N-) seem to be of significance in the formation of SCLC (Johnson et al., 1988). These three oncogenes encode nuclear phosphoproteins that are involved in the regulation of the cell cycle through their DNA binding properties (Nishimura & Sekiya, 1987). They have all been shown to co-operate with *Ha-ras* and *raf* oncogenes in transforming primary rat fibroblasts (Schwab et al., 1985). Although the *myc* family of genes demonstrates a high level of conservation at their DNA level, they are expressed on different chromosomes and have been shown to be mutated by different processes. Similarities in their DNA structure suggest common roles intracellularly for these proteins. However, differential tissue expression during growth, and their differing

complementarity to the *ras* gene suggests that they play unique roles in the development of cells (Zimmerman et al., 1986).

Activation of the *myc* family of oncogenes normally occurs not by mutation, but by gene amplification or over-expression in SCLC cells. One of the first reports of *myc* amplification in SCLC was observed in the variant form of SCLC (van der Hout et al., 1989). *Myc* amplification was seen to correlate with rapid *in vitro* growth, altered morphological characteristics, partial loss of neuroendocrine markers and radioresistance (Carney et al., 1985, Gazdar et al., 1985). Transfection of the *c-myc* oncogene into classical SCLC cells results in the appearance of variant SCLC cell features. Amplification of the *N-myc* and *L-myc* oncogenes has also been described in SCLC, but are not associated with the conversion to a more variant form of SCLC. Amplification of any of the *myc* genes is associated with the patient having undergone prior cytotoxic therapy (Brennan et al., 1991).

Ras Family. The *ras* family of proto-oncogenes (*K-*, *H-* and *N-ras*) encode a 21 kDa membrane associated protein which has been suggested to play a key role in cell signalling (reviewed further in section 1.6.2.3). Mutations in codons 12, 13 and 61 lead to constitutive activation of these proteins. *Ras* oncogene mutations have been shown to exist in a variety of tumour types including colorectal tumours (Bos et al., 1987, Jiang et al., 1989), adenocarcinomas (Rodenhuis et al., 1987 and 1988) and neuroblastomas (Ireland, 1989). This family of proteins is considered to play an important role in the pathogenesis of certain NSCLC tumours types (e.g. adenocarcinomas). Suzuki and co-workers (1990) noted that 27% of adenocarcinomas contained mutations in the *ras* genes, and that 73% of these mutations were in the *Ki-ras* gene isoform. No *ras* mutations were detected in squamous tumour specimens. *Ras* mutations have been detected infrequently in SCLC cells (Kurzrock et al., 1986, Kiefer et al., 1987). However, Mitsudomi and co-

workers (1991) and Suzuki and co-workers (1990) indicated that there were no *ras* mutations in SCLC cells.

1.1.4 Chemotherapy.

Classically, chemotherapeutic reagents were thought to work on the principle that rapidly dividing transformed cells were more sensitive to chemotherapeutic reagents causing selective cytotoxic damage, DNA lesions and cell death. However, it has recently been shown that tumours susceptible to chemotherapy can be relatively slow growing, where as rapidly dividing tumour cells are resistant (Fischer, 1994). Thus a more likely mode of action is now thought to be by causing cell apoptosis (Hannun, 1997, Kerr et al., 1994).

Given that SCLC patients usually present with disseminated disease due to the high metastatic abilities of SCLC cells, strategies of treatment focus on systemic therapy. A study published in 1969 (Veteran's Administration Lung Cancer Study Group) noted that the survival of SCLC patients treated with various single chemotherapy drugs or with radiotherapy treatment, was prolonged. Patients given single-agent cyclophosphamide showed a doubling of their median survival. Further studies using a combined chemotherapy regime, showed an increase in the median survival time for patients with limited disease from 3 -15 months and extensive disease to around 8 months. Whilst many of the new chemotherapy agents are highly active against SCLC both as single reagents (Macbeth et al., 1996) or in combination, combination chemotherapy is usually preferred. Current commonly used combination regimens for the treatment of SCLC are CAV (cyclophosphamide, doxorubicin and vincristine), CAVE (as CAV plus etoposide), VICE (Ifosfamide, carboplatin, etoposide and vincristine) or EP (etoposide and cisplatin).

1.1.4.1 Chemotherapy Agents.

As described previously, a number of different chemotherapeutic drugs are currently being used to treat SCLC patients. These include: -

Cyclophosphamide. This is an alkylating agent which is administered in an inactive form. It becomes activated in the liver where it is metabolised to phosphoramidate mustard. This active metabolite can then bind to DNA and RNA and impair the replication and transcription processes of the cell.

Cisplatin & Carboplatin. Both are platinum analogues thought to cause cytotoxicity by forming cross-linkages in and between DNA strands.

Doxorubicin. Doxorubicin is a cytotoxic anthracycline which is considered to be the most effective chemotherapy drug in the treatment of breast cancer. As with other chemotherapeutic drugs, it is known to concentrate in the cell nucleus where it binds to DNA disrupting its structure.

Etoposide. Etoposide (also known as VP-16) is a semi-synthetic derivative of podophyllotoxin (an antimetabolic agent). This anticancer drug functions by inhibiting cells at the pre-mitotic stage of cell division by inhibiting DNA synthesis. Disruption of entry/progression into early S phase appears to be important in the cytostatic action of etoposide.

A large number of clinically important chemotherapeutic drugs including enzymes that intercalate (e.g. doxorubicin, adriamycin and actinomycin D (Tewey et al., 1984, Pommier & Kohn, 1989, Trask & Muller, 1988)) and do not intercalate (e.g. etoposide and teniposide (Chen et al., 1984)) with DNA, are known to exert their cytotoxic effects via the enzyme topoisomerase II α (Liu et al., 1989, Capranico & Zunino, 1992). Topoisomerase II α is a cell cycle regulated nuclear enzyme that regulates the configuration of cellular DNA. This regulation is essential for DNA replication, recombination, transcription and repair. The cytotoxic effects of chemotherapeutic reagents cause the topoisomerase enzyme to become firmly associated with DNA producing a cleavable complex (Gasser et al., 1986, Endicott & Ling, 1989). The formation of these cleavable complexes ultimately leads to the

production of DNA lesions (Liu et al., 1989), cell cycle delay (Smith et al., 1994, Cummings & Smyth, 1993) and cell death.

1.1.5 Relapse.

Despite initial responsiveness to radio and chemotherapy, SCLC almost invariably relapses and is resistant to further treatment, so that the patient two year survival rate remains between 3-8% (Elias, 1997). In Britain one such study showed that the 2-year survival rate of the 3681 patients entered into a clinical trial was 8.5% for those presenting with limited disease and 2.2% for extensive disease. Additionally, the patients who survive in excess of two years also become more susceptible to other types of lung cancer (Minna et al., 1985).

Results of second-line chemotherapy are poor with response rates of 20 - 25 % and a median survival from further relapse of 3 - 4 months. On relapse, the disease is usually classed as incurable and treatment is offered to try to increase the quality and extend the life span of the patient. The ultimate cause of treatment failure is the tumours' resistance to chemotherapy (Elias, 1997). This type of resistance seems to pre-exist in a small percentage of tumour cells which re-grow after relapse. This type of chemoresistance is therefore known as a clinically acquired multi drug resistance. Researchers are still unsure why cancers become resistant to further treatment even when varied combination chemotherapeutic agents are used.

1.1.6 Cancer Drug Resistance.

Although it is unclear as to what the cause of SCLC multidrug resistance is, a number of proteins have been shown to mediate cellular resistance to different chemotherapeutic reagents. One gene known to aid in cell drug resistance is the multi drug resistance gene (MDR-1). Overexpression of the MDR-1 protein causes resistance to many anticancer agents such as doxorubicin and etoposide by causing a decrease in the intracellular steady-state chemotherapy drug concentration.

The role of MDR-1 in lung cancer has previously been investigated. Levels of expression were shown to be elevated in only a small number of SCLC cell lines and tumours. The level of elevation was negligible in comparison to other tumour types such as colon cancer. It was shown that there was no correlation between the expression of MDR-1 and whether the tumour specimen was from a previously treated or untreated patient, or even if the patient had responded well to chemotherapy. Lai and colleagues (1989) showed that there was no correlation between the mRNA levels of MDR-1 and *in vitro* chemosensitivity. The MDR-1 gene has therefore been concluded as not being involved in the mechanism by which SCLC becomes drug resistant. In contrast to the above conclusions, Kreisholt and co-workers (1998) recently noticed that in a significant number of SCLC tumour biopsies there was a decrease in topoisomerase II α levels, an increase in P-glycoprotein (P-gp) protein levels, and a small increase in multidrug resistance protein (MRP) levels after treatment with either etoposide or teniposide. (Both P-gp and MRP are drug efflux plasma membrane pumps which result in a decrease in intracellular steady-state chemotherapy drug concentrations (Cole et al., 1992, Borst et al., 1993)). The limitations of this study were that protein expression levels alone were assessed and not the actual activity status of the proteins involved.

Alterations in the DNA repair enzyme topoisomerase-II α have been detected in SCLC cell lines presenting resistance to chemotherapeutic reagents (Giaccone et al., 1992b). Nearly all doxorubicin and adriamycin resistant SCLC cell lines have been shown to have decreased topoisomerase II α gene expression, and a reduction in drug-induced DNA breaks (De Jong et al., 1993 and 1990 respectively). Thus, the decrease in DNA lesions may effect the apoptosis levels induced by these chemotherapeutic reagents. However, studies by Supino and co-workers (1993) showed that although there was a small decrease in topoisomerase II α expression in doxorubicin resistant cell lines, this was not sufficient to explain the large increase in drug resistance observed. Supino concluded that alternative unidentified mechanisms must therefore be involved in SCLC cell multidrug resistance.

Finally, tumour responsiveness has also been shown to be dependent upon p53 expression and the activation of cell cycle arrest and apoptosis following exposure to chemotherapeutic reagents (Clarke et al., 1993, Lowe et al., 1993). Therefore, cells that contain p53 deletions/mutations often overcome cell cycle delay and become resistant to chemotherapy.

1.2 Apoptosis.

Nowadays the term apoptosis is widely used to describe the active process by which a cell dies exhibiting a distinct set of biochemical and morphological features (Kerr et al., 1972). This mechanism of cell death enables the cells to die in a manner that will neither harm neighbouring cells, or induce an inflammatory response (Martin, 1993, Fischer, 1994).

Although many of the morphological changes that occur to a cell undergoing apoptosis are well documented, the actual biochemical process and signalling pathways involved are unclear. Morphological changes that occur when a cell undergoes apoptosis include the rounding up of cells and cell shrinkage. As the cell condenses, the nuclear DNA is condensed and ultimately becomes fragmented by specific DNAase enzymes. In addition, alterations in the cell surface lipid composition and receptor expression occur during the apoptotic process. These are important as they enable phagocytic cells such as macrophages to recognise and phagocytose apoptotic cells before the toxic breakdown products, or contents of the cells can injure the surrounding tissue causing inflammation (Savill et al., 1989).

It is clear that a large number of factors influence the commitment of a cell to the death programme in response to a wide variety of apoptotic stimuli. These include cell cycle stage, DNA damage, cell genotype, mutations and the addition of various growth-, survival- and death-promoting factors. Genetic mutations can decrease the

susceptibility of a cell to undergo apoptosis on addition of the correct stimuli. In relation to cancer these mutations may confer an increased growth advantage resulting in tumourigenesis, or decrease the susceptibility of a cell to chemotherapy-induced apoptosis. This knowledge is of interest as tumour mass is the result of the balance between cell proliferation and cell death (via either apoptosis or necrosis), therefore factors affecting the apoptotic rates of tumour cells have a major impact on tumour growth.

1.2.1 Effectors of the Apoptotic Pathway.

Interleukin-1 β Converting Enzyme. Initially, the majority of studies investigating the key intracellular elements involved in the apoptotic process were performed in *Caenorhabditis elegans* (*c. elegans*). The *ced-3* gene was shown by mutational studies to be required for apoptosis in development (Ellis & Horvitz, 1986). Cloning of this gene revealed the presence of a mammalian homologue now known as the Interleukin-1 β converting enzyme (ICE) (Yuan et al., 1993). ICE is now known to be part of a larger family of cysteine proteases which include prICE, CPP32/Yama/Apopain, ICH-2, Flice and others (Thornberry & Lazebnik, 1998) which induce the terminal and irreversible phase of apoptosis characterised by macromolecular breakdown and cellular fragmentation. This family of cysteine proteases is now collectively known as the caspase family (reviewed Porter et al., 1997).

Bcl-2. Another *c. elegans* gene discovered to be important in apoptosis was *ced-9* (Hengartner et al., 1992). *Ced-9* and its mammalian homologue Bcl-2 (B cell leukaemia oncogene 2) are negative regulators of cell death, i.e. increased activation (via phosphorylation) prevents apoptosis. This increased activation often contributes to certain disease types (including cancer) where dysregulation of cell death occurs. Bcl-2 was originally identified from a translocation breakpoint 14;18 (Bcl-2 gene chromosome 18, immunoglobulin promoter chromosome 14) associated with several human B cell lymphomas resulting in increased Bcl-2 expression (Tsujimoto et al.,

1985, Bakhshi et al., 1985). Since its discovery, Bcl-2 has been shown to be a member of a much larger family which includes both anti-apoptotic (including Bcl-X_l) and pro-apoptotic proteins (including Bad and Bax) (Kaufmann, 1997).

Bcl-2 proteins are able to form homodimers within the cell. Another family member Bax (pro-apoptotic Bcl-2 homology protein) can also form homodimers as well as heterodimers with Bcl-2. The balance between the number of Bcl-2 and Bax proteins within a cell has been indicated as the key regulatory step by which different mechanisms confer cellular resistance to apoptosis. Studies in yeast showed that the presence of Bax alone (allowing production of homodimers) caused apoptosis. This could be prevented by the addition of Bcl-2 proteins, but only if they were able to associate with the Bax proteins. The presence of Bcl-2 alone did not have any effect on cell apoptosis levels (Hanada et al., 1995).

1.2.2 Chemotherapy-induced Apoptosis.

Recently, *in vitro* cell apoptosis has been shown to occur in response to many different chemotherapeutic drugs including etoposide, cis-platinum, cyclophosphamide and adriamycin (Kaufmann, 1989, Walker et al., 1991, Hutschscha et al., 1996). The onset of apoptosis seems to be independent of the actual cytotoxic insult, as inhibition of microtubules, nucleotide biosynthesis, direct DNA damage and topoisomerase inhibition (via different agents) all induce cell death (reviewed Hannun, 1997). As SCLC cells in patients are known to become resistant to chemotherapy-induced apoptosis after primary treatment, understanding the underlying mechanisms of chemotherapy-induced apoptosis are key in the ongoing effort to try and understand why SCLC cells become resistant to chemotherapy reagents.

The potential roles of the different known apoptotic mechanisms in chemotherapy-induced apoptosis are unclear. Overexpression of Bcl-2 is able to protect cells from undergoing apoptosis in response to the addition of a wide variety of

chemotherapeutic agents including etoposide, dexamethasone, doxorubicin, actinomycin D (Kamesaki et al., 1993, Fairbairn et al., 1994, Lotem & Sachs, 1994). Addition of etoposide has been shown to result in the cleavage of the anti-apoptotic protein Bcl-2 on administration of the drug to U937 and HL60 cells (Fujita & Tsuruo, 1998). It was shown that Bcl-2 was directly cleaved by the ICE-like family protease CPP32/caspase-3.

Increases in the level of Bcl-2 present in cells is not thought to effect the actual entry of the chemotherapeutic drug into the cell, but rather disable its cytotoxic capabilities. The actual mechanism by which this is achieved is unclear. It is thought that by preventing the cell from replicating, it enables the cell to have time to try and repair any DNA breaks or mutations. Additionally Bcl-2 has been seen to suppress DNA damage by preventing caspase-3 activation (Sentman et al., 1991). The efficiency of Bcl-2 is also dependent upon levels of Bax expression (discussed previously).

Finally, p53 levels are thought to play a role in chemotherapy-induced apoptosis under certain circumstances, as loss of p53 function can lead to a decrease in the chemo- and radio-sensitivity of cells (Lowe et al., 1993, Clarke et al., 1993, Lotem & Sachs, 1993). Conversely, the disruption of p53 has been shown to sensitise breast carcinoma cells from cis-platinum-induced apoptosis (Fan et al., 1995, Eliopoulos et al., 1995). In MCF-7 breast cancer cells, survival assays indicated that when p53 signalling was disrupted, increased cis-platin sensitivity was due to defects in cell cycle G1 checkpoint control, nucleotide excision repair or both.

1.3 The Metastatic Processes Involved in Cancer.

Cancer cells have often been shown to disobey the social organ boundaries within the body and cross into foreign tissues causing the production of metastatic deposits (reviewed Liotta & Stetler-Stevenson, 1991). A number of important events take

place enabling a cancer cell to form the secondary ectopic deposits. These events are often associated with aggressive cancers such as SCLC. The complex cascade of events includes tumour growth and invasion, angiogenesis (formation of new blood vessels within the tumour), intravasation into the lymphatic or blood vessels, adhesion to the vessel wall, extravasation and the ability to grow within a foreign environment (Cleardin, 1998). A number of the above processes require the interaction of cell surface receptors known as integrins, with extracellular matrix (ECM) components.

To enable cells to metastasise, ECM degradation has to take place. A general feature of all carcinomas is the presence of defects within the basement membrane. This is in contrast to benign proliferative disorders which are characterised by a continuous basement membrane. The degradation of ECM proteins is regulated by a family of zinc-dependent enzymes known as matrix metalloproteinases (MMP's) (reviewed MacDougall & Matrisian, 1995, Matrisian, 1992). These enzymes are known to be regulated by a wide variety of factors including growth factors, oncogenes and hormones. A number of recent studies have suggested that certain MMPs co-localise with integrins, and whilst the activation of the integrin heterodimer regulates cell motility, the MMP potentiates matrix degradation. The co-operation between these two processes has therefore been suggested to facilitate tumour invasion and metastasis (Brooks et al., 1996). Studies by Gonzalez-Avila and co-workers (1998) indicated that MMP-2 and MMP-9 (type IV collagenases) were expressed by all lung neoplasms, and that SCLC cell extracts showed the highest enzymatic activity. The presence of these activated MMPs in SCLC cells is thought to help explain why these cells are highly metastatic.

1.4 The Extracellular Matrix.

The extracellular matrix (ECM) is an insoluble meshwork of proteins (Kefalides et al., 1979) whose adhesive interactions with surrounding cells have been shown to be important in the regulation of a large number of cellular processes including

proliferation, migration, survival and differentiation. Many of these processes are important to the ability of a transformed cell to survive and metastasise.

The ECM can be described as a 3D lattice meshwork of collagen and elastin which is able to act as a filter allowing the movement of certain molecules through the matrix aiding in cell / tissue homeostasis. It is able to exert both mechanical and chemical influences on a cell causing changes in cell shape and biochemistry via the plasma membrane (Bissell et al., 1982). The cells in turn are able to regulate ECM production and degradation. Loss of tissue / cell homeostasis, due to a change in ECM - cell receptor association and in ECM composition, is consistently observed in cancer.

In comparison to normal cells, the behaviour of their cancerous counterparts is often altered on adhesion to ECM proteins. A culture of non-malignant primary mammary epithelial cells in a 3-dimensional reconstituted basement membrane matrix stopped proliferating and underwent morphogenesis. In contrast, malignant immortalised mammary epithelial cells continued to proliferate and formed colonies (Peterson et al., 1992). In addition, Roskelley and co-workers (1995) noted that association of cells to ECM proteins was able to cause alterations in cell shape and an increase in intracellular tyrosine phosphorylation (via integrin binding). This led to changes in gene expression in breast epithelial cells. These studies serve to illustrate that adhesion of cells to ECM proteins is able to cause alterations in the behaviour of cells.

1.4.1 Extracellular Matrix Proteins.

1.4.1.1 Collagen IV.

Collagen IV is only one of a much larger family of proteoglycans (Kuhn, 1995) and forms the backbone of the basement membrane. Collagen IV and V are the only types to be found in the basement membrane, and are localised in the basement

membrane of the alveolar walls of capillaries and bronchioles of the lung (Madri & Furthmayr, 1980). Collagen IV is seen to co-localise with laminin within the basement membrane and provide a scaffold on to which other components of the basement membrane are incorporated.

Both collagen IV and V have been shown to be resistant to the classic MMP collagenases which are known to degrade the interstitial collagens I, II and III. This observation is important in the maintenance of the basement membrane cell barrier. Conversely, metastatic tumour cells have been shown to produce a specific MMP that is able to degrade collagen IV alone (Liotta et al., 1979). The production of this MMP possibly enables cancerous cells to degrade the basement membrane allowing metastasis to occur. Metastatic cells have also been shown to have a higher affinity for collagen IV in comparison to other types of collagen (Nicolson et al., 1981, Terranova et al., 1982).

1.4.1.2 Laminin.

The laminins are a major family of biologically active glycoproteins (Timpl et al., 1979) comprising of α , β and γ chains. Laminin is the most abundant glycoprotein in the basement membrane where it is bound to collagen IV. There are a number of receptor binding sites scattered throughout the ligand including sites for integrins $\alpha3\beta1$, $\alpha6\beta1$, $\alpha1\beta1$ and a 67 kDa receptor (Powell & Kleinman, 1997).

Laminin plays a key role in the interactions of tumour cells to the surrounding basement membrane resulting in the regulation of cell adhesion, proliferation, migration and morphology (Terranova et al., 1980, Grover et al., 1983, McCarthy et al., 1983). Adhesion to laminin has been shown to stimulate the production of collagenase IV which goes on to degrade collagen IV. Proteins that inhibit collagenase IV activity and prevent cells from adhering to laminin inhibit malignant cell invasion. This implies that adhesion to laminin and collagen IV degradation, are essential for the formation of tumour metastasis (Reich et al., 1988). Animal tumour

models have shown that on selection and injection of tumour cells that adhere preferentially to laminin, a 10 fold increase in the metastatic ability of the cells was observed (Terranova et al., 1982).

1.4.1.3 Fibronectin.

Fibronectins consist of a large family of heterodimer and polymeric glycoproteins found in the plasma and connective tissue of healthy lungs. Fibronectin is known to be synthesised by connective tissue cells (Hynes, 1986, Yamada, 1989). Fibronectins are now recognised as a family of proteins which modulate key cellular responses via integrin and non-integrin interactions including adhesion, proliferation and differentiation (depending upon cell type).

In humans, the fibronectin family of proteins are all encoded by a single gene which undergoes alternative splicing at the pre-mRNA stage to form 20+ isoforms (Schwarzbauer, 1991) ultimately producing a dimer approximately 250 kDa in size. Two forms of fibronectin are found within the body: pFn - a soluble form found in plasma and often secreted by hepatocytes; cFn - cellular fibronectin secreted mainly by epithelial cells, macrophages and fibroblasts. Both forms are found in the ECM.

Fibronectin is a flexible molecule which contains binding sites for a number of different receptors including $\alpha 5\beta 1$ (classical fibronectin receptor), $\alpha 3\beta 1$ and $\alpha 4\beta 1$. The interaction of fibronectin with integrin receptors has been found to be essential for the assembly of a fibronectin matrix which is required for vertebrate development, wound healing and tumourigenesis (Wu et al., 1995). Fibronectin secretion has also been shown to cause collagen IV degradation and the invasion of certain tumour cell types. Shibata and co-workers (1997) showed that fibronectin caused the production of the matrix metalloproteinase MMP-9 (via an $\alpha 5\beta 1$ integrin-dependent mechanism) leading to the degradation of collagen IV and an increase in ovarian cancer cell invasiveness.

1.4.1.4 Tenascin.

The vertebrate tenascins are a family comprised of 4 members (C-, R-, X- and Y-tenascin (Chiquet-Ehrismann et al., 1994). Tenascin-C was first observed by a number of groups in the early 1980's and the majority of tenascin research has focused on this family member. Structurally tenascin-C is a disulphide-linked hexamer that can be expressed in a number of different isoforms. As with other ECM proteins, tenascins are degraded by members of the MMP family (Siri et al., 1995).

The expression of tenascin-C is very complex and has been shown to dramatically change in pathological conditions including wound healing and cancer (Mackie, 1994). It has been suggested that many processes may influence alterations in tenascin-C expression including cell type and ligand stimulation. These include regulation by transforming growth factor- β (Pearson et al., 1988), fibroblast growth factor (Tucker et al., 1993), interleukin-1 and angiotensin II (Sharifi et al., 1992) (reviews Chiquet-Ehrismann et al., 1995, Rettig et al., 1994). Additionally, glucocorticoids (e.g. dexamethasone) have been shown to down regulate tenascin-C (but increase fibronectin expression) (Chiquet-Ehrismann et al., 1995).

Initial studies were inconclusive as to the adhesive properties of tenascin. Chiquet-Ehrismann (1991) described tenascin as an anti-adhesive protein. However, Lotz and co-workers (1989) as well as Sriramarao and co-workers (1993) said that adhesion to tenascin by different cells is weak and cell type specific. Along with other functional consequences, the binding of tenascin-C by different receptors (including integrins and heparin sulphate proteoglycans) under different circumstances, and on different cell types, has been shown to stimulate / inhibit proliferation, cause neurite outgrowth (in the development of the nervous system) and support morphogenesis in lung development (Chiquet & Wehrle-Haller, 1994).

1.5 Integrins.

Integrins are the major family of cell receptors which enable cells to adhere to ECM proteins. They are activated when bound to immobilised extracellular matrix proteins, or by integrin receptor aggregation. Upon integrin activation a number of cellular processes can occur including alterations in gene expression (Streuli et al., 1995, Lin et al., 1994, Shaw et al., 1990), increases in intracellular free calcium (Schwartz, 1993), increases in cellular pH levels (Schwartz et al., 1991) and phosphoinositide turnover (McNamee et al., 1993). Additionally, integrins play a key role in a number of cellular processes that impact on the development of tumours including the regulation of cell proliferation, apoptosis, cellular motility and invasion, and angiogenesis (Shaw et al., 1997, Bredin et al., 1998, Zhang et al., 1995).

Integrins have been shown to produce a large number of signals that converge upon the growth regulatory pathways and often utilise the same signalling molecules. Many reports (reviewed Schwartz, 1997) suggest that at least three major signalling pathways can be activated on the convergence of integrin and growth factor receptor signalling, these include the phospholipase C (PLC) - protein kinase C (PKC) pathway, the mitogen activated protein kinase (MAP kinase) pathway and the phosphoinositide 3-kinase (PI 3-kinase) pathway.

1.5.1 Integrin Receptors.

Integrins are a widespread family of cell surface receptors that are comprised of a mixture of at least 17 α (120 - 180 kDa) and 8 β (90 - 110 kDa) subunits (review Kumar, 1998). These in turn can form at least 22 different non-covalently linked heterodimers.

Alternative splicing of both the α and β subunits can occur (Hynes, 1992). Each subunit contains a large extracellular domain, a single hydrophobic transmembrane

spanning region and a short cytoplasmic tail (with the exception of $\beta 4$ which has a long cytoplasmic tail). The cytoplasmic tail of the α subunit differs a great deal between the different subunits, but they all function to provide ligand specificity, surface expression and regulate cell adhesion and spreading (Sastry & Horwitz, 1993). The β subunits have a more conserved sequence and are required to target the integrins to the focal contacts enabling signalling pathways to be activated (La Flamme et al., 1994).

1.5.2 Integrin Ligands.

Non-covalently associated integrin hetero-dimers are normally able to bind to more than one ligand type (exception $\alpha 5\beta 1$ binds fibronectin only). Integrins are also known to have different affinities for different ligands depending upon cell type (Kirchhofer et al., 1990, Elices & Hemler, 1989). In addition, although two different integrin dimers may bind to the same ligand, and even to the same specific target sequence, different functional consequences may occur. Zhang and co-workers (1995a) noted that Chinese hamster ovary (CHO) cells normally undergo apoptosis on withdrawal from serum. However, activation of the $\alpha 5\beta 1$ integrin dimer prevented this from occurring. Activation of the $\alpha v\beta 1$ integrin (binds to the same RGD sequence as $\alpha 5\beta 1$), did not prevent apoptosis from taking place.

1.5.3 Integrin Expression on Cancer Cells.

A number of studies have been performed investigating the differences in integrin expression on the surface of cancer cells. Mette and co-workers (1993) noted that in comparison to normal lung cells, the expression of integrins on lung cancer cells was more heterogeneous. Studies by Damjanovich and co-workers (1992) also noted a more heterogeneous population of integrin ligands on lung cancer cells. They also noted that the number of integrins expressed on the cell surface decreased. Further research into the integrin expression of lung cancer cells (Koukoulis et al., 1997) showed that no lung cancer cells (including SCLC cells) expressed the $\alpha 5\beta 1$ integrin

dimer (although normal bronchial epithelial cells did). It has also been noted that lung cancer cells which do not express the $\alpha 5\beta 1$ integrin dimer, are able to bind to fibronectin in an $\alpha 3\beta 1$ -dependent manner (Falconi et al., 1994).

It has long been accepted that the pattern of fibronectin and integrin expression in cancer cell lines is often altered. Malignant cells often express less fibronectin leading to alterations in adhesiveness, motility and morphology (Yamada, 1983, Akiyama et al., 1995). However, addition of exogenous fibronectin is sometimes sufficient to revert the transformed phenotype (Akiyama et al., 1995). Oncogenic transformation of cells has often been associated with a decrease in $\alpha 5\beta 1$ expression leading to a decrease in fibronectin matrix formation (Plantefaber & Hynes, 1989, Wu et al., 1995, Fogerty et al., 1990). Work performed in CHO cells indicated that overexpression of the $\alpha 5\beta 1$ receptor inhibited or abolished tumour progression (Giancotti & Ruoslathi, 1990), whereas reduced expression increased tumourigenicity in nude mice (Schreiner et al., 1991). *Ras*-transformed cells have also been shown to express reduced levels of the $\alpha 5\beta 1$ dimer (Plantefaber & Hynes, 1989).

Initial studies by Stallmach (1994) indicated that colon cancer cells no longer expressed the $\alpha 5\beta 1$ integrin dimer. Conversely, Gong and co-workers (1997) noted that a highly invasive colon cancer cell line expressed an increased level of $\alpha 5\beta 1$ in comparison to the less invasive colon cancer cell lines. This increased expression led to strong adhesion to fibronectin *in vitro*. Injection of colon cells expressing high levels of $\alpha 5\beta 1$ into athymic nude mice resulted in increased tumourigenicity. Further work has shown that key growth-regulating genes are altered in cells that overexpress the $\alpha 5\beta 1$ integrin (Varner et al., 1995). This alteration in integrin expression led to a decrease in cell proliferation rates which could be reversed on adhesion to fibronectin. $\alpha 5\beta 1$ has also been shown to be important in colon cell survival as $\alpha 5\beta 1$ -transfected cells were partially protected from serum withdrawal-induced apoptosis via a Bcl-2-dependent mechanism (Zhang et al., 1995b, O'Brien et

al., 1996). Thus, $\alpha 5 \beta 1$ can act as a regulator of cell growth, tumourigenicity and apoptosis in colon cancer cells.

In relation to other integrin types, many primary and metastatic tumours have been shown to express $\alpha 3 \beta 1$ (Bartolazzi et al., 1994) and cancers of the lung, neck, head, colon and bladder have been shown to express the $\alpha 6$ integrin subunit (Van Waes & Carey, 1992, Liebert et al., 1993, Costantini et al., 1990). In breast carcinomas, poor expression of $\alpha 2 \beta 1$ was shown to induce a more invasive and less differentiated phenotypic cell. Ectopic expression of the integrin heterodimer suppressed this transformed phenotype (Zutter et al., 1995) preventing invasion and differentiation.

1.5.4 Integrin-mediated Cell Survival.

Loss of epithelial and endothelial integrin-mediated cell adhesion to ECM proteins has been shown to trigger detachment-induced apoptosis (Meredith et al., 1993, Boudreau et al., 1995, Frisch & Francis, 1994. Reviewed Frisch & Ruoslati, 1997), which has since been termed 'anoikis' (meaning apoptosis induced by loss of anchorage). However, fully transformed cells are known to be anchorage-independent and overcome anoikis. This anchorage-independence has been shown to play a key role in the ability of tumour cells to metastasise. Overexpression of a number of different signalling proteins has often been linked to anchorage-independence in different cell types. Previous work by Frisch and Francis (1994) showed that transformation of epithelial cells with the oncogenes *ras* or *src* enabled cells which were normally anchorage-dependent to grow in suspension. This oncogenic transformation appeared to enable the cells to stimulate signals normally produced upon integrin activation within the cell. Additionally, Khawja and co-workers (1997) noted that activation of *ras* or *src* could protect epithelial cells from under going cell detachment-induced apoptosis via activation of the lipid/tyrosine kinase phosphoinositide 3-kinase (PI 3-kinase) and one of its downstream targets protein kinase B (PKB) (review of PI 3-kinase and PKB in the regulation of apoptosis Franke et al., 1997). The activation of cell adhesion regulated pathways by

oncogenes such as *integrin-linked kinase (ILK)* and *Bcr-Abl* (Schwartz, 1997) have also been shown to promote anchorage-independent growth (Radeva et al., 1997). Finally, Guan & Shalloway (1992) reported that the protein focal adhesion kinase (FAK) could be activated by both cell adhesion (via integrins) or by oncogenic transformation. FAK expression has since been shown to correlate with the invasive potential of a number of metastatic tumours (Brunton et al., 1997, Jenq et al., 1996, Withers et al., 1996, Owens et al., 1995), and FAK overexpression is known to block anoikis (Frisch et al., 1996a). This implies that FAK maybe important in anchorage-independent growth in certain transformed cells.

Boudreau and co-workers (1995) have shown that breast epithelial cells undergo anoikis via a $\beta 1$ integrin- and ICE-dependent pathway (caspase review Porter et al., 1997). Recently caspases were shown to cause FAK cleavage (Wen et al., 1997) suggesting the presence of a positive feedback loop. On activation of caspases, FAK was cleaved preventing activation of the anti-apoptotic pathway involving PI 3-kinase and PKB. Additionally PKB has been shown to cause phosphorylation (causing inactivation) of the pro-apoptotic protein Bad (Datta, et al., 1997) strengthening the role of PKB it the prevention of apoptosis. (Bad is a pro-apoptotic protein of the Bcl-2 family.)

An alternative mechanism involves the anti-apoptotic protein Bcl-2. Zhang and co-workers (1995a) noted that adhesion of cells to fibronectin via its classical $\alpha 5\beta 1$ receptor caused an increase in Bcl-2 expression, which in turn aided in cell survival. Frisch and co-workers (1996a) noted that MAP kinase-mediated Jun kinase anoikis could be inhibited by expression of Bcl-2. Cardonne and co-workers (1997) investigated the role of the MAP kinase protein MEKK-1 (MAP/ERK kinase kinase 1) in anoikis and noted that loss of anchorage led to MEKK-1 activation (due to protein cleavage, via caspase activation) ultimately leading to activation of Jun kinase. However this process could be blocked by increasing the concentration of Bcl-2 within the cell which led to the inhibition of caspase activation. Loop holes in this alternative hypothesis were highlighted by Khwaja and Downward (1997) who

noted that addition of a dominant negative Jun kinase did not prevent anoikis of epithelial cells on cell detachment.

1.6 Phosphoinositide 3-kinase Signalling.

For highly metastatic cells such as SCLC cells to survive, they have to be able to overcome both serum- and anchorage-dependence to enable them to grow, survive and metastasise throughout the body. The enzyme phosphoinositide 3-kinase (PI 3-kinase) has been shown to be involved in the regulation of many key untransformed and transformed cellular processes including anchorage-independence and survival (Table 1.2).

Table 1.2: Examples of Cellular Functions which Require Phosphoinositide 3-kinase Activation.

Cellular Function	Reference
Proliferation	Cantley et al., 1991, Walker et al., 1998 Carpenter & Cantley, 1996
Transformation	Whitman et al., 1985, Rodriguez-Viciano et al., 1997
Survival	Yao & Cooper, 1995, Franke et al., 1997 Khwaja et al., 1997, Kauffman-Zeh et al., 1997
Secretion and trafficking	Joly et al., 1994 and 1995 Shepherd et al., 1996
Migration	Wennstrom et al., 1994, Kundra et al., 1994
Anchorage-independence	King et al., 1997, Shaw et al., 1997, Meredith et al., 1993, Frisch & Francis, 1994
Morphological Alterations	Kotani et al., 1995 and 1996
Invasion and metastasis	Shaw et al., 1997, Keely et al., 1997

PI 3-kinase is a dual specificity kinase which can phosphorylate both phospholipids and proteins (Carpenter et al., 1993, Dhand et al., 1994), therefore acting as a direct biochemical link between proteins containing kinase activity and products of the phosphoinositide pathway.

Initially PI 3-kinase was shown to phosphorylate the D3 position of the inositol ring of phosphatidylinositol (PI) to produce phosphatidylinositol (3) phosphate (PI3P) (Whitman et al., 1988). Since then, a whole family of enzymes have been discovered (some conserved to higher eukaryotes only) which are capable of phosphorylating the D3 position of the inositol ring including Vps34p and Cpk (Toker & Cantley, 1997). The first described mammalian PI 3-kinase family member was the p85/p110 heterodimer (structural and functional analysis reviewed by Dhand et al., 1994).

Whilst p85/p110 PI 3-kinase can phosphorylate the lipid substrates PI, PI4P, PI(4,5)P₂ *in vitro* with equal efficiency, PI(4,5)P₂ is thought to be its preferred substrate *in vivo*. (Note, Vps34p (yeast protein) is able to phosphorylate PI only, where as Cpk (*c. elegans* protein) can phosphorylate PI and PI4P.) Along with other lipid kinases and phosphatases that are able to phosphorylate and dephosphorylate other positions on the inositol ring, a highly complex family of secondary lipid second messengers is found within the cell (Figure 1.1).

To date 2 p85 isoforms (α and β) (class 1_A) and 4 p110 subunit isoforms have been identified (reviewed Vanhaesebroeck et al., 1997). In addition to the two p85 α and β isoforms, a third p85-like subunit was recently discovered (p101) (class 1_B). This isoform has been shown to be activated by G-protein coupled receptors only, and bind to the p110 γ isoform (Stephens et al., 1997). Of the p110 isoforms, α and β (class 1_A) have been shown to be ubiquitously expressed in all eukaryotic cell types. The γ isoform (class 1_B), has been shown to be exclusively regulated by the G-protein coupled family of receptors, and to date has been shown to be expressed in haemopoietic cell types (Stephens et al., 1994) and osteosarcoma cell lines only (Morris et al., 1995). The more recently discovered δ isoform has so far been shown to be homologous to the α and β isoforms, but is expressed in haemopoietic cell types only.

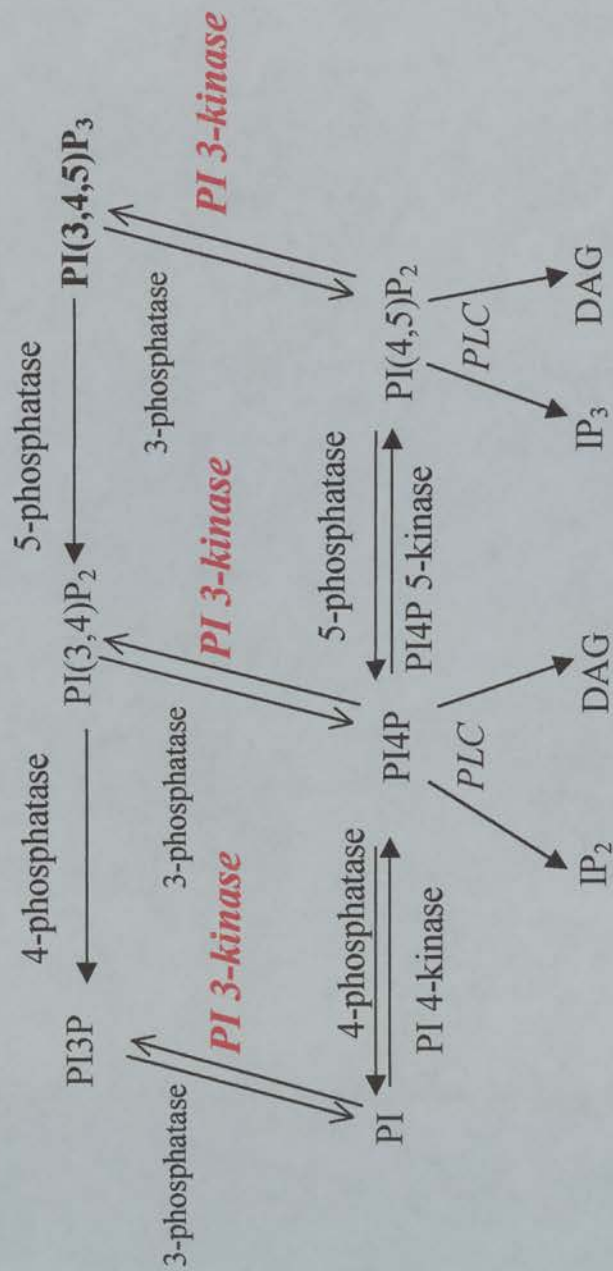


Figure 1.1: Schematic Diagram demonstrating the Phosphoinositide signal transduction pathways.

Abbreviations: PI 3-kinase phosphoinositide 3-kinase; PI phosphoinositol; PLC phospholipase C; DAG 1,2-diacetyl glycerol; IP₃ inositol 1,4,5 trisphosphate; IP₂ inositol 1,4 diphosphate

1.6.1 Activation of Phosphoinositide 3-kinase.

As mentioned previously, PI 3-kinase has been shown to be involved in many different cellular processes (Table 1.2). In view of this, research has shown that PI 3-kinase can be activated by multiple different mechanisms including integrin activation (King et al., 1997, Chen & Guan, 1994), growth factor receptor activation (Carter & Downes, 1992, Chen & Guan, 1994a), G-protein coupled receptor activation (Lopez-Illasaca et al., 1997 and 1998, Morris et al., 1995) and oncogene expression/receptor mutation (Moscatello et al., 1998).

1.6.1.1 Receptor Tyrosine Kinase Activation.

Tyrosine kinase receptors are a large family of single membrane spanning receptors that include growth factors and hormones. Upon activation (due to ligand binding), the receptors oligomerise due to conformational changes in the extracellular regions of the receptors (exception insulin receptor which already exists as a dimer). The adjacent cytoplasmic domains of the receptor subunits interact causing phosphorylation of certain intracellular proteins and/or autophosphorylation of the receptor itself (reviewed Ullrich & Schlessinger, 1990).

The intracellular catalytic tyrosine kinase domains of the receptor provide a number of docking sites for different intracellular proteins including Ras (Kaplan et al., 1990), phospholipase C (PLC γ) (Ronnstrand et al., 1992), the adaptor protein Grb2 (Arvidsson et al., 1994) and PI 3-kinase (Kazlauskas & Cooper, 1990). The phosphotyrosine residues (with motif YxxM) form docking sites for the two SH2 domains of the PI 3-kinase regulatory p85 subunit. (SH2 = src homology 2 domains. These associate with specific phosphorylated tyrosine residues.) This association causes activation of PI 3-kinase. The receptor tyrosine kinase domain catalyses the transfer of the γ -phosphate of ATP to tyrosine residues on PI 3-kinase, and aids in positioning the p110 PI 3-kinase catalytic subunit close to the plasma membrane (which contains the *in vivo* PI 3-kinase lipid substrate PI(4,5)P₂).

1.6.1.2 G-protein Coupled Receptor Activation.

The G-protein coupled family of receptors have a conserved topology within the plasma membrane and consist of a single protein chain which transverses the cell membrane seven times (amino terminal situated on the outside of the cell and the carboxyl terminal inside). Classically, activation of G protein-coupled receptors leads to the formation of $G\alpha$ -GTP and $G\beta\gamma$ subunits. These are able to activate PLC leading to phosphoinositide hydrolysis, the formation of diacylglycerol and IP_3 , and the subsequent release of calcium from intracellular stores.

Looking at the mechanism in more detail it is known that on ligand binding conformational changes are observed in the receptor enabling it to interact with distinct classes of $G\alpha\beta\gamma$ protein heterotrimers (Conklin & Bourne, 1993, Neer, 1995). This triggers exchange of GDP for GTP on the α subunit of the heterotrimer leading to dissociation of the G protein complex from the receptor and the formation of GTP- $G\alpha$ and $G\beta\gamma$ subunits. These subunits then interact with distinct enzymes and ion channels eliciting specific physiological responses within the cell. The GTP associated with the α subunit is then exchanged for GDP causing re-association of the $G\alpha\beta\gamma$ heterotrimer at the plasma membrane.

Activation of PI 3-kinase via the G-protein coupled receptor is not fully understood. The $\beta\gamma$ subunit of the heterotrimer has been shown to associate with the Class 1_B PI 3-kinase (p101-p110 γ) heterodimer causing activation (Stephens et al., 1994, Stephens et al., 1997). Also the possibility of transactivation of a class 1_A PI 3-kinase residing in close proximity to the activated G-protein coupled receptor has been postulated by a number of research groups (see Figure 1.3).

1.6.1.3 Adhesion/Integrin Activation.

Integrin-dependent signalling pathways are known to occur in the majority of cell types. As many of the proteins involved can be stimulated by growth factor receptor

stimulation also, many integrin-associated proteins have been identified. The actual mechanisms involved in integrin signalling are still however poorly understood.

Activation of PI 3-kinase via integrin stimulation is thought to occur by a number of mechanisms including association of the SH2 domains of the p85 subunit with the protein FAK. FAK is known to bind to, and become phosphorylated on β integrin subunit stimulation. PI 3-kinase is then known to associate with the phosphorylated tyrosine residues on FAK (Chen & Guan, 1994, Chen et al., 1996a). This association enables PI 3-kinase to be targeted to the cytoplasmic face of the plasma membrane where its lipid substrates are localised. The p110 catalytic subunit binds with very high affinity to the inter-SH2 region of the p85 subunit and contains intrinsic catalytic activity. A lipid-binding site is also found to be situated in the inter-SH2 region of the p85 subunit and may confer some specificity as to the enzyme substrate preference.

Another mechanism of PI 3-kinase activation involves SH2 - SH3 adaptor protein shc. Shc is known to preside in close proximity to focal adhesion plaques which are formed on the integrin binding of specific heterodimers (Clarke & Brugge, 1995) such as $\alpha 1\beta 1$, $\alpha v\beta 3$ and $\alpha 5\beta 1$ (Wary et al., 1996, Giancotti, 1997). Association with the adaptor protein shc (associates with a limited number of integrin heterodimers (via caveolin binding to the α subunit)) can lead to indirect activation of PI 3-kinase via Grb2 - mSOS and ras (Wary et al., 1996).

1.6.2 Downstream Targets of Phosphoinositide 3-kinase.

Discovery of the pharmacological tools wortmannin (Arcaro & Wymann, 1993, Yano et al., 1993) and LY294002 (Vlahos et al., 1994) have enabled scientists to investigate the role of PI 3-kinase in many different cell types and signal transduction pathways. Wortmannin is a fungal metabolite which has been shown to be a specific non-competitive inhibitor of PI 3-kinase (Arcaro & Wymann, 1993, Ui et al., 1995) by associating with the ATP binding site of PI 3-kinase (Powis et al., 1994, Yano et

al., 1993). It binds irreversibly to the p110 catalytic subunit of PI 3-kinase (Arcaro & Wymann, 1993, Wymann et al., 1996) and has been shown in many cell types to have an IC_{50} of around 3 nM (Powis et al., 1994). LY294002 is structurally different to wortmannin and inhibits PI 3-kinase in an alternative manner. It behaves as a competitive inhibitor for the ATP binding site of PI 3-kinase (Vlahos et al., 1994) with an IC_{50} in the low micromolar range.

Recently, the production of a number of PI 3-kinase dominant negative mutants (Hara et al., 1994) and constitutively active p110 mutants (Hu et al., 1995) has enabled further investigations to be undertaken to try and elucidate specific downstream targets of PI 3-kinase in different cell types (reviewed Duronio et al., 1998). These targets include ras (Hu et al., 1995), PKB (Franke et al., 1995, Burgering & Coffey, 1995), p70^{s6k} (Monfar et al., 1995), protein kinase C family members (Toker et al., 1994), mitogen activating protein kinase signalling pathway members (Lopez-Illasaca et al., 1997), selective Rho/Rac dependent pathway members (Kotani et al., 1995) and Jun N-terminal kinase (Klippel et al., 1996).

These studies have shown that PI 3-kinase is important in a wide range of cellular processes (Table 1.2). The mechanisms by which these varied cellular functions are regulated are not fully understood. However, as research begins to identify key downstream targets of PI 3-kinase, the major role of PI 3-kinase becomes more apparent.

1.6.2.1 Protein Kinase B and PI(3,4,5)P₃-Dependent Kinase 1.

The serine/threonine proto-oncogene protein kinase B (PKB) (also known as Akt or RACPK (related to A and C protein kinase)) was first identified in 1991 (Coffey & Woodgett, 1991, Jones et al., 1991, Bellacosa et al. 1991). Differing research strategies were utilised in the identification of PKB: PCR (Coffey & Woodgett, 1991), low-stringency library screening with a cAMP-dependent kinase probe (Jones et al., 1991) and sequencing of a human cDNA hybridising with *v-akt* (Bellacosa et

al., 1991). To date three separate isoforms have been discovered (α , β and γ). PKB α and β have been shown to be widely expressed within many types of tissue. Highest levels have been shown to be in the brain, thymus and lung (Coffer & Woodgett, 1991, Jones et al., 1991). Expression of the γ isoform is thought to be more selective, with the highest levels found to be in the brain and testes. Low expression levels have also been seen in lung, heart, spleen and skeletal muscle.

Since its discovery, PKB has been shown to be the product of the oncogene *v-akt* and highly expressed in a number of cancers. Overexpression of the PKB β isoform has been seen in ovarian (approximately 12%) (Cheng et al., 1992, Bellacosa et al., 1995), and pancreatic (10%) (Ruggeri et al., 1998, Cheng et al., 1996) cancers. Overexpression of the α isoform has been reported in a small number of breast cancers (3%) (Bellacosa et al., 1995).

PKB Activation. Over the last year, the mechanism of PKB activation has begun to be understood (reviewed Alessi & Cohen, 1998, Downward, 1998, Bellacosa et al., 1998). Whilst association of PKB to PI 3-kinase lipid product PI(3,4,5)P₃ (due to the presence of a plekstrin homology (PH) domain (Shaw, 1996)) causes activation, it is not sufficient for full activation. This suggests that the association is required to induce a conformational change in PKB allowing it to be targeted to the plasma membrane and activated by additional enzymes.

In unstimulated cells, PKB is situated in the cytosol, and upon stimulation it is rapidly translocated to the plasma membrane. The mechanism causing the translocation of PKB to the plasma membrane has been shown to be prevented by inhibitors of PI 3-kinase activity and by deletion of the PH domain of PKB. Conversely, addition of a membrane-targeting sequence to PKB enables it to be activated in unstimulated cells at the membrane (Andjelkovic et al., 1997).

Once located at the plasma membrane, another stage involving the phosphorylation and activation of PKB occurs. A PI(3,4,5)P₃-dependent kinase (PDK1) has been

shown to phosphorylate Thr308 but not Ser473 of PKB α . A serine kinase that has yet to be discovered is still required to phosphorylate Ser473 (hereafter known as PDK2). *In vitro* PDK1 is strongly activated by PI(3,4,5)P₃ (to lesser extent by PI(3,4)P₂), and is known to possess a PH domain (Alessi et al., 1997a). The mechanism of activation of PKB whereby association with PI(3,4,5)P₃ causes a conformational change in PKB enabling PDK1 to phosphorylate a specific residue of PKB (Figure 1.2) is supported by the observation that a PKB α mutant unable to interact with PI(3,4,5)P₃ was not phosphorylated by PDK1 (Stokoe et al., 1997). In addition, PKB α lacking its PH domain could be phosphorylated by PDK1 in the absence of PI(3,4,5)P₃ or PI(3,4)P₂ (Alessi et al., 1997a) as the conformational restraints of the PH domain were removed.

Recent work by Delcommenne and co-workers (1998) has indicated that the integrin associated protein integrin-linked kinase (ILK) (Hannigan et al., 1996) is able to phosphorylate PKB at Ser473. The second kinase, known at this time as PDK2 may therefore possibly be ILK. ILK activation is known to be PI 3-kinase dependent, therefore both PDK1 and 'PDK2' may be regulated by PI 3-kinase.

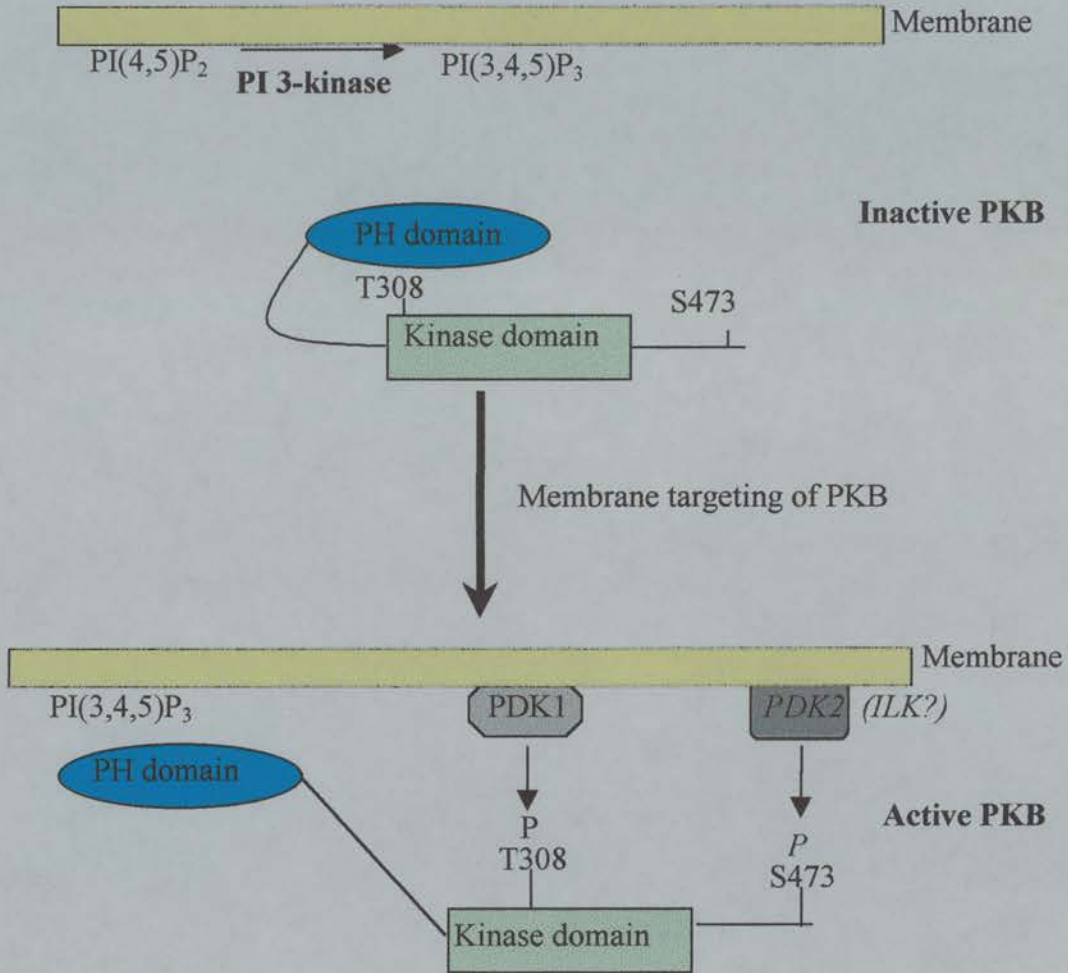


Figure 1.2: Diagram of the Potential Mechanism by which Protein Kinase B is Activated.

Adapted from Alessi & Cohen, 1998.

Abbreviations: PI 3-kinase, phosphoinositide 3-kinase; PH, pleckstrin homology; T308, threonine 308; S473, serine 473; PDK, $PI(3,4,5)P_3$ -dependent kinase.

Downstream of PKB. PKB is thought to be involved in the regulation of the protein glycogen synthase kinase-3 (GSK3) by deactivating the protein via phosphorylation (Sutherland et al., 1993, van Weeren et al., 1998). GSK3 is known to control the synthesis of glycogen via insulin and ultimately regulates the transcription factors AP-1 and cyclic-AMP-responsive element (CREB) (Cross et al., 1995). Recently PKB has also been shown to regulate insulin stimulation of mammalian target of rapamycin (mTOR) (Scott et al., 1998). By artificially activating PKB, they showed that PKB caused an increase in mTOR activity using an *in vitro* kinase assay. mTOR is known to lie upstream of p70^{s6k} (discussed further in section 1.6.2.2). Studies by Burgering and Coffey (1995) also suggested that PKB was responsible for the activation of p70^{s6k} via mTOR.

PI 3-kinase mediated activation of PKB appears to play an important role in the prevention of apoptosis in many cell types (reviewed Downward, 1998, Marte & Downward, 1997, Hemmings, 1997, Eves et al., 1998, Franke et al., 1997). PKB has been shown to be able to phosphorylate the p53 regulated pro-apoptotic Bcl-2 family member Bad (del Peso et al., 1997, Datta et al., 1997) both *in vivo* and *in vitro*. Once phosphorylated (inactive), the adaptor protein 14-3-3 is able to bind to Bad preventing it from being able to heterodimerise with and inhibit the activity of the anti-apoptotic proteins Bcl-2 and Bcl-x_l (Zha et al., 1996, Zundel & Giaccia, 1998, del Peso et al., 1997). The ability of PKB to aid in cell survival has also been shown to synergise with the ability of the *ras* oncogene to promote cell survival via the PI 3-kinase/PKB and not MAP kinase pathway (Khwaja et al., 1997). Given that over a quarter of human malignancies have undergone *ras* mutations (occur very infrequently in SCLC), it is possible that their ability to survive is triggered via a PI 3-kinase / PKB dependent mechanism. However, all these anti-apoptotic signals mediated via PI 3-kinase/PKB do seem to be cell type and stimuli specific. Minshall and colleagues (1996) reported that although activation of PI 3-kinase was able to protect haemopoietic progenitor cells from undergoing apoptosis, this protection was dependent upon the external signal supplied to the cell. PI 3-kinase prevented apoptosis due to IL-3 mediated signals but not IGF-1.

1.6.2.2 Ribosomal Protein S6 Kinase.

The serine/threonine protein kinase ribosomal protein s6 kinase (p70^{s6k}) (reviewed Proud, 1996, Chou & Blenis, 1995, Abraham, 1998) has been shown to be associated with a highly conserved signalling pathway activated in response to many growth factors and neuropeptides. It exists as two isoforms; the p85 (α I) nuclear isoform and the p70 (α II) cytoplasmic isoform. These two proteins are encoded by distinct transcripts differing in their 5' ends derived from the same gene by alternative splicing (Reinhard et al., 1994). The enzyme exists in a basally phosphorylated form in most cell types. On stimulation, it is phosphorylated on a number of novel sites before it becomes fully activated. A number of these mitogen-induced phosphorylation sites have now been identified (Ferrari et al., 1992, Cheatham et al., 1995). It is known that at least 10 serine and threonine residues need to be phosphorylated to enable p70^{s6k} to become active (Dennis et al., 1996).

p70^{s6k} has been shown to be present downstream of PI 3-kinase using a number of different biochemical methods. These include mutation of the PDGF receptor Tyr740/751 residues causing the inactivation of PI 3-kinase, use of PI 3-kinase inhibitors (Chung et al., 1994), and the use of constitutively active PI 3-kinase mutants causing activation of p70^{s6k} (Weng et al., 1995). Additionally, two independent groups have recently shown that p70^{s6k} can be activated in a PI 3-kinase-dependent manner via PDK1 under both *in vitro* and *in vivo* situations (Alessi et al., 1998, Pullen et al., 1998). However, although p70^{s6k} has been shown to be regulated in a PI 3-kinase-dependent manner, it can also be independently regulated by PKC (Tudan et al., 1998) (first demonstrated by Chung et al., 1994). Chung and co-workers showed that the treatment of HepG2 cells with phorbol esters was able to cause p70^{s6k} activation. Additionally, using add-back mutants, activation of p70^{s6k} by PDGF via PI 3-kinase was shown to be PKC-independent, whereas PLC γ activation was dependent upon PKC activation.

Further elucidation of the role of p70^{s6k} in cell signalling has been possible due to the discovery of the p70^{s6k} specific pharmacological inhibitor rapamycin (Price et al., 1992). Rapamycin is an immunosuppressive anti-proliferative macrolide which becomes activated on binding to a specific 12 kDa protein known as FK506-binding protein 12 (FKBP12). The rapamycin-FKBP12 complex inhibits the autophosphorylation of the mammalian target of rapamycin (mTOR), also known as FRAP (FKBP-rapamycin associated protein) (Brown et al., 1995) or RAFT1 (rapamycin-FKBP target 1) (Sabatini et al., 1995).

To date, the association between mTOR and p70^{s6k} is not clear. mTOR has not been shown to directly phosphorylate p70^{s6k}. Given that the addition of rapamycin does cause a decrease in p70^{s6k} phosphorylation, an additional component (probably more than one kinase as p70^{s6k} is phosphorylated at a number of different sites), must lie between mTOR and p70^{s6k}. As mentioned earlier, one potential kinase (causing phosphorylation of the Thr252 residue) is PDK1 (Pullen et al., 1998, Alessi et al., 1998).

Only recently it was shown that mTOR mediated activation of p70^{s6k} can occur by a pathway parallel to the PI 3-kinase pathway. The identification of a p70^{s6k} mutant that was resistant to rapamycin was shown to still be inhibited by wortmannin (Cheatham et al., 1995, Weng et al., 1995). Therefore it seems likely that in certain circumstances/cell types, mTOR may be regulated by a PI 3-kinase/PKB-dependent mechanism, where as under different circumstances, mTOR is activated independently of PI 3-kinase.

The number of potential downstream targets of p70^{s6k} is still not clear. The major known target is the ribosomal S6 protein (part of the S40 ribosomal subunit) which becomes phosphorylated on activation of p70^{s6k} (Lavoinne et al., 1991, Flotow & Thomas, 1992). In response to mitogenic stimuli p70^{s6k} phosphorylates S6 on multiple sites causing activation. These changes enable the S40 ribosomal subunit to translate polysomes (Jefferies & Thomas, 1996). The translation of mRNAs bearing

5'-terminal oligopolypyrimidine ends are known to be preferentially enhanced by S6 phosphorylation. Other rapamycin-sensitive signalling molecules include the initiation factor eIF-4E-binding protein 4E-BP1 (Lin et al., 1995). This protein has been shown to be involved in insulin-mediated translational processes. p70^{s6k} has also been shown to phosphorylate one isoform of the transcription factor, cyclic-AMP-responsive element (CREM) (de Groot et al., 1994). This observation indicates another role for p70^{s6k} in the regulation of mitogen-stimulated transcriptional activities.

1.6.2.3 Small GTPase Proteins (Ras, Cdc 42 and Rac).

These small GTP binding and hydrolysing proteins act as two-state 'molecular switches' (Milburn et al., 1990). A cycle of GTP binding and hydrolysing followed by the dissociation of the resulting GDP, causes alterations in the conformation of the proteins. This alteration enables the proteins to interact with specific downstream targets as part of a signal transduction cascade. The ras GTPase family of proteins includes a number of proteins with a wide variety of cellular functions (Rubins & Dickey, 1993), however they are all activated in the same manner.

Ras. Activation of the 'classical' p^{42/44} MAP kinase signalling cascade can occur via the activation of ras. Ras signals via the cytoplasmic serine/threonine kinase raf (Avruch et al., 1994) which in turn causes phosphorylation of enzymes involved in the classical MAP kinase cascade (section 1.6.2.5). PI 3-kinase has been shown to directly interact with the small GTP-binding protein ras (Rodriguez-Viciana et al., 1994 and 1996 and 1997, Kodaki et al., 1994) due to the presence of a ras binding site on the p110 catalytic subunit of PI 3-kinase. Activation occurs in a GTP-dependent manner and has so far been shown to occur with the p85 α /p110 type of PI 3-kinase only. The positioning of ras and PI 3-kinase in relation to one another along different signalling cascades is not fully established as different groups have published data demonstrating the presence of PI 3-kinase both upstream and down stream of ras (Hu et al., 1992, Rodriguez-Viciana et al., 1994 respectively).

Additionally, due to the fact that in a number of cell types PI 3-kinase activation has been shown to be required for ras-dependent and -independent MAP kinase signalling cascade activation (Cross et al., 1994, Knall et al., 1996), the exact positioning may be cell type and stimuli specific.

In relation to cell survival, apoptosis has been shown to be induced in haemopoietic cells unable to activate the ras signalling pathway (Ihle & Kerr, 1995, Kinoshita et al., 1995). Recent studies have highlighted an association of ras with the anti-apoptotic protein Bcl-2 (Chen & Faller, 1996). As the two proteins are localised to different areas of the cell (Bcl-2 intracellular membranes), ras (inner leaflet of the plasma membrane), it is not clear how these two proteins interact. It has been suggested that other ras family members localise to the intracellular membranes and associate with Bcl-2, or that activated raf-1 is able to translocate to the intracellular membranes and interact with Bcl-2 (Krajewski et al., 1993).

Rac and Cdc 42. *In vitro* PI 3-kinase has been shown to associate with the GTP bound forms of the Ras-like proteins rac and cdc 42 via the breakpoint cluster-related (BCR)-homology region of the p85 PI 3-kinase regulatory subunit (Zheng et al., 1994). Studies by Hawkins and co-workers (1995) indicated that activation of PI 3-kinase via PDGF caused an increase in GTP-bound rac. Inhibition of membrane ruffling by wortmannin has been shown to occur via a rac-dependent signalling mechanism. Given that it is known that cdc 42, rho and rac are regulators of actin cytoskeletal rearrangements (reviewed Nobes & Hall, 1995), further studies have strengthened the link between PI 3-kinase and the small GTP-binding protein rac (reviewed Parker, 1995).

1.6.2.4 Protein Kinase C.

The serine/threonine protein kinases known collectively as the protein kinase C family (PKC) have been implicated in both short and long-term regulation of cellular responses including changes in morphology, differentiation, gene expression and

proliferation. To date three classes of PKC have been discovered which are grouped according to structural variations and biochemical properties: classical (sometimes known as conventional) PKC (cPKC) (α , β 1, β 11 and γ) which are calcium- and diacylglycerol (DAG) or phorbol ester-dependent, novel (nPKC) (δ , ϵ , η and θ) which are phorbol ester or DAG-dependent and atypical (aPKC) (ζ and λ) which do not require calcium or DAG for activation. In addition the PKC μ isoform can be categorised as either novel (due to its structural characteristics) or atypical (due to its functional characteristics).

In relation to PI 3-kinase signalling, Nakanishi and co-workers (1993) initially showed that atypical PKC ζ was activated by the PI 3-kinase product PI(3,4,5)P₃. Further studies have shown that *in vitro* 3-phosphorylated lipid products are able to phosphorylate and activate a number of PKC isoforms also including PKC δ , PKC ϵ and PKC α (Singh et al., 1993, Toker et al., 1994, Palmer et al., 1995). Work performed by Palmer and co-workers (1995) concluded that *in vitro* there seemed to be no PKC isoform specificity, as many PKC isoforms could be activated by the different 3' phosphorylated products of PI 3-kinase.

In vivo few studies have so far been able to link PKC activation to PI 3-kinase. In permeabilised platelets, addition of PI(3,4,5)P₃ was able to cause phosphorylation of the major classical PKC substrate pleckstrin. This activation was inhibitable in whole cells by the addition of wortmannin, but bypassed by the addition of PI(3,4,5)P₃ (Zhang et al., 1995a). Further work by other groups has suggested that other PKC isoforms may also be activated by the lipid products of PI 3-kinase (Akimoto et al., 1996, Moriya et al., 1996). Immunoprecipitation studies have shown that certain PKC isoforms are directly associated with PI 3-kinase *in vivo* (Moriya et al., 1996) and that stimulation of a hemopoietic cell line increased association between the proteins. Additionally, IL-2 stimulation of mouse T cells was shown to cause association of a PKC isoform with PI 3-kinase (Ettinger et al., 1996). In both these studies an increase in PI 3-kinase serine/threonine activity were observed. Finally,

recent work by Le Good and co-workers (1998) has shown that specific PKC family members (e.g. PKC α , δ and μ) are associated with, and can be activated by PDK1.

PKC proteins have been shown to be potentially involved in the suppression of apoptosis. PKC has been shown to directly phosphorylate Bcl-2 and that this phosphorylation has been associated with growth factor-induced suppression of apoptosis (May et al., 1994). (Other investigations have found that Bcl-2 can be phosphorylated in a PKC-independent manner by raf-1 (Blagosklonny et al., 1996, Wang et al., 1994)).

In SCLC cells a number of published investigations have studied the role of PKC enzymes in growth, apoptosis and resistance to chemotherapy. Using the classical SCLC cell line (NCI 209) Barr and colleagues (1997) indicated that expression of the cPKC β II isoform resulted in a positive selection on transfection of the *c-myc* oncogene. This was due to PKC β II expression slowing down cell proliferation rates and improving survival statistics. Additionally, Schuller and colleagues (1992) noted that on addition of the PKC inhibitor dihydropyridine dexniguldipine, certain SCLC and non-SCLC cell lines were prevented from proliferating. Finally, characterisation of H69 cisplatin-sensitive and -resistant cell lines indicated that a reduction of cPKC levels, and an increase in nPKCs may aid in SCLC cell resistance to cisplatin (Basu et al., 1996).

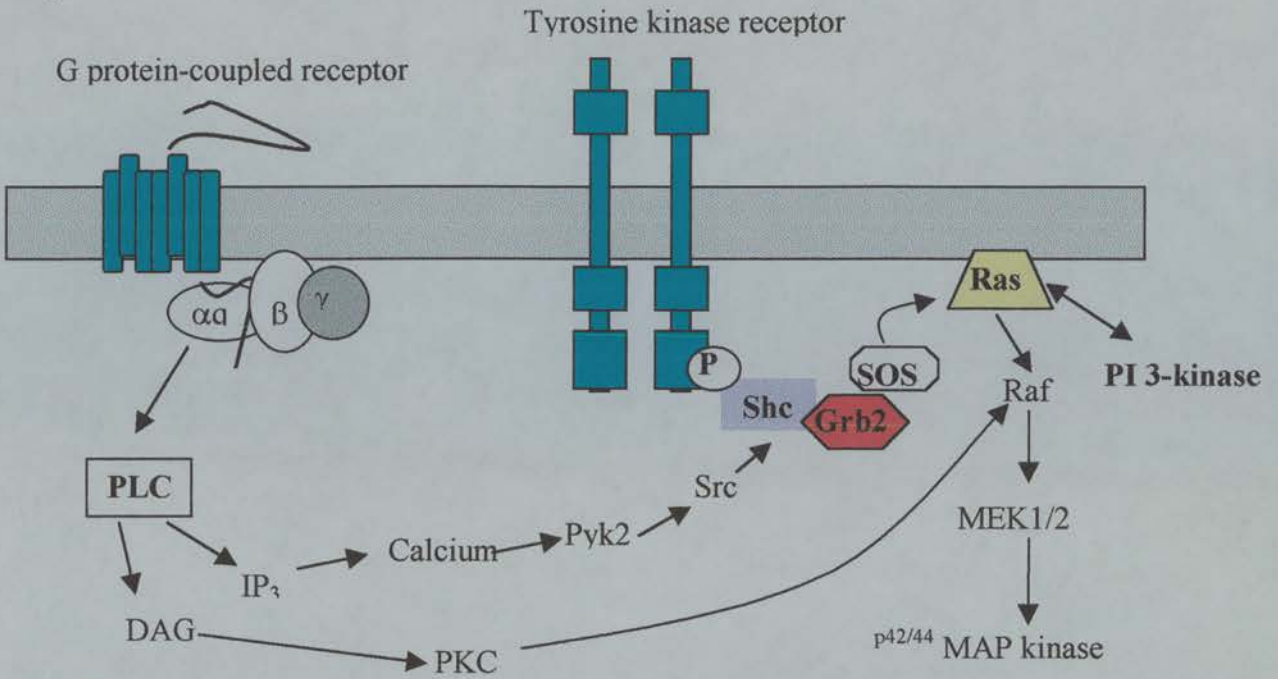
1.6.2.5 Mitogen Activated Protein Kinase Signalling Pathway.

The most studied cytoplasmic signal transduction pathway which involves the sequential phosphorylation of a cascade of protein kinases is the mitogen activated protein kinase cascade (MAP kinase cascade). The proteins are activated by the phosphorylation of both tyrosine and threonine residues by specific kinases. Activation of the 'classical' p^{42/44} MAP kinase signalling cascade (reviewed Marshall, 1995, Davis, 1993) can occur via the activation of ras. Members of the PKC family have also been shown to activate the protein raf-1 both *in vitro* and *in vivo* thereby

activating the MAP kinase cascade. Ras activation of raf-1 causes phosphorylation and activation of the MAPK/ERK kinases (MEK 1 & 2 in mammalian cells) (reviewed Robinson & Cobb, 1997). The MEK kinases then activate the downstream extracellular-signal regulated kinases (ERK 1 & 2) (also known as p⁴⁴/p⁴² MAPK respectively) ultimately leading to the activation of a wide variety of cellular responses including cell proliferation and differentiation.

Until recently, growth factor tyrosine kinase receptor mediated activation was the major mechanism by which the MAP kinase cascade was thought to be activated (Fantl et al., 1993). However, a number of G-protein coupled receptors have recently been shown to cause activation of this cascade (Kahan et al., 1992). Many G_i-protein coupled receptors stimulate MAP kinase via Gαβγ subunits both dependently and independently of their ability to stimulate PLC (Della et al., 1997, Hawes et al., 1995 respectively). The involvement of PI 3-kinase in G_i protein receptor stimulation of the MAP kinase cascade has been demonstrated in COS-7 and CHO cells (Hawes et al., 1996, Lopez-Illasaca et al., 1997 respectively). In COS-7 cells (Monkey kidney cells), Hawes and colleagues (1996) showed that the inhibition of G-protein mediated activation of PI 3-kinase by wortmannin, LY294002 or a dominant negative PI 3-kinase, prevented MAP kinase activation. In these studies PI 3-kinase was shown to be situated upstream of ras and SOS. (SOS is a guanine nucleotide exchange factor that converts ras-GDP to ras-GTP ultimately leading to activation of the MAP kinase signalling pathway). G_q proteins are able to activate the MAP kinase pathway via activation of PKC leading to activation of raf (Faure et al., 1994). (Figure 1.3).

A)



B)

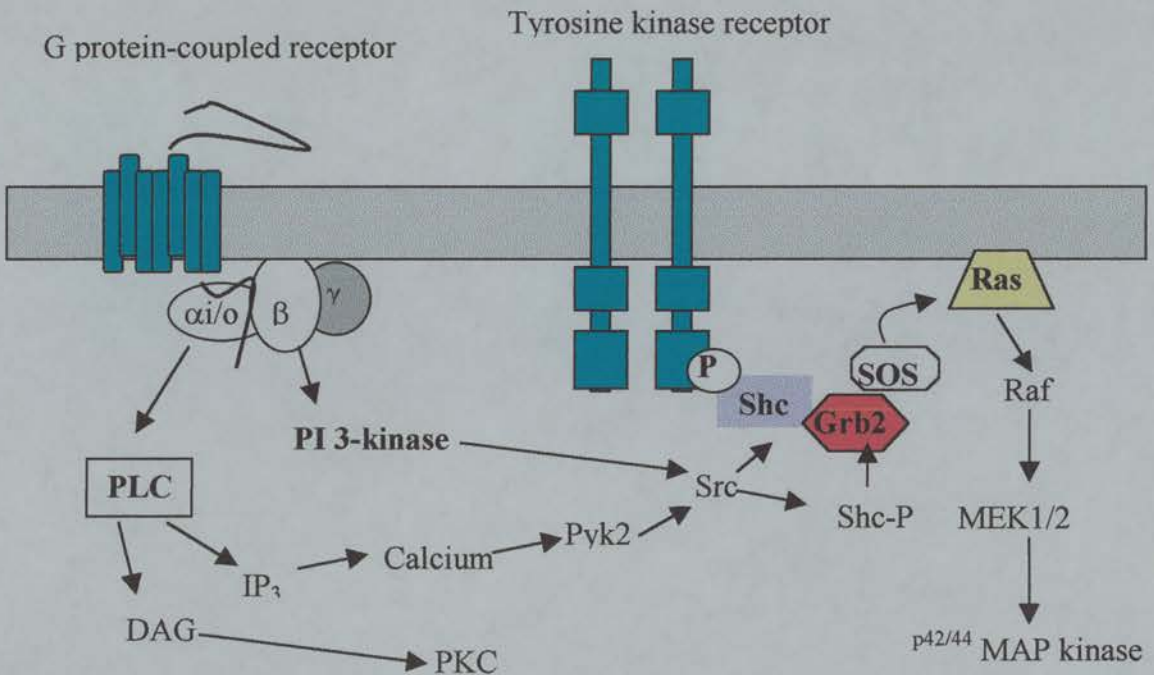


Figure 1.3: Activation of the Classical Mitogen Activated Protein Kinase Cascade.

A) Gq-coupled receptors. B) G i/o-coupled receptors.

Abbreviations: Grb2, growth-factor-receptor-bound protein 2, IP₃, inositol 1,4,5-trisphosphate, MAP kinase, mitogen activated protein kinase, PI 3-kinase, phosphoinositide 3-kinase, PKC, Protein kinase C, Ras, rat sarcoma virus, PLC, phospholipase C, Pyk2, proline rich tyrosine kinase 2, SOS, son of sevenless, MEK, MAP/ERK kinase, DAG, 1,2-diacylglycerol, Shc, SH2-domain-containing α2-collagen related protein and src, rous sarcoma virus.

Activation of G protein-coupled receptors leads to the formation of G α -GTP and G $\beta\gamma$ subunits. These are able to activate PLC leading to phosphoinositide hydrolysis and formation of diacylglycerol and IP₃. The subsequent release of calcium from intracellular stores causes phosphorylation of the FAK-related kinase (PYK2) (Dikic et al., 1996, Lev et al., 1995). Once phosphorylated, PYK2 associates with Grb2 which in turn brings the small molecular weight GTPase adaptor protein mSOS in to the signalling complex leading to ras activation and ultimately MAP kinase activation. PYK2 is also able to activate rac-1 (Deckert et al., 1996, Crespo et al., 1996 and 1997).

Additionally, Schlaepfer and co-workers (1994) showed that the MAP kinase cascade could be activated on integrin binding. On the adhesion of NIH 3T3 fibroblasts to fibronectin, FAK associated with the adaptor molecule Grb2 (a SH2-SH3-SH2 adaptor protein now known to associate with FAK via its NH₂-terminal SH2 domain). Grb2 is then known to associate (via its SH3 domain) with mSOS ultimately leading to MAP kinase activation. This observation was strengthened by reports that adhesion of Swiss 3T3 fibroblasts to fibronectin caused MAP kinase activation (Chen et al., 1994). More recently Schlaepfer and Hunter (1997) also noted that overexpression of FAK led to ras-dependent signalling of the ERK2 MAP kinase enzyme. Finally, another major pathway by which the MAP kinase cascade is thought to be activated via integrins involves the adaptor protein shc. A dominant negative shc protein was shown to suppress ERK activation in response to integrin ligation (Wary et al., 1996).

In SCLC cells Seufferlein and Rozengurt (1996a) indicated that activation of the classical MAP kinase pathway via PKC was important in neuropeptide-mediated cell growth. SCLC colony formation was also abrogated by the addition of the MEK1 inhibitor PD 098059 or the PKC inhibitor GF 109203X. Additionally, Beekman and co-workers (1998) showed that inhibition of basal PKC activity (via the prevention of PLC β activation) inhibited colony formation and receptor stimulated activation of the MAP kinase proteins ERK 1 & 2. Additionally recent work by Cattaneo and co-

workers (1997) indicated that indirect nicotine-mediated MAP kinase activation in SCLC cells was mediated via the release/production of an unknown co-factor. This co-factor then went on to activate MAP kinase via a pertussis toxin-sensitive (inhibit G_i family of $G\alpha$ proteins) and tyrosine kinase-sensitive pathway. Finally, Seckl and Rozengurt (1997) noted that inhibition of neuropeptide-mediated MAP kinase activity (by [D-Arg¹,D-Trp^{5,7,9},Leu¹¹]Substance P), inhibited SCLC tumour growth *in vivo*.

1.6.3 Activation of Parallel Signalling Pathways by Phosphoinositide 3-kinase.

It is now being widely accepted that activation of a specific intracellular signalling molecule may lead to the stimulation of a number of unrelated cellular functions. The reasoning behind why specific cell signalling pathways are switched on/off by different cell stimuli is not fully understood. An example of parallel signalling is described below and relates to work performed by Shaw and co-workers in breast carcinoma cells (1997).

Shaw and co-workers (1997) noted that an increase in PI 3-kinase activity (due to $\alpha\beta 4$ -mediated integrin adhesion), resulted in an increase in migration and invasion. This cellular function was found to be mediated via a PKB- and p70^{s6k}-independent mechanism (as the inhibition of both enzymes did not effect cell invasion). This implied the existence of an alternative role for PI 3-kinase other than cell survival or cell proliferation (via PKB and/or p70^{s6k}). Activation of the small GTPase protein rac was shown to be required for PI 3-kinase mediated invasion. The resulting hypothesis that PI 3-kinase regulates the activity of both PKB and rac via different parallel pathways (each terminating in a different cellular process) was reinforced by Welch and co-workers (1998). Rac activation (via PDGF stimulation of PI 3-kinase or by rac mutation) caused the induction of lamellipodia formation, membrane ruffling and p70^{s6k} activity (previously seen by Chou & Blenis, 1995). Inhibition of rac activity resulted in loss of lamellipodia formation and membrane ruffling and a



depletion in p70^{s6k} activity (approximately 60%), indicating that rac had a regulatory role in p70^{s6k} activity only. Inhibition of p70^{s6k} with rapamycin did not effect the formation of lamellipodia and membrane ruffles. PDGF stimulation caused activation of PKB which could not be inhibited by the addition of a kinase dead rac or rapamycin. Inhibition of PKB inhibited p70^{s6k} activity without effecting rac activation or membrane ruffling. The conclusions drawn from these observations are that PKB and rac activation both occur via PI 3-kinase but are situated on parallel pathways. These two pathways result in differing cell processes (rac cytoskeletal changes, PKB survival etc.) but are linked downstream as both regulate p70^{s6k} activity. The role of rac in membrane ruffling and lamellipodia formation is independent of PKB and p70^{s6k} activity implying the production of two or more signalling pathways emanating from/downstream of rac, one via p70^{s6k} and another leading to cytoskeletal rearrangements.

Figure 1.4 summarises a number of the potential signal cascades which are mediated by PI 3-kinase activation.

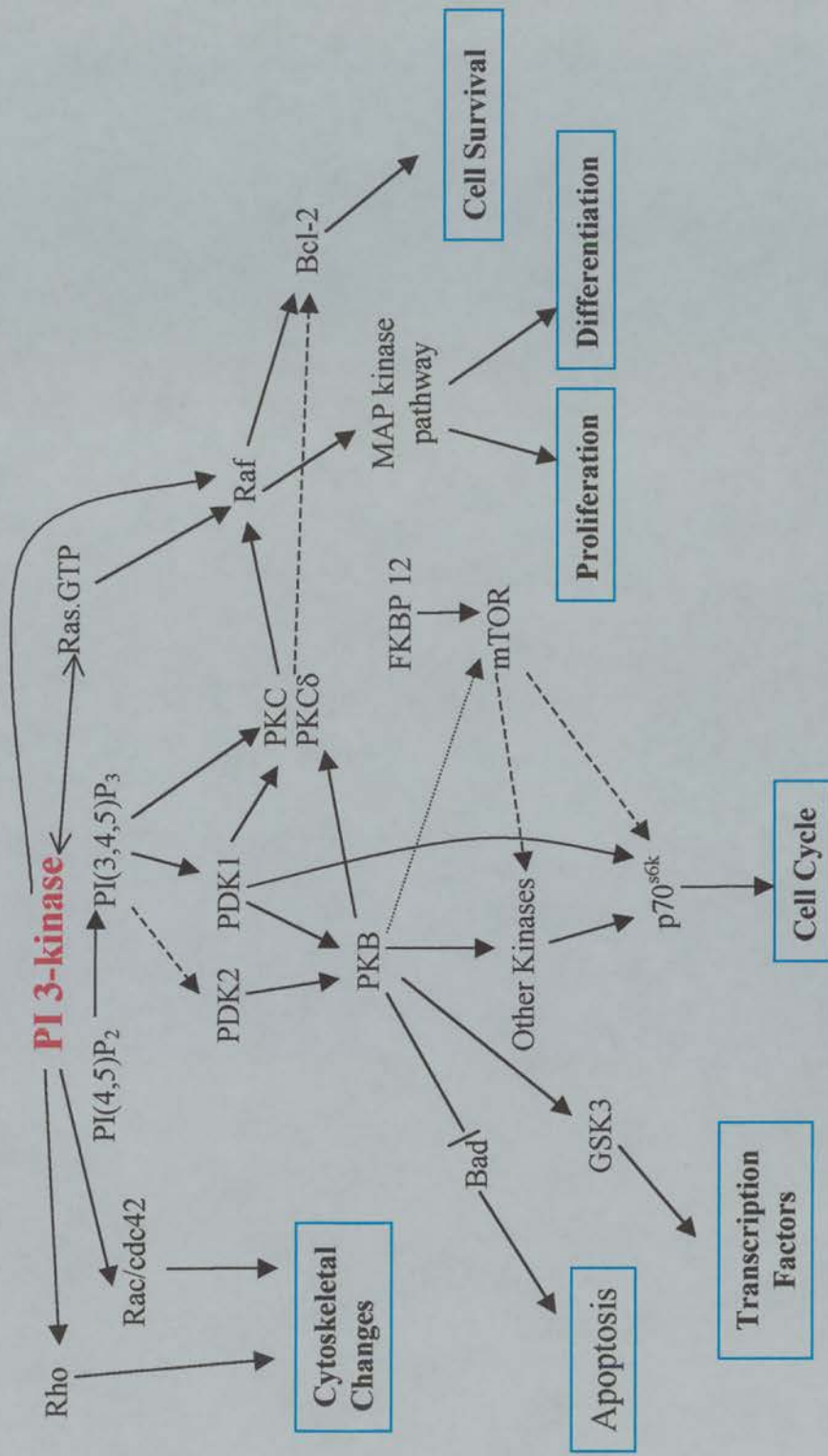


Figure 1.4: Summary of a Selection of the Potential Signalling Pathways Regulated by Phosphoinositide 3-kinase as described in section 1.6.

Abbreviations: MAP kinase, mitogen activated protein kinase; PI(3,4,5)P₃, phosphatidylinositol (3,4,5)-trisphosphate; PI(4,5)P₂, phosphatidylinositol (4,5)-bisphosphate; PI 3-kinase, phosphoinositide 3-kinase; PDK, PI(3,4,5)P₃-dependent kinase; PKB, protein kinase B; PKC, Protein kinase C; Rho, Ras homologous protein; Rac, Ras-related C₃-botulinum toxin substrate, Ras, rat sarcoma virus; p70^{S6K}, ribosomal protein S6 kinase; GSK3, glycogen synthase kinase-3; mTOR, mammalian target of rapamycin and FKBP12, FK506 binding protein 12.

1.7 Plan of Study.

Small cell lung cancer (SCLC) represents approximately 25% of all primary lung cancers. SCLC patients normally present with disseminated disease and therefore chemotherapy has become the mainstay of treatment. Given that widespread metastasis and eventual resistance to chemotherapy are the two main causes of SCLC patient death, these two areas were to be addressed within this thesis. The overall aim of the following research was to try to further our understanding of the complex molecular events which underlie the rapid proliferation times, high metastatic rates and the cause of chemoresistance in SCLC cells.

I) Anchorage-independent growth is essential to allow tumour cells to survive and metastasise *in vivo*. Recent studies have suggested that PI 3-kinase may be critical to the proliferation, survival and metastatic capabilities of different tumour types (Keely et al., 1997, Shaw et al., 1997). Investigations were undertaken to examine the role of PI 3-kinase in SCLC cell growth and tumourigenesis.

II) Despite initial sensitivity to chemotherapy, patients almost invariably relapse and become resistant to chemotherapy so that the two year survival rate is between 3 - 8 % (Elias, 1997). Recently, investigators have suggested that chemotherapeutic agents cause cell death via apoptosis (Chen et al., 1996a, Hannun, 1997). Adhesion to extracellular matrix (ECM) proteins has been shown to protect many cell types from under going apoptosis (Meredith et al., 1993, Frisch & Francis, 1994, Boudreau et al., 1995 and 1996). The extracellular matrix and its protein components (section 1.4) have also been shown to regulate tumour cell proliferation, migration, invasion, survival and differentiation. Therefore, the potential role of ECM proteins in SCLC cell growth and resistance to chemotherapy-induced apoptosis were investigated.

CHAPTER 2

Materials and Methods.

2.1 Small Cell Lung Cancer Cell Lines.

Classical small cell lung cancer (SCLC) cell lines NCI-H345, NCI-H69 and NCI-H510 were purchased from the American Type Tissue Culture Collection (ATCC) (Rockville, USA). All cell lines were of human origin and routinely screened for bacterial, fungal and mycoplasma to ensure they were infection free. Screening was performed by the Department of Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh. Cells were used for experimental purposes at low passage levels (p5 – 50).

Cells were occasionally screened for the presence of typical SCLC cell surface markers (Table 2.1). Cytospins were made (as described in section 2.5.3) of SCLC cells taken directly from culture. Cytospins were air dried and fixed in 3% formaldehyde before being stored at 4°C until required. Slides were examined by the Department of Pathology, University of Edinburgh Medical School as part of a routine screening process.

Table 2.1: Antibody Markers Routinely used to Screen for Small Cell Lung Cancer Cell Surface Markers in the Department of Pathology.

	H69	H510	H345
Chromogranin	+++	++	+
Factor VIII	+	-	-
Vimentin	-	-	-
CAM 5.2	+++	+++	++
NSE	+++	+	+++

+++ strong staining; ++ intermediate staining; + weak staining and - no staining. NSE - neuron specific enolase. All assessments were performed in conjunction with positive/negative controls (tissue/ cells known to positively or negatively express the specific markers in question).

Note: The weak staining observed in H69 SCLC cells of Factor VIII was not expected. This result maybe due to the fact that a rabbit polyclonal antibody was utilised which may have cross reacted with another unknown cell surface marker.

2.2 Cell Culture.

2.2.1 Small Cell Lung Cancer Cell Lines.

Stocks were grown in RPMI 1640 medium containing 25 mM HEPES (Sigma, UK.). They were supplemented with 10% (v/v) foetal calf serum (FCS) (heat-inactivated at 56°C for 1 hr.), 50 U/ml penicillin, 50 µg/ml streptomycin and 5 µg/ml L-glutamine. Cells were grown in 75 cm³ tissue culture flasks (Costar, UK.) in a humidified atmosphere of 5% CO₂/95% air at 37°C . Cells were passaged every 5 – 7 days. For experimental purposes, cells 3 - 5 days post-passage were pelleted (225 x g, 4 mins) at room temperature before being transferred into SITA medium consisting of RPMI 1640 medium with 25 mM HEPES supplemented with 30 nM selenium, 5 µg/ml insulin, 10 µg/ml transferrin, 0.25% (w/v) bovine serum albumin (BSA), 50 U/ml

penicillin, 50 µg/ml streptomycin and 5 µg/ml L-glutamine. Cells were cultured for a further two days prior to use. Alternatively, cells were taken from growth medium, washed thoroughly with quiescent medium and incubated in fresh quiescent medium for 24 hours prior to experimentation. Quiescent medium comprised of RPMI 1640 medium containing 25 mM HEPES which was supplemented with 0.25% (w/v) BSA, 50 U/ml penicillin, 50 µg/ml streptomycin and 5 µg/ml L-glutamine. All stocks of media were stored at +4°C and solutions at -20°C. Once prepared, media were not stored for more than 7 days. Figure 2.1 shows representative photographs of SCLC cell lines growing in liquid culture.

Table 2.2: Small Cell Lung Cancer Cell Line Characteristics.

(Information from ATCC, Gazdar et al., 1980 and Carney et al., 1985a & b.).

Cell Line	H69	H345	H510
Year Submitted	1980	1981	1982
Patient Sex	Male	Male	Male
Age (yrs)	55	64	56
Prior Treatment	Chemo	Chemo	Radio
Source	PE	BM	A
SCLC Class	Classical	Classical	Classical
DDC u/mg	240	98	214
CK-BB µg/mg	2.2	5.8	2.7
NSE ng/mg	817	4075	491
BLI pmol/mg	1.7	4.7	7.4

Abbreviations: Chemo, Chemotherapy, Radio, Radiotherapy (to a brain lesion only), PE, pleural effusion, BM, bone marrow; A, adrenal, DDC, L-dopa decarboxylase (> 1.0 u/mg = elevated), CK-BB, creatine kinase brain isoenzyme (> 0.4 µg/mg = elevated), NSE, neuron specific enolase (> 100 ng/mg = elevated), BLI, bombesin-like immunoreactivity (> 0.1 pmol/mg = elevated).

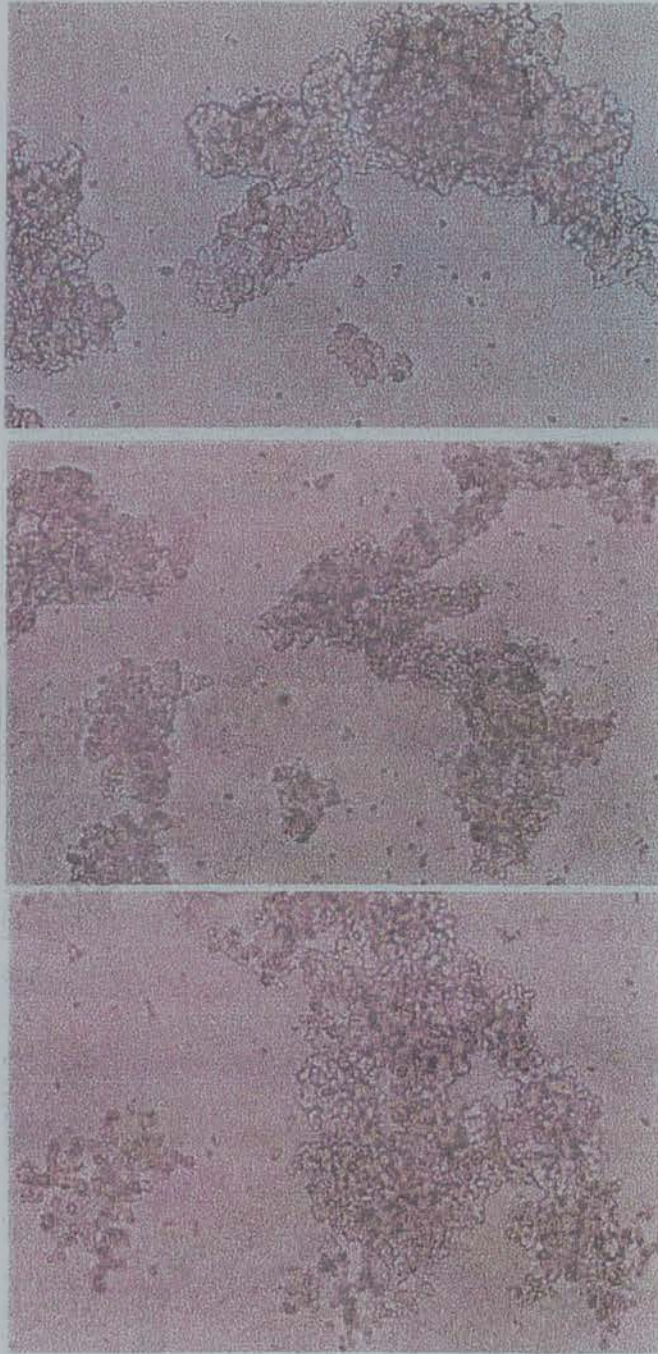


Figure 2.1: Examples of Small Cell Lung Cancer Cell Lines H345 (upper), H69 (centre) and H510 (lower) Growing in Liquid Culture.

Cells were grown in RPMI containing 10% (v/v) foetal calf serum (as described in section 2.2.1). SCLC cells, 3 – 4 days post-passage were photographed using an Olympus inverted light microscope (x 40 magnification).

2.2.2 Swiss 3T3 Mouse Fibroblasts.

Cells were obtained from the European Tissue Culture Collection (Porton Down, UK.). Stocks were grown in 162 cm³ tissue culture flasks (Costar, UK.) in Dulbecco's modified Eagle's medium (DMEM) (Sigma, UK.) supplemented with 10% (v/v) FCS, 50 U/ml penicillin, 50 µg/ml streptomycin and 5 µg/ml L-glutamine. Cells were incubated in a humidified atmosphere of 5% CO₂/95% air at 37°C. Cells were passaged every 4 – 6 days. Whilst passaging the cells, they were washed in DMEM containing no serum, then incubated in pre-warmed trypsin/EDTA (approx. 8 ml/flask) (Sigma, UK.) in a humidified atmosphere of 5% CO₂/95% air at 37°C until the cells began to detach. DMEM containing 10% (v/v) FCS was added (to deactivate trypsin), the cells scrapped and pelleted (225 x g, 4 minutes). Cells were then re-seeded in fresh culture media.

For experimental purposes cells were either lysed directly from culture vessels using phosphoinositide 3-kinase (PI 3-kinase) lysis buffer (see section 2.3.1a) (for western blot analysis), or when grown to sub-confluence (90 - 95% confluence by light microscopy) in 24 well plates (Costar, UK.), they were quiesced for 24 hours in DMEM containing 0.25% (v/v) FCS before use. All stocks of media and trypsin were stored at +4°C. Once prepared, media was not stored for more than 7 days.

2.2.3 Rat Pheocromocytoma Cell Culture.

Cells (PC12s) were a kind gift from Dr G Hoyne, Respiratory Medicine Unit, University of Edinburgh Medical School. Cells were grown in 162 cm³ tissue culture flasks (Costar, UK.) in RPMI 1640 supplemented with 10% (v/v) horse serum (Sigma, UK.), 50 U/ml penicillin, 50 µg/ml streptomycin and 5 µg/ml L-glutamine in a humidified atmosphere of 5% CO₂/95% air at 37°C. Cells were passaged every 5 – 7 days using essentially the same method as described for Swiss 3T3 cells (section 2.2.2) (different cell culture medium was used). For western blot analysis cells were lysed directly from culture vessels using PI 3-kinase lysis buffer (see section 2.3.1a).

All stocks of media were stored at +4°C. Once prepared, media was not stored for more than 7 days.

2.2.4 Other Cell Lines.

Other members of the laboratory who routinely used specific cells for experimentation, provided all other cell types described and utilised in this thesis.

Bovine trachea smooth muscle cells (BTSM) – These primary culture cells were kindly provided by Dr T.R. Walker.

Human alveolar type II epithelial cells / adenocarcinoma cells (A549s) – These cells were purchased from the ATCC and routinely cultured by Ms T Watchorn.

Both of the above cell types was cultured in DMEM supplemented with 10% (v/v) FCS, 50 U/ml penicillin, 50 µg/ml streptomycin and 5 µg/ml L-glutamine.

For western blot analysis, the cells were lysed directly from culture vessels using ice cold PI 3-kinase lysis buffer (section 2.3.1a) before being equilibrated for total protein content.

2.3 *In Vitro* Protein Kinase Activity Assays.

2.3.1 Phosphoinositide 3-kinase Activity Assay.

Section a describes the activity assay used to provide the results described throughout this thesis. Section b describes some of the alterations carried out to achieve this final assay procedure. Section c describes additional experimentation performed to ensure the final assay product was correct.

2.3.1a Assay Procedure.

PI 3-kinase lysis buffer : Stock Solution : 50 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10% (v/v) glycerol and 1% (v/v) Triton X-100. The stock solution was stored at +4°C until required.

Added fresh: 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, 50 μM AEBSF, 5 μg/ml leupeptin, 20 μg/ml aprotinin and 10 μg/ml soybean trypsin inhibitor. Protease inhibitors were stored at -20°C until required.

Sonicated Lipid Substrate: (Prepared on the day.) Phosphatidylinositol (PI) and phosphatidylserine (PS) (Sigma, UK.) (150 μl : 50 μl) was dried under vacuum at room temperature. To the resulting dried lipids, 250 mM HEPES (1 ml) was added and the lipids sonicated on ice using a small probe (soniprep - MSE) (2 x 10 sec). Final stock concentration was 2 mg/ml. The resulting lipid micelle were stored on ice until required.

Essentially, SCLC cells were cultured overnight in quiescent medium, pelleted (225 x g, 4 mins) and washed three times with warmed PBS. Cells (1x10⁷ /ml) were gently disaggregated by two passes through a 21 gauge needle. Cells were then transferred in to 24 well plates (1 ml PBS/ well) and allowed to equilibrate at 37°C for 1 hour. Varying concentrations of wortmannin, DMSO vehicle (final concentration 0.001% v/v) (20 minutes) or specific mitogens (see figure legends) (5 or 10 minutes) were added. After incubation, the cells were immediately transferred into 1.5 ml eppendorfs on ice (Starstedt Ltd, Germany.), pelleted (300 x g, 1 min, 4°C), the resulting supernatant removed and the remaining pellet lysed using 400 μl ice-cold PI 3-kinase lysis buffer (20 minutes on ice). Lysates were clarified by centrifugation at 13000 x g for 10 minutes (4 °C) and supernatants transferred to fresh microfuge tubes. The supernatants were pre-cleared for 1 hour by addition of 50 μl BSA-sepharose beads (Sigma, UK.) in 50 μl lysis buffer. Lysates were pre-cleared prior to immunoprecipitation to limit any non-specific adsorption of proteins to the beads used

later in the immunoprecipitation step. Protein content was measured using a Pierce BCA protein assay kit (section 2.7) (Rockville, USA). Approximately 1 mg of total protein was used per assay point. Lysates were snap frozen by being rapidly immersed in liquid nitrogen and stored at -80°C until required.

The frozen lysates were slowly thawed on ice prior to the addition of the p85-PI 3-kinase antibody ($2.5\ \mu\text{g}/\text{assay point}$). Following a 90 minute incubation at 4°C on an orbital shaker, $30\ \mu\text{l}$ of a 50:50 slurry containing goat anti-mouse IgG agarose beads in lysis buffer was added, and the incubations continued for a further 2 hours. The beads were then pelleted and washed sequentially: once with lysis buffer, once with $100\ \text{mM}$ Tris HCl ($\text{pH } 7.6$), $500\ \text{mM}$ LiCl, and twice with $200\ \text{mM}$ HEPES ($\text{pH } 7.4$), $40\ \text{mM}$ MgCl_2 , $600\ \text{mM}$ NaCl.

PI 3-kinase assays were performed using phosphatidylinositol (PI) as the substrate in a final volume of $200\ \mu\text{l}$ containing: (final concentrations) $50\ \text{mM}$ HEPES ($\text{pH } 7.4$), $10\ \text{mM}$ MgCl_2 , $150\ \text{mM}$ NaCl, $[\text{}^{32}\text{P}]\gamma\text{-ATP}$ ($10\ \mu\text{Ci}$, $3000\ \text{Ci}/\text{mmol}$), $50\ \mu\text{M}$ ATP and $0.2\ \text{mg}/\text{ml}$ sonicated phosphatidylinositol/phosphatidylserine (3:1, w/w). The reactions were carried out for 20 minutes at 37°C , terminated by the addition of chloroform/methanol/ $0.1\ \text{M}$ HCl (40/80/1, v/v/v; $750\ \mu\text{l}$) and a phase-partition achieved by the addition of chloroform ($250\ \mu\text{l}$) and $0.1\ \text{M}$ HCl ($250\ \mu\text{l}$). After centrifugation ($13,000 \times g$, 1 min) the upper phase was aspirated and the lower phase washed twice with synthetic upper phase ($750\ \mu\text{l}$) consisting of MeOH:HCl(1M): CHCl_3 (48 :47 :3 v/v/v).

The resulting chloroform phase was dried under vacuum at room temperature and the lipid products separated by thin layer chromatography (TLC) using the following solvent system: methanol/ chloroform/ conc. ammonia/ dH_2O (10/ 7/ 1.5/ 2.5, v/ v/ v/ v.) (prepared fresh when required). Phosphorylated lipids were identified by autoradiography (TLC plates placed for 1 - 2 days at -70°C , XOMAT film (Kodak)). The radioactive bands that were visualised by autoradiography were excised from the TLC plate and transferred into liquid scintillation vials. Liquid scintillation fluid was

added (3 ml) (Flo-Scint IV (Packard B.V.)) and radioactivity incorporation assessed using a Packard 1900 TR liquid scintillation analyser.

For assays involving LY294002, immunoprecipitates from untreated cells were prepared as above, washed and incubated with varying concentrations of LY294002 for 20 minutes at 37°C before being assayed for PI 3-kinase activity as above. Figure 2.2 demonstrates a representative TLC autoradiograph showing the concentration-dependent inhibition of PI 3-kinase activity on the addition of wortmannin and LY294002. In the results sections, data is expressed as % change in PI 3-kinase activity in comparison to untreated control cells.

In figure 2.2 differences between the amount of 'free ATP' present in each sample was observed on the TLC plate treated with wortmannin. This was due to differences in the efficiency of the phase partition between the aqueous and organic phases. On addition of the synthetic upper phase (aqueous), free ATP would move into the aqueous phase until equilibrium was reached. The aqueous phase would be removed and replaced twice with fresh synthetic upper phase in the attempt to remove any free ATP not utilised during the assay procedure. If any of the aqueous phase was not removed (i.e. was stuck to the side of the eppendorf, or not all the interphase was removed efficiently), free ATP would remain behind. The amount of free ATP present in each sample at this stage does not effect the assay result in any way. On the lower autoradiograph, bands are visualised below the PI3P band. These bands were analysed by HPLC and shown not to be PI3P. They eluted off the SAX 5 column by HPLC at a low $(\text{NH}_4)_2\text{HPO}_4$ concentration. It is thought that these bands were probably due to the presence of impurities in the phospholipid mixture used in the kinase assay.

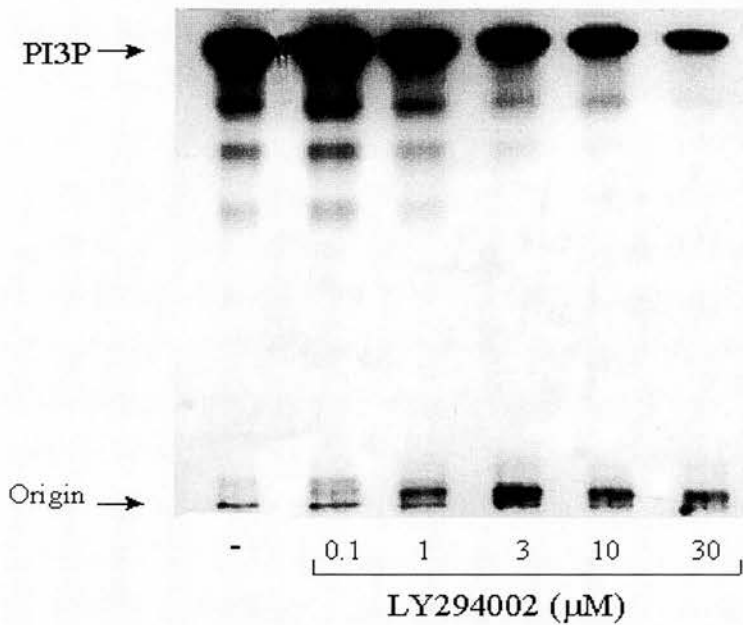
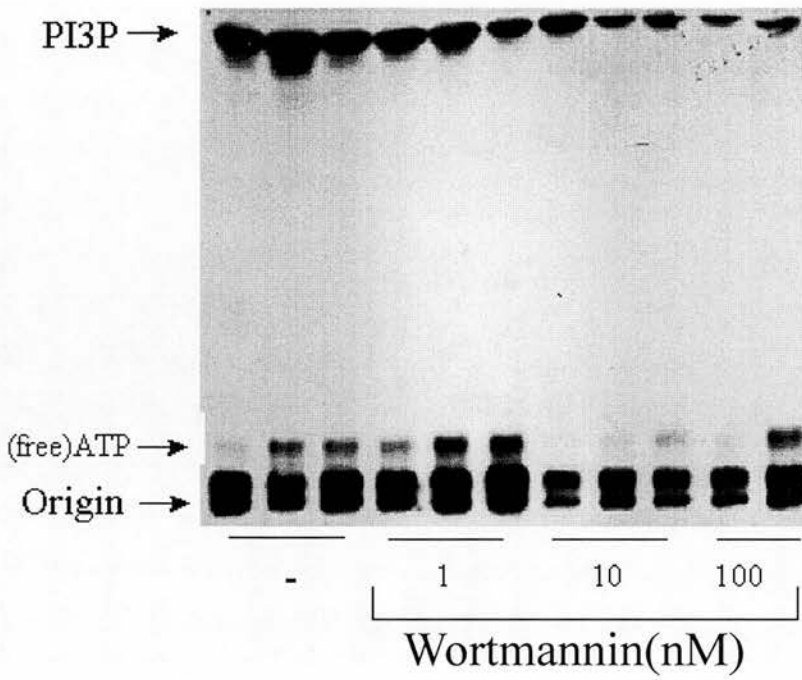


Figure 2.2: Representative Autoradiographs of TLC Plates indicating the Presence of the Lipid Molecule Phosphatidylinositol 3 phosphate (PI3P) on Addition of different Phosphoinositide 3-kinase Inhibitors.

Upper: H345 cells were inhibited with increasing concentrations of wortmannin over 20 minutes. Cells were lysed and immunoprecipitated using a p85-PI 3-kinase antibody. A PI 3-kinase activity assay was performed and the resulting lipid products separated by TLC and autoradiographed as described in section 2.3.1a.

Lower: H345 cells were lysed and immunoprecipitated for p85-PI 3-kinase before being inhibited with increasing concentrations of LY294002 (20 minutes) prior to assaying for PI 3-kinase activity. The resulting lipid products were separated by TLC and autoradiographed.

When assaying for PI 3-kinase activity in the presence of the extracellular matrix protein laminin, 12 well tissue culture plates were coated with laminin (10 $\mu\text{g/ml}$, 500 $\mu\text{l/well}$) overnight at 4°C. Plates were washed in sterile PBS and the cells (1 ml/well) allowed to adhere for 40 minutes. This time frame was chosen as SCLC cells were known to be able to adhere to ECM proteins in 40 minutes by both adhesion and immunofluorescence experiments (see chapter 5). Cells were plated at a concentration of approximately $8 \times 10^6/\text{ml}$ as this number was assessed visually by light microscopy to produce a single monolayer of cells. After adhesion in the presence or absence of wortmannin, cells were transferred onto ice before being centrifuged at 670 x g for 4 minutes at 4°C to enable the cells to pellet on to the base of the wells. Once transferred back onto ice, media was aspirated off and PI 3-kinase lysis buffer added to each well (500 μl). This method was adopted because trying to aspirate the cells out of the wells and in to eppendorfs prior to lysis, caused the strongly adherent cells to remain in the wells. Cells were then lysed on ice, equilibrated for protein and the assay performed as described previously.

2.3.1b Initial Experiments

Undertaken to achieve final assay procedure described in section 2.3.1a.

Initial experiments were undertaken to investigate the possibility that PI 3-kinase would be activated by the addition of various neuropeptides and growth factors that had previously been shown to activate PI 3-kinase in other cell types (described further in results chapter 3).

The original PI 3-kinase activity assay was developed using adherent PC12 cells (Carter & Downes, 1992). Given that SCLC cells grow in suspension, alterations were made to the incubation procedure of the assay. Experiments were performed by placing quiesced SCLC cells (approximately $2 \times 10^7/\text{ml}$, 0.5 ml/well) (24 well tissue culture plate (CorningCostar, New York.)) in a water bath at 37°C, prior to the addition of various mitogens. To terminate the reaction, the tissue culture plates were placed on ice and 0.5 ml of ice cold PI 3-kinase lysis buffer was added to each well.

The assay procedure was then followed essentially as described by Carter & Downes (1992). The first sets of experiments were unsuccessful with no bands/ radioactivity visualised by autoradiography.

Worries about the efficiency of the lysis step were addressed. Dithiothreitol, sodium orthovanadate, and the required protease inhibitors were all freshly added to the other components of the PI 3-kinase lysis buffer to attempt to achieve efficient lysing of the cells.

To try and provide a more effective lysis technique, and to attempt to decrease the initial plating concentration of the SCLC cells, cells were incubated in 1 ml of RPMI/well. After 'stimulation' the plates were transferred on to ice where the samples were transferred into ice-cold eppendorfs prior to being gently pelleted (as described in the final assay system) at 4°C. The resulting RPMI supernatant was removed and 0.5 ml of ice-cold PI 3-kinase lysis buffer was added to each pellet and assay continued as before. This second assay procedure took slightly longer to perform but prevented the lysis buffer from being mixed with warmed RPMI. After these changes, weak bands were visualised on the autoradiographs. Results primarily indicated that on the addition of 10% (v/v) foetal calf serum or insulin, there was no significant increase in PI 3-kinase activity (discussed in chapter 3). However, on the addition of the PI 3-kinase inhibitor wortmannin, PI 3-kinase activity was shown to fall below control levels. (Control cells – received diluent alone.)

To attempt to make the immunoprecipitation stage of the assay more efficient, a PI 3-kinase specific monoclonal antibody was used which recognised the SH3 domain of the p85 regulatory subunit. The primary antibody was a mouse monoclonal antibody. To precipitate this type of antibody research has shown that an anti-mouse IgG agarose is the most efficient.

Results still suggested that the addition of mitogens did not cause a large increase in PI 3-kinase activity. As this was still thought surprising, it was hypothesised that the

initial protein concentration in each assay point may be inaccurate (i.e. the number of cells plated/well varied). Therefore more protein was present in the control samples artificially elevating the basal PI 3-kinase activity levels calculated. As SCLC cells are free floating in culture, investigators are unable to grow the cells to subconfluence in petri/culture dishes. Using adherent cell types, investigators are able to assume that once the petri dishes are subconfluent there will be approximately an equal concentration of protein in each dish. To overcome this possible problem of uneven protein concentration distribution between assay points, an additional step was added to the assay procedure. Following the pre-clearing stage, lysates were assayed for total protein concentration so that each point could be equilibrated (protein assay procedure section 2.7). Even when the samples were equilibrated for total protein concentration, the same assay result was repeatedly obtained. The addition of mitogens did not cause a large increase in PI 3-kinase activity levels above control values. (Note - As experience has increased, it is possible to efficiently aliquot approximately identical cell numbers per well; however protein assays are still regularly used to assess total protein content.)

So far the experiments undertaken had been carried out with cells that were quiesced overnight (before being plated in RPMI for the assay), therefore it seemed unlikely that wortmannin should be able to reduce the PI 3-kinase basal activity levels by such a high degree. To attempt to lower the basal activity levels, cells were quiesced overnight at a lower cell concentration, washed in large volumes of PBS and plated in PBS for the assay. This was to remove any possible trace of exogenous mitogens. This made no difference to the results obtained.

Further changes carried out to try and ensure that the assay procedure was efficient were :

I) Carter & Downes (1992) previously dipped the TLC plates in a solution of 1% potassium oxalate, 2 mM EDTA in 50% methanol/50% water, and dried them at 110°C for 1 - 2 hr prior to use. This was to increase the 'cleanness' of the lipid separations on the TLC plate because they were assaying for the presence of a number

of different lipid products in each sample. In view of the fact that the assay described throughout this thesis investigated only the conversion of the lipid substrate PI to PI3P, this was deemed not necessary. Parallel experiments run on dipped and undipped TLC plates showed no differences in the results obtained.

II) Initially the substrate lipid mixture (PI/PS) was sonicated in PI 3-kinase assay buffer. This resulted in the production of large lipid micelle. To produce smaller lipid micelle, lipids were sonicated in (250 mM HEPES). This resulted in the production of a more uniform small lipid micelle solution.

III) The initial experimentation technique by Carter & Downes (1992) described how the primary antibody was incubated with cell lysates for 2 hours, the secondary antibody was added and the resulting solution incubated overnight. Investigations studying the efficiency of the immunoprecipitation procedure were performed. Results indicated that incubation with the secondary antibody for approximately two hours was as efficient as overnight. This was assessed by western blotting the immunoprecipitates and probing with the PI 3-kinase antibody. Densitometry analysis showed no significant variations between the procedures (data not shown).

IV) As the immunoprecipitation stage now consisted of an 90 minute, and a 2-hour incubation, studies were carried out to see if snap freezing the cell lysates (after assessment for total protein) in liquid nitrogen affected PI 3-kinase activity. In parallel assay investigations, this was shown not to be the case.

Once the above alterations were performed, the assay resulted in the production of a radioactive PI3P lipid product. The result however was still the same (Figure 2.3) - addition of mitogens did not cause a large increase in PI 3-kinase activity levels over basal, and the addition of wortmannin significantly lowered the PI 3-kinase activity level below basal levels.

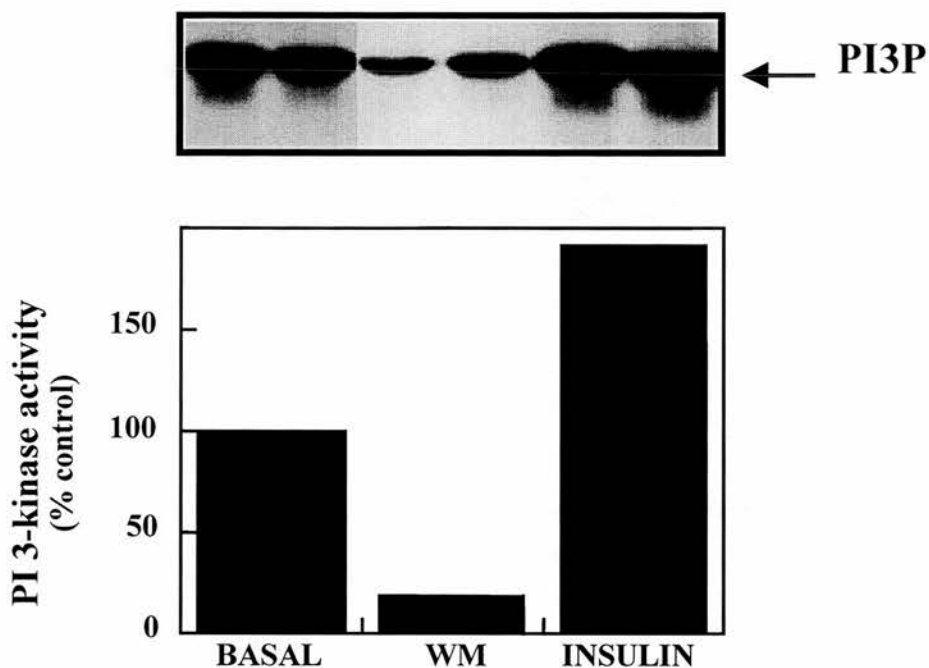


Figure 2.3: Effect of Wortmannin and Insulin on H345 Small Cell Lung Cancer Cell Phosphoinositide 3-kinase Activity.

Cells were pre-incubated with wortmannin (20 minutes) or insulin (5 minutes) prior to cell lysis. Cell lysates were then assayed for PI 3-kinase activity as described in section 2.3.1a. The graph is representative of a single experiment carried out 2 – 4 times out in duplicate, in SCLC cell lines H345, H510 and H69. Basal = control cells that received diluent only. (WM, wortmannin (100 nM) and insulin (1 μ M)).

Above: Representative autoradiograph showing the 3'-phosphorylated product (PI3P) for each condition in duplicate.

Further experiments were undertaken to study basal PI 3-kinase activity levels in a panel of cell types. Results (Chapter 3) indicated that the high basal PI 3-kinase activity levels observed in SCLC cell lines were not artificial. In a number of parallel experiments SCLC cell lines routinely expressed PI 3-kinase basal activity levels significantly higher than other cell types. All cell types were quiesced prior to assaying for activity and each assay point was equilibrated for total protein concentration. In view of the fact that the PI 3-kinase activity assay procedure was now accepted as working correctly, further investigations were performed, and are discussed further in this thesis.

2.3.1c Clarification Studies.

Throughout the studies, checks were carried out to ensure that the band visualised by autoradiography and subsequently excised from the TLC plate at the end of the assay, was phosphatidylinositol 3-phosphate (PI3P). To identify the 3'-phosphorylated lipids by high pressure liquid chromatography (HPLC) they have to be deacylated. After removal of the fatty acid backbone (deacylation), the molecules (glycerolphosphoinositol phosphates) become water soluble and highly negatively charged. These products were then separated by anion-exchange columns with high salt buffers, using a modified method first described by Clarke and Dawson (1981).

Radioactive bands were excised, 25% methylamine in water:methanol:butanol (4:4:1 v/v/v mixture, 3 ml) was added, and the resulting mixture capped tightly and placed at 53°C for 30 minutes with 30 second vortexing every 10 minutes (Methylamine causes cleavage of the fatty acid backbone.) The tube was spun for 3 minutes at approximately 1,000 x g before being centrifugally dried overnight in a speedivac (R10.10 Jouan) at room temperature. Once dry, water (1 ml) and butanol:40-60 bp petrol ether:ethyl formate (20:4:1 v/v/v, 1.2 ml) were added and the solution vortexed. The top layer (containing transacylated lipids) was discarded. The lower phase was again washed with the butanol:40-60 bp petrol ether:ethyl formate mix (20:4:1 v/v; 1.2 ml). The final lower phase that was obtained contained the cleaved lipid head

group. This solution was separated using a HPLC strong anion exchange (SAX) 5 (5 micron pore size) column with a $(\text{NH}_4)_2\text{HPO}_4$ gradient (Figure 2.4) (1 ml fractions were collected at 1 minute intervals). Authentic tritiated standards were used as markers. The tritiated markers were generated by Dr T.R. Walker, Rayne laboratory, University of Edinburgh from metabolically labelled ^{13}C astrocytes and characterised by Dr I. Batty, Biochemistry Department, University of Dundee.

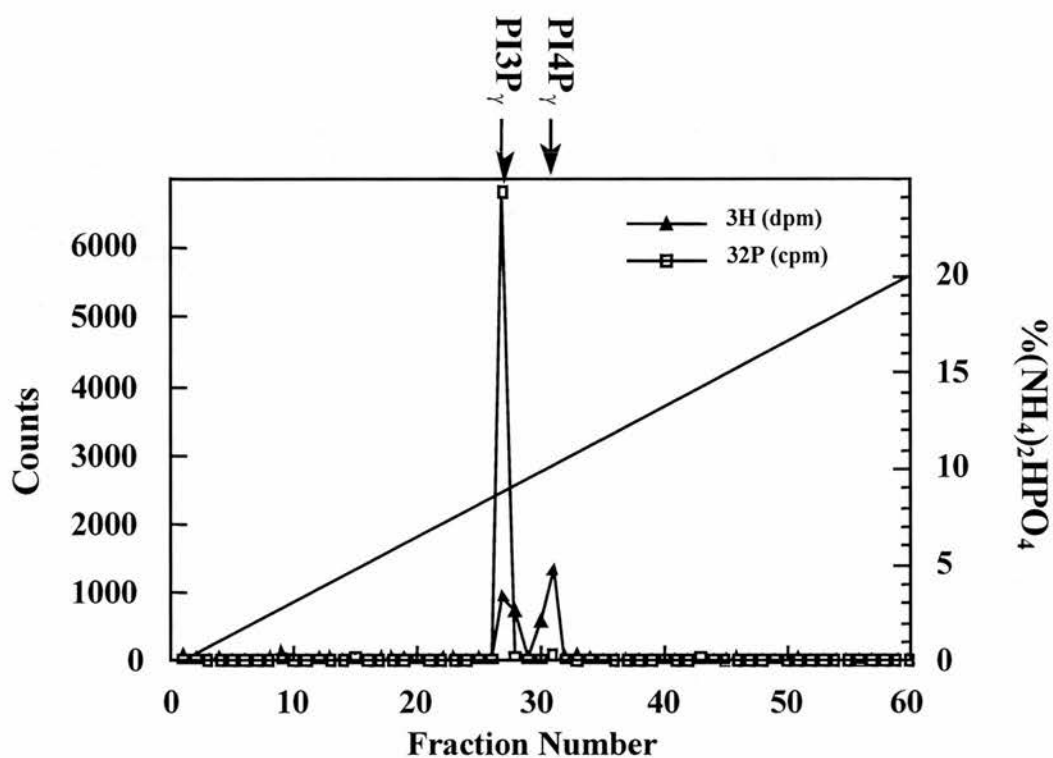


Figure 2.4: Representative HPLC trace of the 3-phosphorylated Lipid products formed during a Phosphoinositide 3-kinase Activity Assay.

Areas of a TLC plate were excised which corresponded to the radioactive sections of the plate. The lipid products were separated from the silicon plate before being deacylated and analysed by HPLC on a $(\text{NH}_4)_2\text{HPO}_4$ gradient (see section 2.3.1c).

2.3.2 Ribosomal Protein S6 Kinase Activity Assay.

p70^{s6k} lysis buffer : Stock Solution : 50 mM Tris/HCl (pH 8.0), 120 mM NaCl, 20 mM NaF, 5 mM EGTA, 1 mM EDTA and 10 mM sodium pyrophosphate (stored at 4°C until required).

Freshly added: 10 mM *p*-nitrophenyl phosphate, 1 mM benzamidine, 0.1 mM PMSF and 1% (v/v) Nonidet P40.

Activation of Pansorbin: Pansorbin (crude extract of protein A from *Staphylococcus aureus*) was aliquoted into 1.5 ml eppendorfs (1 ml/eppendorf) and centrifuged (2,000 x g) for 10 minutes. The supernatant was removed and the pellet resuspended in PBS containing 10% (v/v) β-mercaptoethanol and 3% (v/v) SDS. The solution was vortexed and boiled for 15 minutes before being centrifuged at 2000 x g for a further 10 minutes. The resulting pellet was resuspended in the original volume (1 ml) in p70^{s6k} lysis buffer and stored at -20°C until required.

Washing Buffer : 25 mM HEPES (pH 7.4), 20 mM β-glycerophosphate, 20 mM MgCl₂, 3 mM EGTA, 0.2 mM sodium orthovanadate and 2 mM dithiothreitol. The buffer was made fresh when required.

The p70^{s6k} activity assay performed was modified from a procedure previously described by Scott and co-workers (1996). SCLC cells cultured overnight in quiescent medium were washed in prewarmed PBS before being transferred into 1.5 ml eppendorfs (Starstedt Ltd, Germany). The cells were preincubated at 37°C in a shaking thermomixer for 30 minutes before the addition of various concentrations of wortmannin, LY294002, DMSO vehicle (final concentration 0.001% v/v) (20 minutes) or specific mitogens (see figure legends) (5 or 10 minutes). After incubation, the cells were immediately pelleted (~ 300 x g, 1 min, 4°C), the resulting supernatant removed and the pellet lysed using 400 µl ice-cold lysis buffer for 30 minutes on ice. Lysates were clarified (13000 x g, 10 minutes at 4°C) and equal quantities of the protein equilibrated supernatants (approximately 0.4 mg/assay point),

were incubated with 2 μg polyclonal anti-p70^{s6k} antibody at 4°C on a shaking platform. After 90 minutes the lysates were further incubated with 25 μl of activated pansorbin.

After 60 minutes incubation in the presence of pansorbin (4°C), the immunoprecipitates were washed twice in lysis buffer and twice in the same buffer without detergent. This was followed by two washes in washing buffer prior to the pellets being incubated in washing buffer containing 100 μM S40 substrate peptide, 10 μM adenosine 3' 5'-cyclic monophosphate-dependent protein kinase inhibitor and [³²P] γ -ATP (10 μM ; 1 $\mu\text{Ci}/\text{condition}$, 3000 Ci/mmol) in a final volume of 30 μl . (The S40 peptide substrate was comprised of a 9 amino acid sequence (KKRNRTLTK) that is specifically recognised by the p70^{s6k} and not the related p90^{rsk} protein). The *in vitro* kinase assay was performed over 20 minutes at 30°C in a shaking thermomixer. The reaction was terminated by the addition of 10 μl of 300 mM phosphoric acid before 30 μl of the resulting mixture was spotted onto P81 chromatography paper (3 x 3 cm squares) (Whatmann). The papers were washed twice with 0.5% (v/v) phosphoric acid and then allowed to dry and being transferred into liquid scintillation vials and 3 ml of liquid scintillation fluid added (Flo-Scint IV (Packard)). Radioactivity incorporation was assessed using a Packard 1900 TR liquid scintillation analyser. The phosphorylation of the S40 substrate peptide was quantified by liquid scintillation counting as an index of enzymatic activity. Results are expressed as % change in enzyme activity in comparison to untreated cells.

2.4 Assessment of Small Cell Lung Cancer Cell Growth.

Two methods were utilised under liquid culture conditions to assess SCLC cell growth:

[³H]-thymidine incorporation provides an estimate of the relative number of cells that are undergoing DNA synthesis in comparison to other cells incubated under a variety of different conditions. The cells are known to incorporate the thymidine during the S phase of the cell cycle. Therefore you are looking at changes in the relative number of cells that are undergoing cell division within the time frame of the experiment.

The liquid growth assay is a more indirect method used to assess cell proliferation. In this type of experiment you are assessing the total number of cells present in your culture. This method therefore takes into account not only the increase in cell number due to cell division, but also the decrease in cell number due to cell death via apoptosis/necrosis. Additionally, a small percentage of the cells counted may be undergoing apoptosis whilst still being intact.

2.4.1 [³H]-Thymidine Incorporation.

SCLC cells 3-5 days post passage, were cultured in serum-free SITA medium for two days before being centrifuged at 225 x g for 3 minutes, washed and resuspended in SITA medium. Cells were then disaggregated into an essentially single cell suspension by two passes through a 21 gauge needle. Viability was judged by trypan blue exclusion to be > 95%. Cell number was determined using a Coulter cell counter (Coulter electronics, UK.) before the cells (5×10^4 /ml) were aliquoted into round-bottomed 96 well plates (Gibco BRL, UK.) (200 μ l/well). Plates were incubated for approximately 4 hours in a humidified atmosphere of 5% CO₂/ 95% air at 37°C prior to the addition of inhibitors (see appropriate figure legends). Cells were then

incubated under the same conditions for 48 or 72 hours prior to the addition of [³H]-thymidine (20 – 30 Ci/mmol, 0.1 µCi/well). Cells were incubated in the presence of [³H]-thymidine for a further 24 hours. Finally the cells were harvested onto GF-B glass fibre filters using a TOMTEC cell harvester before [³H]-thymidine incorporation was measured using a Wallac (1205) beta plate counter. All results are expressed as % inhibition of [³H]-thymidine incorporation with respect to control cells (treated with diluent only).

When assaying for [³H]-thymidine incorporation in the presence of the ECM protein collagen IV, a slightly altered procedure was used. Flat bottomed 96 well plates (Gibco BRL, UK) were coated overnight at 4°C with various concentrations of collagen IV (50 µl/well). The next day the wells were washed with sterile PBS to remove excess collagen IV. SCLC cells were then added to the wells and incubated as described above. Prior to [³H]-thymidine incorporation assessment, plates were shaken using a benchtop vortex fitted with a plate holder adaptor head to loosen the adherent cells. Cells were further loosened using a yellow tip (2 passes). Finally, [³H]-Thymidine incorporation was assessed as described above. At the end of the assay procedure, each plate was studied by light microscopy to ensure the majority of cells had been removed.

2.4.2 Liquid Growth Assay.

Cultures of SCLC cells at five days post-passage, were grown for two days in SITA medium. Prior to experimentation, cells were centrifuged at 225 x g (4 minutes) and washed twice in SITA medium before being resuspended in fresh SITA. Cells were gently disaggregated by two passes through a 21 gauge needle into an essentially single cell suspension as judged by light microscopy. Viability was determined by trypan blue exclusion on a haemocytometer and judged to be > 95%. Cells (1 x 10⁵/ml) were seeded into 24 well plates in SITA medium and incubated for 4 hours in a humidified atmosphere of 5% CO₂/ 95% air at 37°C prior to the addition of various inhibitors (see appropriate figure legends). Cells were further incubated under the

same conditions before being removed at various times, disaggregated into a single cell suspension using a 21 gauge needle (two passes) (modified from (Carney et al., 1980)) and cell number determined using a Coulter Cell Counter. Results are expressed as % inhibition of cell number with respect to control cells (treated with diluent only), or as fold increase in cell number with respect to initial number of cells plated..

2.4.3 Clonogenic Assay.

Previous work by Freedman and Shin (1974) has demonstrated that the ability of SCLC cells to form colonies in semi-solid media directly correlates to the tumourigenicity of the cancer *in vivo*. This assay not only assesses the cells' ability to proliferate, but also to survive within a 3-dimensional environment in a single cell suspension.

SCLC cells 3-5 days post passage, were centrifuged at 225 x g for 4 minutes, washed and resuspended in SITA medium. Cells were then disaggregated into a single cell suspension by two passes through a 21 gauge needle. Viability was judged by trypan blue exclusion to be greater than 95%. Cell number was determined using a Coulter cell counter, before 1×10^4 cells (per ml) were mixed with SITA medium containing 0.3% (w/v) agarose and the specific agonist/inhibitor at the concentrations indicated (see relevant figure legends). This was layered over a solid base of 0.5% (w/v) agarose in SITA medium containing the same concentration of the relevant agonist/inhibitor, in 35 mm plastic dishes (CorningCostar, UK.). The cultures were incubated in a humidified atmosphere of 5% CO₂/ 95% air at 37°C for 21 days prior to being stained with the vital stain nitroblue tetrazolium. Colonies of >120 µm diameter (16 cells) were counted by light microscopy (x40).

2.5 Assessment of Small Cell Lung Cancer Cell Apoptosis.

2.5.1 Acridine Orange, Ethidium Bromide Staining.

Washed SCLC cells ($2 \times 10^5/\text{ml}$) were seeded into 96 well plates in (200 μl) and incubated in a number of different culture mediums for a defined time. A mixture of ethidium bromide (100 $\mu\text{g}/\text{ml}$) and acridine orange (100 $\mu\text{g}/\text{ml}$) (1:1 v/v; 1 μl) diluted in PBS (pH 7.2) was added to each well. The percentage of cells undergoing apoptosis was then assessed using fluorescent microscopy (x 40) (BH2 Olympus) as described previously (Cotter & Martin, 1996).

Table 2.3: Assessment Guidelines for Calculation of Cell Apoptosis Levels Using Acridine Orange and Ethidium Bromide Staining.

Cell Stage	
Viable Cells	Bright green nucleus with intact structure.
Early Apoptotic	Bright green nucleus showing condensation of chromatin as dense areas in the nucleus
Late Apoptotic	Orange nucleus showing condensation of chromatin as dense orange areas.
Necrotic	Orange nucleus with intact structure

Information from Cotter & Martin, 1996.

Essentially acridine orange and ethidium bromide excite green and orange fluorescence bands respectively. Acridine orange is able to penetrate through an intact plasma membrane therefore healthy cells appear green. When both acridine orange and ethidium bromide enter the cell (late apoptotic and necrotic cells) the ethidium bromide fluoresces stronger and the cells appear orange.

2.5.2 ELISA Cell Death Detection.

SCLC cells were washed and quiesced for 24 hours prior to gentle disaggregation into an essentially single cell population by 2 passes through a 21 gauge needle. Approximately 5×10^5 cells/ml were plated out into 48 well plates (500 μ l/well). Cells were incubated at 5% CO₂/ 95% air, 37°C for 2 - 3 hours before addition of the inhibitors (see relevant figure legends). After 24 hours cells (150 μ l) were removed for cytocentrifugation (see following method), and from the remaining cells 200 μ l were removed and assessed for apoptosis using a Cell Death Detection ELISA plus kit (Boehringer and Mannheim, Germany).

The assay is a photometric enzyme-immunoassay which detects the presence of cytoplasmic histone-associated-DNA-fragments which are produced during programmed cell death (apoptosis). Essentially, the cells were washed and lysed before the lysates were transferred into the streptavidin-coated ELISA plates provided. A mixture of anti-histone (biotinylated) and anti-DNA (peroxidase-conjugated) mouse monoclonal antibodies were added to the lysates and incubated over 2 hours. During this period the anti-histone antibodies associated with the histone components of the nucleosomes. These in turn became attached to the streptavidin-coated plates (via its biotinylation). The anti-DNA antibodies then reacted and associated with the DNA components of the nucleosomes. After removal of any unbound antibody, the amount of nucleosomes bound to the plate was quantified and determined photometrically using the substrate supplied (ABTS^R). Results are expressed as the increase in absorption (405 nm) of treated cells in comparison to untreated cells (positive and negative controls are also provided with the kit).

2.5.3 Morphological Assessment by Cytocentrifugation.

Cells that were treated with various inhibitors over 24 hours (see section 2.5.2) were harvested from each well (150 μ l approximately 7.5×10^4) and cytocentrifuged (300 x g, 3 min) (Shandon, Cytospin 3) on to glass slides. The resulting slide preparations

were air dried, fixed in methanol (5 minutes) and allowed to air dry again before being stained with May-Grunwald-Giemsa stain. Covers slips were added to each slide using DPX. Cell morphology was examined by objective light microscopy (x40), and apoptotic SCLC cells defined as smaller cells containing more a darkly stained condensed nuclei. For each condition examined, slides were prepared from triplicate incubations and a total of at least 500 SCLC cells were counted over a minimum of five random fields per slide. Figure 2.5 shows two representative SCLC cytopsins.

SCLC cells are mitotic cells (i.e. are continually dividing). One of the characteristics of SCLC cells when spun for cytological examination is that mitosis is particularly noticeable. Concerns are often expressed as to the visual differences between apoptotic and dividing cells. During the dividing procedure the cells do condense but the chromatin structure is more noticeable. The nuclei of dividing cells do not stain as darkly as apoptotic nuclei, and often more cytoplasm is noticeable around the nucleus.

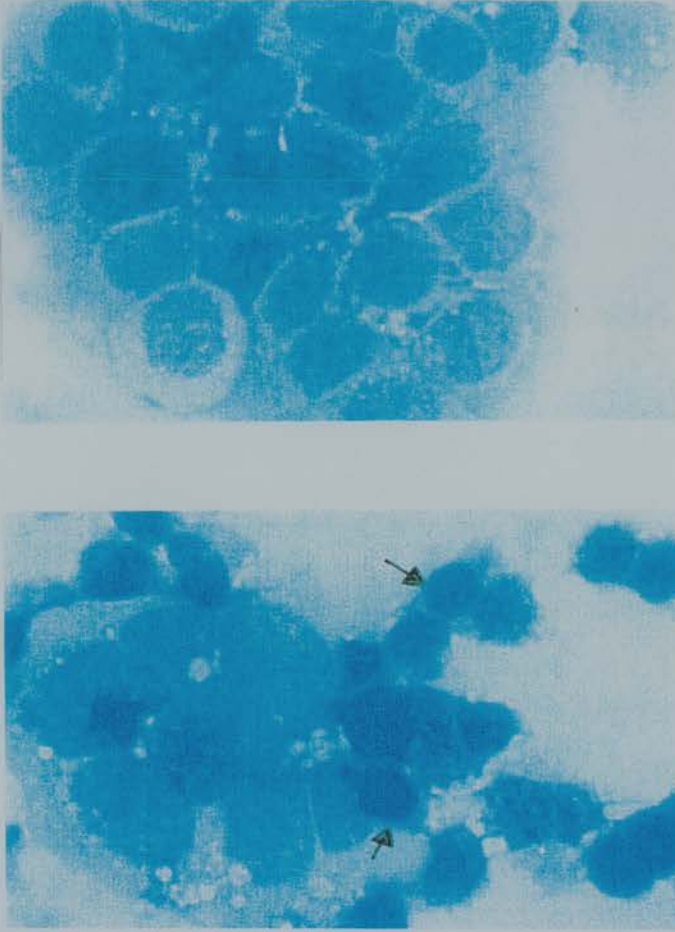


Figure 2.5: Representative Cytocentrifugation Slides which were used to assess Small Cell Lung Cancer Cell Apoptosis levels.

Cells were assayed and cytocentrifuged (as described in material and methods section 2.5.3). The photographs above show representative SCLC cytopsins.

UPPER: A cytopsin of control SCLC cells incubated over 48 hours in quiescent medium.

LOWER: A cytopsin of SCLC cells treated with tyrphostin-25 (25 μ M) over 48 hours prior to assessment for apoptosis.

NOTE the apoptotic SCLC cells are more condensed and darkly stained (\rightarrow).

2.6 Immunoblotting.

Cell lysates were assessed using western blotting techniques to identify the presence of a variety of different proteins throughout this thesis. Cell pellets were lysed on ice in PI 3-kinase lysis buffer (50 μ l) (section 2.3.1a) for 30 minutes before the protein concentration of each cell lysate was measured using a Pierce BCA protein assay (Section 2.7). Once equilibrated for total protein, the whole cell lysates were separated by SDS-PAGE.

2 x Sodium dodecyl sulfate (SDS)-PAGE Sample Buffer : 250 mM Tris HCl (pH 6.8), 4% SDS (v/v), 10% glycerol (v/v), 0.006% bromophenol blue (v/v) and 2% β -mercaptoethanol (v/v).

10% SDS Polyacrylamide Gels :

Resolving gel preparation: acrylamide solution (30%) (29% : 1% acrylamide : N',N'-methylenebisacrylamide in dH₂O) (8.33 ml), separating gel buffer (1.5 M Tris base (pH 8.8), 10% SDS) (6.25 ml), dH₂O (10.38 ml), 25% ammonium persulphate (100 μ l) and temed (20 μ l).

Stacking gel preparation: acrylamide solution (30%) (1.5 ml), stacking gel buffer (1.0 M Tris base (pH 6.8), 10% SDS) (2.5 ml), dH₂O (5.7 ml), 25% ammonium persulphate (50 μ l) and temed (10 μ l).

Gel Electrophoresis Buffer: 25 mM Tris base (pH 8.3), 250 mM glycine, 0.1% SDS.

Blotting Buffer: 210 mM glycine, 24.7 mM Tris base and 20% methanol in dH₂O.

Washing Buffer: (Tris buffered saline (TBS)). 20 mM Tris/HCl (pH 7.4), 150 mM NaCl and 0.2 % (v/v) Tween 20.

When immunoblotting whole cell lysates, 20 µg of total protein was solubilised in an equal volume of 2x SDS-PAGE sample buffer at 95°C for 3 minutes. Samples were cooled, any undissolved material was pelleted (13,000 x g, 1 min) before equal amounts of protein (20 µl) were resolved on 10% SDS polyacrylamide gels using a tris-glycine gel electrophoresis buffer (Atto Electrophoresis gel apparatus, 80 - 120 volts, 3 - 4 hours at room temperature (or until broad range molecular weight markers (Sigma, UK.) leading front reached the base of the gel)).

The separated proteins were transferred on to nitrocellulose membranes (HybondC, Amersham UK.) in blotting buffer (Biorad Mini Protean II apparatus, 100 volts, 1 hour at 4°C). The resulting filters were blocked using 5% (w/v) non-fat milk in washing buffer overnight at 4°C to prevent any antibodies binding to the nitrocellulose membranes in a non-specific manner. Blots were then incubated with primary antibody diluted in blocking buffer (washing buffer containing 5% (w/v) non-fat milk) for 1 hour at room temperature on a rocking platform. Between antibody incubations, filters were washed in washing buffer (3 x 10 minutes) to ensure thorough removal of any unbound antibody. After washing, the secondary antibody (appropriate HRP-conjugated antibody) was incubated with the nitrocellulose membrane for approximately 1 hour at room temperature on a rocking platform. Both the primary and secondary antibodies were normally used at a 1:1000 in blocking buffer (see figure legends for exact dilutions). Finally, the immunoreactive bands were identified using enhanced chemiluminescence (ECL) (Amersham, UK.). ECL is a high resolution, non-radioactive method of detecting specific immobilised antigens that are conjugated (indirectly or directly), to horseradish-peroxidase labelled antibodies.

To ensure that equal protein loading of gels occurred, duplicate gels were run and stained for protein when blotting protein samples. Figure 2.6 represents an example of a gel run and analysed for total protein. The protein bands were stained with comassie blue (consisting of 0.25% (w/v) comassie blue in destain solution) for 1 hour on a shaking platform. Excess stain was then removed from the gel by sequential

washes of the gel in destain (40% (v/v) methanol, 10% (v/v) acetic acid in dH₂O) and the gel dried overnight under vacuum.

2.7 Protein Concentration Assessment.

The assay kit was purchased from Pierce, IL, USA. Essentially the protein present within the test solution causes the reduction of copper²⁺ - copper¹⁺ in an alkaline medium. The copper¹⁺ is then detected colourmetrically (purple) by bicinchoninic acid (BCA) (Smith et al., 1985). The colour is produced by the chelation of BCA to copper¹⁺ (2 : 1 ratio) and the protein concentration is assessed using a spectrometer at 562 nm. The assay is able to measure protein concentrations accurately between 20 µg/ml and 2 mg/ml. The protein concentrations in each sample are calculated in comparison to BSA standards made in the lysis buffer in which the samples to be tested were lysed. The assays were carried out by incubating the mixtures at 37°C for 30 minutes before allowing the plates to cool to room temperature and reading the results on an automated plate reader (Dynatech, MR5000, Dynatech UK.). Each assay point was read in triplicate and the average absorbance calculated.

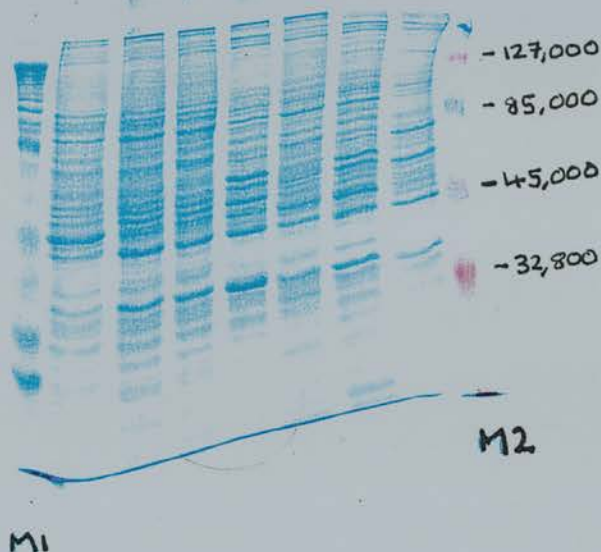


Figure 2.6: Representative SDS-polyacrylamide Gel Stained to Visualise all the Protein Bands present in Whole Cell Lysates of a Number of Different Cell Types.

A representative dried SDS-polyacrylamide (10%) gel stained with coomassie blue (0.25% w/v) (section 2.6.1). Left - Right: Broad range molecular weight markers (M1), H510, H345, H69, A549, Swiss 3T3, PC12, BTSM, kaleidoscope molecular weight markers (M2). Abbreviations BTSM - bovine trachea smooth muscle cells.

2.8 Tissue Immunohistochemistry.

Small cell cancer lung resection specimens were collected from pathology files (1989-1994) of the Department of Pathology, University of Edinburgh. Representative sections were cut from formalin fixed and paraffin embedded blocks, dewaxed in xylene and rehydrated through graded alcohols to water. Antigen unmasking was performed by either pre-incubating sections in 0.1% trypsin (pH 7.6) at 37°C for 15 minutes (fibronectin, laminin and tenascin) or microwave-oven boiling for 15 minutes in 0.1 M citrate (pH 6.0) (collagen IV).

ECM expression was demonstrated using antibodies against fibronectin (1:100), laminin (1:100, LAM-89) tenascin (1:100, BC-24) and collagen IV (1:100, Col-94). All were purchased from Sigma UK. and were mouse monoclonal antibodies with the exception of fibronectin (rabbit polyclonal). Sections were processed (by Dr R.C. Rintoul) using a Techmate 500 (Dako, UK.). Antibody detection was performed using a multilink streptavidin biotin complex method and visualisation achieved using a di-amino benzidine chromagen method as per manufacturer's instructions (Dako, UK.). The slides were counterstained using Harris' haematoxylin, dehydrated through graded alcohols and mounted in xylene. Negative controls (sections processed as above except the primary antibody was omitted) were incubated each time the procedure was performed. Sections were used as their own positive controls by ensuring that areas of each section that would normally be positive for each ECM (e.g. blood vessel walls) were indeed positive. All slides were reviewed independently by two people. The staining was graded on a semi-quantitative scale as no staining, focal staining or extensive (> 50% of section area) staining.

2.9 Adhesion Assay.

Laminin (Ln), fibronectin (Fn) (Gibco BRL, UK.) and collagen IV (C IV) (Sigma, UK.) were used as substrates in the serum-free adhesion assays. All ECM proteins were aliquoted and stored as per manufacturers instructions (-80°C). Fibronectin is known to be very unstable, so when the protein was required it was flash thawed at 37°C prior to use. (This was to prevent the protein from immunoprecipitating out of solution.)

Cells (5×10^5 /ml) 3-5 days post passage were washed twice in RPMI and disaggregated into essentially a single cell population (by two passes through a 21-gauge needle) in quiescent medium. Tissue culture plates (96 well) were previously incubated overnight with different ECM proteins (laminin, 10 µg/ml, fibronectin, 20 µg/ml and collagen IV, 10 µg/ml) and poly-L-lysine (25 µg/ml) (50 µl/well). The following day, the plates were washed with sterile PBS to remove any unbound proteins before being blocked with 1 mg/ml BSA (1 - 2 hrs at 37°C). After washing the plates with sterile PBS, the SCLC cells (100 µl) were added to 96 well tissue culture plates (CorningCostar UK.) and were allowed to attach for 45 mins at 37°C. The non-adherent cells were then gently removed by aspirating off the media and gently washing the wells with PBS. The remaining cells were fixed with 3% formaldehyde for 1 hr at room temperature prior to centrifugation (225 x g, 5 mins). The formaldehyde was removed and the attached cells were stained with 1% methylene-blue. After 5 minutes, the wells were washed with distilled water to remove any excess dye. 0.1N HCl (100 µl) was added/well to release the internal dye, and the resulting solution read at 630 nm on an automated plate reader (Dynatech, MR5000, Dynatech UK.). The attachment of SCLC cells to wells coated with 25 µg/ml poly-L lysine were designated as 100% adhesion. (Poly-L-lysine causes enhanced non-specific charge-related adhesion.)

Note: - In experiments when inhibitors were added, see figure legends for concentrations and the time the cells were exposed to inhibitors prior to the adhesion assay being performed. Times varied due to the efficiency of the specific inhibitor at entering the cell and inhibiting the enzyme in question.

2.10 Confocal Microscopy.

Glass-cover slips were coated with specific ECM proteins or poly-L-lysine (10 µg/ml) overnight at 4°C. This was carried out by covering a large petri dish in clingfilm. Approximately 50 µl (depending upon size of coverslip) of the coating matrix solution was added to the clingfilm to form a small 'mound' of matrix solution. Coverslips were gently placed on the top of the solutions enabling the lower face of the coverslip to be in contact with the matrix solution. After overnight incubation at 4°C, the coverslips were removed and placed in 12 or 24 well culture plates with the matrix covered face of the coverslip facing upwards. The coverslips were washed in SITA to remove any unbound ECM proteins prior to the addition of SCLC cells to the wells.

Cells were gently disaggregated by passing twice through a 21-gauge needle. The essentially single cell population of SCLC cells (in SITA) was allowed to attach to the matrix coated coverslips for 45 mins in a humidified atmosphere of 5% CO₂/95% air, at 37°C. Unattached cells were gently removed, fresh SITA added (1 ml /well) and the attached cells were allowed to adhere for a defined time. Finally, the SITA medium was removed and the cells were fixed (3% para-formaldehyde in PBS) for 20 mins, washed before being quenched (50 mM NH₄Cl/PBS) for 10 mins to mop up any excess formaldehyde present. Cells were permeabilised using 0.1% TX-100 (v/v) in calcium and magnesium free (CMF)-PBS for 4 mins. Permeabilised cells were blocked using 0.2% (w/v) fish skin gelatin/CMF-PBS (3 x 5 mins) before being stained with rhodamine phalloidin (1 : 400 dilution, Molecular Probes Europe BV)

for 20 mins in the dark at room temperature for actin filament studies. (All the above procedures were carried out in culture dishes.)

For the studies investigating the expression of tyrosine phosphorylated proteins (anti-phosphotyrosine antibody 4G10, 1 : 150 dilution), the coverslips were inverted on clingfilm on top of the primary antibody (diluted in blocking buffer) for 30 mins - 1 hour at room temperature (50 µl/coverslip). This was followed by three consecutive washes of 5 minutes with blocking buffer in culture plates. The slides were finally incubated inverted on clingfilm again with the secondary antibody (anti-mouse FITC, 1 : 25 dilution in blocking buffer) for 30 mins - 1 hour before being washed in sterile PBS and air dried.

At the end of either staining procedure, the coverslips were mounted onto glass slides (matrix and cell covered face facing the glass slide) using DPX. Immunofluorescence was scanned using a BioRad MRC 600 laser confocal microscope with a Zeiss 63X objective or by fluorescence microscopy using a Olympus BH2 microscope.

Note: When cells were incubated in the presence of specific inhibitors, before being allowed to adhere to the matrix coated coverslips, they were incubated in sterile tissue culture flasks (5 ml cells/ flask) in the presence of the inhibitor for a defined time (see appropriate figure legends) in a humidified atmosphere of 5% CO₂/95% air, at 37°C. Cells were then washed in fresh SITA (containing the inhibitor), before being gently disaggregated into an essentially single cell population. Cells were finally allowed to adhere to the matrix coated coverslips (in media still containing the inhibitor). Once the non-adhered cells were removed fresh SITA (containing the inhibitor) was added and the experiment continued as described previously.

2.11 Chemosensitivity Assay.

Cells grown in SITA 3-5 days post-passage were washed, resuspended in SITA medium and seeded (5×10^5 cells/200 μ l/well) in 96 well plates in the presence or absence of ECM (laminin or collagen IV 10 μ g/ml and fibronectin 20 μ g/ml, each well coated overnight at 4°C, 50 μ l matrix solution/well). Serial dilutions of the cytotoxic agent were added to each well 1 - 2 hrs after seeding. When tyrphostin-25 (25 μ M) was utilised, it was added along side the cytotoxic agent. After three days in a humidified atmosphere of 5% CO₂/95% air, at 37°C, the cells were assayed for either total cell number or apoptosis levels. When assaying for percentage apoptosis, the cells detached from the plates, aspirated and cytospun onto glass slides, fixed in methanol and stained using May-Grunwald-Geimsa stain (described in section 2.5.3), or spun down directly onto the microtitre plate, fixed and stained for counting and photography. Cell number was calculated by counting the number of cells in 5 random fields of an inverted light microscope at 100X magnification. The total number of cells present in the well was then calculated mathematically as the size of the whole well and each field counted was known.

NOTE: In every experiment each condition was carried out in quadruplicate and in duplicate tissue culture plates.

In the experiments described throughout this thesis, the chemotherapy reagent cyclophosphamide was often utilised. As cyclophosphamide is a pro-drug which has to be metabolised in the liver before it becomes active (section 1.1.4.1), the active form (cis-4-sulfoethylthio-cyclophosphamide) was utilised in the experiments described within this thesis.

2.12 Flow Cytometric Analysis of Integrin Expression.

Monoclonal antibody labelling of intact cells was performed using an indirect immunofluorescence technique. Briefly, cells (1×10^5) were added to a 96-well flexible assay plate and washed in phosphate buffered saline containing 0.2% (w/v) BSA and 0.1% (w/v) sodium azide (EPICS buffer). Cells were then incubated in 50 μ l of saturating concentrations of primary antibodies (1 : 500 dilution) in EPICS buffer, α v (13C2, Dr Mike Horton, St Bartholemew's Hospital), α 2 (AK7), α 3 (11G5), α 4 (HP2/1), α 5 (SAM-1) α 6 (AF-10 (IgG2b)), β 3 (PM6/13), β 2 (MHM23, Dako, UK.) or β 1 (mAb 13 (rat IgG, Becton Dickinson, UK.)). All antibodies were purchased from Serotec UK. unless otherwise stated. Cells were incubated for 30 min on ice, washed twice in EPICS buffer and incubated with 1:25 dilution of FITC conjugated anti-mouse secondary antibody (Dako, UK.) (with the exception of the β 1 antibody where a FITC conjugated anti-rat secondary antibody was used) for 30 min on ice. Cells were washed and resuspended in EPICS buffer and analysed by flow cytometry using an EPIC profile II (Coulter Electronics, UK.). All photomultiplier settings were standardised throughout the study. Measured mean fluorescence intensity (arbitrary units (AU)) was recorded for each sample.

2.13 Materials.

Monoclonal p85-SH3 PI 3-kinase antibody, and the S40 peptide substrate were from TCS (Buckingham, UK.). Polyclonal p70^{s6k} antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Adenosine 3' 5'-cyclic monophosphate-dependent protein kinase inhibitor, tyrosine kinase inhibitors genistein and tyrphostin-25, MAP kinase (MEK 1) inhibitor PD 098059 and LY294002 were from Calbiochem-Novabiochem Corp (Nottingham UK.). Mouse monoclonal phosphotyrosine antibody 4G10 was purchased from Affinity UK. [³²P] γ -ATP (3000 μ Ci/mmol), [6-³H]-

thymidine (20-30 Ci/mmol) and Hybond C nitrocellulose membranes were purchased from Amersham International (Amersham UK.). CIS-4-sulfoethylthio-cyclophosphamide (pro-drug of cyclophosphamide) was purchased from Astra Pharmaceuticals (UK). Cell culture antibiotics (Penicillin and Streptomycin), Trypsin/EDTA (1X) (in Hanks buffered salt solution without calcium and magnesium), 30% acrylamide solution and the extracellular matrix proteins fibronectin and laminin were obtained from Gibco BRL (UK.). Horse radish peroxidase (HRP)-conjugated antibodies were obtained from Dako UK. The May-Grunwald-Giemsa stain (Diff Quick solution: Solution I – Eosin G in phosphate buffer pH 6.0 and solution II – Thiazine blue in phosphate buffer pH 6.0) was purchased from Baxter healthcare Ltd, Scotland, UK..

PKB α , γ , PH domain antibodies were a kind gift from Dr D.R. Alessi and Professor P Cohen, University of Dundee, UK. The integrin function blocking antibody P5D2 was kindly given to us by Dr. P. Hughes, Scripps Research Institute, La Jolla, USA.

All reagents not specified along with RPMI 1640, BSA agarose, IgG protein agarose, foetal calf serum, collagen IV, chemotherapy reagents, cytochalasin D, rapamycin and wortmannin were purchased from Sigma (Poole, UK.). All reagents were of the purest grade available.

2.14 Statistical Analysis and Pharmacological Definitions.

All statistical analysis carried out within this thesis used the InStat computer package (version 3.0). One way analysis of variance (ANOVA) was performed followed by the Student-Newman-Keuls multiple correlation post test. (ANOVA compares the means of 3 or more groups of data. Student-Newman-Keuls test compares the mean of each set of data with all the others.)

All IC_{50} concentrations and curve fits were calculated using the Macintosh Curve Fit Package – Kaleidograph version 2.3.1.

Definitions of pharmacological abbreviations used in this thesis:

IC_{50} - Where a compound causes an inhibitory response, the IC_{50} is the molar concentration which produces 50% of its maximum possible inhibition.

EC_{50} - The molar concentration of an agonist which produces 50% of the maximum possible response for that agonist.

CHAPTER 3

Phosphoinositide 3-kinase Activity is Elevated in Small Cell Lung Cancer Cells and Regulates Ribosomal Protein S6 Kinase Activity *In Vitro*.

3.1 Introduction.

The majority of cancer patients die as a result of tumour invasion and secondary metastatic deposits. Thus, the ability of cancer cells to grow in an inappropriate environment is central to cancer-related death. The actual mechanisms of how SCLC cells overcome anchorage-dependence and metastasise are unclear.

Research to date has revealed that a large number of neuropeptides are secreted by SCLC cells (reviewed Moody & Cuttitta, 1993), and that these neuropeptides promote SCLC clonal growth (Carney et al., 1987, Sethi & Rozengurt, 1992). This ability to grow in the absence of exogenously provided mitogens, via autocrine growth loops, enables SCLC cells to become serum-independent and survive in an inappropriate environment (i.e. whilst metastasising).

Studies performed in different cell types have shown that overexpression of focal adhesion kinase (FAK), *ras* and *src* can lead to anchorage-independence, serum-independence and cell transformation (Frisch et al., 1996a, Ihle & Kerr, 1995, Frisch & Francis, 1994, Khwaja et al., 1997). Results showed that after constitutive activation of *ras*, MDCK cells were able to survive when detached from tissue culture plates (Khwaja et al., 1997). Detachment-induced apoptosis was also

abrogated in epithelial cells when they were transformed with either *v-Ha-ras* or *v-src* (Frisch & Francis, 1994). However, in SCLC cells, none of these proteins have been shown to be either overexpressed or constitutively active (Tallett et al., 1996, Suzuki et al., 1990, Budde et al., 1994). Therefore, other proteins, yet to be identified, must regulate SCLC cell anchorage-independence and aid in SCLC cell transformation.

Previous studies have shown that activation of the lipid/tyrosine kinase phosphoinositide 3-kinase (PI 3-kinase) (reviewed section 1.6) is required to enable many processes involved in tumourigenesis to occur. These include cell proliferation, inhibition of apoptosis and anoikis, and invasion (Yao & Cooper, 1995, Shaw et al., 1997, Keely et al., 1997, Khwaja et al., 1997). Shaw and co-workers noted that activated PI 3-kinase was required to enable breast carcinoma cells to become invasive. Additionally, Keely and co-workers showed that PI 3-kinase activation was required to enable transformed breast epithelial cells to become motile and invasive. Finally, Khwaja and co-workers (1997) showed that adhesion to ECM proteins caused an increase in PI 3-kinase activation levels in MDCK cells. Detachment of these cells led to anoikis. However, in genetic mutation studies, constitutively activated PI 3-kinase could prevent anoikis from occurring via a PKB-dependent mechanism.

Thus, in response to the above observations, we went on to examine the potential role of PI 3-kinase in regulating different cellular functions in SCLC cells. The aim of the work presented in this chapter was to investigate whether the expression and activation of PI 3-kinase was significantly altered in any way in comparison to different cell types, and to identify key downstream targets of PI 3-kinase in SCLC cells.

3.2 Results.

3.2.1 Expression of Phosphoinositide 3-kinase in Small Cell Lung Cancer Cells.

The expression of the p85 α subunit of PI 3-kinase was examined in a number of different cell types. Swiss 3T3 cells are often used as model cells to investigate the mechanisms behind SCLC cell proliferation and survival, and thus provided a comparable cell system in which to examine the relative expression of PI 3-kinase. A549 cells are derived from a different lung cancer type (adenocarcinoma) and were used to examine the possibility that differences observed might relate to more than one type of lung cancer. Rat pheochromocytoma cells (PC12) cells were tested along side SCLC cells, as they are of neuroendocrine origin. SCLC cells are known to exhibit a large number of neuroendocrine markers.

Using western blotting techniques, the presence of the p85 α PI 3-kinase subunit in SCLC cells was demonstrated (Figure 3.1). In comparison to Swiss 3T3 cells, A549 cells and PC12 cells, high levels of p85 α PI 3-kinase were observed under normal culture conditions in SCLC cell lines H69 and H345. Slightly lower levels of PI 3-kinase expression were seen in the SCLC cell line H510.

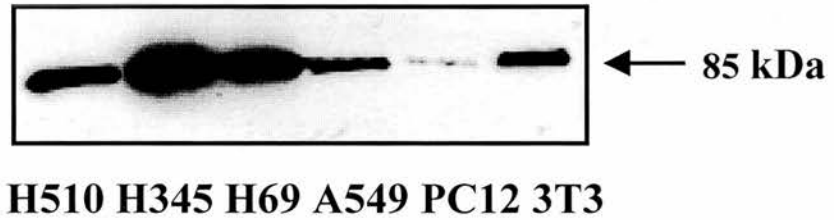


Figure 3.1: Western Blot of the p85 α Protein Subunit of Phosphoinositide 3-kinase.

Cells were grown 3 – 5 days post passage prior to being lysed in PI 3-kinase lysis buffer. Lysates were equilibrated for protein, separated by SDS-PAGE and probed with a p85-SH3 PI 3-kinase mouse monoclonal antibody (1:1000 dilution). The blot represents one experiment carried out in duplicate (n = 4).

Abbreviations: H69, H345, H510 – SCLC cell lines, A549, adenocarcinoma cell line, PC12, rat pheochromocytoma cell line, 3T3, Swiss 3T3 mouse fibroblasts.

3.2.2 Activity Levels of Phosphoinositide 3-kinase in Small Cell Lung Cancer Cell Lines under Different Culture Conditions.

Figure 3.1 demonstrates the relatively high expression levels of p85 α PI 3-kinase in SCLC cells, but does not provide any information on its activity status. The ability of PI 3-kinase to be activated in response to the addition of various neuropeptides or growth factors was examined in the SCLC cells. Quiesced/ basal SCLC cells were classified as cells grown overnight in quiescent (Q) medium (section 2.1), before being washed and incubated in warmed PBS prior to experimentation. This procedure was performed to remove any exogenous or autocrine growth factors which may stimulate the cells, as it has been proposed that SCLC cells proliferate by autocrine/paracrine growth factor and neuropeptide stimulation. Given that addition of growth factors which have previously been shown to stimulate p85 α /p110 PI 3-kinase activity in other cell types should theoretically result in the activation of PI 3-kinase in SCLC cells, foetal calf serum was initially used as it contains a variety of mitogenic factors known to stimulate PI 3-kinase including LPA, NGF and IGF-1 (Zhang et al., 1995, Vemuri & Rittenhouse, 1994, Soltoff et al., 1996, Carter & Downest, 1996, Kotani et al., 1994). However, PI 3-kinase activity levels could only be increased by a modest amount in H69 ($51 \pm 19.4\%$ (mean \pm S.D., $n = 2$)) and H510 ($82.9 \pm 22\%$ (mean \pm SEM, $n = 4$)) SCLC cell lines in comparison to quiesced SCLC cells upon the addition of 10% (v/v) foetal calf serum over 10 minutes.

The neuropeptide insulin has been shown to be important in the proliferative properties of many SCLC cell lines (Bepler et al., 1988, Nakanishi, 1988). Addition of insulin (1 μ M) for 5 minutes was unable to induce a large increase in the activation of PI 3-kinase. A modest increase in PI 3-kinase activity was observed in all three SCLC cell lines including H345 cells (e.g. average PI 3-kinase activity per mg of total protein; Basal = 12080 cpm and insulin (1 μ M) = 17680 cpm (corresponds to a 46% increase in PI 3-kinase activity above control values)).

In view of the above results, further studies were performed under basal conditions to establish that SCLC PI 3-kinase activity levels were elevated. SCLC cells were grown in either quiescent growth medium (devoid of all growth factors and neuropeptides) or in serum-free SITA medium (supplied with a number of known key proliferation stimulators of SCLC cells). Under these conditions, similar levels of PI 3-kinase activity were seen (Figure 3.2). The activity levels of PI 3-kinase per mg of total protein were SITA 55140 ± 3143 cpm, quiescent 49685 ± 4173 cpm and 10% (v/v) FCS 83405 ± 16181 cpm (mean \pm SD, n=2 in duplicate) for H69 SCLC cells.

Further investigations have examined the basal levels of PI 3-kinase activity in a larger number of SCLC cell lines (H69, H345, H510, DMS79 and LS274) (Moore et al., 1998). Elevated basal PI 3-kinase activity levels were observed in all the SCLC cell lines examined in comparison to a number of different cell types including Swiss 3T3 fibroblasts and A549 cells (Figure 3.3). All cells were quiesced and washed in PBS prior to experimentation to remove all exogenous mitogens.

Thus, under strict conditions where every effort was made to remove the presence of all exogenous/autocrine growth factors and neuropeptides (by extensive washing and the incubation of SCLC cells in PBS), high basal PI 3-kinase activity levels were observed in SCLC cells only. These results suggested that the elevation in PI 3-kinase activity was not caused by the external stimulation of growth factor/G-protein coupled receptors in SCLC cells. This was corroborated by the observation that the PI 3-kinase activity levels of SCLC cells incubated in either quiescent or serum-free SITA medium were not statistically different. However, elevated PI 3-kinase basal activity levels could be increased further upon the addition of 10% (v/v) FCS (approximately 50% increase).

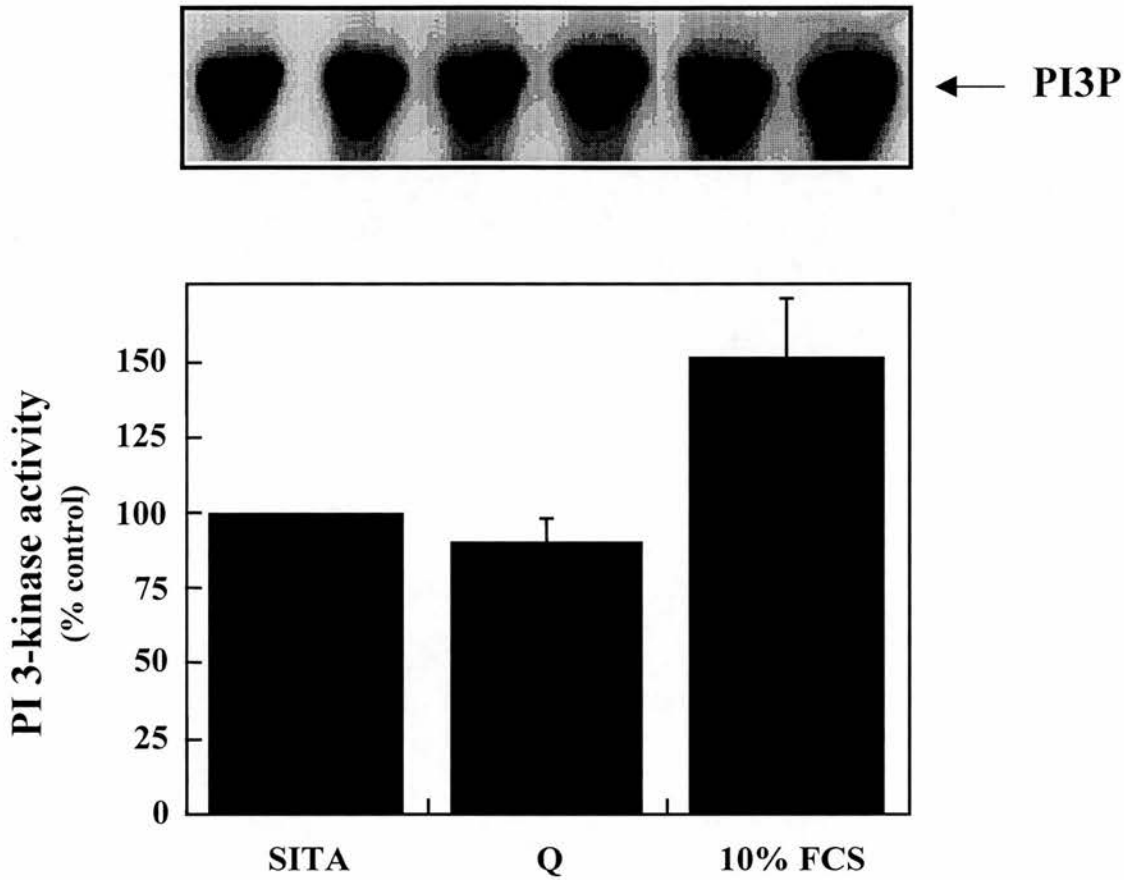


Figure 3.2: Changes in Phosphoinositide 3-kinase Activity levels in H69 Small Cell Lung Cancer Cells under Different Culture Conditions.

Cells were washed and cultured in either serum-free SITA or quiescent medium (Q) overnight prior to being re-washed, plated in warmed PBS and assayed for PI 3-kinase activity. 10% FCS indicates cells were cultured in Q medium and then stimulated with 10% (v/v) FCS for 10 minutes prior to lysis. 3-phosphorylated lipids were resolved using TLC, autoradiography and quantified by liquid scintillation counting. Results are the mean \pm SD. of two independent experiments carried out in duplicate. Representative autoradiograph shows the 3'-phosphorylated lipid product (PI3P) for each condition in duplicate. Results are expressed as % change in activity in comparison to SITA (control). SITA activity levels were taken to equal 100% PI 3-kinase activity.

Average cpm/mg total protein/min 2757 cpm (SITA = 100%), 2484 ± 209 cpm (Q) and 4171 ± 810 cpm (10% (v/v) FCS).

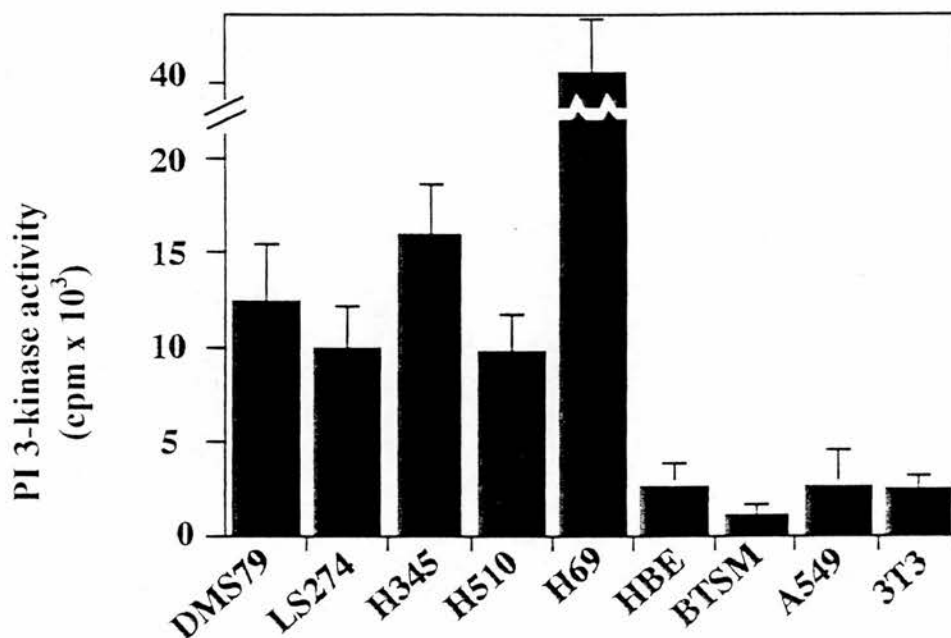


Figure 3.3: Phosphoinositide 3-kinase Activity in a Number of Different Cell Types.

Cells were quiesced before being washed in PBS and lysed in PI 3-kinase lysis buffer. After the lysates were equilibrated for protein concentration, they were assayed for PI 3-kinase activity. Results are the mean cpm \pm S.D. of 2 independent experiments performed by Dr R.C. Rintoul.

Abbreviations: HBE, human bronchial epithelial cells, 3T3, Swiss 3T3 fibroblasts, BTSM, bovine trachea smooth muscle cells, A549, human type II alveolar epithelial cells. All other cell types were SCLC cell lines.

3.2.3 Inhibition of Phosphoinositide 3-kinase Activity in Small Cell Lung Cancer Cells using Wortmannin.

Due to the above observation that PI 3-kinase is activated under basal cell culture conditions in SCLC cells, the ability to inhibit this activity was examined using a well documented and specific PI 3-kinase inhibitor, wortmannin. Wortmannin is a non-competitive inhibitor of PI 3-kinase which is able to inhibit PI 3-kinase activity by irreversibly binding to the ATP binding site present on the p110 catalytic subunit of the p85/p110 heterodimer (Arcaro & Wymann, 1993, Wymann et al., 1996).

Pretreatment of the SCLC cells with increasing concentrations of wortmannin (DMSO < 0.001% (v/v)) for 20 minutes prior to cell lysis caused a concentration-dependent inhibition of basal PI 3-kinase activity in all three cell lines (graphs with representative autoradiographs for H69 and H345 cell lines shown - Figure 3.4). The IC_{50} values for wortmannin were 8.0 ± 2.1 nM (mean \pm S.D., $n = 2$), 0.27 ± 1.1 nM (mean \pm SEM, $n = 3$) and 6.55 ± 1.49 (mean \pm SEM, $n = 4$) for H69, H345 and H510 SCLC cells respectively. The IC_{50} values obtained using wortmannin are similar to those previously published (Powis et al., 1994 ($IC_{50} = 3$ nM)). Maximal inhibition was observed with the addition of 100 nM wortmannin ($75 \pm 3.3\%$, $82 \pm 7\%$ and $79.33 \pm 10.33\%$ for H345, H69 and H510 SCLC cells respectively).

3.2.4 Inhibition of Phosphoinositide 3-kinase Activity in Small Cell Lung Cancer Cells using LY294002.

To clarify that basal SCLC cell PI 3-kinase activity could be inhibited, an alternative PI 3-kinase inhibitor was utilised. LY294002 is structurally different to wortmannin and inhibits PI 3-kinase activity in an alternative manner (Vlahos et al., 1994). LY294002 is a competitive inhibitor of PI 3-kinase activity, whilst wortmannin is a non-competitive inhibitor. LY294002 associates with a region of PI 3-kinase common to the agonist (ATP in this case) without activating the enzyme. Whilst competitive inhibitors normally associate with their putative binding site,

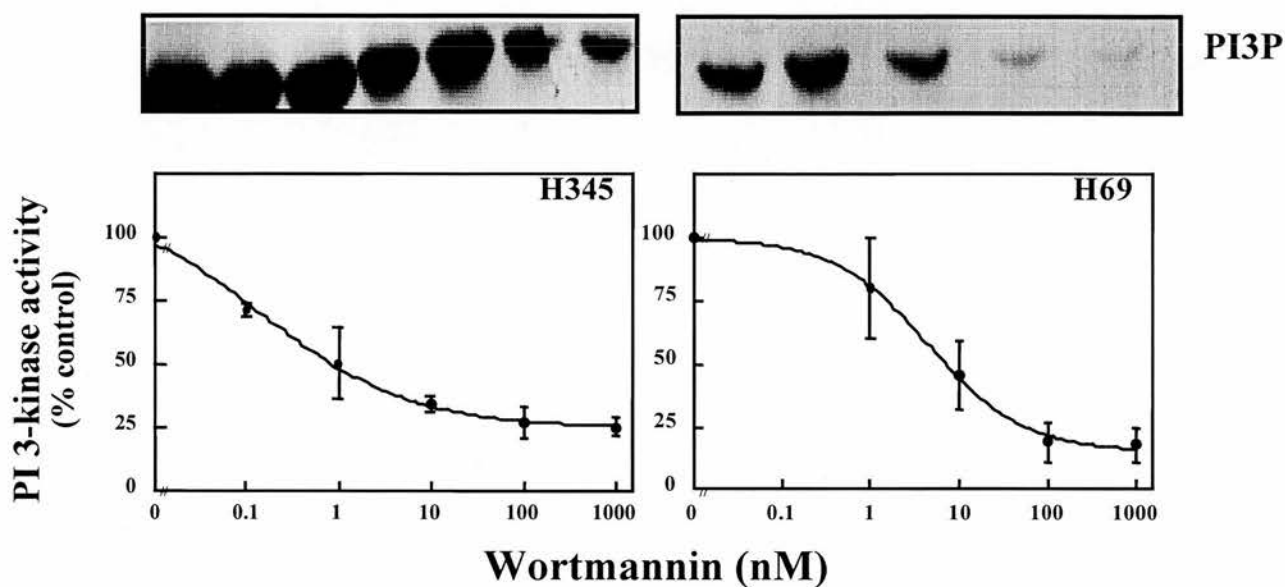


Figure 3.4: Effect of Wortmannin on Phosphoinositide 3-kinase Activity in Small Cell Lung Cancer Cells H345 (left) and H69 (right).

Pre-washed SCLC cells were inhibited with increasing concentrations of wortmannin for 20 minutes prior to lysis and assaying for PI 3-kinase activity (see section 2.3.1a) using phosphoinositide (PI) as the substrate. Values shown are the mean \pm SD (H69) and SEM (H345) for 2 – 3 independent experiments carried out in duplicate. Basal (control) PI 3-kinase activity was taken as 100% (no inhibitor added). 3-phosphorylated lipids were resolved by TLC, visualised by autoradiography and quantified by liquid scintillation counting. Above, representative autoradiographs showing the 3-phosphorylated lipid product (PI3P) are shown for each condition.

non-competitive inhibitors covalently bind to their target forming a stronger bond. The competitive association/binding of an inhibitor can often be overcome by increasing the concentration of agonist (ATP) with respect to the inhibitor. This results in an increased probability of the agonist binding to the site in relation to the inhibitor. Also continual washing of the enzyme following immunoprecipitation may cause dissociation and loss of the inhibitor.

Pretreatment of the SCLC cells with LY294002 had no effect on basal PI 3-kinase activity (Figure 3.5). However, addition of LY294002 to PI 3-kinase immunoprecipitates caused a concentration-dependent inhibition of PI 3-kinase activity (Figure 3.6). As with the inhibition of SCLC cell PI 3-kinase using wortmannin, LY294002 produced IC_{50} values for all three cell lines similar to those previously published (Vlahos et al., 1994 (1.4 μ M)). The IC_{50} values for the inhibition of PI 3-kinase activity by LY294002 in H69, H345 and H510 cells were $3.4 \pm 1.4 \mu$ M (mean \pm SEM, $n = 5$), $0.85 \pm 0.41 \mu$ M (mean \pm SEM, $n = 4$) and $1.27 \pm 0.55 \mu$ M (mean \pm SD, $n = 2$) respectively. LY294002 (100 μ M) was maximally effective causing $98 \pm 0.9\%$, $98 \pm 3\%$ and $95 \pm 1.46\%$ inhibition of PI 3-kinase activity in H345, H69 and H510 SCLC cells respectively.

In summary, PI 3-kinase activity was shown to be elevated under basal growth conditions in all SCLC cell lines examined. Under these conditions, the PI 3-kinase activity levels observed in SCLC cells were significantly higher than in any of the other cell types examined. However, this elevated activity could be significantly inhibited upon the addition of either wortmannin or LY294002. These data taken together suggests that in SCLC cells PI 3-kinase is constitutively activated. This is the first time PI 3-kinase has been shown to be constitutively active in any human cancer cell line.

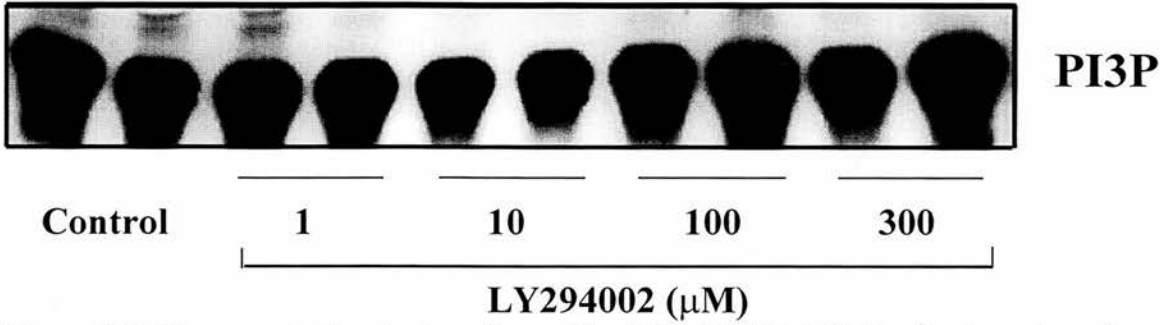


Figure 3.5: Representative Autoradiograph of the PI3P Lipid Product produced in a Whole Cell Phosphoinositide 3-kinase Activity Assay in the Presence of Increasing Concentrations of LY294002.

SCLC cells were pre-incubated with increasing concentrations of LY294002 for 20 minutes prior to cell lysis. Lysates were assayed for PI 3-kinase activity as described in the materials and methods section. Autoradiograph shows the formation of the 3'-phosphorylated lipid product (PI3P) from a single experiment representative of at least 6 experiments carried out in duplicate.

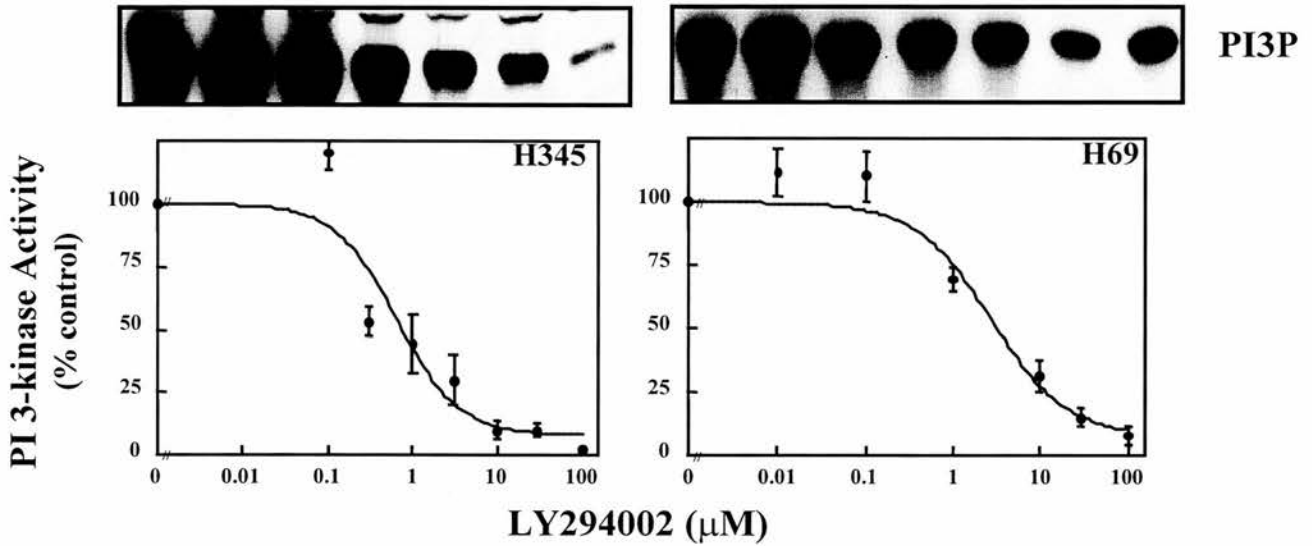


Figure 3.6: Concentration-dependent Inhibition of Phosphoinositide 3-kinase Activity by LY294002 in Small Cell Lung Cancer Cell Lines H345 (left) and H69 (right).

Untreated SCLC whole cell lysates were immunoprecipitated using a p85-PI 3-kinase antibody (2.5 $\mu\text{g}/\text{mg}$ protein). The immunoprecipitates were washed and treated with increasing concentrations of LY294002 immediately prior to assaying for *in vitro* phosphoinositide 3-kinase activity using PI as the lipid substrate. The resulting lipid products were separated by TLC before [^{32}P] γ -ATP incorporation was assessed by liquid scintillation counting. Values shown are mean \pm SEM for 4 – 5 independent experiments performed in duplicate. Untreated SCLC cell lysate activity was taken to equal 100% (control value). Above: representative autoradiographs showing the 3-phosphorylated lipid product (PI3P) for each condition.

To investigate whether the presence of this constitutive PI 3-kinase activity in SCLC cells resulted in the increased activity of potential downstream signalling targets, ribosomal protein s6 kinase (p70^{s6k}) activity was assessed. This enzyme has been shown to reside downstream of PI 3-kinase in many cell types (Chung et al., 1994, Weng et al., 1995) using a number of different techniques including the use of pharmacological inhibitors as well as enzyme and receptor mutants (section 1.6.2.2).

3.2.5 Expression of p70^{s6k} in Small Cell Lung Cancer Cells.

Using western blotting techniques, the serine/threonine protein p70^{s6k} was shown to be expressed in all three SCLC cell lines (Figure 3.7).

Seufferlein and Rozengurt (1996) have previously shown that in the SCLC cell lines, p70^{s6k} is constitutively phosphorylated, and that this phosphorylation could be inhibited by the addition of rapamycin. However, research by different research groups has shown that p70^{s6k} contains a number of phosphorylation sites and exists in various phosphorylation states. It is not until a number of crucial sites are phosphorylated that the enzyme becomes active (Ferrari et al., 1992, Pearson et al., 1995, Moser et al., 1997). Therefore, in the case of p70^{s6k}, phosphorylation levels as a whole may not reflect enzymic activity. To investigate the activity of p70^{s6k} in SCLC cells, an *in vitro* kinase assay technique was utilised.

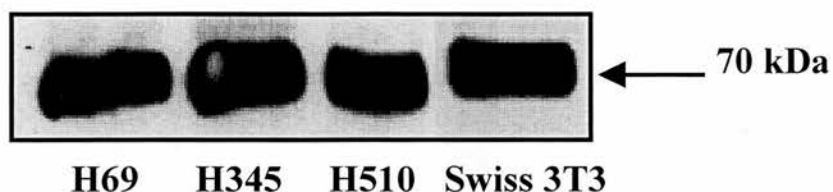


Figure 3.7: Western Blot Demonstrating the Expression of p70^{s6k} in Small Cell Lung Cancer Cell Lines H69, H345 and H510 and Swiss 3T3 Fibroblasts.

Cells were grown 3 – 5 days post passage prior to lysis, protein equilibration and separation by gel electrophoresis. Levels of p70^{s6k} were assessed using a rabbit polyclonal anti-p70^{s6k} antibody (1:1000 dilution) followed by an anti-rabbit HRP conjugate (1:1000) (Dako, UK.). The blot was visualised by ECL. Autoradiograph is representative of a single experiment carried out four times.

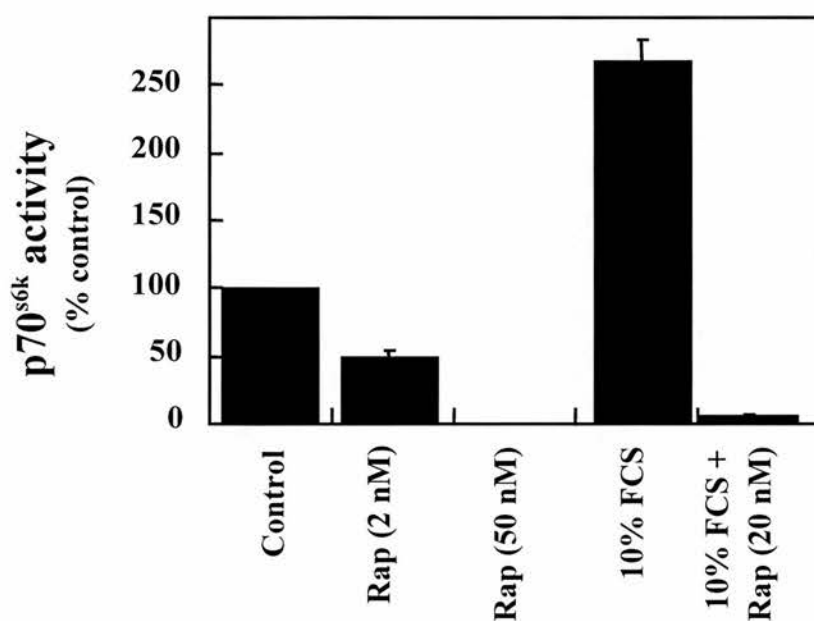


Figure 3.8: Effect of Rapamycin and Foetal Calf Serum on p70^{s6k} Activity in Small Cell Lung Cancer Cells.

Cells were incubated with either rapamycin (Rap) (20 min), 10% (v/v) FCS (10 min) or in combination before being lysed and assayed for p70^{s6k} activity using the S40 peptide as the enzyme substrate. Results are expressed as % activity of p70^{s6k} in relation to cells treated with diluent only (control = 100% (DMSO < 0.001% v/v)). Each bar is representative of the mean (\pm range) of a single experiment carried out in duplicate of at least 3 experiments in H345 SCLC cells.

3.2.6 p70^{s6k} Activity in Small Cell Lung Cancer Cells.

Initial results demonstrated the presence of a significant level of p70^{s6k} activity in basal SCLC cells which could be both stimulated by the addition of 10% (v/v) FCS and maximally inhibited by the addition of rapamycin (Figure 3.8). Prior addition of rapamycin to incubated whole cells could prevent the stimulation of p70^{s6k} by serum. Addition of rapamycin to the immunoprecipitated p70^{s6k} enzyme did not inhibit enzyme activity confirming that its mechanism of action is to inhibit an upstream regulator of the enzyme (Chou & Blenis, 1995) (Figure 3.9).

3.2.7 Regulation of p70^{s6k} Activity by Phosphoinositide 3-kinase.

To investigate whether p70^{s6k} was situated downstream of PI 3-kinase in SCLC cells, p70^{s6k} *in vitro* kinase assays were performed in the presence of wortmannin and LY294002. Cells were pre-treated with increasing concentrations of wortmannin or LY294002 for 20 minutes prior to cell lysis. This resulted in a concentration-dependent inhibition of p70^{s6k} activity by both inhibitors in all three SCLC cells lines tested (Figure 3.10). The IC₅₀ values in the three SCLC cell lines tested were 1.3 ± 0.73 nM (mean ± SEM, n = 4), 14.6 ± 5.5 nM (mean ± SEM, n = 3) and 4.1 nM (n = 1) for wortmannin, and were 0.9 ± 0.68 μM (mean ± SEM, n = 4), 4.2 ± 1.2 μM (mean ± SEM, n = 3) and 1.0 μM (n = 1) for LY294002 in H69, H345 and H510 cells respectively. These results are similar to the IC₅₀ values obtained using both wortmannin and LY294002 to inhibit PI 3-kinase activity (Table 3.1). Whilst performing these experiments, it was noticed that both LY294002 and wortmannin were unable to fully inhibit p70^{s6k} activity. The addition of either inhibitor was only able to achieve between 65 and 70% inhibition of p70^{s6k} activity at maximally effective concentrations.

Additional experiments were performed to clarify that both LY294002 and wortmannin are unable to inhibit p70^{s6k} directly. The incubation of either inhibitor

with immunoprecipitated p70^{s6k} was shown to have no effect on enzyme activity (Figure 3.9).

In summary, p70^{s6k} activity is regulated in a PI 3-kinase -dependent and -independent manner. However, inhibition of mTOR activation completely prevents p70^{s6k} activation even in the presence of constitutive PI 3-kinase activity and mitogenic cell stimulation.

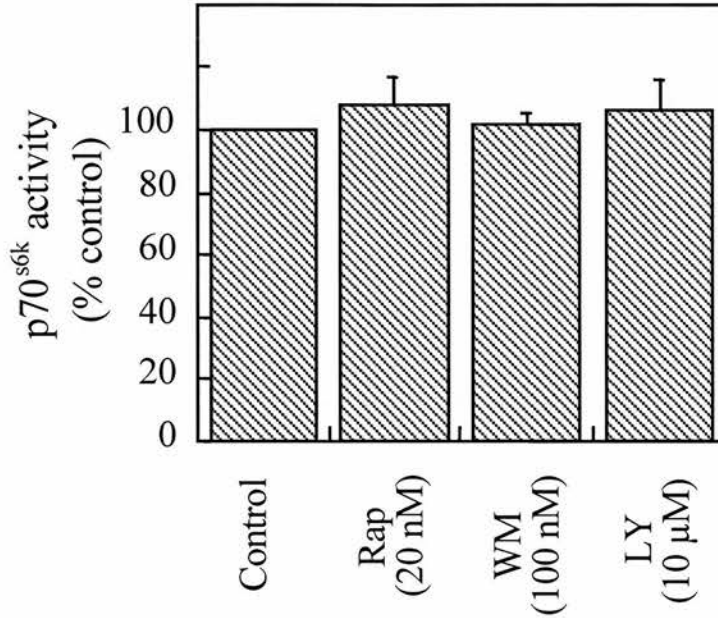


Figure 3.9: Effect of Various Inhibitors on Immunoprecipitated p70^{s6k} Activity. H345 SCLC cells were washed and lysed prior to immunoprecipitation of p70^{s6k} using a p70^{s6k} rabbit polyclonal antibody (2 μg/assay point). Immunoprecipitates were incubated with specific inhibitors (Rap, rapamycin; WM, wortmannin or LY, LY294002) for 20 minutes before assaying for p70^{s6k} activity. In comparison to control values (cell lysates treated with diluent alone (DMSO < 0.01% (v/v))), results are expressed as the mean (± range) of a single experiment carried out in duplicate (n = 3).

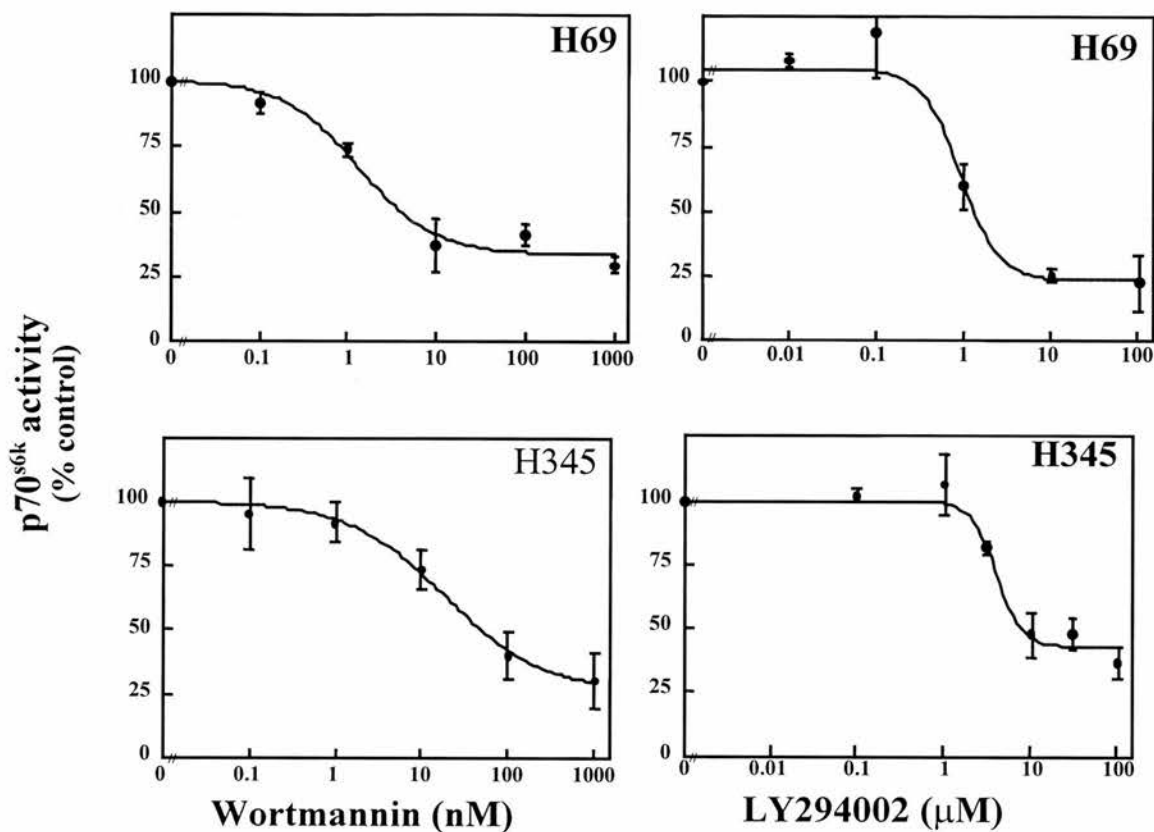


Figure 3.10: Ribosomal p70^{s6k} Activity in Small Cell Lung Cancer Cells in the Presence of Wortmannin and LY294002.

Well washed H69 (upper) and H345 (lower) SCLC cells were inhibited with increasing concentrations of Wortmannin (left) and LY294002 (right) for 20 minutes prior to lysis. Cell lysates were immunoprecipitated using a p70^{s6k} antibody (2.0 μg/assay point) and assayed for p70^{s6k} activity using a S40 synthetic peptide substrate. Results are expressed as mean values ± SEM for 3 – 4 independent experiments carried out in duplicate. Control cells (cells received diluent alone (DMSO < 0.01% (v/v))) were expressed as 100% activity. (Basal/ control H69 activity level was 4317 ± 346 cpm with non-specific background counts of 698 ± 158 cpm. For H345 cells basal/ control level was 4417 ± 897 cpm with non-specific background levels of 392 ± 90 cpm.)

3.2.8 Presence of Protein Kinase B Isoforms in Small Cell Lung Cancer Cell Lines H69, H345 and H510.

Over the last few years protein kinase B (PKB) has been shown to be situated downstream of PI 3-kinase in many different cell types (discussed in section 1.6.2.1). PKB is a product of the *v-akt* oncogene and has been shown to be overexpressed in a number of different cancers including ovarian and breast cancer (Cheng et al., 1992, Bellacosa et al., 1995).

Western blot analysis was performed to investigate the presence of the different PKB isoforms in SCLC cells (Figure 3.11). Whole cell lysates were separated by SDS-PAGE and western blotted using polyclonal antibodies to the different isoforms of PKB. Antibodies raised against the pleckstrin homology (PH) domain of PKB recognised a number of proteins upon western blotting. This may be due to the polyclonal antibody recognising the PH domains of other proteins within the cell. To date, over 100 different proteins are known to contain PH domains (Shaw, 1996).

In view of the fact that only the α and γ PKB isoforms were initially shown to be expressed in the SCLC cells (Figure 3.11), further experimentation investigating the presence of the PKB β isoform was performed using a higher concentration of the PKB β antibody. Using an elevated concentration of the PKB β antibody (8 $\mu\text{g/ml}$), a band was identified at the molecular weight corresponding to PKB β (approx. 57 kDa) (Figure 3.12). In addition, other proteins were detected by this antibody at the higher concentration. This may be due to the polyclonal PKB antibody recognising other epitopes on a variety of proteins.

In protein equilibrated samples, similar levels of PKB α were observed in both the SCLC and Swiss 3T3 cells (Figure 3.13). Two bands were consistently observed in SCLC cell line H510, which may reflect the presence of an alternatively spliced variant.

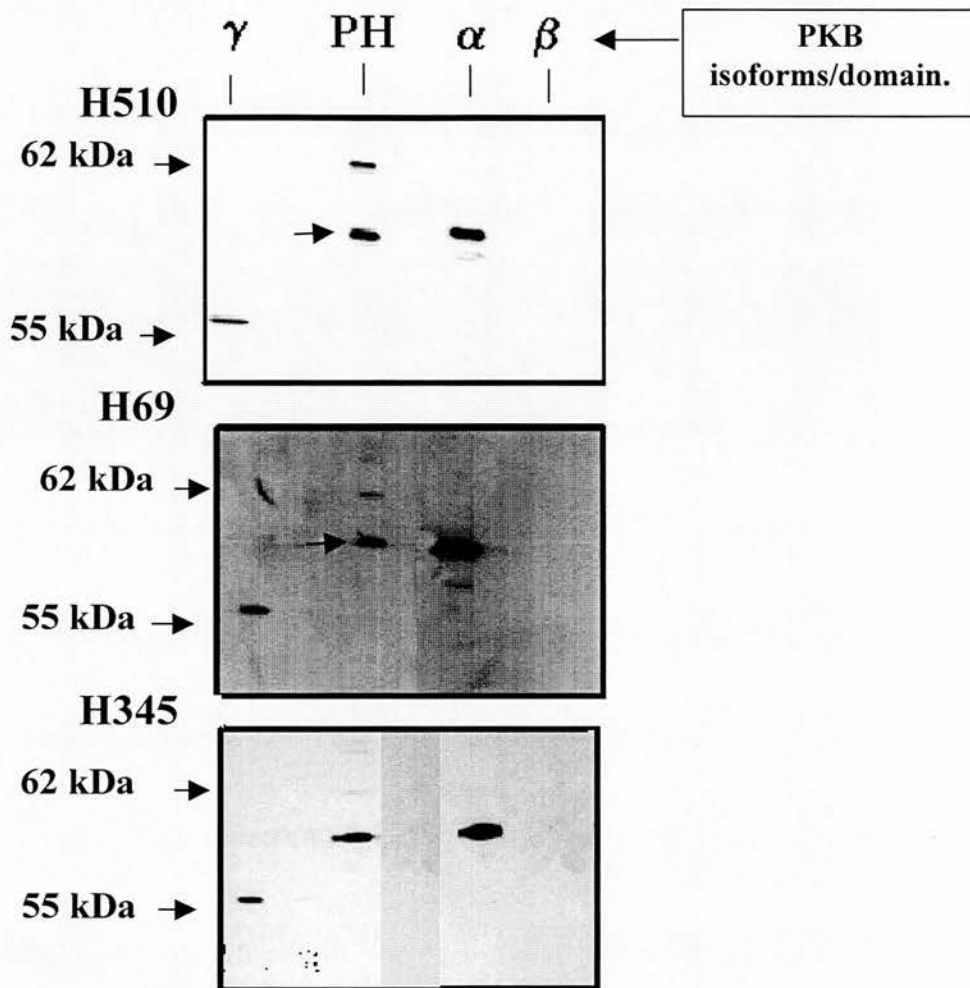


Figure 3.11: Western Blots Demonstrating the Presence of the Different Protein Kinase B Isoforms in Small Cell Lung Cancer Cells.

Cells grown for 3 – 5 days post passage were lysed in PI 3-kinase lysis buffer. Proteins were separated by SDS-PAGE and the resulting nitrocellulose blots were visualised with various antibodies to the different protein kinase B isoforms (α , β , γ and PH – antibody recognising the pleckstrin homology domain present in PKB). Primary antibodies (sheep polyclonal) were used at a concentration of 2 $\mu\text{g/ml}$. The secondary antibody (1:1000) was a donkey anti-sheep HRP (SAPU UK.). (Note ‘ \rightarrow ’ corresponds to the PKB isoform present in that specific lane.) Results are representative of individual experiments carried out at least 3 times in each SCLC cell line.

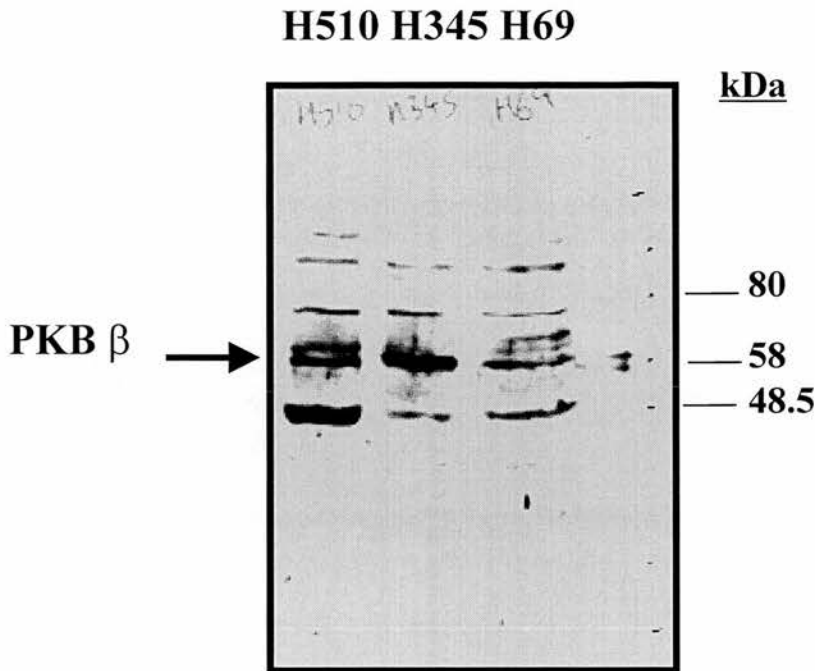


Figure 3.12: Western Blot of Small Cell Lung Cancer Cells Probed with a Protein Kinase B β Antibody.

Lysates were produced as previously described (section 2.6), and probed with a sheep polyclonal anti-PKB β antibody (8 μ g/ml) (secondary antibody donkey anti-sheep HRP (1:1000)). Immunoreactive bands were visualised by ECL. Western blot is representative of an individual experiment performed 2 times.

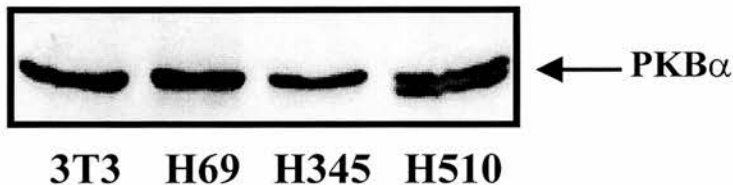


Figure 3.13: Expression of Protein Kinase B α in Small Cell Lung Cancer Cell Lines H69, H345 and H510 and Swiss 3T3 cells.

Cells were cultured for 3 – 4 days in liquid culture media prior to whole cell lysis. Cell lysates were equilibrated for protein, and proteins separated by SDS – PAGE. Levels of PKB α present were assessed using a PKB α sheep polyclonal antibody (0.15 μ g/ml). (Secondary antibody donkey anti-sheep 1:1000 (SAPU UK.)). Bands were visualised by ECL. The western blot is representative of an individual experiment carried out three times in each SCLC cell line.

3.3 Discussion.

3.3.1 Phosphoinositide 3-kinase.

Initial experiments revealed high protein levels of the p85 α regulatory subunit of PI 3-kinase in the SCLC lines examined. This led us to examine the activity status of this enzyme in SCLC cells. The observation that the basal PI 3-kinase activity levels seen in all three SCLC cell lines were sustainable after washing and incubation in warmed PBS (devoid of all growth factors), suggested that this activity was independent of any exogenous mitogenic stimulus including autocrine growth factor stimulation.

Further studies have shown that basal PI 3-kinase activity levels in SCLC cells are at least 4 fold higher than in Swiss 3T3 mouse fibroblast cells, A549 cells and BTSM cells when equilibrated for total protein. The basal activity levels of PI 3-kinase in Swiss 3T3 cells could be markedly lowered by thorough washing and incubation of cells in quiescent media (personal communication Dr R.C. Rintoul).

A concentration-dependent inhibition of basal PI 3-kinase activity was achieved using the PI 3-kinase inhibitors wortmannin and LY294002. These inhibitors were shown to be maximally effective at concentrations of 100 nM for wortmannin and 100 μ M for LY294002. The IC₅₀ values obtained using wortmannin and LY294002 were similar to those previously reported in other cell types; 0.27 - 11.1 nM and 0.85 - 3.4 μ M for wortmannin and LY294002 respectively in the three SCLC cell lines (Table 3.1). This data indicates that in unstimulated SCLC cells, PI 3-kinase activity is elevated, and the inhibitors wortmannin and LY294002, are selectively able to significantly inhibit this elevated PI 3-kinase activity.

These results demonstrate for the first time, the presence of constitutively active PI 3-kinase in any human cancer cell.

Table 3.1: IC₅₀ Values for Wortmannin and LY294002 on PI 3-kinase, PKB* and p70^{s6k} Activity.

Assay	H69	H345	H510
PI 3-kinase			
Wortmannin (nM)	8.0 ± 2.1	0.27 ± 1.1	6.55 ± 1.49
LY294002 (μM)	3.4 ± 1.4	0.85 ± 0.41	1.27 ± 0.55
Protein Kinase B *			
Wortmannin (nM)	2.0 ± 0.6	ND	ND
LY294002 (μM)	2.7 ± 0.1	ND	ND
p70^{s6k}			
Wortmannin (nM)	1.3 ± 0.73	14.6 ± 5.5	4.1
LY294002 (μM)	0.9 ± 0.68	4.2 ± 1.2	1.0

* Experiments performed by Dr. R.C. Rintoul (presented in Moore et al., 1998). (ND = not determined.)

In view of the fact that PI 3-kinase mediates a large number of key cellular responses including cell proliferation and cell survival (Table 1.2), understanding the cellular consequences of this elevated activity in SCLC cells may help in identifying specific targets to aid in the treatment of SCLC (initial functional results shown in chapter 4).

There are a number of possible reasons why PI 3-kinase is constitutively activated in SCLC cells. Previous studies performed in different cell types, have shown that PI 3-kinase can be activated by a number of intracellular signalling intermediates including FAK, src and ras (Chen & Guan, 1994b, Rodriguez-Viciano et al., 1994 and 1997). These three proteins have been shown to cause anchorage-independence and transform cells when overexpressed or mutated (Frisch & Francis, 1994, Frisch et al., 1996, Khwaja et al., 1997, Guan & Shalloway, 1992). PI 3-kinase may therefore be activated in SCLC cells due to high levels of any of these three proteins leading to anchorage-independence and cell transformation. However, under basal conditions, Tallett and colleagues (1996) have shown that FAK is not tyrosine

phosphorylated and therefore activated in SCLC cells. Further studies show that there is no evidence to suggest the presence of activated *ras* mutations in SCLC cells (Mitudomi et al., 1991, Suzuki et al., 1990). *pp60src* activity has also been shown to be low in SCLC cells (Budde et al., 1994).

In view of the above data, it can be hypothesised that the elevated basal PI 3-kinase activity observed in the SCLC cells maybe due to a novel mechanism of PI 3-kinase activation, or due to the mutation of a receptor/ligand which is constitutively switched on causing PI 3-kinase activation. An example of receptor mutation was described by Moscatello and colleagues (1998). Moscatello observed that on transfection of NIH 3T3 cells with a mutated epidermal growth factor receptor, PI 3-kinase became increasingly activated. However, this increased activation could still be inhibited on the addition of either wortmannin or LY294002. Finally, there is the possibility that the PI 3-kinase enzyme itself could be mutated in the SCLC cells resulting in constitutive PI 3-kinase activity, enabling the enzyme to be switched on with or without an activation signal. Sequencing of the p85/p110 heterodimer present in SCLC cells may reveal an enzyme which is either missing some regulatory sites, or some conformational restraints causing constitutive activity. Further experimentation is required to determine why PI 3-kinase is constitutively activated in SCLC cells.

3.3.2 Phosphoinositide 3-kinase Inhibitor Specificity.

Wortmannin is a fungal metabolite which is known to display anti-inflammatory and immunosuppressive effects in experimental animal models. Wortmannin was initially shown to inhibit neutrophil cellular responses to receptor activation (Baggiolini et al., 1987). However, further experimentation indicated that the fungal metabolite was a specific inhibitor of PI 3-kinase activity ($IC_{50} = 3$ nM). It has since been shown to bind irreversibly to the p110 catalytic subunit of PI 3-kinase, and remain associated after repeated washing (Yano et al., 1993), suggesting that it is able to covalently bind to PI 3-kinase (Thelen et al., 1994). Initial research

demonstrated that a short pre-incubation with micromolar concentrations of wortmannin caused the inhibition of the myosin light chain (IC_{50} 1.9 μ M) (Nakanishi et al., 1992). Additionally, Protein kinase C, cAMP-dependent protein kinase, cGMP-dependent protein kinase and calmodulin-dependent protein kinase II were shown to be minimally effected by concentrations of wortmannin as high as 10 μ M. Protein kinase A, C and G were subsequently reported to be unaffected by concentrations of wortmannin up to 1 μ M (Okada et al., 1994, Yano et al., 1993). In view of these data wortmannin was said to be a specific inhibitor of PI 3-kinase at nanomolar concentrations.

Since these first studies were performed, it has been suggested that wortmannin can cause inhibition of other proteins in the nanomolar range including a novel PI 4-kinase β protein with an IC_{50} of approximately 150 nM (Meyers & Cantely, 1997) and PLA_2 (Cross et al., 1995). Brunn and colleagues (1996) reported that wortmannin inhibited the activity of the protein mammalian target of rapamycin (mTOR) (also known as FRAP) (discussed in section 1.6.2.2). mTOR contains a PI 3-kinase-related catalytic domain (Helliwell et al., 1994). However, mTOR has not been shown to exhibit lipid kinase activity. Brunn and colleagues addressed the idea that mTOR may act as a protein kinase within the cell, and that serine autokinase activity of the enzyme may be inhibited by the addition of wortmannin (Brown et al., 1995). Wortmannin, directly added to purified mTOR from rat brain extracts was shown to cause a concentration-dependent inhibition of mTOR autokinase activity (IC_{50} around 200 nM). However, a maximal inhibition of autokinase activity was not achieved. After further experimentation, Brunn concluded that mTOR activity was inhibited by wortmannin in the nanomolar range, but that mTOR was over a 100 fold less sensitive to wortmannin than the p85/p110 PI 3-kinase protein. Conversely Brown and colleagues (1995) showed that in Jurkat T cells, rapamycin was able to prevent mTOR autophosphorylation (therefore prevent p70^{s6k} activation) (IC_{50} between 10 and 15 nM), where as the addition of wortmannin (50 nM and 500 nM) was unable to affect autophosphorylation of mTOR. In view of this recent data, it can be concluded that wortmannin does have a degree of selectivity for PI 3-kinase

over any of the other proteins examined to date, but at high nanomolar concentrations it may lose its specificity.

Initial studies investigating the potential biological uses of the naturally occurring bioflavonoid quercetin revealed that the compound was a potent inhibitor of PI 3-kinase activation (Matter et al., 1992). However, quercetin had previously been shown to inhibit other enzymes such as PI 4-kinase (Nishioka et al., 1989). Using quercetin as a model compound, several similar compounds were synthesised by Vlahos and co-workers (1994) whilst attempting to synthesise a more selective PI 3-kinase inhibitor. One of the compounds synthesised, LY294002, was shown to be more selective towards PI 3-kinase as it did not inhibit cytosolic MAP kinase, p70^{s6k}, PKA, DAG kinase or pp60^{src} activity at concentrations up to 35-fold greater than the IC₅₀ for PI 3-kinase inhibition (IC₅₀ of 1.4 µM). In view of this data, LY294002 has been classified as a PI 3-kinase specific inhibitor at low micromolar concentrations.

3.3.3 Ribosomal Protein S6 Kinase.

The hypothesis that p70^{s6k} may lie downstream and be regulated by the PI 3-kinase in SCLC cells was investigated. Primarily p70^{s6k} was shown to be expressed in all three SCLC cell lines examined. To further the observation that p70^{s6k} is constitutively phosphorylated in SCLC cells (Seufferlein & Rozengurt, 1996), the activity of p70^{s6k} in basal SCLC cells was seen to be elevated as rapamycin was able to fully inhibit basal p70^{s6k} activity. Results suggested that, although p70^{s6k} was highly phosphorylated and active under basal conditions, additional activation occurred in the presence of FCS. Addition of 10% (v/v) FCS to SCLC cells caused a 50 - 100% increase in PI 3-kinase activity, whereas addition of an identical amount of FCS for the same period of time caused over a 150% increase in p70^{s6k} activity. This suggests that either an alternative signalling pathway maybe involved in regulating p70^{s6k} independently of PI 3-kinase activation, or that the signal emanating from PI 3-kinase becomes amplified further downstream. The suggestion that p70^{s6k} might be activated by both PI 3-kinase-dependent and -independent signalling pathways was

strengthened by the observation that concentrations of both LY294002 and wortmannin that were able to maximally inhibit PI 3-kinase activity, were only able to partially inhibit p70^{s6k} activity where as rapamycin could fully inhibit p70^{s6k} activity. A similar observation was made by Chung and co-workers (1994). In HepG2 cells they showed that wortmannin (up to 1 μ M) could not fully inhibit p70^{s6k} activity, where as rapamycin could.

From the observations described in this chapter, it could be hypothesised that the mTOR signalling pathway acts parallel to the PI 3-kinase regulated pathway in SCLC cells. Parallel activation of these two signalling pathways has previously been shown to occur by Cheatham and co-workers (1995) and Weng and co-workers (1995). A rapamycin resistant p70^{s6k} was shown be sensitive to wortmannin-mediated inhibition of enzyme activity. Upstream regulators of the mTOR signalling pathway have yet to be established. It is therefore possible that the alternative signalling pathway causing an increase p70^{s6k} activity in response to FCS may signal via mTOR. The presence of this residual p70^{s6k} activity could also be due to an alternative pathway activating p70^{s6k}, e.g. the protein kinase C (PKC) pathway. In other cell types, PKC isoforms have been shown to activate p70^{s6k} (Tudan et al., 1998, Chou & Blenis, 1995, Chung et al., 1994) independently of PI 3-kinase. Chung and co-workers (1994) showed that PLC-dependent activation of p70^{s6k} was PKC-dependent, where as PI 3-kinase-mediated p70^{s6k} activation was PKC-independent. The suggestion that a PLC/PKC signalling pathway may regulate p70^{s6k} independently of PI 3-kinase is strengthened by the observation that phorbol esters have been shown to cause p70^{s6k} activation in cultured cells (Erikson, 1991). Further experiments will have to be performed using SCLC cells to discover the additional pathway(s) able to stimulate p70^{s6k} in a PI 3-kinase-independent manner.

It must be noted that previous experiments studying the phosphorylation patterns of p70^{s6k}, have shown that mTOR activity is required to phosphorylate specific serine residues on p70^{s6k} before the enzyme becomes fully active (Chou & Blenis, 1995). This is demonstrated by the observation that rapamycin is able to fully inhibit p70^{s6k}

activity even when PI 3-kinase is constitutively activated. In relation to this observation, it could be hypothesised that the mTOR signalling pathway is required to be active to allow p70^{s6k} to be basally phosphorylated. On further phosphorylation (e.g. via PI 3-kinase) the protein becomes fully activated allowing downstream targets to be stimulated/inhibited.

3.3.4 Protein Kinase B.

Initial experiments confirmed the presence of all three isoforms of PKB in SCLC cells. In addition to the observations of constitutive PI 3-kinase activity in SCLC cells, high basal activity levels of PKB α have been reported in H69 SCLC cells in comparison to Swiss 3T3 cells (Moore et al., 1998). As in the case of PI 3-kinase, this basal activity could only be increased by 1.5 - 2 fold with the addition of 10% (v/v) foetal calf serum. Additionally, the potent SCLC mitogen IGF-1 (100 ng/ml) could only increase PKB α levels by 1.5 - 2 fold. This stimulation was relatively small in comparison to 5 - 10 fold increases in PKB α kinase activity in Swiss 3T3 cells using IGF-1 (Dr R.C. Rintoul personal communication).

The addition of either wortmannin or LY294002 was able to completely abolish the activation of basal PKB α in H69 cells with an IC₅₀ value of 2.0 ± 0.6 nM and 2.7 ± 0.1 μ M respectively (mean \pm SEM, n = 3) (Moore, et al., 1998, experiments performed by Dr. R.C. Rintoul). Complete inhibition of PKB α was observed at concentrations of wortmannin and LY294002 specific to PI 3-kinase inhibition. This suggested that the elevated PKB α activity in basal SCLC cells is most likely a direct downstream consequence of constitutive PI 3-kinase activity, and is entirely PI 3-kinase-dependent. Thus studies performed by Moore and colleagues show the presence of a constitutively activated signalling pathway in SCLC cells which emanates from PI 3-kinase and involves both PKB and p70^{s6k}.

The presence of p70^{s6k} downstream of PKB remains controversial. Burgering and Coffey (1995) demonstrated that stimulation of PKB causes activation of p70^{s6k}

upstream of the rapamycin inhibition site. More recently PKB has been shown to activate p70^{s6k} in co-transfection assays (Reif et al., 1997, Kohn et al., 1996). However, there is contradictory evidence that PKB is unable to phosphorylate p70^{s6k}, by the failure of a kinase dead PKB to prevent p70^{s6k} stimulation (Burgering & Coffey, 1995).

Additional data indicating that PKB and not p70^{s6k}, mediates the PI 3-kinase-dependent survival of rat-1 fibroblasts and neurons (Dudek et al., 1997, Kauffmann-Zeh et al., 1997, Kulik et al., 1997), and that PKB and p70^{s6k} are differentially regulated by calcium (Conus et al., 1998) suggests that either these two proteins can be differentially regulated by various signalling input, or that PKB activates p70^{s6k} when cells have been stimulated and the intracellular calcium levels are appropriate for the activation of both enzymes. Another reason may be that these observed variations in how PKB potentially regulates p70^{s6k} activation is due to alterations in the signalling cascades of different cell types. Finally PKB and p70^{s6k} may both be situated on the same pathway as well as parallel pathways within a cell, and depending upon the stimulation or environmental factors surrounding the cell, either pathway may become dominant.

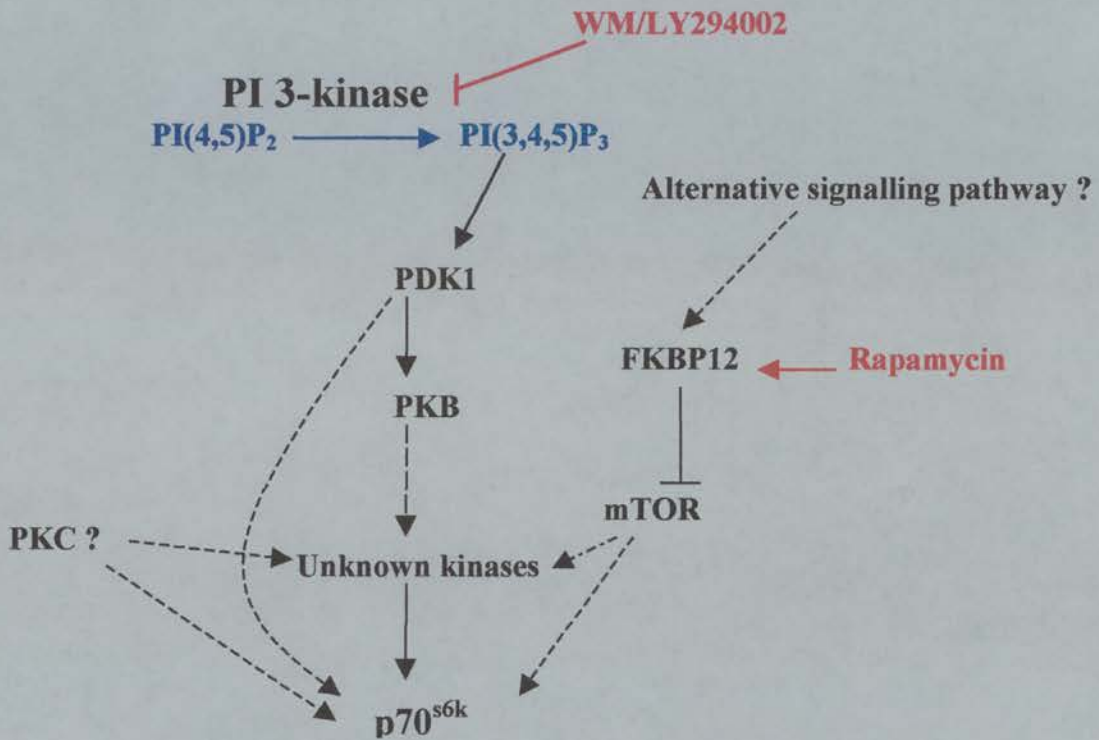


Figure 3.14: Potential Signalling Pathways Originating from Phosphoinositide 3-kinase which cause p70^{s6k} Activation in Small Cell Lung Cancer Cells.

Abbreviations: Phosphoinositide 3-kinase, PI 3-kinase; wortmannin, WM; PI(3,4,5)P₃-dependent kinase, PDK1; protein kinase B, PKB; protein kinase C, PKC; FK506 binding protein 12, FKBP12 and mammalian target of rapamycin, mTOR.

3.4 Summary.

In this chapter it has been reported that the anchorage-independent SCLC cell lines H69, H345 and H510 exhibit constitutive PI 3-kinase activity which results in elevated PKB and p70^{s6k} activity. This increased activity was shown not to be due to the proposed auto/paracrine growth factor loops thought to underlie SCLC cell survival and proliferation. These data represent the first description of constitutively activated PI 3-kinase in any human cancer. Constitutive activation of this enzyme which is known to be activated both in an integrin-dependent manner and by a number of proteins which are able to cause anchorage-independence, may account for the anchorage-independent phenotype of SCLC cells, and help explain the highly metastatic nature of this human cancer.

CHAPTER 4

Role of Phosphoinositide 3-kinase in Small Cell Lung Cancer Cell Proliferation and Survival.

4.1 Introduction.

The enzyme PI 3-kinase has been shown to be important in the regulation of a wide range of cellular processes including mitogenesis and apoptosis (reviewed Table 1.2, Duronio et al., 1998, Vanhaesebroeck et al., 1997, Carpenter & Cantley, 1996). Activated PI 3-kinase was shown to be required to enable cells to proliferate in response to PDGF receptor stimulation (Carpenter & Cantley, 1990, Parker & Waterfield, 1992). Additionally, Yao and Cooper (1995) noted that to rescue PC12 cells from undergoing apoptosis in response to serum deprivation, PI 3-kinase activation was required. Finally, IGF-1 has been shown to prevent fibroblasts from undergoing UV-induced apoptosis. This was shown to be mediated via a PI 3-kinase-dependent mechanism as the addition of wortmannin blocked the anti-apoptotic effect (Kulik et al., 1997).

Research has demonstrated a number of possible cellular functions for the cytosolic protein p70^{s6k}. The inhibition of p70^{s6k} activation is known to effect the proliferative and cell cycle properties of a number of different cell types. Using the immunosuppressant rapamycin, inhibition of p70^{s6k} activity causes G1 arrest in T-lymphocytes and delays the entry of fibroblasts into the S phase of the cell cycle (Chung et al., 1992, Kuo et al., 1992, Price et al., 1992). In relation to the apoptotic capabilities of p70^{s6k}, p70^{s6k} has been shown to exhibit either no effects or anti-

apoptotic effects in different cell types. Studies by Yao and Cooper (1996) noted that p70^{s6k} inhibition did not effect the apoptosis levels of serum-independent cell types. Conversely Shi and co-workers (1995) showed that the addition of rapamycin enhanced cell apoptosis in T cells, HL-60 cells and fibroblasts. Cells were also seen to be more sensitive to cis-platin-induced apoptosis when p70^{s6k} activity was inhibited.

In view of the fact that the balance between cell proliferation and cell death regulates tumour mass, the role of these two cellular functions was investigated in SCLC cells by the addition of the specific inhibitors LY294002 and rapamycin.

4.2 Results.

4.2.1 Proliferation.

4.2.1.1 Effect of Exogenous Growth Factors on Small Cell Lung Cancer Cell Growth in Liquid Culture.

SCLC is a cancer which displays both serum- and anchorage-independence *in vitro*. SCLC cells have been shown to synthesise and secrete a large number of peptides (reviewed Moody & Cuttitta, 1993, Belper et al., 1988) and hormones (Sorenson et al., 1981) which sustain SCLC cell growth via autocrine and paracrine growth loops (Carney et al., 1987, Sethi & Rozengurt, 1992).

The requirement of specific growth factors for the growth of SCLC cells *in vitro* has been extensively studied (Simms et al., 1980, re-evaluated and revised Nakanishi, 1988). Nakanishi (1988) concluded that the growth factors selenium, insulin and transferrin (SIT) are required to allow efficient proliferation of SCLC cells in liquid culture to occur.

Figure 4.1 demonstrates that the presence of selenium, insulin, transferrin (plus bovine serum albumin, A) (SITA) reduced the length of the lag phase of SCLC cells growing in liquid culture, in comparison to cells grown in the absence of these growth factors. To carry out these experiments, the cells were plated at a relatively low density to allow the experiment to run over a number of days without the cells exhausting the media. In view of the fact that the SCLC cells used in this study have doubling times of between 55 and 72 hours, it took 2 – 3 days before the full effect of the proliferative environment could be assessed. The results obtained suggested the possibility that, the cells plated in the absence of all exogenous growth factors were required to synthesise more of their own ‘autocrine’ growth factors to mimic the SITA growing conditions. However, the results show that although the number of

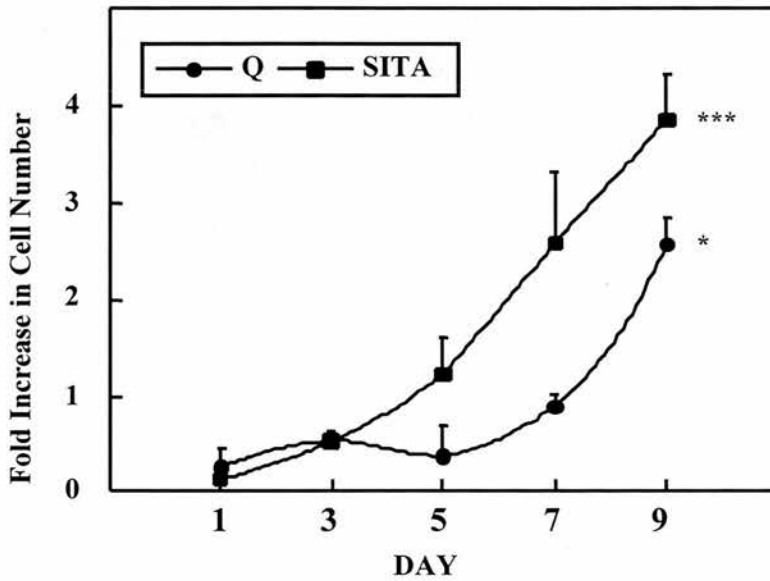


Figure 4.1: Increase in Small Cell Lung Cancer Cell Number with the Addition of Specific Mitogens.

H345 SCLC cells were incubated overnight in either quiescent (Q) media or serum-free SITA medium. Cells were washed and disaggregated into an essentially single cell population (cell viability was assessed by trypan blue exclusion > 95%). Cells were plated (500 μ l/well, 2×10^5 /ml), in 24 well culture dishes in either quiescent or SITA media. Cells were incubated in a humidified atmosphere, 5 % CO₂/95% air at 37°C. After a defined time, cells were removed and gently disaggregated. Cells (200 μ l) were removed for apoptotic assessment (Figure 4.5) and remaining cell number counted using a Coulter Counter. Results are expressed as fold increase in cell number in comparison to the number of cells initially plated. Each point is the mean (\pm SEM) of 3 independent experiments carried out in triplicate. * p<0.05, *** p<0.001 (Student-Newman-Keuls ANOVA post test).

cells plated in SITA medium increased by the greatest amount, cells in the absence of the exogenous growth factors were still able to significantly increase in number over 9 days.

4.2.1.2 Small Cell Lung Cancer Cell Proliferation is Phosphoinositide 3-kinase-dependent.

The importance of PI 3-kinase activity in SCLC cell proliferation was investigated using the PI 3-kinase specific inhibitor LY294002.

Figure 4.2a shows the concentration-dependent inhibition of [³H]-thymidine incorporation in H510 SCLC cells 72 (left) and 96 (right) hours after treatment with LY294002. Addition of 100 μ M LY294002 caused maximal inhibition of DNA synthesis ($95.8 \pm 2.15\%$ and $95.7 \pm 2.34\%$ mean \pm SEM, $n = 3$ for 72 and 96 hours respectively). The IC_{50} values obtained for LY294002 were $22.1 \pm 7.4 \mu$ M and $30.2 \pm 11.2 \mu$ M for 72 and 96 hours respectively (mean \pm SEM, $n = 3$). LY294002 was also shown to inhibit H69 SCLC cell proliferation by preventing [³H]-thymidine incorporation (Figure 4.2b) (IC_{50} at 96 hours, $15.6 \pm 1.22 \mu$ M (mean \pm S.D., $n = 2$ of replicates of five)).

These studies demonstrated that SCLC cell proliferation in liquid media is dependent upon signalling pathways which emanate from the constitutively activated enzyme PI 3-kinase. In view of the knowledge that PI 3-kinase is constitutively activated in SCLC cells, it could be hypothesised that the proliferation signal in SCLC cells is permanently switched on. This may help to explain why SCLC cells are the fastest growing lung cancer cell type.

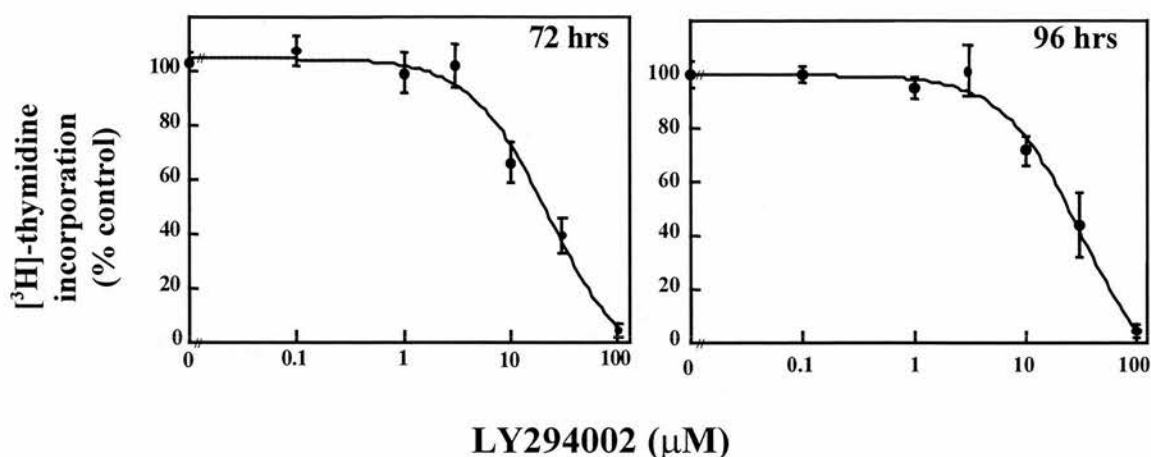


Figure 4.2a: Effect of Increasing Concentrations of LY294002 on [³H]-Thymidine Incorporation in H510 Small Cell Lung Cancer Cells.

SCLC cells were grown for 24 hours in quiescent medium before being washed and re-plated in SITA medium (5×10^4 /ml, 200 μl/well) in the presence of increasing concentrations of LY294002. After 48 (left) or 72 hours (right), [³H]-thymidine was added (0.1 μCi/well) and the cells incubated for a further 24 hours. Cells were finally harvested and [³H]-thymidine incorporation counted on a beta plate reader (see section 2.4). Each point represents the mean \pm SEM percentage [³H]-thymidine incorporation in comparison to control cells (received diluent alone = 100%) of three independent experiments carried out in replicates of five. (average control cpm = 46817)

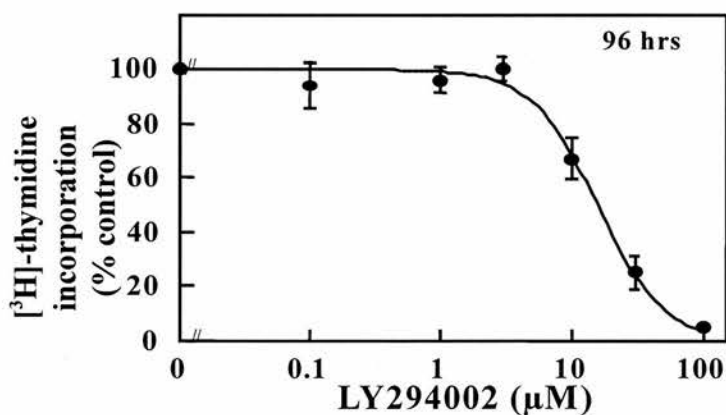


Figure 4.2b: Effect of Increasing Concentrations of LY294002 on [³H]-Thymidine Incorporation in H69 Small Cell Lung Cancer Cells.

SCLC cells were grown for 24 hours in quiescent medium before being washed and re-plated in SITA medium (5×10^4 /ml, 200 μl/well) in the presence of increasing concentrations of LY294002. After 72 hours [³H]-thymidine was added (0.1 μCi/well) and the cells incubated for a further 24 hours. Cells were finally harvested and [³H]-thymidine incorporation counted on a beta plate reader (section 2.4.1). Each point represents the mean \pm S.D. percentage [³H]-thymidine incorporation in comparison to control values (diluent alone = 100%) of two independent experiments carried out in replicates of five.

4.2.1.3 Phosphoinositide 3-kinase-dependent Small Cell Lung Cancer Cell Proliferation is Mediated via Both p70^{s6k}-independent and -dependent Signalling Pathways.

Results described in chapter 3 indicate that in SCLC cells, p70^{s6k} activity is regulated in a PI 3-kinase-dependent and -independent manner. Using the p70^{s6k} specific inhibitor rapamycin, p70^{s6k} has been shown to reside downstream of PI 3-kinase in many cell types, and has been shown to be important in the regulation of cell proliferation and the cell cycle (Chung et al., 1992, Marx et al., 1995). In view of these observations, the relative contribution of p70^{s6k} in the PI 3-kinase mediated SCLC cell proliferation pathway was investigated.

Previous studies by Seufferlein and Rozengurt (1996) assessed the role of p70^{s6k} in SCLC cell growth in liquid culture (via inhibition of the enzyme using rapamycin). They indicated that addition of rapamycin alone caused a slight concentration-dependent inhibition in cell number (IC₅₀ concentration around 0.3 - 1 nM and maximal inhibition concentration of around 10 nM). A maximally effective concentration was however only able to achieve 55% growth inhibition in liquid culture. (Experiments were performed over 7 – 11 days).

Figure 4.3 shows that in further studies in SCLC cell line H69, a maximally effective concentration of rapamycin (100 nM) caused a 33 - 39% inhibition in [³H]-thymidine incorporation over 3 – 4 days. At a concentration close to the IC₅₀ (20 μM), LY294002 caused a 50.4 ± 5.55% and 46 ± 9.45% inhibition of [³H]-thymidine incorporation at 72 and 96 hours respectively (mean ± SEM, n = 3). In combination, LY294002 (20 μM) and rapamycin (100 nM) caused a 64.3 ± 5.7% and 65.3 ± 6.35% inhibition of [³H]-thymidine incorporation at 72 and 96 hours respectively.

These results indicate that addition of a maximally effective concentration of rapamycin significantly inhibited DNA synthesis in SCLC cells grown in liquid culture in comparison to cells grown in the absence of any inhibitor. This inhibition

of [³H]-thymidine incorporation could be significantly increased by the co-incubation of SCLC cells with LY294002 (IC₅₀) and rapamycin (max). However, this inhibition was shown not to be additive. Thus, the data implies that PI 3-kinase regulates SCLC cell proliferation in serum-free liquid culture. Downstream of PI 3-kinase the signalling pathway splits with a proportion of the proliferation signal acting through p70^{s6k}.

4.2.1.4 Inhibition of Small Cell Lung Cancer Cell Colony Formation by LY294002.

One of the key characteristics of a cells transformed phenotype is its anchorage-independent ability to proliferate. The majority of SCLC cells grow in liquid culture as free floating aggregates highlighting their transformed phenotype. The ability of transformed SCLC cells to form colonies in semi-solid medium has been well documented and a positive correlation between cloning efficiency, the histological involvement and the invasiveness of SCLC tumour specimens has been reported (Freedman & Shin, 1974).

Figure 4.4 highlights the ability of SCLC cells to form colonies in semi solid medium. This ability is enhanced by the addition of different neuropeptides. This corresponds to the previous work carried out by Sethi and Rozengurt (1991 and 1992) showing that neuropeptides stimulate SCLC cell colony formation by activating multiple autocrine and paracrine growth loops. Addition of LY294002 (10 µM) to SCLC cells both in the presence and absence of additional neuropeptides inhibited colony formation. Thus, active PI 3-kinase is required to enable SCLC cells to grow and survive in a semi-solid media environment. This suggests that PI 3-kinase activation is necessary to enable SCLC cells to become tumourigenic in an *in vivo* environment.

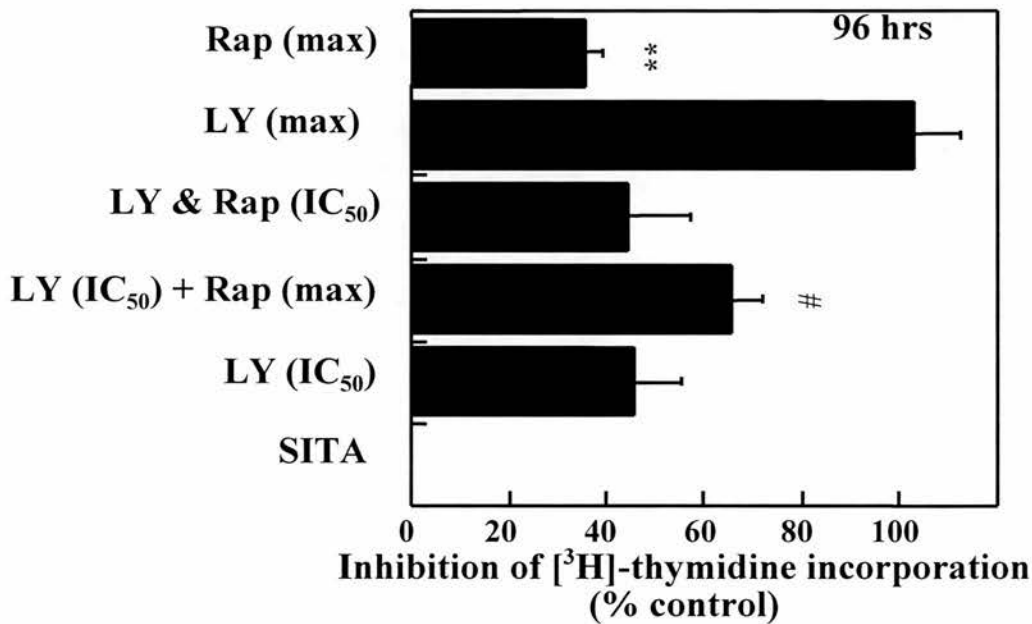
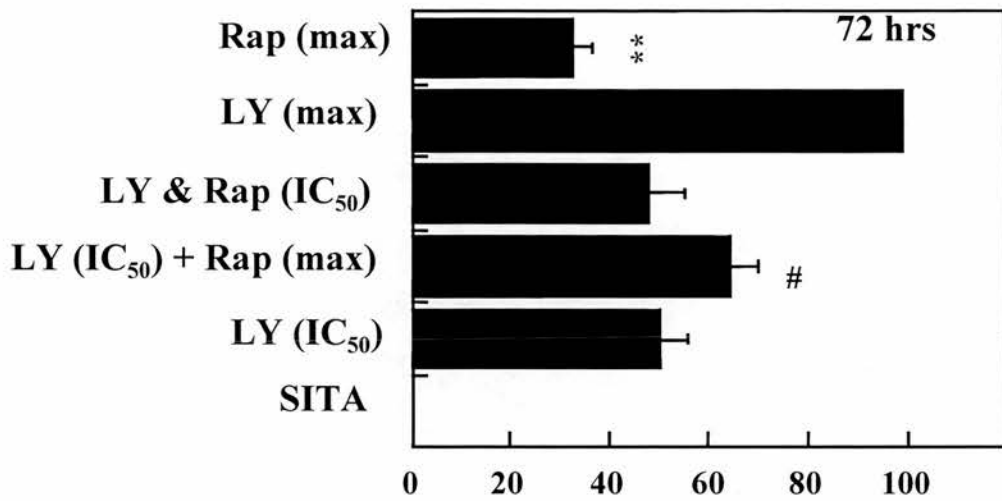


Figure 4.3: Effects of LY294002 and Rapamycin Either Alone or in Combination on Small Cell Lung Cancer Cell [³H]-thymidine incorporation.

H69 SCLC cells 3 – 5 days post passage were incubated in quiescent medium for 24 hours before being washed and incubated (5×10^4 /ml, 200 μ l/well) in serum-free SITA medium for 48 (upper) or 72 (lower) hours in the presence of various inhibitors. [³H]-thymidine (0.1 μ Ci/well) was added and the cells incubated for a further 24 hours prior to being assessed for [³H]-thymidine incorporation (see section 2.4.1) in comparison to control cells that received diluent alone (SITA). Each point represents the mean (\pm SEM) of three independent experiments carried out in replicates of five. Average counts for the control values were 35156 cpm (72 hours) and 43067 cpm (96 hours). Abbreviations: LY - LY294002, IC₅₀ 20 μ M, max 100 μ M, Rap - rapamycin IC₅₀ 2 nM, max 100 nM. In comparison to SITA - ** $p < 0.01$. In comparison to Rap (max) # $p < 0.05$ (Student-Newmann-Keuls ANOVA test).

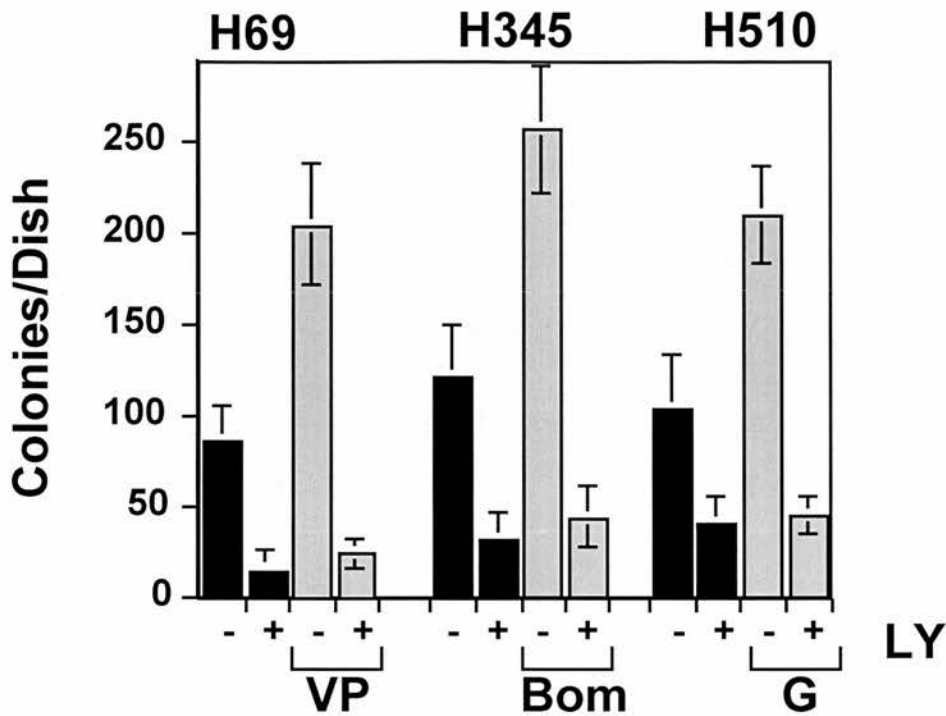


Figure 4.4: Small Cell Lung Cancer Cell Clonal Growth is Dependent upon Phosphoinositide 3-kinase Activity.

Cells were washed and plated in SITA medium (1×10^4 cells/ml) containing 0.3% agarose on top of a base of 5% agarose in culture medium (see section 2.4.3). Both layers contained either no additional neuropeptides (black bars) or vasopressin, 50 nM (VP), bombesin, 50 nM (Bom), or gastrin, 100 nM (G) (grey bars), in the absence (-) or presence (+) of LY294002, 10 μ M (LY). After 21 days colonies > 16 cells were counted. Results are expressed as the mean number of colonies/dish for 2 independent experiments carried out in triplicate (\pm S.D.)

4.2.2 Cell Survival.

4.2.2.1 Role of Exogenous Growth Factors in Small Cell Lung Cancer Cell Survival.

In order to assess the role of certain growth factors on SCLC cell survival, SCLC cell apoptosis levels were examined in serum-free SITA media and quiescent media over a period of 9 days. Figure 4.5 demonstrates that in SCLC cell line H345, the addition of selenium, insulin and transferrin (SIT) did not effect the apoptosis levels over the first three days. However, from day 5, the rate of apoptosis in quiesced cells was higher than the levels observed in the cells incubated in SITA medium. These results show that in the presence of specific growth factors, the levels of SCLC cell apoptosis in liquid culture are lowered.

When examining the results in figure 4.5 alongside the parallel growth studies (Figure 4.1), it can be seen that the cells in SITA exhibit both a decrease in apoptosis levels as well as an increase in growth rates in comparison to cells grown in quiescent media. However, when studying the increases in absolute live cell number in both SITA and quiescent medium (taking into account apoptosis rates), cells in either media significantly increased in number over time. In conclusion, these experiments indicate that the presence of selenium, transferrin and insulin enables SCLC cells in liquid culture to increase in number at a faster rate than cells grown in the absence of these growth factors. However, in the absence of these exogenous growth factors, SCLC cells still have the ability to survive and grow. This observation highlights their ability to survive and grow in a foreign environment in the absence of exogenous mitogens. (e.g. once the SCLC cells have metastasised around the body).

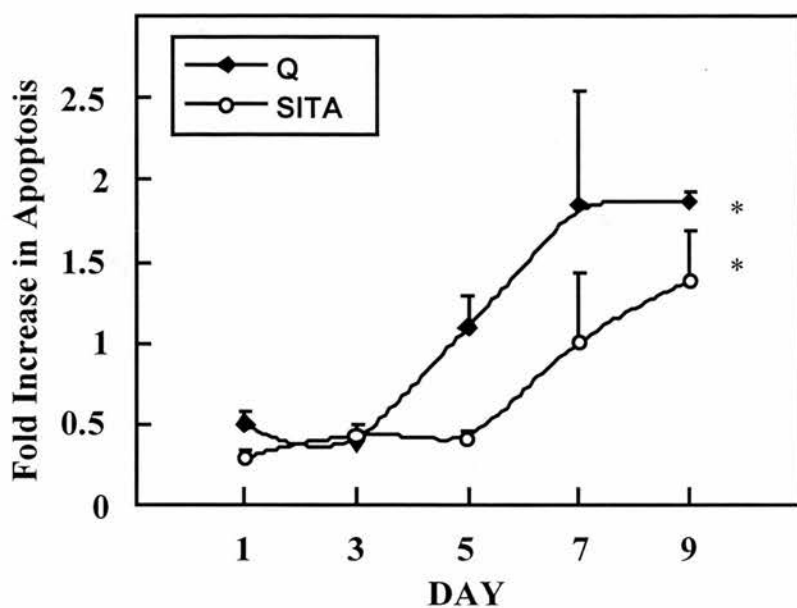


Figure 4.5: H345 Small Cell Lung Cancer Cell Apoptosis Levels over 9 days Under Different Culture Conditions.

H345 cells ($2 \times 10^5/\text{ml}$, $500 \mu\text{l}/\text{well}$) were washed thoroughly prior to being incubated in the presence of either quiescent medium (Q) or serum free SITA medium over 9 days and assessed for apoptosis. Apoptosis was assessed by the addition of ethidium bromide ($100 \mu\text{g}/\text{ml}$) and acridine orange ($100 \mu\text{g}/\text{ml}$) (1:1 v/v; $1 \mu\text{l}$) to the SCLC cells ($200 \mu\text{l}$) and examined using fluorescent microscopy as described in section 2.5.1. Each point represents the fold increase in apoptosis in relation to the levels observed at day 0 (between 8 & 11 %). Each point is the mean (\pm SEM) of three experiments carried out in triplicate. * $p < 0.05$ (Student-Newman-Keuls ANOVA post test).

As expected there was a small basal turnover of apoptosis observed in SCLC cells (basal apoptosis at day 0 = 8 – 11 %). This basal apoptosis was observed in the cultured cells during all of the following experiments but remained between approximately 1 and 8%.

4.2.2.2 Small Cell Lung Cancer Cell Survival is Modulated in Part by Phosphoinositide 3-kinase.

The results described in this chapter have so far indicated that PI 3-kinase plays a crucial role in regulating SCLC proliferation and tumourigenicity. As tumour mass is a balance between cell growth and death (via apoptosis and necrosis), the role of PI 3-kinase in SCLC cell apoptosis was assessed. PI 3-kinase has also been shown to inhibit apoptosis (and anoikis) in many cells types (often via PKB activation) (Downward, 1998, Eves et al., 1998, Khwaja et al., 1997, Franke et al., 1997).

The effect of the PI 3-kinase inhibitor LY294002 on SCLC apoptosis was assessed using two different experimental methods. Primarily, cells were quiesced for 24 hours prior to experimentation. After 24 hours figure 4.6 demonstrates that addition of LY294002 caused a concentration-dependent increase in the level of H69 SCLC cell apoptosis as assessed by cytocentrifugation (morphological assessment) (upper) and ELISA (immunoassay assessment of DNA fragmentation) (lower). (During the experiment cell viability was assessed by trypan blue exclusion to be > 95%.) In comparison to control cells (received only diluent), results show that LY294002 causes a 300 - 400 % increase in apoptosis as assessed by morphological techniques (section 2.5.3). The mean control apoptosis rates as assessed by cytocentrifugation were between 1.5 and 3.0 % for the two independent experiments.

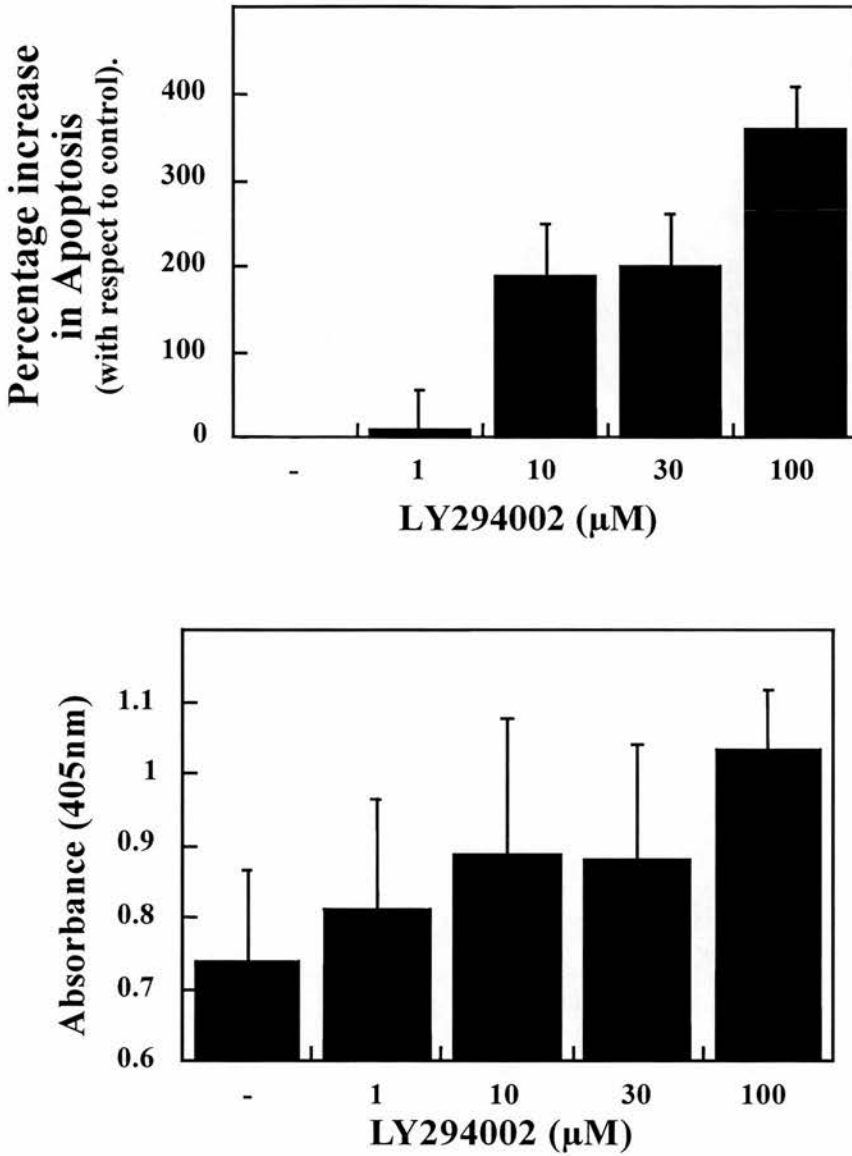


Figure 4.6: Effect of LY294002 on Small Cell Lung Cancer Cell Apoptosis.

SCLC cells, 3 – 5 days post-passage were quiesced overnight, washed thoroughly and plated in quiescent media ($2.5 \times 10^5/500 \mu\text{l/well}$) in the presence of increasing concentrations of LY294002. After 24 hours, cells ($150 \mu\text{l}$) were removed and assessed for apoptosis by cytocentrifugation (upper). From the remaining cells $200 \mu\text{l}$ was removed and assessed for apoptosis by ELISA (lower) (see section 2.5). Results are expressed as percentage increase (upper) and increase in absorbency (lower) in relation to control cells (-) cultured in quiescent medium (received diluent only). The results show the mean \pm SD. for two separate experiments performed in triplicate. Student-Newman-Keuls ANOVA not performed as $n = 2$ (see section 2.14).

The effects of LY294002 were confirmed using an ELISA based technique. The antibodies utilised in the ELISA based assay bind to histones and the cleaved single and double-stranded DNA in the cells. Results highlighted in figure 4.6 (lower) show that on the addition of increasing concentrations of LY294002, an increase in DNA fragmentation was observed (occurs after cell-induced cell death).

4.2.2.3 Role of Ribosomal p70^{s6k} in Phosphoinositide 3-kinase-mediated Small Cell Lung Cancer Cell Apoptosis.

It was noted earlier that the proliferative effect of PI 3-kinase in SCLC cells is regulated via a p70^{s6k}-dependent and -independent pathway. Yao and Cooper (1996) showed that inhibition of p70^{s6k} in both serum-dependent and -independent cells had no effect on cell survival. Therefore, to examine the role of p70^{s6k} in the apoptotic response in SCLC cells, rapamycin was added using a maximally effective concentration causing 100% inhibition of enzyme activity. Apoptosis was assessed by ELISA (section 2.5.2) in the presence of LY294002 (10 μ M), rapamycin (100 nM) and tyrphostin-25 (25 μ M). Figure 4.7 demonstrates that in H69 cells, rapamycin caused an increase in apoptosis levels. This increase was similar to the increase observed using the IC₅₀ concentration of LY294002. However, a maximally effective concentration of LY294002 is able to further increase apoptosis levels (Figure 4.6). This implies that the increase in SCLC cell apoptosis emanating from PI 3-kinase inhibition is mediated via both a p70^{s6k}-dependent and -independent mechanism.

The effect of both LY294002 and rapamycin on basal SCLC cell apoptosis levels were however less than the increases in apoptosis levels caused by the tyrosine kinase inhibitor tyrphostin-25 (Figure 4.7 and 4.8). This suggests that signalling pathways not involving either PI 3-kinase or p70^{s6k} are also involved in the control of SCLC apoptosis.

4.2.2.4 Effect of Increased Exposure of Small Cell Lung Cancer Cells to LY294002.

Tallett and colleagues (1996a) recently reported that addition of the protein tyrosine kinase inhibitor tyrphostin-25, caused an increase in SCLC cell apoptosis. This increase was both concentration and time-dependent (maximal inhibition seen after approximately 72 hours). Figure 4.8 shows that LY294002 was unable to produce a further increase in apoptosis level over an extended time period (72 hours). This shows that LY294002 causes SCLC cell apoptosis within 24 hours, and that the rate of apoptosis is constant over time.

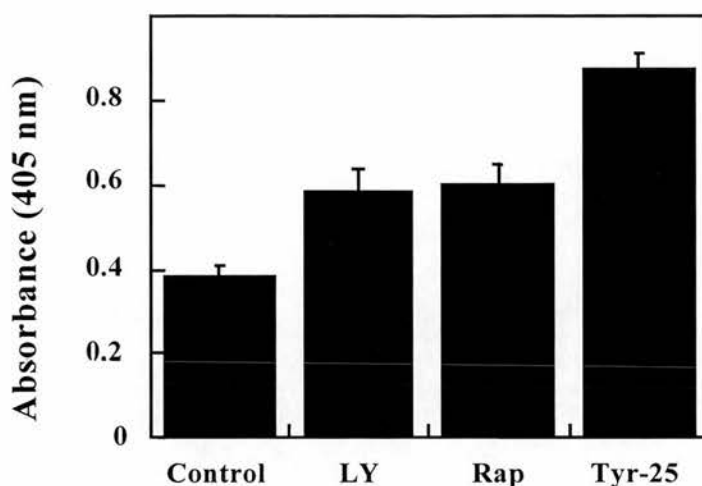


Figure 4.7: Effect of the Phosphoinositide 3-kinase Inhibitor (LY294002), p70^{s6k} Inhibitor (Rapamycin) and Tyrosine Kinase Inhibitor (Tyrphostin-25) on Apoptosis in H69 Small Cell Lung Cancer Cells.

Quiescent cells ($2.5 \times 10^5/500 \mu\text{l/well}$) were washed and plated in fresh quiescent media in the presence of LY294002 (LY) $10 \mu\text{M}$, rapamycin (Rap) 100 nM and tyrphostin-25 (Tyr-25) $25 \mu\text{M}$ for 24 hours. Cells were then assayed for apoptosis using the ELISA method (see section 2.5.2). Each bar represents the mean (\pm S.D.) of two separate experiments performed in triplicate.

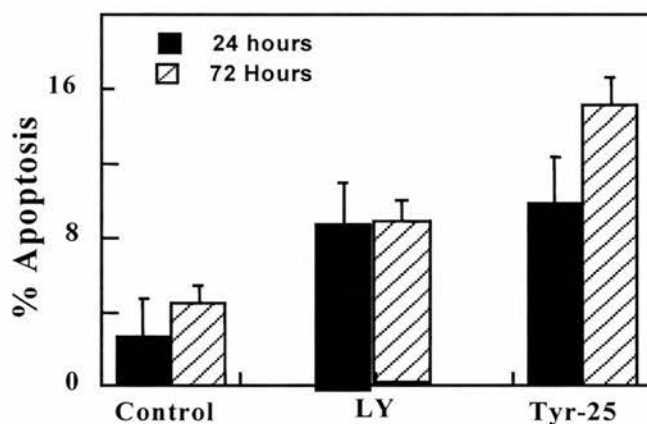


Figure 4.8: The Effect of Increasing the Exposure Time of Small Cell Lung Cancer Cells to Different Inhibitors on Apoptosis.

Cells (H345) ($2.5 \times 10^5/500 \mu\text{l/well}$) were washed and incubated in quiescent media in the presence of various inhibitors LY294002 (LY) $10 \mu\text{M}$, or Tyrphostin-25 (Tyr-25) $25 \mu\text{M}$. After 24 and 72 hours, cells ($100 \mu\text{l}$) were removed and assessed for apoptosis by cytocentrifugation (see section 2.5.3). Each bar represents the mean of 3 – 4 independent experiments (\pm SEM) carried out in triplicate.

4.3 Discussion.

4.3.1 Proliferation.

SCLC cells have the fastest growth rates of the four main lung cancer cell types. Discovery of a potential signalling pathway that regulates SCLC cell growth, would be of benefit in the design of new therapies in the treatment of SCLC.

LY294002 caused a concentration-dependent inhibition of SCLC cell [^3H]-thymidine incorporation. A maximally effective concentration of LY294002 (100 μM) which is able to cause approximately 100% inhibition in PI 3-kinase activity, fully inhibited SCLC cell proliferation as assessed by [^3H]-thymidine incorporation studies. The slight increases in the LY294002 IC_{50} values obtained from the proliferation studies (chapter 4) in comparison to the enzyme activity studies (chapter 3, Table 3.1) may be due to the fact that the enzyme activity experiments were carried out in cell free conditions.

In the presence of a maximally effective concentration of rapamycin, the inactivation of p70^{s6k} caused a significant decrease in [^3H]-thymidine incorporation (SCLC cell proliferation). Additionally, this inhibition in [^3H]-thymidine incorporation was shown to be regulated upstream by a PI 3-kinase-dependent mechanism. This was demonstrated by the observation that the addition of a maximally effective concentration of rapamycin and LY294002 (IC_{50}) together did not cause an additive inhibitory effect on [^3H]-thymidine incorporation in comparison to the effects observed in the presence of rap (max) and LY294002 (IC_{50}) alone. Parallel studies were performed investigating the role of p70^{s6k} and PI 3-kinase in SCLC liquid growth studies (Moore et al., 1998). Similar results were obtained showing that PI 3-kinase-dependent cell growth was mediated downstream by both p70^{s6k} -dependent and -independent signalling pathways. Over 50% of the growth signal was shown to be able to bypass the requirement of p70^{s6k} activation. The observation that PI 3-

kinase-regulated signalling bifurcates upstream of p70^{s6k} has previously been suggested by Chung and co-workers (1994). Wortmannin was shown to inhibit insulin-dependent GLUT4 translocation (Kanai et al., 1993), where as inhibition of p70^{s6k} using rapamycin had no effect on this process. This suggested that PI 3-kinase was able to mediate events independently of p70^{s6k} activation even though wortmannin was still able to inhibit p70^{s6k} activation.

There are a number of possible signal transduction pathways which may regulate SCLC cell growth in liquid culture via a PI 3-kinase-dependent mechanism. In addition to p70^{s6k} being involved, three other pathways have been identified in different cell types and under different cell conditions. Firstly, calcium-independent and atypical PKC isoforms have been shown to be activated by the 3'-phosphorylated lipid products of PI 3-kinase (section 1.6.2.4, Nakanishi et al., 1993, Toker et al., 1994 and 1995, Zheng et al., 1995) leading to cell growth. A second pathway involves the activation of the small protein Rac. Rac activation has been shown to cause activation of the JNK/stress activated protein kinase cascade leading to DNA synthesis (Hawkins et al., 1995, Klippel et al., 1996, Kyriakis et al., 1995, Minden et al., 1995). Finally, the MAP kinase pathway has been shown to be activated by PI 3-kinase in a number of different cell types. PI 3-kinase has also been shown to be required for MAP kinase activation via the neuropeptide and hormones vasopressin, insulin-like growth factor 1 and insulin (DePaolo et al., 1996, Cross et al., 1994, Uehara et al., 1995).

There are number of mechanisms by which cells may activate the MAP kinase pathway in a PI 3-kinase-dependent manner. PI 3-kinase is known to associate with ras via its p110 catalytic domain leading to co-activation of the MAP kinase signalling pathway. PI 3-kinase may also mediate the activation of the MAP kinase cascade via the serine-threonine protein raf independently of ras. The cytosolic protein raf may associate with the 3'-phosphorylated lipid products of PI 3-kinase via its cysteine rich domain. These lipids are associated with the plasma membrane causing targeting of raf to the plasma membrane where it can associate with other

downstream proteins causing activation of the MAP kinase pathway (Leevers et al, 1994). Another potential mechanism whereby PI 3-kinase may cause raf activation is via association with members of the PKC family. The PKC isoforms ϵ and α have been shown to cause MEK-1 activation via raf (Cai et al., 1997).

King and co-workers (1997) showed that adhesion of COS-7 cells to fibronectin (not poly-L-lysine), caused integrin activation resulting in the accumulation of 3'-phosphorylated phospholipids, as well as activation of PKB, Raf-1, MEK-1 and ERK-1. Addition of either LY294002 or wortmannin caused over a 80% inhibition of these kinases, but was unable to inactivate ras suggesting that PI 3-kinase was present downstream of ras in the MAP kinase signalling cascade when stimulated by integrins. Cross and co-workers (1994) also showed that addition of wortmannin to rat skeletal L6 cells blocked the activation of the MAP kinase pathway between ras and raf. Additionally, previous work by McIlroy and co-workers (1997) has shown that both p70^{s6k} and the MAP kinase signalling pathways are involved in serum stimulated, PI 3-kinase-mediated regulation of DNA synthesis in CHO cells using the selective pharmacological inhibitors rapamycin and PD 098059. Finally Grammer and Blenis (1997) suggested that PI 3-kinase might have a role in the MEK-dependent and -independent activation of the MAP kinase cascade via growth factor stimulation. In kinetic studies, the initial activation of MAP kinase by PDGF was shown to be MEK-dependent and PI 3-kinase-independent. Further studies carried out in Swiss 3T3 cells, suggested that prolonged activation of MAP kinase by PDGF mainly occurred via a MEK-independent mechanism. This stimulation of MAP kinase was however seen to be dependent upon PKC and PI 3-kinase activation. Grammer and Blenis also showed that a number of different cell types (e.g. T-lymphocytes and NIH 3T3 cells) were dependent upon PI 3-kinase stimulation for the activation of MAP kinase.

Initial observations (Moore, unpublished observations) have indicated that serum-free SCLC cell proliferation is partially mediated via a MAP kinase-dependent pathway. In H69 SCLC cells, addition of the MEK1 specific inhibitor PD 098059

(Alessi et al., 1995, Dudley et al., 1995) caused a maximal inhibition of SCLC growth after 8 days of $46 \pm 6 \%$ (mean \pm SEM, $n = 3$), in comparison to cells that received diluent alone (DMSO $< 1\%$ (v/v)). DMSO at a concentration of 1% (v/v) was shown not to effect SCLC growth in liquid culture. The mean IC_{50} obtained from these experiments was $2.7 \pm 2.3 \mu\text{M}$. A similar observation was seen by Seufferlein and Rozengurt (1996a), where a maximally effective concentration of PD 098059 ($20 \mu\text{M}$), inhibited SCLC cell growth in liquid culture by approximately 50% . Previous work by Alessi and co-workers (1995) has shown that PD 098059 is a selective non-competitive inhibitor of MEK1 activation in both whole cell and cell free assay systems. In an *in vitro* cell free cell assay system, PD 098059 was shown to have an IC_{50} between $2 - 7 \mu\text{M}$. To date, a link between the MAP kinase pathway and PI 3-kinase has not been investigated in SCLC cells. However, $p^{42}\text{MAPK}$ activation has been shown to occur via the stimulation of G-protein coupled receptors (Seufferlein & Rozengurt, 1996a); and G-protein coupled receptor-mediated neuropeptide SCLC cell colony formation has been shown to be inhibited by the addition of LY294002 (chapter 4).

The above observations along with the results described in this chapter, indicate that although both the $p70^{\text{s6k}}$ and the MAP kinase cascade have been repeatedly shown to be important in cell growth, the potential upstream enzyme PI 3-kinase maybe critical to SCLC cell growth. It was only on addition of the PI 3-kinase inhibitor LY294002, that SCLC cell proliferation was shown to be fully inhibited.

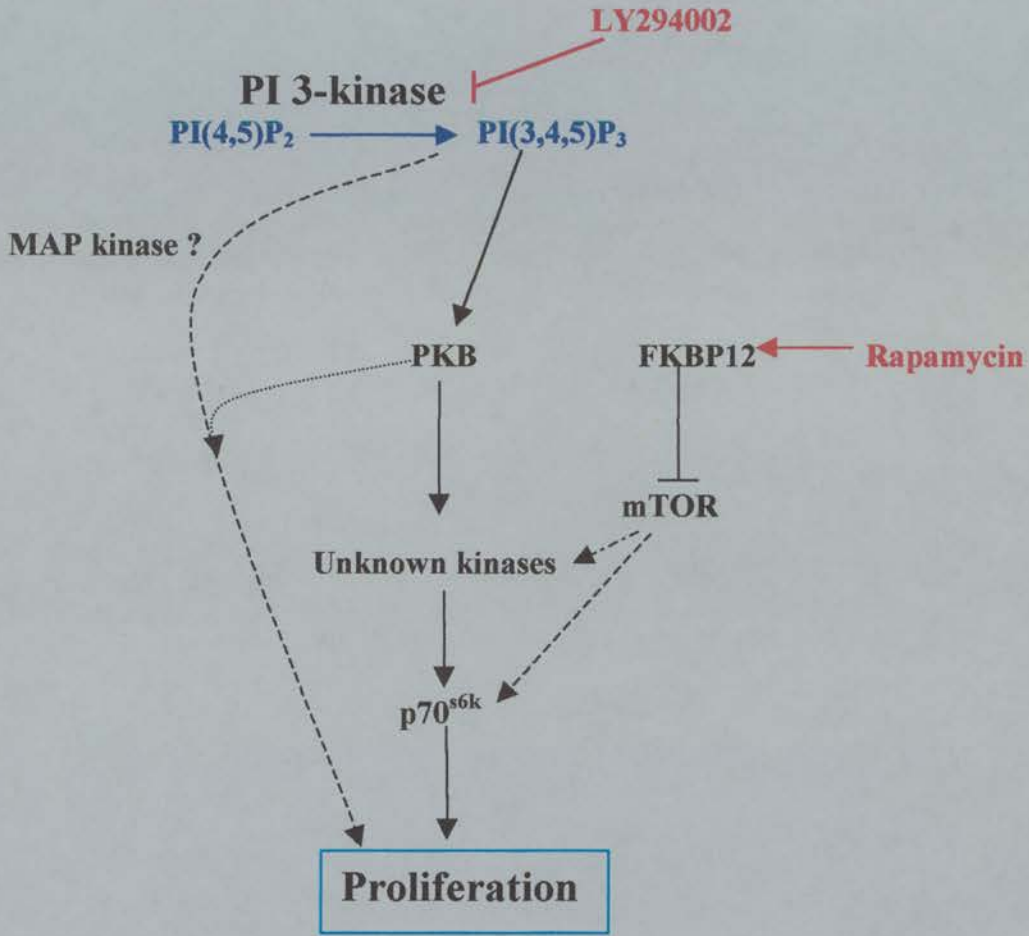


Figure 4.9: Potential Signalling Pathway(s) mediated by Phosphoinositide 3-kinase, Causing Small Cell Lung Cancer Cell Proliferation.

Abbreviations: phosphoinositide 3-kinase, PI 3-kinase, protein kinase B, PKB, MAP Kinase, mitogen activated protein kinase, FK506 binding protein 12, FKBP12 and mammalian target of rapamycin, mTOR.

Finally, studies have shown that there is a positive correlation between SCLC cell cloning efficiency *in vitro* and the invasiveness of SCLC cells *in vivo* (Freedman & Shin, 1974). It appears from the data described in this chapter, that active PI 3-kinase may play a crucial role in sustaining the clonal growth of SCLC cells *in vitro*. From this it can be hypothesised that PI 3-kinase plays a key role in the invasiveness of SCLC cells *in vivo* and that inhibition of this enzyme (shown to be basally active in SCLC cells) may inhibit the tumourigenicity of this highly metastatic tumour *in vivo*. Work performed in breast cancer cells has shown that PI 3-kinase activation is required to enable the cells to migrate on ECM proteins and become invasive in character (Keely et al., 1997, Shaw et al., 1997). Thus, the role of PI 3-kinase in SCLC cell migration and invasion needs to be investigated further.

4.3.2 Cell Survival.

In many cell types (e.g. PC12 cells, MDCK cells and CHO cells), PI 3-kinase has been linked to an anti-apoptotic mechanism of cell survival (Yao & Cooper, 1995 and 1996, Kennedy et al., 1997, Khwaja et al., 1997). The downstream protein kinase PKB has been studied over the past 3 - 4 years in greater detail, and its role in cell survival has become apparent. PI 3-kinase regulation of cell survival appears often to be mediated via PKB (review Marte & Downward, 1997, Hemmings, 1997, Eves et al., 1998, section 1.6.2.1). Conversely, studies performed by Yao and Cooper (1996) noted that serum-independent cells did not require PI 3-kinase activation for protection against apoptosis by growth factor withdrawal.

Results showed that in SCLC cells, a maximally effective concentration of LY294002 (~100% inhibition of PI 3-kinase activity and proliferation (100 μ M)), caused approximately a 400% increase in the basal apoptosis levels of H69 SCLC cells (1.5 – 8% and 3 – 12 % in two separate independent experiments). This modest but consistent increase in apoptosis suggests a small but significant role for PI 3-kinase in basal SCLC cell survival.

Experiments using the phosphotyrosine kinase inhibitor tyrphostin-25, have previously shown that tyrosine kinase activation is required for SCLC cell survival (Tallet et al., 1996a), cell proliferation and clonal growth (Seckl & Rozengurt, 1996). Tyrphostins are a series of compounds that specifically act as competitive inhibitors at the protein tyrosine kinase substrate subsite (Gazit et al., 1989). They selectively inhibit both membrane bound (e.g. EGF and PDGF receptors) (Lyll et al., 1989, Bilder et al., 1991), and cytosolic protein tyrosine kinases (e.g. pp60src) (Anafi et al., 1992, O'Dell et al., 1991). Addition of tyrphostin-25 caused increased SCLC cell apoptosis in comparison to LY294002. This suggests that additional tyrosine kinase enzymes (not present downstream of PI 3-kinase) may also aid in the regulation of basal SCLC cell apoptosis. Additional apoptosis experiments described in the following chapter show that the addition of chemotherapeutic agents such as etoposide and doxorubicin can induce much higher levels of SCLC cell apoptosis. For example etoposide (50 $\mu\text{g/ml}$) and doxorubicin (100 μM), are able to induce apoptosis levels of 75% in H345 and H510 SCLC cells respectively over 3 days (Figure 5.15). Additionally, etoposide (25 $\mu\text{g/ml}$) was seen to induce apoptosis levels of approximately 80% (Figure 5.16) in H69 SCLC cells over 3 days. This data suggests that the inhibition of PI 3-kinase does not effect a major signalling pathway that is required for SCLC cell survival in liquid culture.

An alternative explanation may be that in SCLC cells 'cross talk' between the major signalling cascades involved in the onset of cell apoptosis occurs. If one specific pathway is blocked, alternative signals from other pathways become involved preventing apoptosis. It is only when a number of the major pathways are inhibited that a larger percent of cells undergo apoptosis. This hypothesis relates to the recent observation noted by Murray (1998). Addition of wortmannin (100 nM) over 6 hours did not cause an increase in neutrophil apoptosis levels as assessed by morphology ($2.7 \pm 0.7\%$) (Basal rate $1.7 \pm 0.6\%$). Addition of $\text{TNF}\alpha$ (12.5 ng/ml) over 6 hours caused $7.4 \pm 2.3\%$ apoptosis in neutrophils. The addition of wortmannin and $\text{TNF}\alpha$ in combination, greatly increased neutrophil apoptosis levels ($30.6 \pm 2\%$) (mean \pm SEM, n = 3). This suggested that in neutrophils, inhibition of

PI 3-kinase alone is insufficient to activate an apoptotic signalling pathway. However, in combination with TNF α , the TNF α apoptotic pathway is activated along with the activation of a pro-apoptotic, or suppression of an anti-apoptotic signal causing a marked increase in neutrophil apoptosis.

In addition, the reasoning behind why LY294002 only has a modest effect upon SCLC cell apoptosis levels, but is able to completely inhibit PI 3-kinase activation and SCLC cell growth in liquid culture may be answered by a recent observation by Moore and colleagues (1998). Recent studies (Moore et al., 1998) have shown that addition of LY294002 causes an inhibition in the number of cells undergoing cell division. An increase in the number of cells in the G1 phase of the cell cycle was observed after the cells were incubated with LY294002 over 24 hours. This indicates that the large anti-proliferative effects of LY294002 noted, may be due to a combination of effects; the PI 3-kinase inhibitor is able to cause SCLC cell cycle delay and a modest increase in apoptosis levels. However, due to the presence of constitutively activated PI 3-kinase in SCLC cells, it is unlikely that the cells will ever be in an environment where activity is suppressed *in vivo*. Therefore, SCLC cells will continually proliferate, and this phenomenon may go some way to describing why SCLC has the highest growth rate of the four main types of lung cancer.

Variations occurred in the 'fold increase' in apoptosis observed on the addition of LY294002 throughout the cell survival studies. These variations may have been due to the different experimental techniques applied in studying the cells. Also morphological assessment of apoptosis can be difficult and variations may have occurred due to more than one person counting the cytocentrifugation slides. (This allowed us to say that although the increase was small it was however consistent.) Basal alterations in cell apoptosis also may have played a part in causing slight inter-experimental variations.

4.4 Summary.

SCLC cells are known to be able to survive and grow in the absence of exogenous growth factors. However, SCLC cell proliferation *in vitro* requires the activation of PI 3-kinase. This proliferation pathway signals via a p70^{s6k}-dependent and -independent mechanism, with the majority of the signal bypassing p70^{s6k}. As PI 3-kinase is constitutively activated in SCLC cells, they are able to continually grow and this may account for the rapid doubling times seen in SCLC cells.

In this chapter it was demonstrated that constitutive PI 3-kinase activity regulates proliferation, tumorigenicity and in part apoptosis. Recent studies by Shaw and co-workers (1997) and Keely and co-workers (1997), indicate that PI 3-kinase activation induces integrin-mediated cell motility and invasiveness. Given that serum- and anchorage-independent survival are important in the maintenance of a transformed phenotype, and to the ability of a cell to metastasis within the body, an understanding of the mechanisms regulated by this constitutive PI 3-kinase activity could be of benefit in designing novel therapies for the treatment of SCLC.

CHAPTER 5

Extracellular Matrix Proteins: Protection of Small Cell Lung Cancer Cells from Chemotherapy-Induced Apoptosis.

5.1 Introduction.

Studies have shown that after primary chemotherapy treatment, the majority of SCLC patients relapse within 1 year. On relapse, SCLC cells become resistant to further chemotherapy treatment, thus the patient two year survival rate remains less than 5% (Smyth et al., 1986). The mechanisms underlying why SCLC cells become resistant to chemotherapy are unclear. It is now thought that chemotherapy reagents exert their cytotoxic effects by causing cells to undergo apoptosis (Hannun, 1997, Walker et al., 1991, Kaufmann, 1989). Therefore, it may be hypothesised that after primary treatment, SCLC cells become resistant to chemotherapy-induced apoptosis. In view of the fact that tumour mass is a balance between cell proliferation and cell death (apoptosis and necrosis), the factors effecting this balance will have a profound effect on tumour growth.

In untransformed epithelial and endothelial cells, cell adhesion to ECM proteins has been shown to protect the cells from undergoing apoptosis (Meredith et al., 1993, Boudreau et al., 1995, Frisch & Francis, 1994). The integrin family of receptors are

the major extracellular matrix receptor. On activation, integrins stimulate a large number of intracellular signalling processes including tyrosine phosphorylation, cell migration, cell survival and regulation of the cell cycle (Hynes, 1992, Guadagno et al., 1993, Khawaja et al., 1997). Tumour cells are known to adhere to different ECM proteins (Terranova et al., 1982). This adherence (via integrins) has been shown to cause the activation of a number of intracellular signalling pathways including the stimulation of cell migration on the ECM proteins and cell invasion (Shaw et al., 1997, Keely et al., 1997, Bredin et al., 1998, Shibata et al., 1997).

In view of the above information, studies were performed to investigate if ECM proteins were expressed within the local environment of SCLC cells *in vivo*, and to examine if this local micro-environment could provide an anti-apoptotic signal, or suppress an apoptotic signal resulting in SCLC cell resistance to chemotherapy.

5.2 Results.

5.2.1 Expression of Extracellular Matrix Proteins *In Vivo*.

5.2.1.1 The Presence of Increased Amounts of Extracellular Matrix Proteins around Small Cell Lung Cancer Cells *In Vivo*.

SCLC lung sections were stained using an immunohistochemical technique (section 2.8) for the presence of ECM proteins laminin, fibronectin, collagen IV and tenascin. Normal areas of the lung showed staining for laminin and collagen IV in the basement membranes of alveoli, blood vessels and bronchial glands as expected. Fibronectin stained weakly positive in a diffuse fashion throughout the pulmonary interstitium without accentuation along the basement membrane (Figure 5.1a). The ECM protein tenascin was not present in normal lung tissue.

In SCLC lung tissue specimens, ECM protein staining fell under three categories, either no staining, extensive staining (> 50% of section area) or focal staining. Extensive and focal areas of dense staining were observed for collagen IV, fibronectin and tenascin (Figure 5.1a & b). In summary, between 90 and 100% of the tissue specimens examined (23 specimens) had either focal or extensive staining for fibronectin, collagen IV or tenascin (Sethi et al., 1999). Staining for these three ECM proteins was mainly observed in areas of patient reactive tissue. This was seen as extensive areas of scarring or as stromal bands delineating packets of SCLC tumour cells. SCLC lung sections also possessed thickened alveolar basement membranes in comparison to normal lung tissue. These membranes showed increased expression of laminin, fibronectin and collagen IV.

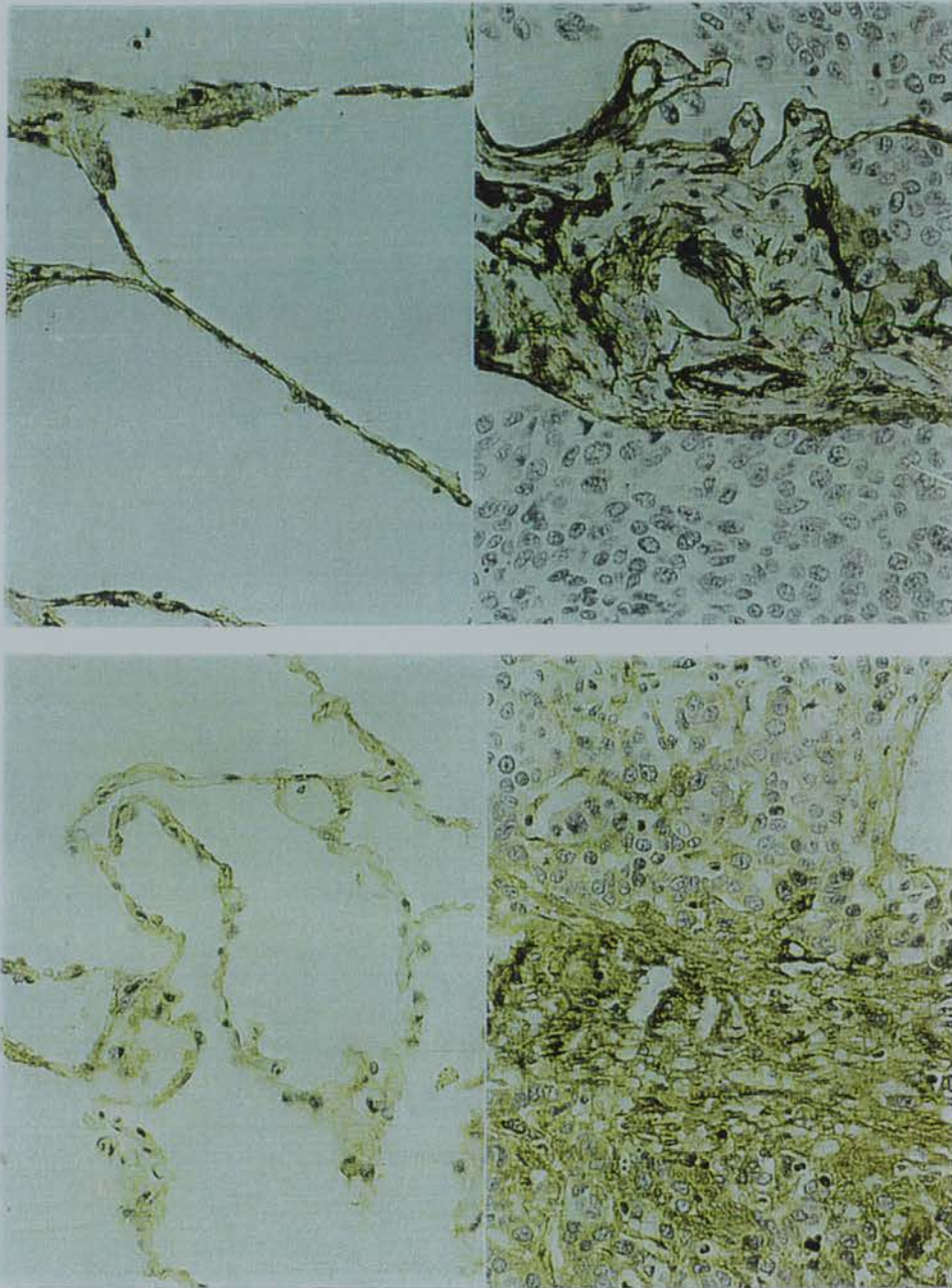


Figure 5.1a: Photographs of Small Cell Lung Cancer and Normal Lung Sections Stained for Collagen IV (upper) and Fibronectin (lower).

Lung sections of normal (left) and SCLC (right) lung tissues were stained using immunohistochemical techniques (section 2.8) (primary antibodies 1: 100 dilution). Brown staining shows strong immunoreactivity and marks the presence of the extracellular matrix protein being studied. (x40 magnification.)

Sections were processed by Dr R.C. Rintoul.

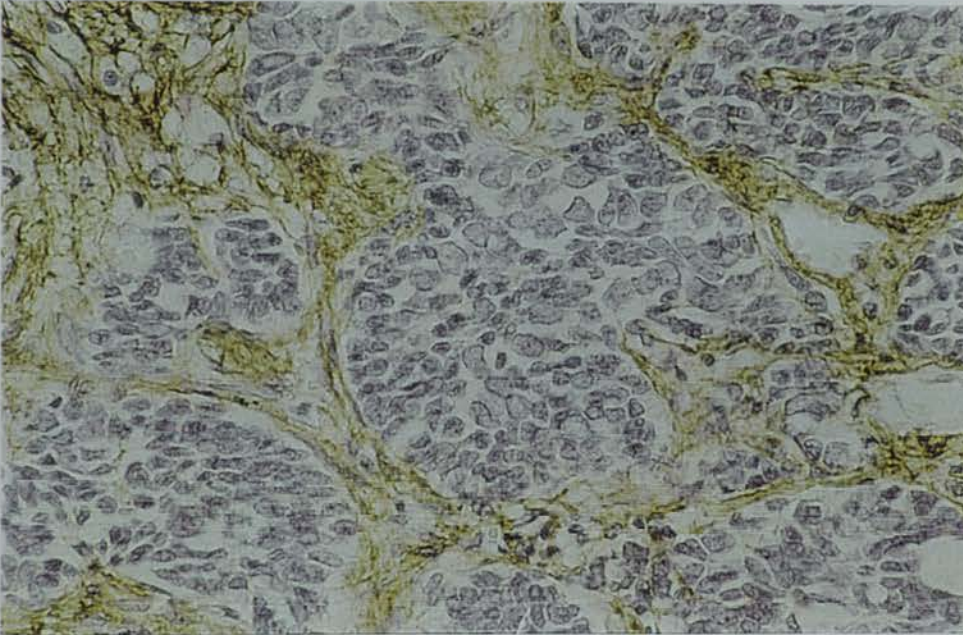
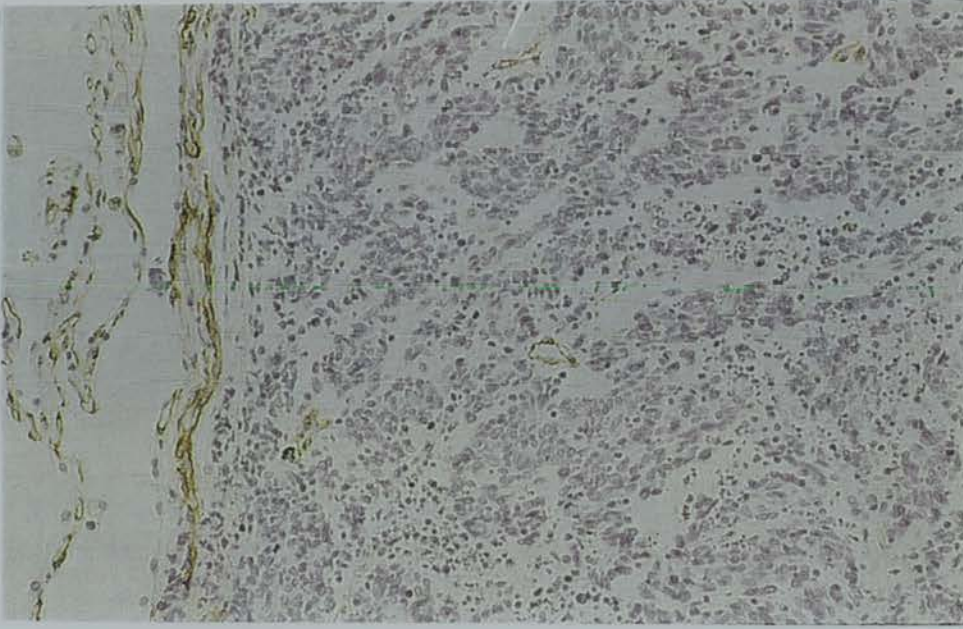


Figure 5.1b: Photographs of Small Cell Lung Cancer Lung Sections Stained for Laminin (upper) and Tenascin (lower).

SCLC lung tissues were stained using immunohistochemical techniques (section 2.8) for the presence of laminin (upper) and tenascin (lower) (primary antibodies 1: 100 dilution).

Brown staining shows strong immunoreactivity and marks the presence of the extracellular matrix protein being studied.

Sections were processed by Dr R.C. Rintoul.

Further results highlighted by Sethi and colleagues (1999), showed that a proportion of the SCLC cells in the SCLC lung tissue samples stained positive for either fibronectin or laminin (26% and 16% respectively). This observation suggested that a percentage of the cells were able to synthesise and potentially secrete both laminin and fibronectin into the surrounding tissue. Thus, *in vivo*, SCLC cells were shown to be surrounded by an ECM rich micro-environment. The physiological relevance of this observation was examined further.

5.2.2 Regulation of the Adhesion of Small Cell Lung Cancer Cells to Extracellular Matrix Proteins *In Vitro*.

5.2.2.1 Adherence of Small Cell Lung Cancer Cells to Extracellular Matrix Proteins *In Vitro* is Mediated via a $\beta 1$ Integrin-dependent Mechanism.

Adhesion of cells to ECM proteins is widely accepted to occur via the binding of integrin dimers to specific polypeptide sequences on proteins. To clarify the presence of different integrin subunits on the SCLC cell lines H69 (Figure 5.2), H345 and H510, the relative expression of the integrin subunits was assessed by flow cytometry (section 2.12). As shown previously by Falcioni and colleagues (1994) and Feldman and colleagues (1991), high expression of the $\beta 1$ integrin subunit was observed in comparison to the other β integrin subunits examined (Table 5.1).

When studying the α subunits (Table 5.1), $\alpha 3$ was highly expressed on all three cell lines. Similar levels of αv were observed in the three cells lines tested. $\alpha 6$ expression was significantly elevated in all three SCLC cell lines examined in comparison to background fluorescence levels. Only H510 cells expressed significant amounts of the $\alpha 2$ subunit. Expression levels of all the other α subunits examined were similar to background control levels (2.4 ± 0.2 AU). In conclusion, the SCLC cell lines predominately expressed integrin dimers that were involved in the binding of laminin ($\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 1$), fibronectin ($\alpha 3\beta 1$ and $\alpha v\beta 1$) and

collagen ($\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 1$) (Hynes, 1992, Elices et al., 1991, Vogel et al., 1990).

Table 5.1: Integrin Subunit Expression on Small Cell Lung Cancer Cell Lines H69, H345 and H510.

Integrin subunit	Relative Expression Values (AU)		
	H69	H345	H510
$\beta 1$	30.8 ± 3.5	17.1 ± 1.4	42.3 ± 3.9
$\beta 2$	5.0 ± 0.3	1.4 ± 0.2	4.2 ± 0.4
$\beta 3$	6.8 ± 0.5	6.8 ± 0.6	4.5 ± 0.7
$\beta 4$	6.7 ± 0.9	4.6 ± 0.5	6.0 ± 0.1
$\beta 5$	5.6 ± 0.8	3.8 ± 0.2	6.8 ± 0.6
$\beta 6$	5.6 ± 0.6	5.9 ± 0.4	2.5 ± 0.1
$\alpha 1$	4.3 ± 0.2	6.1 ± 0.5	4.9 ± 0.8
$\alpha 2$	2.3 ± 0.3	7.1 ± 0.6	17.4 ± 2.2
$\alpha 3$	36.4 ± 2.5	38 ± 2.2	30 ± 1.8
$\alpha 4$	5.7 ± 0.4	6.5 ± 0.3	3.2 ± 0.2
$\alpha 5$	3.0 ± 0.2	2.4 ± 0.4	4.4 ± 0.4
$\alpha 6$	32.4 ± 2.8	22.5 ± 2.5	17.1 ± 2.9
αv	20.6 ± 3.1	14.7 ± 2.8	26.3 ± 1.2

Expression of the different integrin subunits was determined by flow cytometric analysis using an indirect immunofluorescent technique (section 2.12). Each value represents the mean fluorescence intensity (AU – arbitrary units) \pm SEM of 3 – 4 independent experiments carried out in duplicate. (Background control AU = 2.4 ± 0.2)

In response to the above observations, all SCLC cells lines were shown to adhere to laminin, fibronectin and collagen IV in a dose dependent manner (Figure 5.3). The EC_{50} values for the three ECM proteins were 20 $\mu\text{g/ml}$ (fibronectin), 10 $\mu\text{g/ml}$ (laminin) and 12 $\mu\text{g/ml}$ (collagen IV) in H69 SCLC cells.

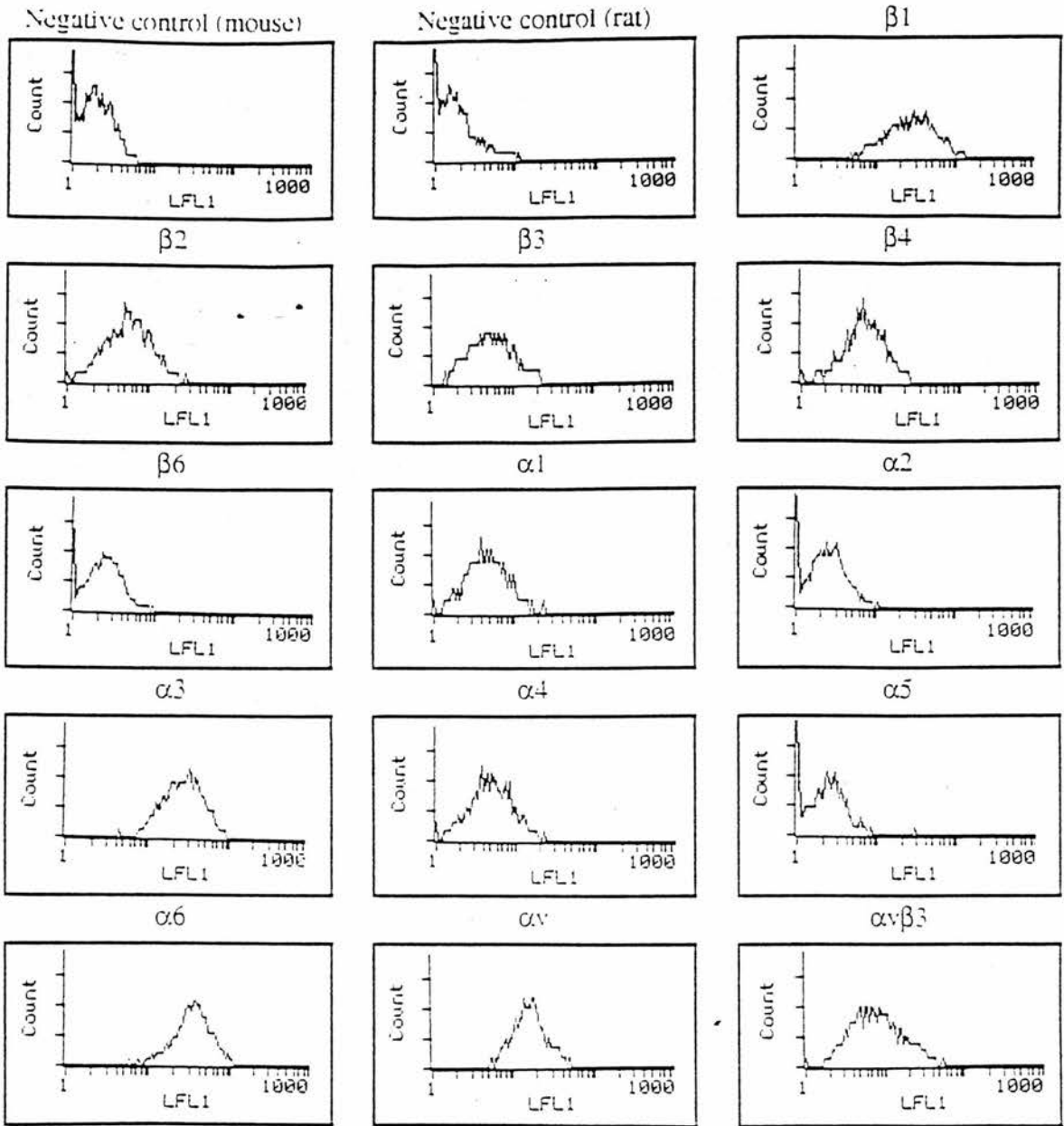


Figure 5.2: Representative Examples of Integrin Profiles as Assessed by Flow Cytometry for Small Cell Lung Cancer Cell Line H69.

Cells (1×10^6 /ml, 100 μ l/well) were washed in PBS containing azide (EPICS buffer) and incubated sequentially with saturating concentrations of the primary integrin antibodies (1: 500 dilution) for 30 minutes on ice. Cells were washed in EPICS buffer and then incubated with species-specific FITC conjugated secondary antibodies (1: 25 dilution) for 30 minutes on ice. Labelled cells were examined by flow cytometric analysis. The above histograms are representative of a single experiment carried out 3 – 4 times in each cell line (H69, H345 and H510).

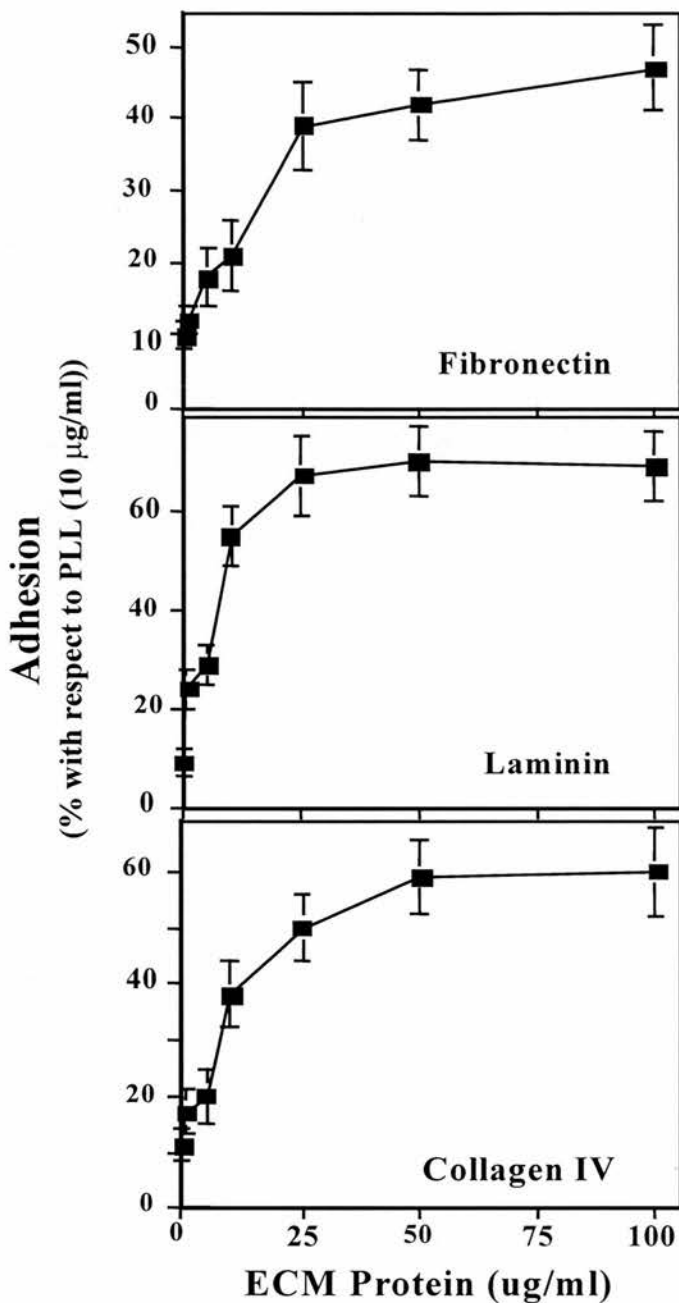


Figure 5.3: Adhesion of H69 Small Cell Lung Cancer Cells to Different Extracellular Matrix Proteins.

Cells (5×10^5 /ml, 100 μ l/well) were washed in RPMI medium and plated on culture dishes previously coated with increasing concentrations of ECM proteins. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂/95% air for a period of 40 minutes before the non-adherent cells were removed. The remaining cells were fixed (3% formaldehyde) and stained with 1% methylene blue. Residual dye was removed and the cells lysed to release the internal stain. The absorbance values were read at 630 nm, and the relative number of adhered cells was calculated in relation to cells adhered to 10 μ g/ml of poly-L-lysine (PLL) (taken to be 100% adherence). Each graph represents the mean (\pm SEM) of 3 – 6 independent experiments carried out in duplicate.

Further experimentation confirmed that the SCLC cells adhered to the different ECM proteins in a $\beta 1$ integrin-dependent manner. Addition of a function $\beta 1$ -blocking antibody (P5D2) to the SCLC cells before plating them on to ECM coated microtitre plates (section 2.9), prevented the cells from adhering (Figure 5.4). The non-specific adhesion of SCLC cells to poly-L-lysine (PLL) and BSA was unaffected. Therefore, although SCLC cells are known to be anchorage-independent *in vitro*, they are able to adhere to different ECM proteins in a $\beta 1$ integrin-dependent manner.

Residual adherence of the SCLC cells to collagen IV was noted in the presence of the $\beta 1$ -function blocking antibody. This may be due to the SCLC cells being able to bind to collagen IV in a non- $\beta 1$ -mediated fashion, or due to contaminants in the collagen IV (extracted from human placenta (Sigma, UK)).

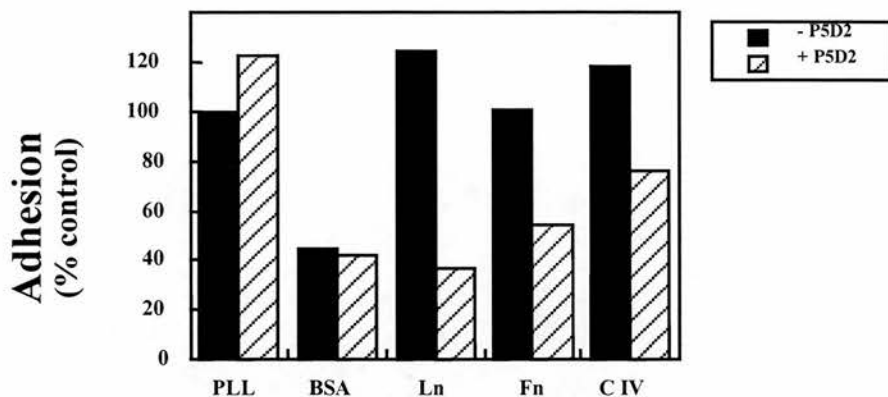


Figure 5.4: Effect of the $\beta 1$ Integrin Function Blocking Antibody (P5D2) on H510 Small Cell Lung Cancer Cell Adhesion to Different Extracellular Matrix Proteins.

H510 cells (5×10^5 /ml, 100 μ l/well) were added to BSA blocked (1 mg/ml BSA, 1 hour, 37°C) microtitre plates which were previously coated with different extracellular matrix proteins overnight at 4°C (50 μ l/well). Cells were added to the 96 well microtitre plate in the presence and absence of the $\beta 1$ function blocking antibody P5D2 (10 μ g/ml). Cells were allowed to adhere for 45 minutes prior to the removal of non-adhered cells and fixing of the remaining cells using 3% formaldehyde. Adhered cells were stained with 1% methylene blue, lysed and analysed using a plate reader at 630 nm. Absorbance of poly-L-lysine (in the absence of P5D2) (25 μ g/ml) was taken to be equal to 100% adhesion (control). Each bar is representative of the mean one experiment performed 3 – 4 times in duplicate. Abbreviations: - poly-L-lysine (25 μ g/ml), PLL, Bovine Serum Albumin (1 mg/ml), BSA, Laminin (10 μ g/ml), Ln, fibronectin (20 μ g/ml), Fn, collagen IV (10 μ g/ml), C IV.

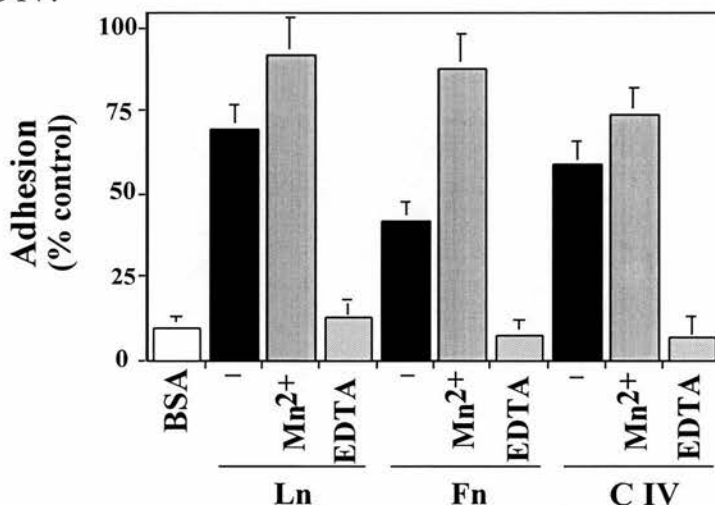


Figure 5.5: Effect of the Divalent Cation Manganese and EDTA on H69 Small Cell Lung Cancer Cell Adhesion to Extracellular Matrix Proteins.

As described above, cells were incubated on pre-coated microtitre plates in the presence of Manganese (Mn^{2+}) (1 mM) or EDTA (1.8 mM). Cells were allowed to adhere for 45 minutes prior to washing, fixing and staining. Once lysed, the percentage of cells adhered to the wells was assessed colourmetrically in comparison to SCLC cells adhered to poly-L-lysine (10 μ g/ml) (control). Each bar represents the mean (\pm SEM) of 3 – 6 independent experiments carried out in duplicate. Abbreviations: - Laminin (10 μ g/ml), Ln, Fibronectin (20 μ g/ml), Fn, collagen IV (10 μ g/ml), C IV.

5.2.2.2 The Adhesion of Small Cell Lung Cancer Cells to Different Extracellular Matrix Proteins is Dependent on the Presence of Functional Cations.

The cation-dependence of integrins has been studied by many groups (reviewed Humphries, 1996). The cations magnesium and manganese have been implicated in the stimulation of ligand binding, whereas calcium has been seen to have an inhibitory effect. Studies have found that the majority of integrins contain a number of cation binding sites (these differ between integrins), which exhibit distinct preferences to the different types of cations available.

To investigate the role of cations in SCLC cell adhesion to ECM proteins, cells were co-incubated with the divalent cation manganese and the cation chelator EDTA. Figure 5.5 demonstrates that the adhesion of H69 SCLC cells to ECM protein laminin, collagen IV and fibronectin increased in the presence of manganese (1 mM). Addition of EDTA (1.8 mM), inhibited integrin-induced adhesion of SCLC H69 cells to the ECM proteins (Figure 5.5). These experiments were carried out in quiescent cell culture medium (RPMI 1640, 25 mM HEPES, Sigma, UK.) which contains both calcium (0.65 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$) and magnesium (0.54 mM $\text{Mg}(\text{SO}_4) \cdot 7\text{H}_2\text{O}$). This enabled the cells to adhere in the presence of no additional cations (-). Addition of manganese to the cell culture medium enhanced the binding ability of the SCLC cells to the different ECM proteins. This may have been due to changes in the different integrin heterodimer preferences and affinities to the manganese cations in comparison to the magnesium cations already present.

These observations were in agreement with the previous studies performed by Elices and co-workers (1991). These studies highlighted the different affinities displayed by the $\alpha 3\beta 1$ integrin on binding to fibronectin in the presence of calcium (low binding), magnesium (6.6 fold increase) and manganese (30 fold increase). Binding of the H69 SCLC cells to collagen I was shown to decrease in the presence of calcium. Addition of magnesium or manganese caused a 1.2 and 1.7 fold increase in

$\alpha 3\beta 1$ binding respectively. All cations caused optimal binding at a concentration between 0.5 - 1 mM.

5.2.2.3 Inhibition of Specific Intracellular Signalling Molecules has No Effect on Small Cell Lung Cancer Cell Adhesion to Extracellular Matrix Proteins.

To investigate if the inhibition of intracellular signalling proteins or cytoskeletal structure organisation could effect SCLC cell adhesion to ECM proteins, various enzyme/protein inhibitors were added to cell cultures prior to, and during the adhesion assay. PI 3-kinase and tyrosine kinase activation has been shown to be elevated on cell adhesion to different ECM proteins (Schwartz, 1997). To investigate if the stimulation of these intracellular proteins was a requirement for cell adhesion, or a consequence of cell adhesion, cells were incubated in the presence of specific enzymic inhibitors and plated onto different ECM proteins. Results indicated that the addition of the PI 3-kinase inhibitor wortmannin (100 nM), and the tyrosine kinase inhibitors tyrphostin-25 (25 μ M) and genistein (100 μ M) did not effect ECM induced SCLC cell adhesion (Figure 5.6).

To investigate if a functional actin cytoskeleton was required to allow the SCLC cells to adhere, SCLC cells were incubated in the presence of the inhibitor cytochalasin D. Cytochalasin D causes capping of the f-actin filaments and therefore prevents actin polymerisation. Pre-incubation of the SCLC cells with cytochalasin D (200 μ M) was unable to prevent the cells from adhering to the ECM proteins.

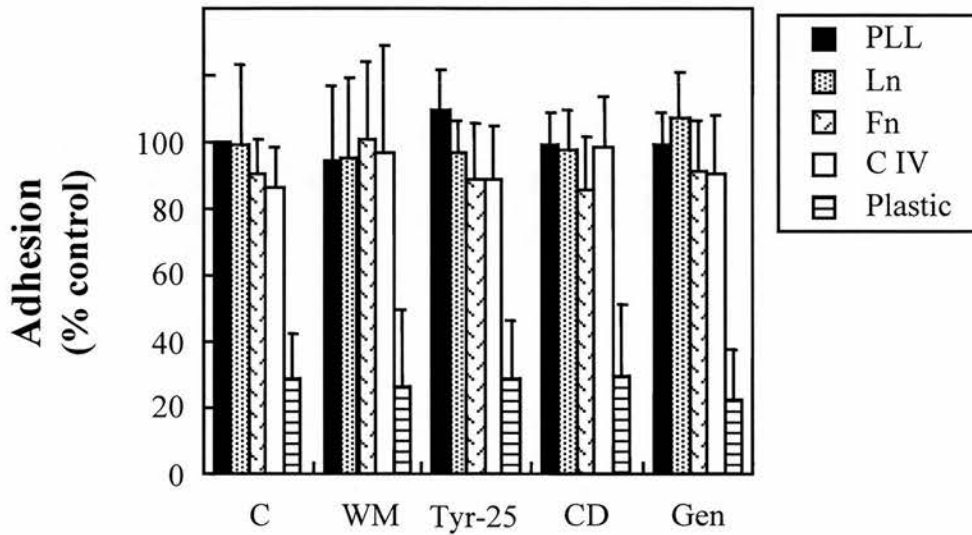


Figure 5.6: Adhesion of H69 Small Cell Lung Cancer Cells to Different Extracellular Matrix Proteins in the Presence of Various Inhibitors.

Cells were assessed for adhesion to different matrices poly-L-lysine PLL (25 $\mu\text{g/ml}$), laminin Ln (10 $\mu\text{g/ml}$), fibronectin Fn (20 $\mu\text{g/ml}$), collagen IV C IV (10 $\mu\text{g/ml}$) and plastic as described in section 2.9. Before, and during the washing and assaying procedure, cells were incubated in the presence of either no inhibitor (C), wortmannin, WM (100 nM) (1 hour before plating), tyrphostin-25, Tyr-25 (25 μM) (24 hours prior to plating), cytochalasin D, CD (20 μM) (1 hour before plating) or genistein, Gen (100 μM) (24 hours prior to plating). Results are expressed as the percentage change in adhesion in comparison to poly-L-lysine (25 $\mu\text{g/ml}$, 100% adhesion (control)), in the presence of diluent alone (DMSO < 0.01% (v/v)). Each bar is representative of the mean (\pm SEM) of four independent experiments carried out in duplicate.

5.2.3 Morphological Alterations in Small Cell Lung Cancer Cells on Adhesion to Extracellular Matrix Proteins.

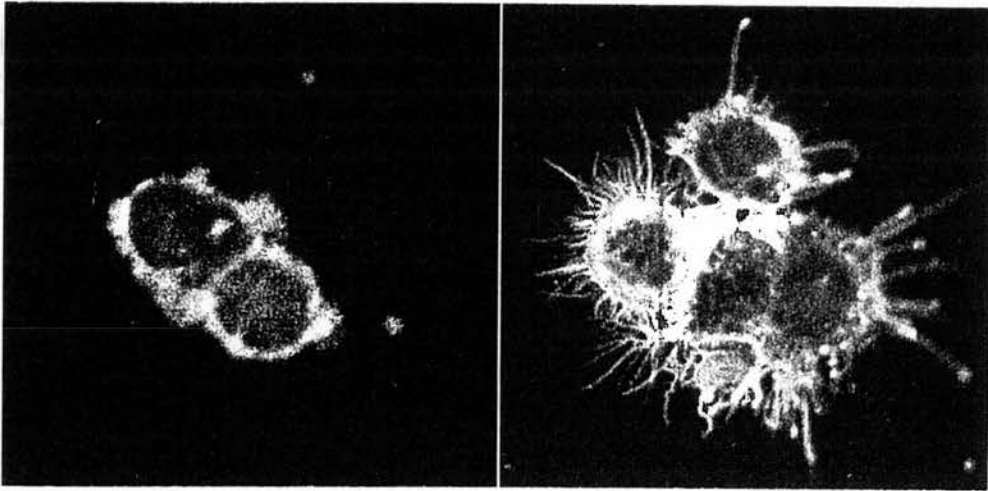
Whilst studying the adhesive capabilities of the SCLC cells, the adherence of H69 (and H345) SCLC cells to both laminin (Figure 5.7) and collagen IV (Figure 5.8) was shown to induce the formation of membrane ruffles and microspikes. This change was noticeable after 40 minutes. The ability of the SCLC cells to spread and produce neurite-like processes (microspikes) was unable to occur on non-specific cell adhesion to poly-L-lysine (Figure 5.7). Immunostaining of f-actin using rhodamine phalloidin showed the presence of a large number of actin filaments within the cells. H510 SCLC cell spreading was much less pronounced on adherence to laminin or collagen IV in comparison to H69 and H345 SCLC cells.

The addition of cytochalasin D prevented the cells from forming membrane ruffles and microspikes (Figure 5.8). This observation was in agreement with previous studies in other cell types (particularly fibroblasts). It has been shown that cell protrusions such as membrane microspikes or lamellipodia are dependent upon the dynamic remodelling of the actin cytoskeleton.

The presence of the tyrosine kinase inhibitor genistein also prevented the formation of the large microspikes and membrane ruffles (Figure 5.9). Cells adopted a more rounded morphology as though they were unable to flatten out along the ECM. As the intracellular protein Rho is known to cause the production of microspikes (Nobes & Hall, 1995) and regulate genistein-sensitive proteins (Ridely & Hall, 1994), this observation was not surprising.

In summary, the data shows that SCLC cell lines H345 and H69 adhere to laminin and collagen IV in a $\beta 1$ integrin-dependent manner causing the cells to flatten and spread on the ECM proteins. Cell spreading was observed in the formation of membrane ruffles, and microspikes (Figure 5.7). Activated tyrosine kinases and a mobile actin cytoskeleton are required to enable the SCLC cells to produce these

neurite-like processes. However, further investigations are required before the functional relevance of these morphological alterations has been properly established.



Poly-L-lysine

Laminin

Figure 5.7: Confocal Image of Small Cell Lung Cancer Cells Adhered to Poly-L-lysine (Left) and Laminin (Right).

H69 SCLC cells were incubated in SITA medium before being gently disaggregated and allowed to adhere to cover slips (previously coated with either poly-L-lysine (10 $\mu\text{g/ml}$) or laminin (10 $\mu\text{g/ml}$) overnight at 4°C). Cells were allowed to adhere for 45 minutes before gently aspirating off any unadhered cells and adding fresh SITA medium. The cells were then allowed to adhere and spread for a total of 4 hours before being fixed, permeabilised and stained using rhodamine phalloidin (1 : 400) to visualise the actin structure within the cell. Confocal imaging was used to visualise the actin structure near the base of the cells where they adhered to the coating matrix. Each picture is representative of a single independent experiment carried out at least 10 times.

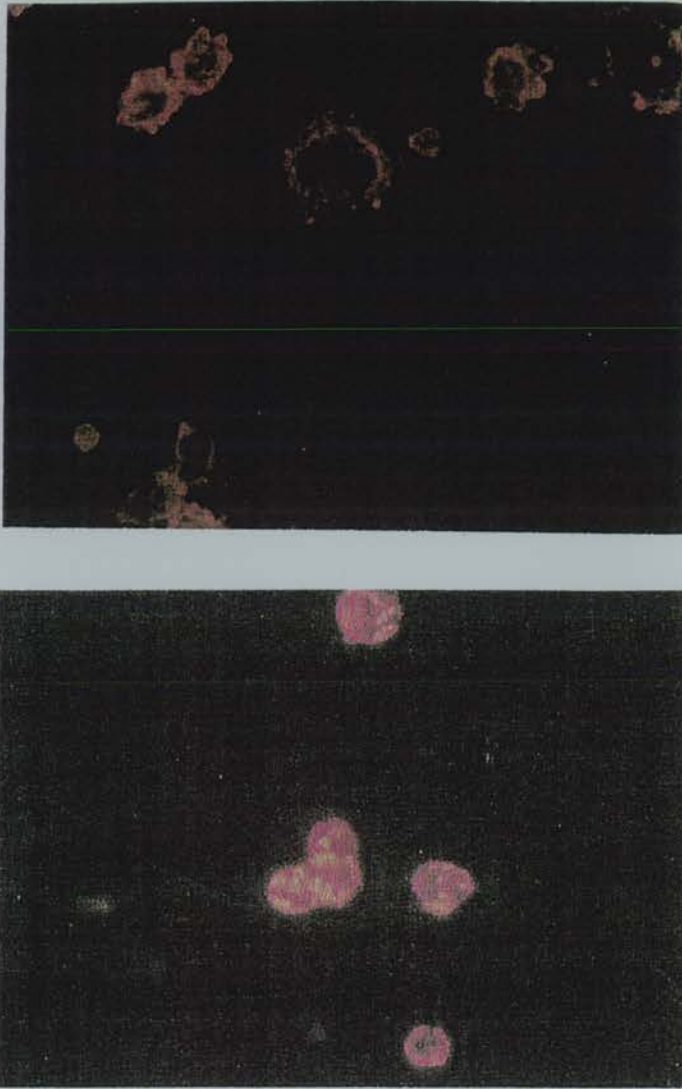


Figure 5.8: Immunofluorescence Image of Small Cell Lung Cancer Cells H69 Adhered to Collagen IV in the Presence of Cytochalasin D.

SCLC cells were incubated in small sterile culture flasks in the absence or presence of the inhibitor cytochalasin D (200 μM) for one hour. Cells were then washed, disaggregated and plated onto the coverslips pre-coated in collagen IV (10 $\mu\text{g}/\text{ml}$) in SITA medium in the absence (upper) and presence (lower) of cytochalasin D. Cells were allowed to adhere for 45 minutes before non-adhered cells were gently aspirated off and the remaining cells allowed to attach and spread for a total of 4 hours in the absence or presence of cytochalasin D (200 μM). After 4 hours, coverslips were washed, fixed, permeabilised and stained using rhodamine phalloidin (1: 400). Cells were examined under fluorescence microscopy (BH2 Olympus microscope). Image taken using a x 40 objective. Each photograph is representative of single independent experiments carried out 4 times in SCLC cells lines H345 and H69.

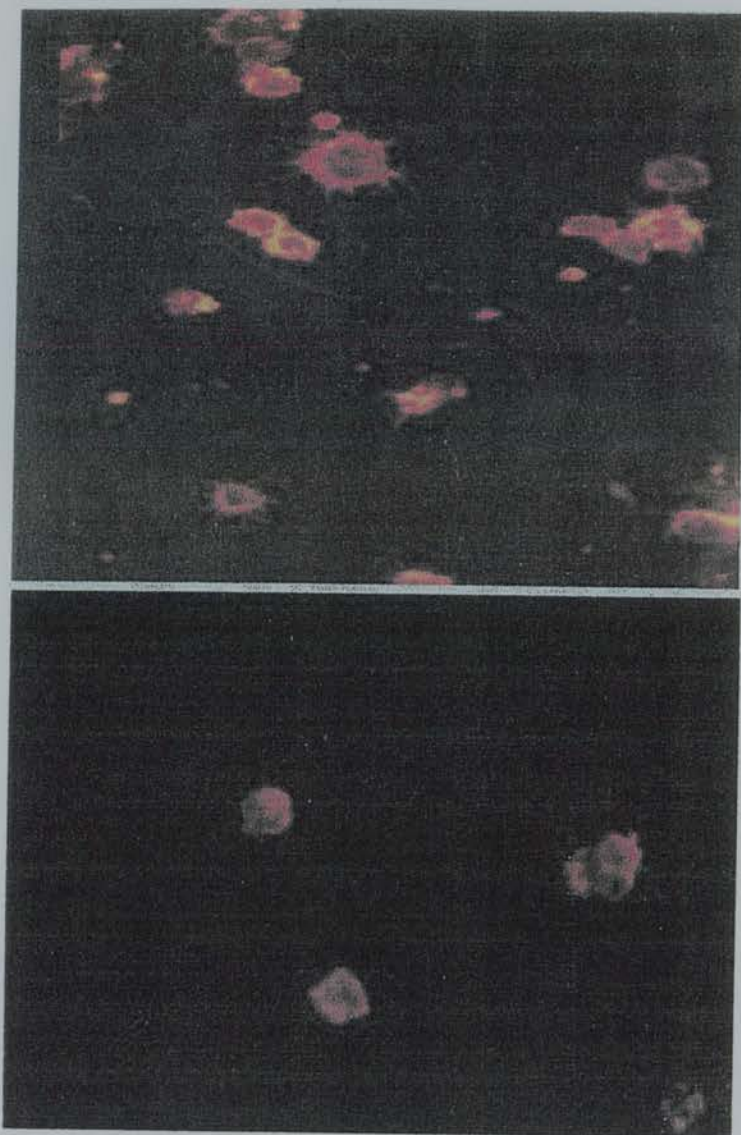


Figure 5.9: Immunofluorescence Images of H345 Small Cell Lung Cancer Cells Adhered to Laminin in the Absence (upper) or Presence (lower) of phosphotyrosine Kinase Inhibitor Genistein.

Cells in SITA medium containing either genistein (100 μ M) or diluent only, were incubated in sterile culture flasks for 24 hours in a humidified atmosphere 5% CO₂/95% air at 37°C. Cells were allowed to adhere (in the presence/absence of genistein) to coverslips pre-coated with laminin (10 μ g/ml) for 45 minutes in a humidified atmosphere 5% CO₂/95% air at 37°C. Non-adhered cells were gently removed and the remaining cells incubated for a total of 4 hours in fresh SITA in the absence (upper) or presence (lower) of genistein (100 μ M). Cells were fixed and permeabilised, before being stained with rhodamine phalloidin (1: 400 dilution) and visualised by immunofluorescent microscopy. Images were photographed using a x 40 objective lens (BH2 Olympus microscope). Results are representative of a single independent experiment performed 4 times.

5.2.4 Increase in Phosphoinositide 3-kinase Activity on Adhesion to Laminin.

The protein PI 3-kinase has been shown to be activated on cell adhesion to different ECM proteins (King et al., 1997, Khwaja et al., 1997, Chen et al., 1996, Wary et al., 1996). The attachment of MDCK cells to matrix led to the rapid elevation of PI 3-kinase lipid products (Khwaja et al., 1997). COS-7 cells were also seen to adhere to the matrix protein fibronectin causing increased PI 3-kinase activity (King et al., 1997). Additionally, Shaw and co-workers (1997) showed that PI 3-kinase activity was required to enable breast carcinoma cells to invade surrounding tissue. In view of the fact that SCLC cells are highly metastatic and are able to adhere to different ECM proteins, experiments were performed to investigate if SCLC cell adhesion to laminin was able to further increase SCLC cell constitutive PI 3-kinase activity.

Adhesion of the SCLC cells to the ECM protein laminin was shown to cause an increase in basal PI 3-kinase activity levels ($143 \pm 83\%$ (H69, mean \pm SEM, $n= 3$) and $128 \pm 25\%$ (H510, mean \pm S.D., $n = 2$), Figure 5.10 preliminary observations). Addition of the PI 3-kinase inhibitors wortmannin and LY294002 was still able to inhibit PI 3-kinase activity to below basal levels.

These observations indicate that although SCLC cells are anchorage-independent and the enzyme PI 3-kinase is constitutively activated within the cells, adhesion to laminin (via a $\beta 1$ integrin-dependent mechanism) causes PI 3-kinase activity levels to be elevated further.

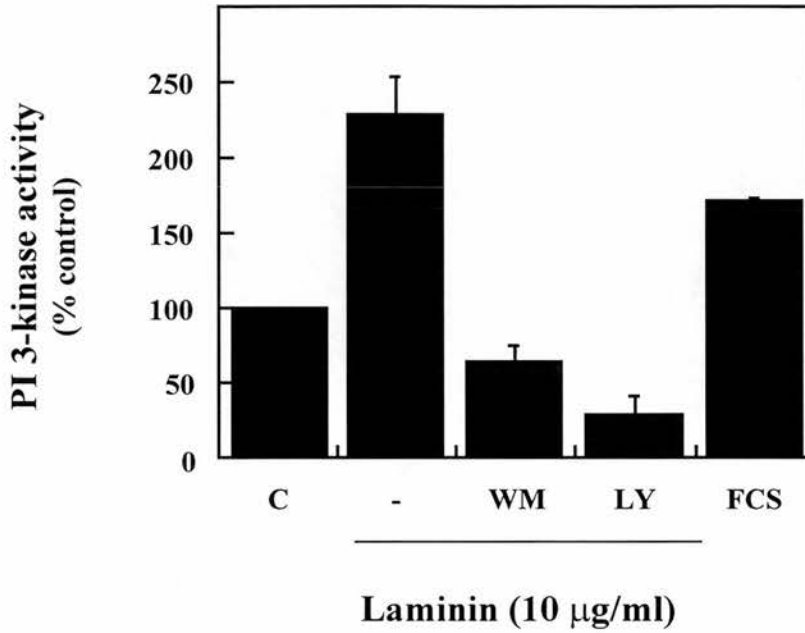


Figure 5.10: Phosphoinositide 3-kinase Activity in Small Cell Lung Cancer Cell Line H510 on Adherence to Laminin, in the Presence or Absence of Wortmannin and LY294002.

Quiesced H510 SCLC cells (approximately 8×10^6 /ml) were washed in warmed PBS before being disaggregated into an essentially single cell population. Cells were allowed to adhere to 12 well culture dishes pre-coated with laminin (10 μg/ml, 500 μl/well, overnight at 4°C) or no coating matrix (control, C) for a total of 40 minutes. Cells were incubated in the presence of wortmannin (WM, 100 nM, 20 minutes) and 10% (v/v) foetal calf serum (FCS, 5 minutes). Cells were lysed in PI 3-kinase lysis buffer before the assay was performed as previously described in section 2.3.1a. As with previous PI 3-kinase experiments LY294002 (LY, 100 μM) was only added immediately prior to carrying out the actual *in vitro* kinase assay.

Results are expressed as the mean percentage PI 3-kinase activity with respect to control cells (plated in the absence of laminin and received diluent alone (DMSO < 0.001% (v/v))). Each bar represents the mean (\pm S.D.) of two independent experiments carried out in duplicate.

5.2.5 The Effect of Small Cell Lung Cancer Cell Adhesion on Cell Growth and Tumourigenicity.

5.2.5.1 The Effects of Small Cell Lung Cancer Adhesion to Extracellular Matrix Proteins on Cell Proliferation.

Whilst the adhesion of SCLC cells to laminin and other ECM proteins has been reported by a number of groups, its relationship with SCLC cell growth is unclear. Initial experiments were undertaken to corroborate previous findings by Giaccone and co-workers (1992a) and Fridman and co-workers (1990). Integrin-mediated adhesion on its own is said not to be sufficient to induce cell mitogenesis. Giaccone and co-workers (1992a), reported no change in the proliferation doubling times of SCLC cells plated on laminin by MTT assay, cell counting and [³H]-thymidine incorporation. Fridman and co-workers (1990) also noted that adhesion of SCLC cells to laminin did not alter the growth rate of the cells in both MTT and [³H]-thymidine assays. Nakanishi (1988) demonstrated that elevated levels of laminin (30 µg/ml) actually had an inhibitory effect on SCLC cell proliferation.

Preliminary studies indicated that the adhesion of H510 SCLC cells to laminin (10 µg/ml) did not effect SCLC [³H]-thymidine incorporation (e.g. SITA = 100 % [³H]-thymidine incorporation, laminin (10 µg/ml) = 94.4 ± 7.5% [³H]-thymidine incorporation, mean ± S.D., n=2 from replicates of five).

As a number of studies have examined the role of laminin in SCLC cell proliferation, we went on to examine the role of the basement membrane protein collagen IV in SCLC cell proliferation. As with laminin, SCLC cells had previously been shown to adhere to collagen IV via a β1 integrin-dependent mechanism and produce membrane ruffles and microspikes on adherence. Results described in figure 5.11 indicate that SCLC cell proliferation is not significantly increased on adhesion to collagen IV. Proliferation studies indicated that in comparison to control cells

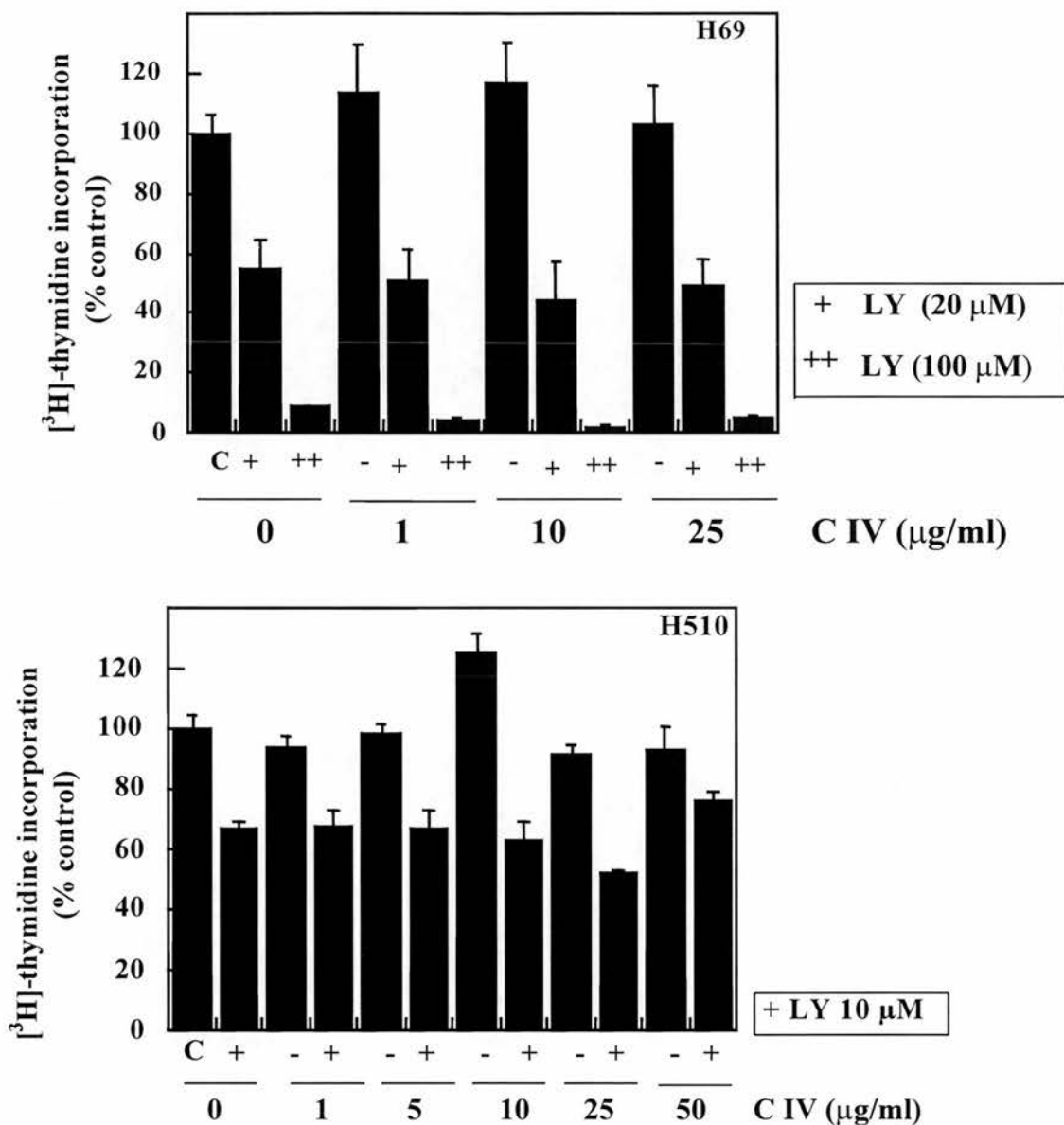


Figure 5.11: Effects of Small Cell Lung Cancer Cell Adhesion to Collagen IV on [³H]-Thymidine Incorporation in the Absence and Presence of Phosphoinositide 3-kinase Inhibitor LY294002.

H69 (upper) and H510 (lower) SCLC cells (1×10^5 /ml, 200 µl/well) were incubated in flat-bottomed 96 well microtitre plates previously coated with collagen IV (C IV) overnight at 4°C (50 µl/well) at various concentrations. Cells were incubated at 37°C in a humidified atmosphere 5% CO₂/95% air for 48 hours in the absence and presence of LY294002. [³H]-thymidine (0.1 µCi/well) was added, and the cells incubated for a further 24 hours prior to assessment for [³H]-thymidine incorporation. Results are expressed as the mean percentage [³H]-thymidine incorporation in comparison to control cells that received diluent alone. UPPER: Each bar represents the mean (\pm SEM) of 3 independent experiments carried out in replicates of five. Average basal counts per minute (cpm) = 40160 cpm. LOWER: Each bar represents the mean (\pm range) of one experiment carried out in replicates of five. Average basal cpm = 41078.

(plated in the absence of collagen IV), lower concentrations of collagen IV (1 - 10 $\mu\text{g/ml}$) caused a slight increase in [^3H]-thymidine incorporation, but this increase was not statistically significant ($p > 0.05$). In H69 SCLC cells 100% adhesion (control cells) was increased to $117 \pm 13 \%$ in the presence of collagen IV (10 $\mu\text{g/ml}$).

LY294002 was added to SCLC cells in the presence of collagen IV to investigate if cell adhesion effected the ability of LY294002 to fully inhibit cell proliferation as PI 3-kinase had previously been shown to be elevated further on $\beta 1$ integrin-dependent adhesion to ECM proteins. LY294002 was still able to inhibit [^3H]-thymidine incorporation in the presence of collagen IV. Addition of 20 μM LY294002 (close to the IC_{50} value obtained in Figure 4.2) caused an average 55% inhibition in [^3H]-thymidine incorporation and 100 μM LY294002 95% inhibition.

5.2.5.2 Increases in Small Cell Lung Cancer Cell Colony Formation in the Presence of Different Extracellular Matrix Proteins and Neuropeptides.

The ability of cells to form colonies in semi-solid medium has been shown to correlate with their tumourigenic capabilities (Freedman & Shin, 1974). Figure 5.1 demonstrates the increased deposition of ECM proteins in the micro-environment surrounding SCLC cells *in vivo*. In view of this, studies were performed to investigate if this increased ECM deposition may aid in SCLC cell tumourigenicity, and help explain why SCLC cells are highly metastatic.

The addition of neuropeptides to SCLC cells grown in semi-solid agar is known to enhance colony formation (Sethi & Rozengurt, 1991 and chapter 4). Figure 5.12 demonstrates an increase in the number of colonies formed by SCLC cells H69 in the presence of ECM proteins laminin and fibronectin. This increase in colony number was furthered by the addition of neuropeptides. Laminin and fibronectin caused a 3.2 and 2.2 fold increase in colony formation respectively. Bradykinin and galanin a 2.5 and 2.4 fold increase respectively. When added along side laminin and fibronectin,

bradykinin and galanin caused a 4.5 and 3.6 fold increase in colony formation respectively. The exact mechanisms underlying this synergistic increase in colony formation are unclear, however, the presence of both ECM and neuropeptides either alone or in combination significantly increase the tumourigenic properties of SCLC cells.

Previously, the ability of lung cancer cells to form colonies in semi-solid medium was shown to increase in the presence of matrigel (Yoshida, et al., 1997). Additionally preliminary studies by Fridman and co-workers (1990) examined the effect of matrigel on SCLC cell tumour growth in an *in vivo* environment. They subcutaneously injected a combination of SCLC cells and matrigel into athymic nude mice. Results confirmed the hypothesis that the presence of ECM alongside SCLC cells aids in SCLC cell tumourigenicity, as the resulting tumours in the mice co-injected with matrigel grew more rapidly.

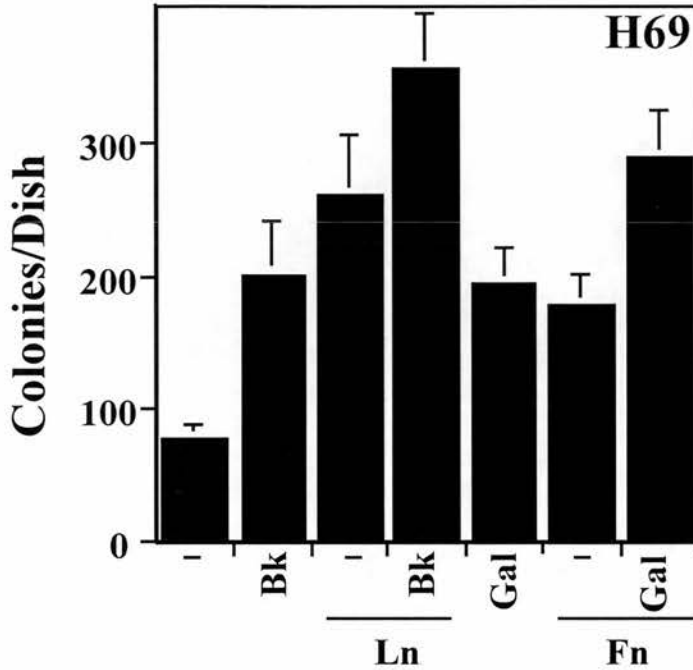


Figure 5.12: The Effect of Extracellular Matrix Proteins Fibronectin and Laminin on H69 Small Cell Lung Cancer Colony Formation in the Presence and Absence of Neuropeptides Bradykinin and Galanin.

H69 cells (1×10^4 /ml) were plated in agarose (3%) as described in section 2.4.3, in the presence and absence of laminin (Ln) ($10 \mu\text{g/ml}$) or fibronectin (Fn) ($20 \mu\text{g/ml}$) and the neuropeptides bradykinin (BK) (10 nM) or galanin (Gal) (50 nM). After 21 days incubation at 37°C , cells were stained with a vital stain (tetrazolium blue). Colony number was assessed by counting colonies >16 cells in size by light microscopy. Each bar represents the mean (\pm S.D.) of two experiments performed in triplicate.

5.2.6 Resistance to Chemotherapy.

The majority of chemotherapy reagents are thought to trigger cell death by causing cells to undergo apoptosis (Hannun, 1997). The adhesion of different cell types to ECM proteins or to $\beta 1$ integrin antibodies, has been shown to protect cells from undergoing apoptosis (known as anoikis) (Meredith et al., 1993, Frisch & Francis, 1994). On serum withdrawal, endothelial cells were prevented from undergoing apoptosis when immobilised on integrin $\beta 1$ antibodies, or when treated in suspension with the tyrosine phosphatase inhibitor sodium pyrophosphate (Meredith et al., 1993). Studies in epithelial cells indicated that if the epithelial cell-matrix interaction was disrupted, the cells underwent anoikis. This could be prevented by overexpression of the anti-apoptotic protein Bcl-2, by cellular transformation with *v-Ha-ras* or *v-src*, or by treatment with phorbol esters (Frisch & Francis, 1994). In view of some of the above observations that $\beta 1$ integrin/ECM interactions aided in cell survival, we went on to investigate whether the presence of, and adhesion to, ECM proteins was able to effect SCLC cell number / apoptosis levels when incubated in the presence of different chemotherapeutic agents.

5.2.6.1 Effects of Chemotherapeutic Agents on Small Cell Lung Cancer Cell Number *In Vitro*.

Our initial results showed that in the presence of chemotherapeutic agents, the number of surviving cells was significantly higher when the SCLC cells were plated in the presence of different ECM proteins (Figure 5.13). When H345 SCLC cells were adhered to Fn, the IC_{50} value of Cyclophosphamide increased from 1 μM to 200 μM , and IC_{50} value of Cis-Platinum from 92 $\mu g/ml$ to 450 $\mu g/ml$. This suggested that the adhesion of SCLC cells to the ECM proteins triggered a signalling pathway that either aided in SCLC cell proliferation (thought not to be the case (Figure 5.11)), or in the prevention of chemotherapy-induced SCLC cell death. In view of these

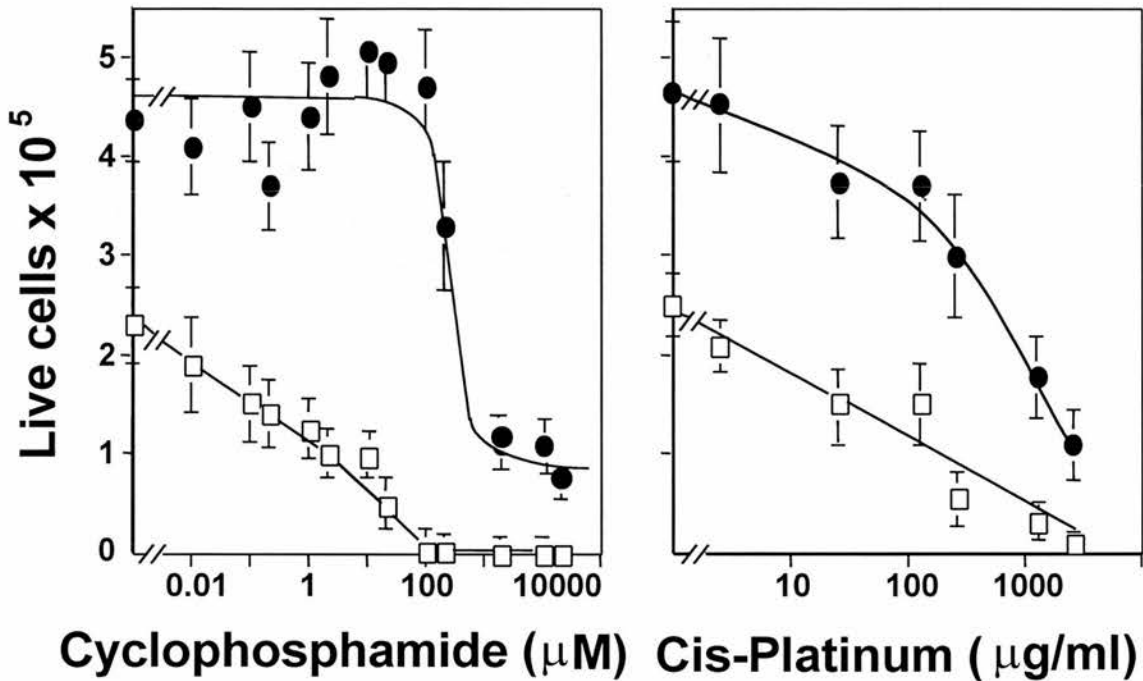


Figure 5.13: Changes in Small Cell Lung Cancer Cell Number when Adhered/Not-Adhered to Fibronectin in the Presence of Chemotherapy Reagents.

H345 cells ($5 \times 10^5/\text{ml}$, $200 \mu\text{l}/\text{well}$) were plated on microtitre plates pre-coated with no coating matrix (\square) or fibronectin (\bullet) ($20 \mu\text{g}/\text{ml}$, overnight at 4°C). Cells were allowed to adhere for 1 – 2 hours prior to the addition of increasing concentrations of Cyclophosphamide (μM) (left) or Cis-Platinum ($\mu\text{g}/\text{ml}$) (right). After 3 days incubation ($5\% \text{CO}_2/95\% \text{air}$, 37°C), total live cell number was assessed as described in the materials and methods section (section 2.11). Each point represents the mean (\pm SEM) of three independent experiments performed in quadruplicate.

results, the role of ECM adhesion on SCLC cell chemotherapy-induced apoptosis was investigated.

5.2.6.2 Extracellular Matrix Proteins Protect Small Cell Lung Cancer Cells from Apoptosis via a β 1 Integrin-dependent Mechanism Resulting in Increased Resistance to Chemotherapeutic Agents.

Figure 5.14 indicates that *in vitro* adhesion of SCLC cells to different ECM proteins protects them from undergoing chemotherapy-induced apoptosis. In comparison to the apoptosis levels observed in SCLC cells in the absence of any chemotherapy agents, lower levels were seen in the presence of both etoposide and cyclophosphamide when the cells were plated onto ECM proteins (except at very high concentration levels). For example, the addition of etoposide to H345 SCLC cells in the absence of any ECM protein produced an IC_{50} of 12.5 μ g/ml. This concentration caused approximately 50% apoptosis. Plating the SCLC cells onto ECM proteins prior to the addition of etoposide (12.5 μ g/ml), caused a decrease in the percent apoptosis observed (+ laminin 10%, + fibronectin 20%). The IC_{50} values of etoposide in the presence of these ECM proteins were also raised (+ laminin IC_{50} 63 μ g/ml, and + fibronectin IC_{50} 58 μ g/ml).

When comparing the protective effects of the different ECM proteins in the presence of various chemotherapy reagents, no major differences between the relative protective effects of each of the ECM proteins could be found. This indicated that adhesion to any of the ECM proteins examined was able to prevent SCLC cells from undergoing apoptosis in the presence of various chemotherapy reagents.

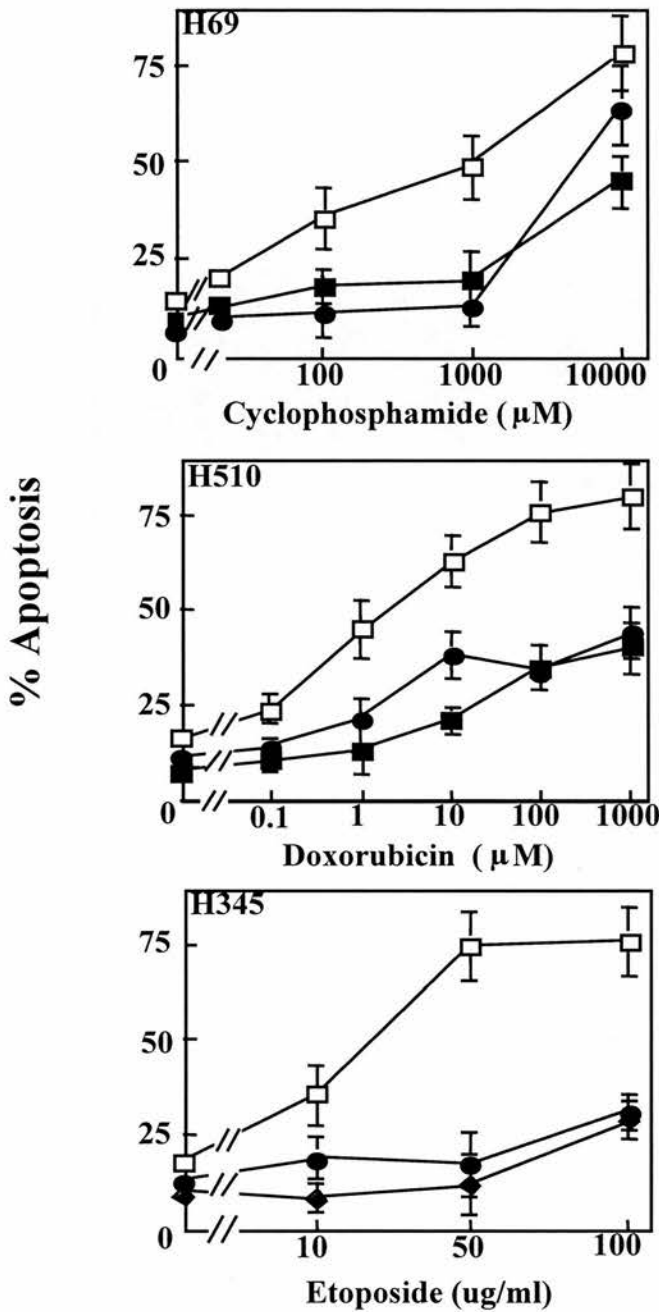
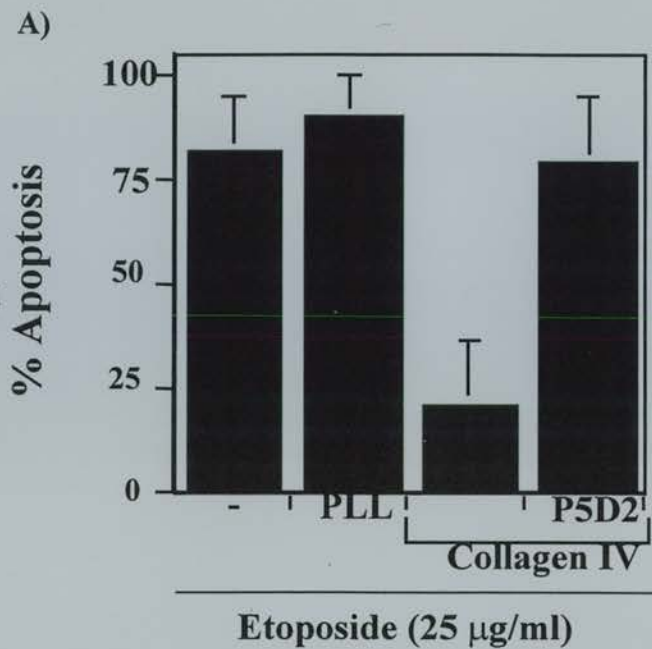


Figure 5.14: Effect of Extracellular Matrix Proteins on Chemotherapy-induced Apoptosis in Small Cell Lung Cancer Cells.

Approximately ($5 \times 10^5/\text{ml}$, $200 \mu\text{l}/\text{well}$) cells were washed and plated in serum-free SITA medium into 96 well microtitre plates (pre-coated with either fibronectin ($20 \mu\text{g}/\text{ml}$) (\bullet), laminin ($10 \mu\text{g}/\text{ml}$) (\blacklozenge), collagen IV ($10 \mu\text{g}/\text{ml}$) (\blacksquare) or in the absence of any ECM protein (\square)). Cells were allowed to adhere over approximately 1 – 2 hours before chemotherapy reagents were added at various concentrations. Cells were incubated for a further 3 days in a humidified atmosphere ($5\% \text{CO}_2/95\% \text{air}$, 37°C) prior to being assessed for apoptosis morphologically by cytocentrifugation (section 2.4). Each point represents the mean (\pm SEM) of 2 – 4 experiments carried out in quadruplicate.

Further investigations were performed to assess if this protective effect was β 1 integrin-mediated. This hypothesis was suggested as it had previously been shown (Figure 5.4) that SCLC cells adhered to the ECM proteins in a β 1-dependent manner. In the presence of a β 1-function blocking antibody (P5D2), SCLC cells incubated in the presence of collagen IV could not be protected against etoposide-induced apoptosis. Additionally, the non-specific adhesion of SCLC cells to poly-L-lysine could not protect the cells from undergoing chemotherapy-induced apoptosis (Figure 5.15). Thus, SCLC cell resistance to chemotherapy-induced apoptosis must be a consequence of either the stimulation a cell survival signal, or the repression of a pro-apoptotic signal that is dependent upon β 1-integrin activation.

During these experiments, differences between the relative resistance of the different SCLC cell lines to various chemotherapy reagents was noted. An example of this can be seen in figures 5.14 and 5.15. Etoposide, at a concentration of 25 μ g/ml caused approximately 56% apoptosis in SCLC cell line H345 (Figure 5.14), and approximately 80% apoptosis in SCLC cell line H69 (Figure 5.15). These differences were thought to be a cell specific phenomenon. Although the differences could not be explained experimentally, the effect of the ECM proteins remained the same. They were all able to cause a 3 - 6 fold decrease in apoptosis levels in any of the SCLC cell lines examined in the presence of chemotherapeutic reagents.



B)

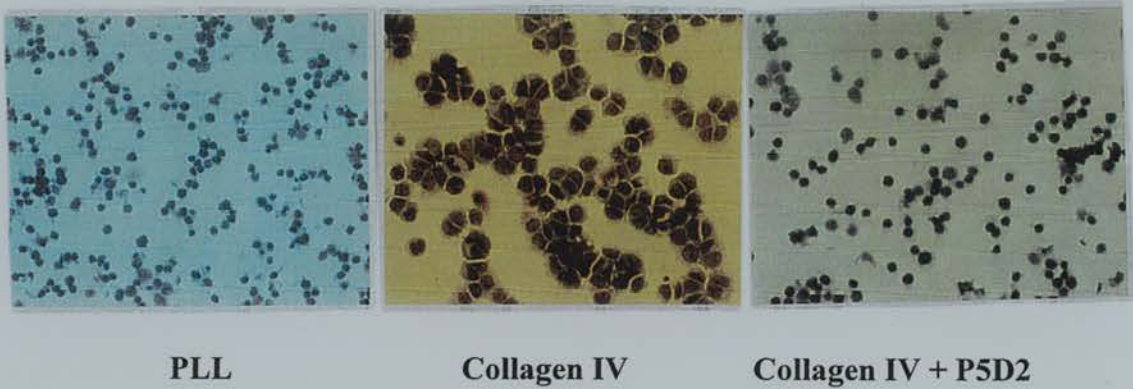


Figure 5.15: Extracellular Matrix Protein Mediated Small Cell Lung Cancer Cell Resistance to Chemotherapy-Induced Apoptosis is Mediated via a $\beta 1$ Integrin-dependent Mechanism.

A) H69 SCLC cells were plated in 96 well microtitre plates (5×10^5 /ml, 200 μ l/well) pre-coated with either collagen IV (10 μ g/ml), poly-L-lysine (25 μ g/ml) (PLL), or no coating matrix (-). SCLC cell apoptosis was assessed in the presence and absence of the $\beta 1$ integrin functional blocking antibody P5D2 (1 : 25 dilution) (added to cells as plated into microtitre plates). After allowing the cells to adhere for 1 – 2 hours, etoposide (25 μ g/ml) was added and the cells incubated for 3 days (5% CO₂/95% air, 37 °C) before being assessed for apoptosis using morphological techniques (section 2.5.3). Each bar represents the mean of 3 experiments carried out in quadruplicate (\pm SEM). B) Representative cytocentrifugation samples of H69 SCLC cells under the above conditions (A) are shown stained with May-Grunwald-Giemsa stain and examined using an Olympus BH-2 microscope.

5.2.6.3 Extracellular Matrix Protein-mediated Small Cell Lung Cancer Cell Resistance to Chemotherapy-induced Apoptosis Requires the Activation of Tyrosine Kinase Enzymes.

Attempts were made to begin to understand the signalling mechanism(s) behind this apparent $\beta 1$ integrin-mediated resistance to chemotherapy-induced apoptosis. Initial observations by Sethi and colleagues (1999) indicated that on adhesion to ECM proteins, there was an increase in the number of tyrosine phosphorylated proteins in SCLC cells. Additionally, Tallett and colleagues (1996a) had previously demonstrated the requirement of tyrosine kinase activity in the suppression of SCLC cell apoptosis in the absence of ECM protein adhesion. Finally, Meredith and co-workers (1993) noted that in endothelial cells, detachment-induced apoptosis could be prevented by the addition of the tyrosine phosphatase inhibitor sodium pyrophosphate. This indicated that tyrosine kinase activation may be stimulated on cell adhesion to $\beta 1$ integrins. Thus, the role of tyrosine kinase activation in SCLC cell resistance to chemotherapy-induced apoptosis was investigated.

As previously demonstrated, the presence of Ln, Fn and C IV caused an increase in the IC_{50} values of etoposide (Figure 5.16). This inhibition of chemotherapy-induced apoptosis was abolished by the addition of the tyrosine kinase inhibitor tyrphostin-25. For example, in the presence/absence of Fn, the IC_{50} values for etoposide were 18 $\mu\text{g/ml}$ and 3 $\mu\text{g/ml}$ respectively. In the presence of tyrphostin-25, the IC_{50} values were lowered to 4.9 $\mu\text{g/ml}$ and 3.9 $\mu\text{g/ml}$ +/- Fn respectively. Therefore, the results indicated that on adhesion to ECM proteins, the intracellular signalling mechanism which enables the SCLC cells to become resistant to chemotherapy-induced apoptosis, requires tyrosine kinase activation.

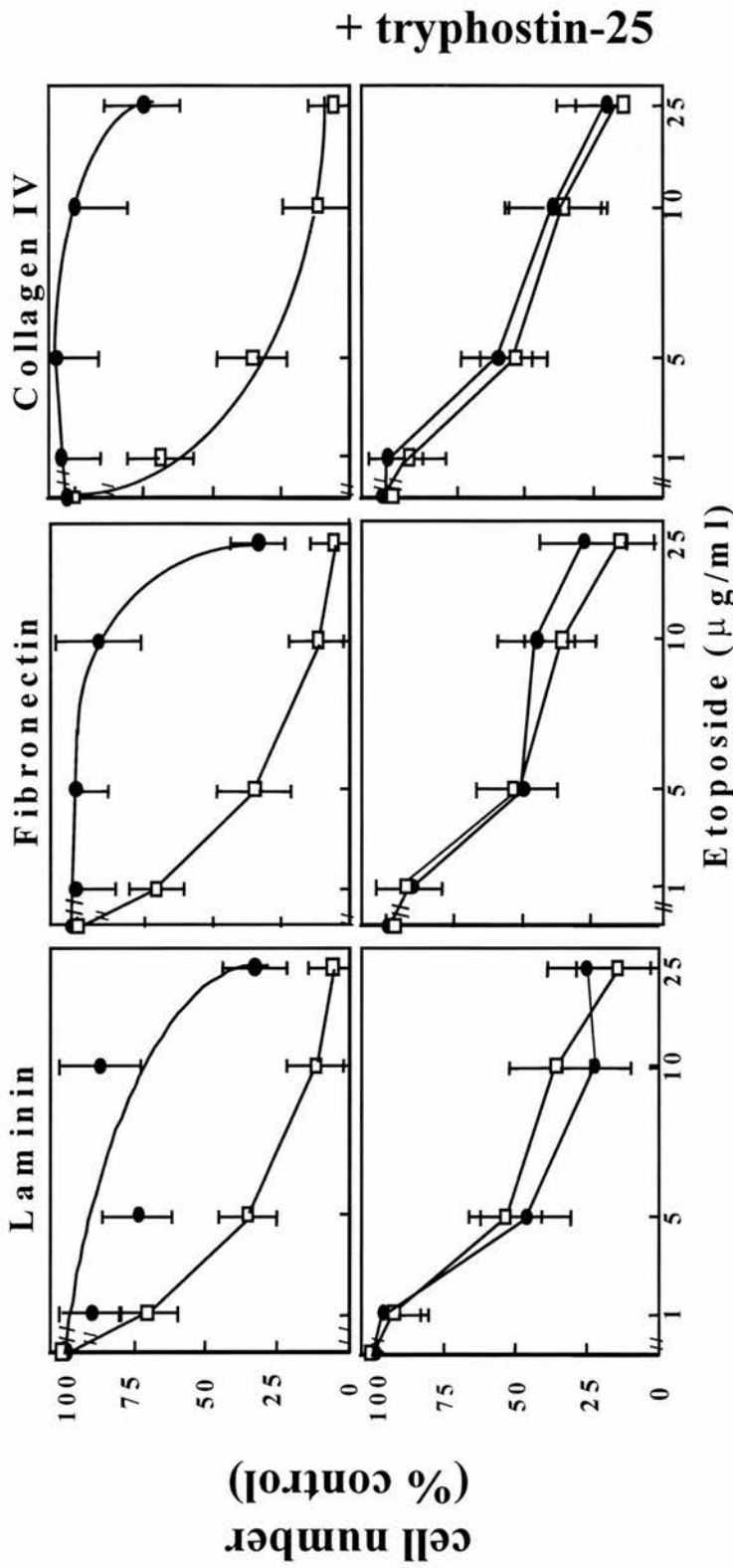


Figure 5.16: The Effect of Tyrrphostin-25 on Extracellular Matrix Mediated Small Cell Lung Cancer Cell Resistance to Chemotherapy-induced Apoptosis.

The chemosensitivity of H69 cells was studied on adherence to the extracellular matrix proteins laminin (10 µg/ml), fibronectin (20 µg/ml) and collagen IV (10 µg/ml) (●) or no coating matrix (□), in the absence, (upper row) or presence, (lower row) of the tyrosine kinase inhibitor Tyrrphostin-25 (25 µM). H69 SCLC cells (5–10 x10⁴/ml, 200 µl/well) were plated in the absence (upper) or presence (lower) of tyrrphostin-25. Cells were allowed to adhere for 1 – 2 hours before the addition of increasing concentrations of etoposide. After 3 days incubation at 37°C in a humidified atmosphere of 5% CO₂/95% air, cell numbers were assessed in comparison to the total number of cells adhered to each type of ECM protein in the absence of etoposide. Each point is representative of the mean (± SEM) of 3 independent experiments carried out in quadruplicate.

5.3 Discussion.

5.3.1 Extracellular Matrix Protein Expression.

In the initial studies we established the increased presence of a number of different ECM proteins in the local environment surrounding SCLC cells *in vivo*. These results corresponded to previous studies performed by Wetzels and co-workers (1992), where they showed that all the SCLC tissue specimens they examined were surrounded by the ECM protein laminin. Paakko and co-workers (1990) also demonstrated the presence of both laminin and collagen IV around all types of lung cancer tissues including SCLC. Further work has indicated that both the primary tumours and their matched metastatic lymph nodes express elevated levels of ECM proteins (Sethi et al., 1999) suggesting that these observations are characteristic of SCLC throughout the body.

The origin of these ECM proteins has yet to be established. However, preliminary observations described by Sethi and colleagues (1999) suggest that *in vivo*, SCLC cells themselves are capable of synthesising and secreting ECM proteins, and thereby altering their own microenvironment. This was in agreement with studies by Mastroianni and co-workers (1993), who noted that 15% of the SCLC tissue samples they studied were positive for intracellular cytoplasmic staining of laminin. Other possibilities include the suggestion that the ECM proteins may be produced as a consequence of autocrine and paracrine interactions involving fibroblasts and other surrounding cells such as epithelial cells.

An important observation was the presence of the ECM protein tenascin in SCLC lung tissue. The exact function of tenascin is not clear, however, it is known to be expressed in adult tissues which are actively remodelling, where it is often co-expressed with different matrix metalloproteinases (Jones et al., 1997). In the presence of tenascin, the adhesion of fibroblasts to fibronectin has been shown to

cause alterations in gene expression (Tremble et al., 1994). Tremble suggested that co-expression of fibronectin and tenascin may regulate the expression of genes involved in cell invasion and tissue remodelling. Roskelley and co-workers (1995) also noticed that the adhesion of breast epithelial cells to ECM proteins caused alterations in cell gene expression. These data imply that the adhesion of SCLC cells to ECM proteins may cause alterations in gene expression possibly leading to SCLC invasion and metastasis.

Tenascin expression has been shown to increase in the presence of the cytokine transforming growth factor β (TGF- β). TGF- β has been shown to be secreted by a number of tumour cell types including breast (Butta, et al., 1992) and colon (Coffey et al., 1986). TGF- β has previously been shown to be constitutively secreted by SCLC cell line NCI-N417. Further studies by Fischer and co-workers (1994) confirmed that 4/8 of the SCLC cell lines examined secreted TGF- β . This work is in agreement with our initial observations indicating that TGF- β is secreted by all three SCLC cell lines examined (H69, H345 and H510 (personal communication Dr. R. Streiter)).

TGF- β has been shown to be a key mediator in wound healing as it causes an increase in collagen formation in experimental wounding models (Sporn et al., 1983). Additionally, TGF- β is able to regulate the alternative splicing of fibronectin at the mRNA level (Borsi et al., 1990). This can lead to the production of other ECM proteins such as laminin and tenascin. Finally, secretion of TGF- β by wound heterophils and macrophages causes the increased deposition of ECM proteins (Keski-Oja et al., 1988, Sporn et al., 1987).

In view of these data, it can be hypothesised that SCLC cells, in conjunction with surrounding cells, may secrete a number of growth factors and cytokines which cause the formation of an ECM rich environment around SCLC cells *in vivo*.

5.3.2 Integrin Profiling and Adhesion to Extracellular Matrix Proteins.

ECM proteins are known to associate with cells via the integrin family of cell surface receptors. SCLC cells have previously been shown to possess laminin receptors (Feldman et al., 1991, Pellegrini et al., 1994) and adhere to laminin and matrigel (a multi-protein preparation rich in laminin, collagen IV, heparan sulphate proteoglycan, TGF- β , and IGF-1) (Fridman et al., 1990). However, to understand the role of integrins in tumour behaviour, information on the pattern of integrin expression is required in relation to normal cell counterparts. SCLC cells were shown to express the same integrin subunits as bronchial epithelial cells, although they do not express the $\alpha 5$ integrin subunit (Koukoulis et al., 1997). Decreases in $\alpha 5 \beta 1$ expression have often been observed to correlate with increased cancer cell aggressiveness (Giancotti & Ruoslahti, 1990, Schreiner et al., 1991), suggesting that the loss of $\alpha 5 \beta 1$ integrin expression may aid in the metastatic capabilities of SCLC cells *in vivo*. To further the hypothesis that the integrin profile of SCLC cells is favourable towards a metastatic cell type, expression of the integrin dimers $\alpha 2 \beta 1$, $\alpha 3 \beta 1$, and $\alpha v \beta 3$ (Schadendorf et al., 1993, Danen et al., 1994) have been shown to be present on a wide variety of malignant melanomas (including the SCLC cell lines examined). Studies by Vink and co-workers (1993) showed that $\alpha 2 \beta 1 / \alpha v \beta 3$ integrin expressing cells colonised the lungs, whereas cells expressing both the $\alpha 2 \beta 1$ and $\alpha 6 \beta 1$ integrin heterodimers colonised both the lungs and extrapulmonary sites. Additionally, the $\beta 1$ integrin has previously been shown to be important in the angiogenic properties of teratoma growth (Bloch et al., 1997). Migration and invasion of a number of SCLC cell lines has also been shown to be dependent upon $\beta 1$ -mediated integrin signals (Bredin et al., 1998). Thus, the data described above suggests that SCLC cells express an integrin profile which is favourable towards the production of a metastatic cancer.

The results obtained in this chapter suggest that to allow SCLC cells to adhere to ECM proteins, a functional $\beta 1$ integrin is required. However, the activation of

certain intracellular proteins are not required to enable the cells to adhere. These results agree with previous studies performed by Tachibana and co-workers (1996). They showed that the adherence of SCLC cell line CADO LC6 was β 1 integrin-dependent but did not require tyrosine kinase activity. This was concluded, because addition of the phosphotyrosine inhibitor herbimycin A could not prevent adhesion. Additionally, observations by Shaw and colleagues (1997) showed that breast carcinoma cells adhered to ECM proteins in a PI 3-kinase-independent manner.

5.3.3 Alterations in Small Cell Lung Cancer Cell Characteristics on Adhesion to Extracellular Matrix Proteins.

Classification of lung cancers has always been complex. Pathologists have often observed the presence of mixed SCLC/NSCLC morphological characteristics in patients. These mixed NSCLC-like changes have frequently been observed after chemotherapy and are often linked with chemoresistance and a poorer prognosis (Matthews & Gazdar, 1981, Radice et al., 1982, Piehl et al., 1988). Studies carried out by a number of laboratories have shown that the induction of NSCLC phenotypical characteristics can occur on the oncogenic transformation of SCLC cells (Doyle, 1993). Transfection and transformation of SCLC cells with *c-myc* and *v-Ha-ras* were associated with a move towards a more large cell morphology/NSCLC phenotype. Additionally, Barr and co-workers (1996) showed that transfection of the *myc* or *ras* oncogene, cell aggregation and attachment to laminin aided in the transition between SCLC and NSCLC phenotypes. These transitions could however be reversed on re-suspension of these cells suggesting that a number of factors such as gene expression, and cell adhesion/environment are key to phenotypic transformation.

As NSCLC cells are known to be adherent under tissue culture conditions, the adhesion of SCLC cells to ECM proteins may cause a shift towards a more NSCLC-like phenotype. However, studies by Sethi and colleagues (1999) showed that on adhesion to ECM proteins, there were no alterations in the expression levels of the

neuroendocrine markers chromogranin A, dopa-decarboxylase and neuron-specific enolase. Fridman and co-workers (1990) also indicated that there were no alterations in the expression of specific NSCLC proteins on the adherence of H345 SCLC cells to laminin. Thus, the data suggests that the ability of SCLC cells to transform into a more NSCLC-like phenotype requires more than just adhesion. A number of additional factors such as an alteration in cell gene expression are required. The suggestion that all lung cancer cell types originate from bronchial epithelial cells, and that NSCLC cell types such as adenocarcinoma cells may be formed from SCLC cells after additional genetic alterations, was hypothesised by Mabry and co-workers (1991). They suggested that normal maturation of a bronchial cell led to the production of a pluripotent undifferentiated cell. This cell was then able to differentiate into epithelial or endocrine cell types. As a consequence of chronic injury these cells were hypothesised to lead to unrestrained growth and autocrine growth loop activation (hyperplasia). Eventually transformation may occur due to genetic alterations. These included deletion of a section of the 3p chromosome and the oncogenic transformation of other oncogenes including *ras* and *myc*.

5.3.4 Small Cell Lung Cancer Growth and Survival.

Initial results indicated that adhesion to either collagen IV or laminin was unable to significantly induce SCLC growth above control levels. These results were in line with previous observations by Fridman and co-workers (1990), Giaccone and co-workers (1992a) and Nakanishi (1988). In contrast, the presence of ECM proteins laminin and fibronectin were able to induce SCLC tumourigenicity in synergy with neuropeptide-mediated SCLC clonal growth in semi-solid media. This indicates that the presence of increased ECM deposition around SCLC cells *in vivo* aids in SCLC tumourigenicity producing a more aggressive cell phenotype.

Further results demonstrated that SCLC cell resistance to chemotherapy is due to the cells becoming resistant to chemotherapy-induced apoptosis when adhered to ECM proteins. As shown by Meredith and co-workers (1993), ECM-mediated prevention

of apoptosis occurred via a $\beta 1$ integrin-dependent mechanism. This observation was strengthened by the fact that adhesion to poly-L-lysine was unable to rescue the cells from chemotherapy-induced apoptosis. Fridman and co-workers (1990) first observed laminin-induced chemoresistance in SCLC cells. The data described in this chapter goes on to show that this initial observation is a more general property of a number of ECM proteins that are found to be expressed in elevated amounts around the SCLC cells *in vivo*. Also this protection from chemotherapy-induced apoptosis occurs via a $\beta 1$ integrin-mediated signalling pathway that requires tyrosine kinase activation.

Further studies examining ECM-induced cell survival mechanisms have shown that both matrix adhesion, and *ras* transformation in suspended cells, are both able to prevent anoikis/apoptosis via a PI 3-kinase-dependent mechanism (Khwaja et al., 1997). It may therefore be hypothesised that the constitutively activated PI 3-kinase present in SCLC cells is able to mimic *ras* transformation and enable SCLC cells to become anchorage-independent. However, further investigations are required before the potential role of PI 3-kinase in chemotherapy-induced apoptosis has been established; although it was noted that on adhesion to laminin PI 3-kinase activation was elevated.

Studies by Simizu and co-workers (1996 and 1998), and Tallet and colleagues (1996), have shown that in SCLC cells grown in liquid culture, the induction of apoptosis can be triggered via the inhibition of tyrosine kinase activation. This induction of apoptosis was shown to be mediated via a caspase-3-like protease. Caspase-3 is a member of the ICE protease family, and exhibits high percentage homology and substrate specificity with the pro-apoptotic protein ced-3 (Nicholson et al., 1995). Caspase-3 is known to induce apoptosis via the cleavage of poly(ADP)ribose polymerase (PARP). Further work by Simizu and co-workers (1998) revealed that the activation of caspase-3 induced an increase in the expression levels of the pro-apoptotic protein Bax. Thus, these data suggests that one of the possible signalling mechanisms able to induce SCLC cell apoptosis is dependent

upon the inhibition of tyrosine kinases and the activation of ICE-like protease caspase-3.

Chemotherapy-induced apoptosis has been shown to be inhibited by the prevention of PARP cleavage (Nicholson et al., 1995, Chen et al., 1996, Dubrez et al., 1996). When examining chemotherapy-induced apoptotic mechanisms, etoposide was shown to cause DNA damage leading to cell cycle delay and activation of caspase-3. Data described by Sethi and colleagues (1999) indicates that cell cycle delay and caspase activation can be prevented by the inhibition of tyrosine phosphatase activation, and by the adhesion of SCLC cells to ECM proteins. This inhibition of etoposide-induced apoptosis was dependent upon tyrosine kinase activation and a β 1 integrin-mediated signal. Thus, from the data described by both Simizu and co-workers (1998) and Sethi and colleagues (1999), it can be hypothesised that etoposide-induced cell cycle delay is regulated by tyrosine kinase activity. On adhesion to ECM proteins, additional tyrosine phosphorylation/activation occurs resulting in cell cycle initiation. The activation of caspase-3 becomes abrogated leading to a down-regulation of cellular apoptosis. It can therefore be hypothesised that due to this process of events, SCLC cells surrounded by ECM proteins *in vivo* are able to escape chemotherapy-induced apoptosis.

Thus, from the *in vivo* and *in vitro* data described in this chapter, it seems likely that the adhesion of SCLC cells to ECM proteins plays a critical role in SCLC cell tumourigenicity and resistance to chemotherapy-induced apoptosis. Understanding how the local environment is formed around SCLC cells *in vivo*, and the exact mechanism which enables specific proteins to be activated/inhibited on adhesion to ECM, may be of benefit when designing novel therapies for the treatment of SCLC.

CHAPTER 6

Final Discussion.

One of the main causes of death in cancer patients is the development of secondary metastatic deposits at sites remote from the primary tumour. SCLC has the highest metastatic potential of any of the solid tumours with over 90% of patients having widespread metastases at initial presentation. When first diagnosed, patients respond well to chemotherapy. However, despite this initial sensitivity to chemotherapy, the tumour almost invariably relapses and becomes resistant to further chemotherapy treatment; thus the patient two year survival rate remains between 3 - 8% (Elias, 1997). In this thesis we aimed to begin to unravel possible signalling mechanisms which regulate SCLC cell growth, tumourigenicity and resistance to chemotherapy-induced apoptosis.

6.1 Major Findings of this Thesis.

The novel findings described within this thesis are that in SCLC cells the lipid/tyrosine kinase PI 3-kinase is constitutively activated resulting in high levels of p70^{s6k} activity. Inhibition of PI 3-kinase activity was shown to fully inhibit SCLC cell proliferation in liquid culture, and prevent both basal and neuropeptide-stimulated colony formation in semi-solid medium. We show that PI 3-kinase-mediated proliferation is regulated by at least two signalling cascades, with one of these pathways signalling via p70^{s6k}. The observed inhibition of SCLC cell growth in liquid culture has since been shown to be regulated by a combination of factors. We show that inhibition of PI 3-kinase activity causes a small but consistent increase in SCLC cell apoptosis. This observation, coupled with the recent observation (Moore et al., 1998) that PI 3-kinase inhibition causes a delay in the SCLC cell cycle at G1,

helps to explain why the PI 3-kinase inhibition prevents SCLC cells from undergoing mitosis. Thus in SCLC cells, the downstream signalling pathways regulated by the constitutively activated PI 3-kinase, are important in SCLC cell anchorage-independent growth and tumourigenicity.

Studies investigating the mechanism(s) underlying SCLC cell resistance to chemotherapy-induced apoptosis, indicated that *in vivo*, SCLC cells were surrounded by an extensive stroma of ECM proteins. *In vitro* SCLC cells were shown to adhere to these ECM proteins predominantly via a $\beta 1$ integrin-dependent mechanism causing the cells to become more resistant to chemotherapy-induced apoptosis. This resistance was shown to involve activated tyrosine kinase enzymes, but the exact enzymes have yet to be established. This survival effect could not be mimicked by the non-specific adhesion of SCLC cells to poly-L-lysine. Additionally, the presence of ECM proteins was shown to increase the tumourigenic properties of the SCLC cells by increasing the number of clones formed in semi-solid medium. Thus, despite SCLC cells being anchorage-independent, the adhesion of SCLC cells to ECM proteins via a $\beta 1$ integrin-dependent mechanism, protected the cells from chemotherapy-induced apoptosis, and resulted in enhanced tumourigenicity.

6.2 Discussion.

Results from chapter 3 indicate that PI 3-kinase is basally activated by a mechanism yet to be established. This is the first time PI 3-kinase has been shown to be constitutively activated in any human cancer. In view of this novel finding, the role of PI 3-kinase in SCLC cell growth and survival was investigated further.

Previous studies have indicated that the proliferation of SCLC cells is driven by multiple autocrine and paracrine growth loops involving calcium mobilising neuropeptides (Sethi & Rozengurt, 1991 and 1992). This mitogenic signalling is thought to enhance SCLC cell tumourigenicity (clonal growth studies) and explain

why the tumour is serum-independent. A wide range of mitogenic signalling cascades have been shown to involve PI 3-kinase (Chung et al., 1994 (PDGF), Vemuri & Rittenhouse, 1994 (NGF), Kotani et al., 1994 (IGF-1), DePaolo et al., 1996, Cross et al., 1994, Uehara et al., 1995 (vasopressin, IGF-1 and insulin), reviewed Toker & Cantley, 1997). Given that PI 3-kinase is constitutively activated in SCLC cells, it may be hypothesised that the presence of PI 3-kinase may explain the serum-independence observed in these cells. The constitutive activation of PI 3-kinase observed in quiescent SCLC cells was shown to be stimulated further upon the addition of FCS or insulin. In view of this, the combination of constitutive PI 3-kinase activation and the presence of autocrine mitogens may explain why SCLC cells are the fastest growing lung cancer cell type. The growth signal within the cell may be permanently switched on due to PI 3-kinase activation. However, when the cells are stimulated via autocrine growth promoting mitogens, the cells become fully activated maximising their growth signal without the requirement for exogenously added mitogens.

The major role of PI 3-kinase in SCLC cell growth was highlighted by the observations that on the inhibition of PI 3-kinase activity, SCLC cells were unable to proliferate in liquid culture. This growth signal was seen to signal via p70^{s6k} and at least one other pathway independently of p70^{s6k}. Studies have shown that inhibition of the MAP kinase signalling cascade in SCLC cells causes approximately a 50% inhibition in cell proliferation in liquid culture. PI 3-kinase has been shown to regulate MAP kinase activation in a number of cells types (Hawes et al., 1996, Lopez-Illasaca et al., 1997, King et al., 1997). King and co-workers (1997) noted that PI 3-kinase and PKB regulated integrin-mediated MAP kinase signalling. They suggested that this occurred either via the PI 3-kinase-dependent activation of PKC causing an increase in raf-1 activation, or by the 3-phosphorylated lipid products of PI 3-kinase activating raf-1. Hawes and co-workers (1996) demonstrated that the inhibition of PI 3-kinase activation in COS-7 cells prevented MAP kinase activation. One hypothesis could therefore be that downstream of PI 3-kinase the proliferation signal splits with one pathway signalling via p70^{s6k} and the other via MAP kinase.

Further experimentation is required before it can be established whether in SCLC cells, MAP kinase activation can be regulated by growth factor/integrin-dependent PI 3-kinase activation and whether this underlies the signalling processes that mediate SCLC cell growth.

Transformation of adherent cells using the cytoplasmic oncogenes *ras* and *src* has been shown to enable cells to grow in suspension (become anchorage-independent) (Frisch & Francis, 1994, Khwaja et al., 1997). Epithelial cells have been seen to overcome anchorage-dependent apoptosis (anoikis). The mechanism underlying this observation was shown to involve the proteins PI 3-kinase and PKB (Khwaja et al., 1997). Given that both *ras* and *src* are not highly expressed or activated in SCLC cells (Mitsudomi et al., 1991, Suzuki et al., 1990, Budde et al., 1994), the presence of constitutively activated PI 3-kinase in SCLC cells may mimic *ras* or *src* transformation. PI 3-kinase has been shown to be stimulated on integrin activation (King et al., 1997, Chen & Guan, 1994b), therefore constitutive PI 3-kinase activity may cause the stimulation of signalling cascades within the SCLC cells which are normally activated on integrin binding enabling the cells to become anchorage-independent.

This hypothesis was strengthened by recent evidence indicating that growth factors alone were unable to induce DNA synthesis in NIH 3T3 cells in the absence of integrin activation (Renshaw et al., 1997). This appeared to result from the inability of growth factors to activate the MAP kinase pathway in the absence of integrin activation and cause the stimulation and production of integrin-mediated second messenger signals. In NIH 3T3 cells Renshaw and co-workers (1997) noted that activation of the classical MAP kinase signalling cascade could only trigger DNA synthesis when the cells were allowed to adhere. In transformed cells (transfected with constitutively active *ras* or *raf* mutants), the activity of p⁴²MAPK remained low in suspended cells in comparison to the activity observed in untransfected cells that were allowed to adhere. However, the p⁴²MAPK activity in the suspended transformed cells was approximately 40% higher than that measured in serum-

stimulated untransformed cells. Thus, using this analogy, it could be hypothesised that in SCLC cells, the constitutive activation of PI 3-kinase may mimic the transformation changes observed by Renshaw. Renshaw suggested that the presence of the *ras* transformation overcame the requirement for cell adhesion by causing the production of integrin-mediated second messengers in the absence of integrin activation. Hence for SCLC cells in suspension, the constitutive activation of PI 3-kinase may cause a stimulation of the 'integrin-mediated' downstream signalling second messengers. This enables the SCLC cells to maintain their 'growth signal' in the absence of integrin adhesion and become anchorage-independent.

Preliminary investigations into the composition of the micro-environment surrounding SCLC cells *in vivo*, indicated that due to possible auto and paracrine interactions, SCLC cells were surrounded by a micro-environment enriched in different ECM proteins. Although SCLC cells are anchorage-independent, in the presence of different ECM proteins, they were shown to adhere via a β 1 integrin-dependent mechanism. On adhesion, an increase in the tyrosine phosphorylation of intracellular proteins (Sethi et al., 1999) was observed. Additionally, the adhesion of the SCLC cells to ECM proteins was shown not to cause a significant increase SCLC cell proliferation in liquid growth media, but cause the production (or inhibition) of a signal which resulted in an increase in SCLC resistance to chemotherapy-induced apoptosis. Moreover, SCLC cell PI 3-kinase activity was shown to increase on adhesion to Laminin. This final observation mimics the observation by Renshaw and co-workers (1997) who noted a 2.5 fold increase in p⁴²MAPK activity in adhered transformed cells in comparison to non-adhered transformed cells. In view of this, the activation of additional signalling proteins (including PI 3-kinase) on SCLC adhesion to ECM proteins may therefore enable SCLC cells to become resistant to chemotherapy-induced apoptosis.

Further studies both described in chapter 5 and performed by Sethi and colleagues (1999), have highlighted that the mechanism underlying SCLC cell resistance to chemotherapy-induced apoptosis is regulated by a tyrosine kinase-dependent

mechanism. The adhesion of SCLC cells to an ECM protein via a $\beta 1$ integrin-dependent mechanism, protected the SCLC cells from etoposide-induced caspase-3 activation and apoptosis by activating tyrosine kinase signalling downstream of DNA damage (as adhesion did not significantly alter either the expression or activity of topoisomerase II). Activation of the tyrosine kinase-dependent signalling mechanisms abrogated cell cycle arrest at the G2/M and S phase. Additional work indicated that the caspase inhibitor Z-VAD prevented the cleavage of pro-caspase-3 and therefore chemotherapy-induced apoptosis. The addition of Z-VAD did not effect DNA damage or cell cycle delay indicating the caspase-3 activation was a downstream event in etoposide-induced SCLC cell death.

Therefore, although SCLC cells are thought of as anchorage-independent, this may not be strictly true. SCLC cells may be able to survive and proliferate whilst in suspension (e.g. whilst moving around the body in the blood stream, or lymphatic system), however to maximise their tumourigenic and metastatic capabilities, they may prefer to be adhered to proteins such as ECM proteins via integrin-dependent mechanisms. For a cell to invade and metastasis it has to adhere and degrade ECM (Cleardin, 1998). This enables the cells to intravasate from the primary tumour site into the blood stream/lymphatic system. From there the cells would then have to be able to adhere to the vessel wall and extravastate and form a secondary ectopic deposit. Tumour cells are known to migrate via integrin-dependent mechanisms (Bredin et al., 1998, Fridman et al., 1990), become more invasive in the presence of laminin (Shaw et al., 1997, Keely et al., 1997) and become increasingly tumourigenic both *in vitro* and *in vivo* (chapter 5, Fridman et al., 1990) in the presence of laminin or matrigel.

Thus, the constitutive activation of PI 3-kinase may be mimicking the effects of an 'activated oncogene' enabling the SCLC cells to become both serum- and anchorage-independent in culture. The activation of PI 3-kinase in SCLC cells appears to be crucial in a number of important cellular functions and may regulate the cell growth, anchorage-independence, serum-independence and tumourigenic properties of SCLC

cells. Along with the ECM/integrin-mediated cell signalling events described above, these observations may help explain why partial responses and the local reoccurrence of SCLC is often seen after chemotherapy.

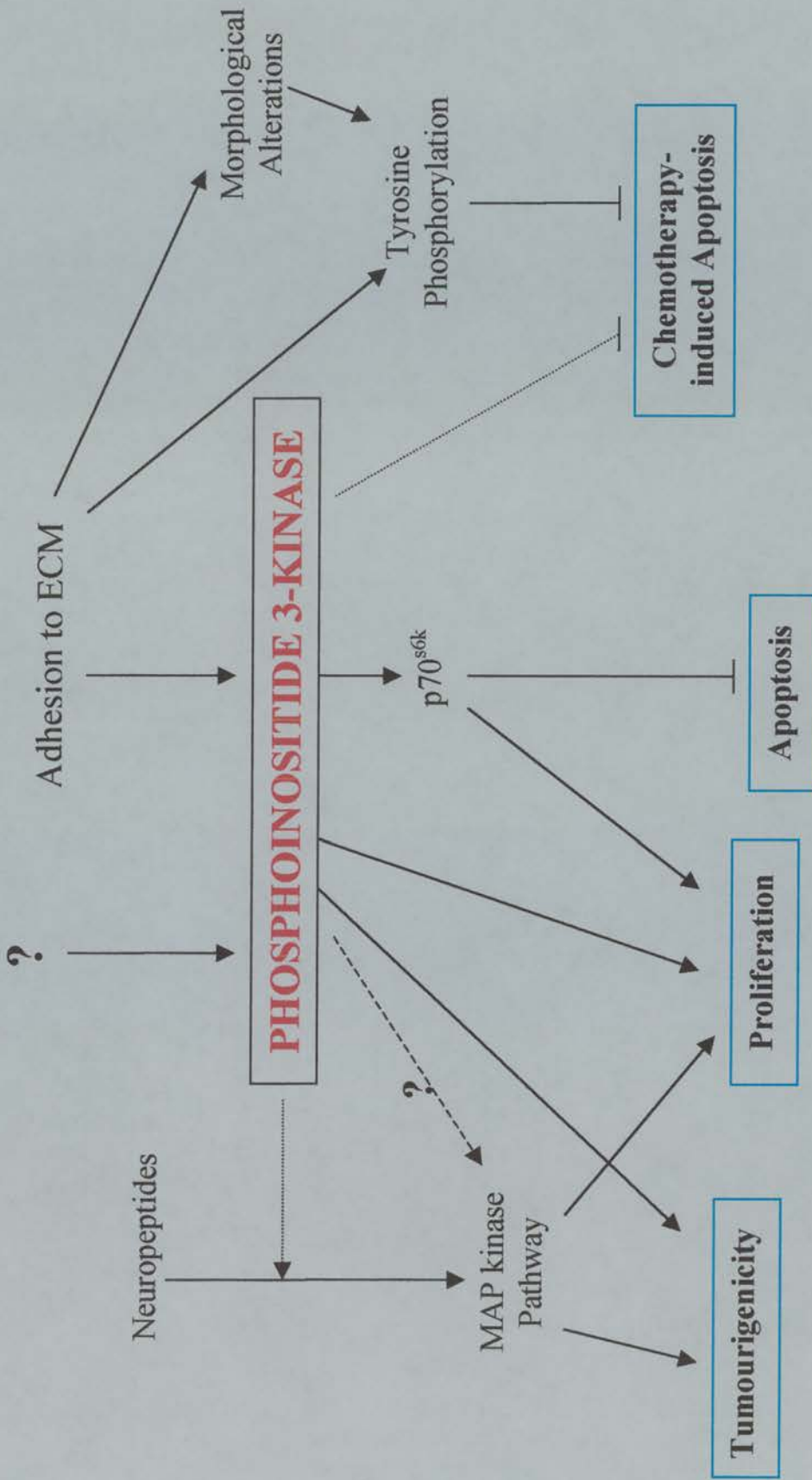


Figure 6.1 Summary of the Possible Signalling Pathways Emanating from PI 3-kinase Activation in Small Cell Lung Cancer Cells.

6.3 Future Directions.

Many questions still need to be addressed in relation to the role of PI 3-kinase in this highly aggressive and metastatic cancer.

i) The use of different strategies to clarify our published observations.

The work presented throughout this thesis has centred upon experiments utilising a number of different pharmacological inhibitors. As discussed with respect to the PI 3-kinase inhibitors wortmannin and LY294002 (section 3.3.2), there have been a number of published cases suggesting that as research progresses, these pharmacological inhibitors are seen to be less enzyme specific. To overcome this, a number of groups now perform experiments in parallel with enzyme inhibitor experiments using molecular genetic approaches. For example when investigating PI 3-kinase, researchers have used dominant negative and constitutively activated PI 3-kinase enzyme mutants to investigate/clarify specific observations they have seen. Researches suggest that using these more direct approaches which target specific proteins, there is less chance of non-specific effects from occurring, like the inhibition of a related protein. To clarify our pharmacologically based observations that PI 3-kinase is constitutively activated in SCLC cells and is required for growth, a dominant negative PI 3-kinase mutant should be transfected into SCLC cell lines and the results assessed in parallel to the data described within this thesis.

ii) The mechanism underlying the constitutive activation of PI 3-kinase.

This question is hard to address as there are many possible reasons as to why PI 3-kinase is constitutively activated in SCLC cells under quiesced basal conditions (section 3.3).

Polymerase chain reactions may be performed using primers to both the p85 and p110 subunits of PI 3-kinase. From the sequences obtained you could potentially sequence the subunits to try to identify any possible mutations. Theoretically a

mutation in the p85 regulatory subunit may cause the removal of an inhibitory constraint. A mutation in either subunit may result in a conformational alteration leading to enzyme activation.

Localisation studies may be performed to address the question is PI 3-kinase constitutively activated due to the catalytic subunit being targeted to the plasma membrane in the absence of any exogenous stimulation. This targeting could be due to mutations within the enzyme itself, or in the receptor at the plasma membrane. Immunohistochemical methods may be used. Given that the actual percentage of PI 3-kinase in all cells is very low, small changes in localisation maybe hard to visualise. Thus, using specific lysis techniques, separation of different compartments of SCLC cells maybe achieved. Activity levels of PI 3-kinase may then be assessed in each cellular fraction. This may give a more accurate picture of the localisation of active PI 3-kinase within the SCLC cells.

iii) The role of PI 3-kinase in MAP kinase activation.

To investigate if in SCLC cells, the MAP kinase pathway is regulated upstream by PI 3-kinase, co-incubation assays could be performed. MAP kinase activity assays maybe undertaken in the presence and absence of PI 3-kinase inhibitors to investigate if either wortmannin or LY294002 could inhibit either neuropeptide-stimulated, or basal MAP kinase activity levels.

The effect of LY294002 and PD 098059 on SCLC cell proliferation rates may be also investigated by incubating the cells in the presence of each inhibitor both alone and in combination.

iv) The role of PI 3-kinase in ECM colony formation.

Inhibition of PI 3-kinase was shown to prevent basal and neuropeptide-stimulated colony formation. To investigate if the increase in colony formation in the presence of different ECM proteins was mediated by PI 3-kinase, experiments where SCLC

cells in semi-solid medium were incubated with LY294002 and ECM alone, and in combination would have to be performed.

v) Further elucidation of the mechanism underlying ECM-mediated resistance to chemotherapy-induced apoptosis.

Given that resistance to chemotherapy is one of the major causes of SCLC patient death, any information on how this may occur is invaluable in the search for novel therapeutic drugs. Using inhibitors to specific proteins further chemotherapy assays maybe performed. This technique is only of use if the inhibitors are known to be enzyme specific. Given that PI 3-kinase has been shown to be stimulated on SCLC cell adhesion to ECM proteins, the possible role of this protein on SCLC cell resistance to chemotherapy-induced apoptosis should be assessed. In the case of p70^{s6k} (which was shown by Shi and co-workers (1995) to be important in cis-platin-induced apoptosis), rapamycin could be utilised to investigate the possible role of p70^{s6k} as it is still thought to be an enzyme specific inhibitor.

Publications Arising From Thesis

Moore, S.M., Rintoul, R.C., Walker, T.R., Chilvers, E.R., Haslett, C., and Sethi, T. (1998). The presence of a constitutively active phosphoinositide 3-kinase in small cell lung cancer cells mediates anchorage-independent proliferation via a protein kinase B and p70s6k-dependent pathway. *Cancer Res.* 58, 5239-5247.

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The Presence of a Constitutively Active Phosphoinositide 3-Kinase in Small Cell Lung Cancer Cells Mediates Anchorage-independent Proliferation via a Protein Kinase B and p70^{S6k}-dependent Pathway¹

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ABSTRACT

Small cell lung cancer (SCLC) is characterized by early and widespread metastases. Anchorage-independent growth is pivotal to the ability of tumor cells to survive and metastasize *in vivo* and under *in vitro* conditions allows transformed cells to form colonies in semisolid medium. Here we report that of five SCLC cell lines tested, all exhibited high basal constitutive phosphoinositide 3-kinase (PI 3-kinase) activity, which results in high basal protein kinase B (PKB) and ribosomal p70 S6 kinase activity (p70^{S6k}). Inhibition of PI 3-kinase activity markedly inhibited SCLC cell proliferation in liquid culture as a result of stimulating apoptosis and promoting cell cycle delay in G₁. Furthermore, PI 3-kinase inhibition reduced basal SCLC cell colony formation in agarose semisolid medium that could not be overcome by the addition of neuropeptide growth factors. Thus, constitutive PI 3-kinase activity in SCLC cells plays an important role in promoting the growth and anchorage independence of SCLC. This is not due to activating *ras* mutations or increased basal *src* or focal adhesion kinase activity. These data represent the first description of constitutively activated PI 3-kinase/PKB in any human cancer. Constitutive activation of these integrin-dependent signaling events provides a molecular explanation for the anchorage-independent growth of SCLC cells and may account for the nonadherent phenotype and highly metastatic nature of this aggressive cancer. Up-regulation of the PI 3-kinase/PKB pathway may therefore represent a novel target for therapeutic intervention in SCLC.

INTRODUCTION

Lung cancer is the commonest fatal malignancy in the developed world. SCLC⁺ constitutes 25% of all lung neoplasms and is characterized by early and widespread metastasis with a 5-year survival of 3-8% (1). The aim of our work has been to devise novel and more rational therapeutic strategies based on a better understanding of the molecular events that are responsible for metastasis and sustaining the growth and preventing the death of SCLC cells.

The molecular mechanisms regulating SCLC cell proliferation and apoptosis are beginning to be elucidated. We have shown recently that tyrosine phosphorylation and tyrosine kinase activation is an important mitogenic signal in SCLC cells and that the regulation of the level of tyrosine phosphorylation may represent a critical determinant of whether SCLC cells survive and proliferate or die by apoptosis (2).

However, little is known about the precise nature of the tyrosine kinases involved or the downstream signaling pathways that may be involved in these responses.

The PI 3-kinase family of enzymes phosphorylate inositol containing phospholipids on the 3' position of the nonpolar inositol head group. Although PI 3-kinase can phosphorylate PI, PI (4)P, and PI (4,5)P₂ *in vitro*, PI (4,5)P₂ is believed to be the preferred substrate *in vivo* generating the second messenger PI (3,4,5)P₃ (3-6). PI 3-kinase is an enzyme that acts as a direct biochemical link between a phosphoinositide pathway and a number of proteins containing intrinsic or associated tyrosine kinase activities including the receptors for insulin and colony-stimulating factor and the products of the oncogenes *v-src* and *v-abl* (7). Phosphorylation of specific tyrosine residues on activated polypeptide growth factor receptors activates the p85-p110 PI 3-kinase heterodimeric complex via an SH2 domain of the p85 regulatory subunit (8). In addition, adhesion to extracellular matrix stimulates the integrin-dependent interaction of the p85 PI 3-kinase complex with FAK, causing increased accumulation of PI (3,4)P₂ and PI (3,4,5)P₃ (9). Thus, PI 3-kinase is a critical component of signaling pathways that can be activated by a variety of growth factors, oncogenes, chemokines, cell surface receptors, and integrins. Use of the selective PI 3-kinase inhibitors wortmannin (10) and LY294002 (11) and dominant-negative or constitutively active mutants of PI 3-kinase have shown that this enzyme plays a key role in a variety of distinct cellular functions including the mitogenic response, apoptosis, intracellular vesicle trafficking/secretion, and regulation of actin and integrin function (reviewed in Ref. 12). Furthermore, recent studies suggest that integrin-mediated PI 3-kinase activation is important for cell migration and can promote carcinoma invasion (13, 14).

In view of these important physiological functions, much interest has focused on potential downstream effectors of PI 3-kinase. Evidence is accumulating to show that PI 3-kinase is a crucial mediator of ribosomal S6 kinase (p70^{S6k}) activation in response to serum and growth factors (15). Activation of p70^{S6k} regulates a wide variety of cellular processes involved in the mitogenic response including protein synthesis, translation of specific mRNA species, and progression from G₁ to S phase of the cell cycle (16-18). Seufferlein and Rozenfurt (19) demonstrated recently that p70^{S6k} is constitutively phosphorylated in SCLC cells and that the p70^{S6k} inhibitor rapamycin inhibits SCLC cell proliferation. Rapamycin inhibits p70^{S6k} indirectly, forming a complex with FK-506-binding protein, which in turn interacts with RAFT/mTOR, a lipid kinase that is a putative upstream regulator of p70^{S6k} (17).

The serine/threonine proto-oncogene PKB is the cellular homologue of the transforming oncogene product v-Akt (20). PKB has been shown to be activated by growth factors, oncogenes, and integrins, signaling through PI 3-kinase (reviewed in Ref. 21). PKB has been shown to be overexpressed in 12% of ovarian, 3% of breast, and 10% of pancreatic cancers, where it has been associated with a poor prognosis and increased tumorigenicity (22, 23). However, the activity state of PKB in tumor cells has not been examined previously.

The ability of cancer cells to grow in the absence of cell adhesion to extracellular matrix has been shown to correlate closely with

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⁴The abbreviations used are: SCLC, small cell lung cancer; PI, phosphoinositide; PI (4)P, PI (4,5)P₂, and PI (3,4,5)P₃, phosphatidylinositol 4-phosphate, 2L,4,5-phosphate, and PI 3,4,5-phosphate, respectively; SH, Src homology; FAK, focal adhesion kinase; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; PKB, protein kinase B; BTSM, bovine tracheal smooth muscle; FBS, fetal bovine serum; p70^{S6k}, ribosomal S6 kinase; MAP, mitogen-activated protein.

PI PHOSPHATIDYLINOSITOL
PI (4)P PHOSPHATIDYLINOSITOL 4-PHOSPHATE
PI (4,5)P₂ PHOSPHATIDYLINOSITOL 4,5-DIPHOSPHATE
PI (3,4,5)P₃ PHOSPHATIDYLINOSITOL 3,4,5-TRIPHOSPHATE

CONSTITUTIVE PI 3-KINASE ACTIVITY IN SCLC CELLS

AGSF: 4-(2-AMINOETHYL)-
BENZENESULFONYL FLUORIDE

tumorigenicity in animal models (24). The main cause of death in patients with cancer arises as a result of either primary tumor invasion or secondary metastatic deposits. Therefore, the ability of tumor cells to survive and grow in inappropriate environments is central to cancer-related death.

The induction of complete oncogenic transformation appears to require both serum and anchorage-independent growth (25). Constitutive stimulation of growth factor pathways by ectopic growth factor expression is mitogenic rather than oncogenic, resulting in benign hyperplasia in animal models (26-28). *In vivo*, autocrine growth factor expression can also be associated with accelerated or serum-independent growth of otherwise normal cells (29). Our previous work suggests that the unrestrained proliferation of SCLC cells is driven by multiple autocrine and paracrine growth loops involving calcium-mobilizing neuropeptides (30). SCLC cells grow as nonadherent, free-floating aggregates *in vitro*; however, the mechanisms resulting in anchorage-independent growth of SCLC cells are unclear. The fact that integrins transmit intracellular signals that protect cells from apoptosis suggests that constitutive activation of these downstream second messengers may mediate anchorage independence. Transformation of adherent cells by cytoplasmic oncogenes such as *ras* or *src* is accompanied by the ability to grow in suspension. Cell detachment-induced apoptosis does not occur in epithelial cells expressing activated *src* or *ras* (31). These oncogenes appear to provide constitutively activated signals, mimicking those initiated by ligand-bound integrins, thereby overcoming the induction of apoptosis initiated by cell detachment from extracellular matrix. A recent study has shown that the ability of *ras* and *src* to protect epithelial cells from cell detachment-induced apoptosis is mediated through PI 3-kinase and PKB (32). However, neither high *src* activity nor activating *ras* mutations have been found in SCLC cells (33-35).

These preliminary findings suggest that the PI 3-kinase pathway may be sensitive to oncogenic conversion and could be important in the development of human cancers. The role of PI 3-kinase-mediated signal pathways in regulating the growth, apoptosis, and anchorage independence of SCLC cells is unknown. Here we demonstrate that all five SCLC cell lines tested have high basal constitutive PI 3-kinase activity. This results in high levels of basal PKB and p70^{src} activity. Furthermore, our results suggest that PI 3-kinase activity plays an important role in promoting the growth and anchorage independence of SCLC cells and may account for the highly metastatic and aggressive nature of this tumor. We believe that this is the first description of constitutively active PI 3-kinase/PKB in a human cancer.

MATERIALS AND METHODS

Cell Culture. SCLC cell lines NCI-H345, NCI-H69, and NCI-H510 and human alveolar type II epithelial cells (A549) were purchased from the American Type Tissue Culture Collection (Rockville, MD). Swiss 3T3 cells were from the European Cell Culture Collection (Porton Down, United Kingdom). SCLC cell lines LS274 and DMS79 were kind gifts from Professor J. Smyth (ICRF, Edinburgh, Scotland). BTSM cells were established in primary culture in our laboratory. 16HBE140 cells were originally from D. Gruenert (University of California, CA). All SCLC cell lines were grown in RPMI 1640 with 25 mM HEPES supplemented with 10% (v/v) heat-inactivated FBS. BTSM, A549, and Swiss 3T3 cells were grown in DMEM containing 10% (v/v) FBS. 16HBE140 cells were cultured in MEM supplemented with 10% FBS and 1% nonessential amino acids. All media contained 50 units/ml penicillin, 50 µg/ml streptomycin, and 5 µg/ml L-glutamine, and cells were cultured in a humidified atmosphere of 5% CO₂/95% air at 37°C.

For experimental purposes, SCLC cells 5 days after passage were transferred into SITA medium consisting of RPMI 1640 with 25 mM HEPES supplemented with 30 nM selenium, 5 µg/ml insulin, 10 µg/ml transferrin, 0.25% (w/v) BSA, 50 units/ml penicillin, 50 µg/ml streptomycin, and 5 µg/ml

L-glutamine and cultured for another 2 days before use. Alternatively, cells were taken from growth medium, washed thoroughly with quiescent medium, and incubated in fresh quiescent medium for 24 h before experimentation. Quiescent medium comprised RPMI 1640 with 25 mM HEPES supplemented with 0.25% (w/v) BSA, 50 units/ml penicillin, 50 µg/ml streptomycin, and 5 µg/ml L-glutamine. BTSM cells were quiesced for 48 h before use in DMEM containing 0.5% FBS. 16HBE140 and A549 cells were quiesced for 24 h in serum-free MEM and DMEM, respectively. Cell viability was confirmed by trypan blue exclusion and was always >95%.

PI 3-Kinase Activity Assay. Adherent cells were washed three times with PBS at 37°C, transferred to ice, and lysed using ice-cold lysis buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM sodium PP_i, 100 mM sodium fluoride, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0.5 mM DTT, 1 mM sodium orthovanadate, 50 µM AEBSF, 5 µg/ml leupeptin, 20 µg/ml aprotinin, and 10 µg/ml soybean trypsin inhibitor. SCLC cells (1 × 10⁷) that had been washed three times with PBS at 37°C were transferred to 24-well plates in 1 ml of PBS and allowed to equilibrate at 37°C for 1 h before the addition of varying concentrations of wortmannin or DMSO vehicle (final concentration, 0.1% v/v) for 20 min. Cells were then transferred to ice, pelleted, and lysed. Lysates were clarified by centrifugation at 13,000 × g for 10 min. For each assay point, 1 mg of total protein from the supernatant was incubated with 2.5 µg of p85-PI 3-kinase antibody for 90 min at 4°C. Thirty µl of goat anti-mouse IgG agarose beads in lysis buffer were added for an additional 2 h. The beads were then pelleted and washed sequentially with: lysis buffer, 100 mM Tris-HCl, and 500 mM LiCl (pH 7.6), once; and twice with 200 mM HEPES, 40 mM MgCl₂, 600 mM NaCl (pH 7.4). PI 3-kinase assays were performed using phosphatidylinositol as substrate in a final volume of 200 µl containing: (final concentrations) 50 mM HEPES (pH 7.4), 10 mM MgCl₂, 150 mM NaCl, [γ-³²P]ATP (10 µCi, 3000 Ci/mmol), 50 µM ATP, and 0.5 mg/ml sonicated phosphatidylinositol:phosphatidylserine (3:1, w/w). Reactions were carried out for 20 min at 37°C and terminated by the addition of chloroform:methanol:0.1 M HCl (40:80:1, v/v/v; 750 µl), and a phase-partition was achieved by the addition of chloroform (250 µl) and 0.1 M HCl (250 µl). After centrifugation, the lower phase was washed twice with synthetic upper phase, dried, and the lipids were separated by TLC. Phosphorylated lipids were identified by autoradiography and radioactivity quantified by liquid scintillation counting. For assays involving LY294002, immunoprecipitates from untreated cells were prepared as above, washed, and incubated with varying concentrations of LY294002 for 20 min at 37°C before PI 3-kinase activity assays as above.

p70^{src} Activity Assay. p70^{src} activity was assayed essentially as described by Scott *et al.* (36) with some minor modifications. Cells cultured in SITA medium were washed and plated as outlined above before the addition of varying concentrations of inhibitors at 37°C for 20 min. Cells were solubilized for 30 min at 4°C in a buffer containing 50 mM Tris/HCl (pH 8.0), 120 mM NaCl, 20 mM NaF, 5 mM EGTA, 1 mM EDTA, 10 mM sodium PP_i, 10 mM β-glycerophosphate, 20 mM MgCl₂, 3 mM EGTA, 0.2 mM sodium orthovanadate, and 2 mM dithiothreitol and then incubated in the same buffer containing 100 µM S40 substrate peptide, 10 µM adenosine 3',5'-cyclic monophosphate-dependent protein kinase inhibitor and [γ-³²P]ATP (10 µM; 1 µCi, 3000 Ci/mmol) in a volume of 30 µl for 20 min at 30°C. The reaction was terminated by the addition of 10 µl of 300 mM phosphoric acid, and the mixture was spotted onto P81 chromatography paper. The papers were washed twice with 0.5% (v/v) phosphoric acid, allowed to dry, and phosphorylation of the S40 substrate peptide was quantified by liquid scintillation counting as an index of enzymatic activity.

PKB Activity Assay. SCLC cells (1 × 10⁶) cultured in quiescent medium for 24 h before experimentation were washed three times in PBS and aliquoted as outlined above before the addition of varying concentrations of inhibitor for 20 min. Cells were then lysed using 250 µl of ice-cold buffer containing 50 mM Tris/HCl (pH 7.5), 0.1% (v/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 M sucrose, 50 mM sodium fluoride, 10 mM sodium β-glycerophosphate, 5 mM

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sodium PP_i, 1 mM sodium orthovanadate, 0.1% (v/v) 2-mercaptoethanol, 1 μM microcystin, 5 μg/ml leupeptin, 20 μg/ml aprotinin, and 10 μg/ml soybean trypsin inhibitor. Lysates were clarified by centrifugation at 13,000 × g for 10 min, and PKB was immunoprecipitated by incubating the lysates (containing 150 μg of protein) for 90 min at 4°C with 1 μg of anti-PKBα antibody pre-conjugated to 15 μl of protein G-Sepharose in lysis buffer. Immunoprecipitates were washed twice with lysis buffer containing 0.5 M NaCl and once with a buffer consisting of 50 mM Tris/HCl (pH 7.5), 0.03% (v/v) Brij-35, 0.1 mM EGTA, and 0.1% (v/v) 2-mercaptoethanol. PKB activity was assayed by incubating washed immunoprecipitates at 30°C for 20 min in a thermomixer in 30 μl of kinase assay buffer (50 μM Crosside, 50 mM Tris/HCl, 0.1 mM EGTA, 20 μM adenosine 3',5'-cyclic monophosphate-dependent protein kinase inhibitor, 20 mM MgAc, 0.2 mM ATP, 1 μM microcystin, and [γ-³²P]ATP (1.0 μCi, 3000 Ci/mmol). The assays were terminated by placing 40 μl of assay mixture onto P81 chromatography paper and washing four times with 0.5% (v/v) phosphoric acid and once with acetone. Radioactive incorporation was quantified by liquid scintillation counting.

Immunoblotting. Cell pellets were lysed at 4°C in PI 3-kinase lysis buffer (see above) for 30 min. Lysates were clarified by centrifugation at 13,000 × g for 10 min at 4°C. Twenty μg of protein were solubilized in SDS-PAGE sample buffer and resolved on 10% gels. The proteins were transferred to nitrocellulose membranes, blocked using 5% (w/v) nonfat milk in TBS-Tween [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.02% (v/v) Tween 20] overnight at 4°C and then incubated sequentially with primary and secondary antibodies diluted in blocking buffer for 1 h each at room temperature. Primary antibodies (used at a 1:1000 dilution) were the same as those used in the activity assays. The secondary antibodies were species appropriate horseradish peroxidase-conjugated antibodies. Immunoreactive bands were identified using ECL according to the manufacturer's instructions.

Liquid Growth Assay. Cultures of SCLC cell lines H345, H69, and H510 at 5 days after passage were grown for 2 days in SITA medium. Before experimentation, cells were washed twice and resuspended in fresh SITA medium before being gently disaggregated by two passes through a 21-gauge needle into an essentially single-cell suspension as judged by light microscopy. Viability was determined by trypan blue exclusion on a hemocytometer. Cells (1 × 10⁵) were seeded into 24-well plates in SITA medium and incubated for 4 h in a humidified atmosphere of 5% CO₂/95% air at 37°C before the addition of inhibitors (see figure legends); cells were then further incubated under the same conditions before being removed at various times and disaggregated into a single-cell suspension using a 21-gauge needle, and cell number was determined using a Coulter Cell Counter (Coulter Electronics, United Kingdom).

Clonogenic Assay. SCLC cells, 3–5 days after passage, were washed and resuspended in SITA medium. Cells were then disaggregated into a single-cell suspension by two passes through a 21-gauge needle. Viability was judged by trypan blue exclusion. Cell number was determined with a Coulter cell counter and 1 × 10⁴ viable cells mixed with SITA containing 0.3% (w/v) agarose and

agonist/antagonist at the concentrations indicated (see figure legend) and layered over a solid base of 0.5% (w/v) agarose in SITA with agonist/antagonist at the same concentration, in 35-mm plastic dishes. The cultures were incubated in humidified 5% CO₂/95% air at 37°C for 21 days and then stained with the vital stain nitroblue tetrazolium. Colonies of >120-μm diameter (16 cells) were counted with a microscope.

Morphological Assessment of SCLC Cell Apoptosis. SCLC cells (1 × 10⁵) were seeded into 96-well plates in 200 μl of SITA medium and incubated in the presence or absence of 10 μM LY294002, 100 nM wortmannin, or appropriate diluent for 24 h. One μl of an ethidium bromide (100 μg/ml):acridine orange [100 μg/ml; 1:1 (v/v)] mixture was added to each well, and the percentage of cells undergoing apoptosis was assessed using fluorescent microscopy as described previously (37).

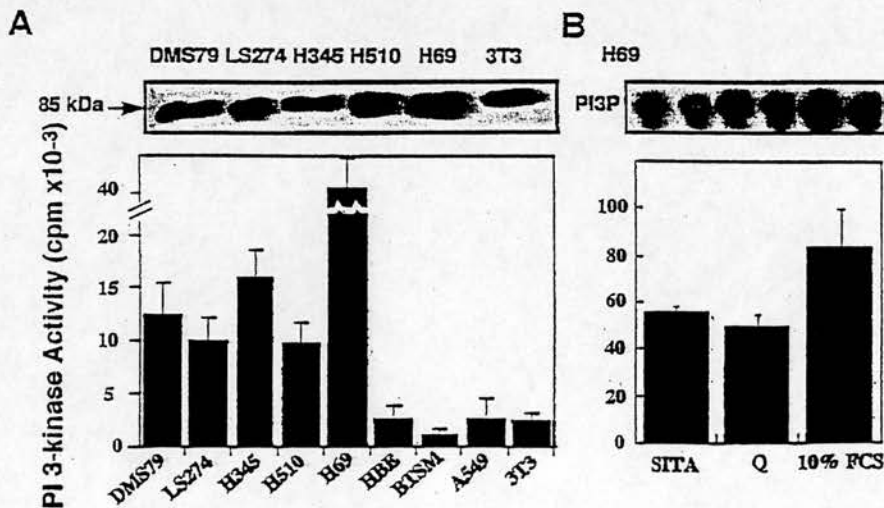
Flow Cytometric Assessment of Cell Cycle Phase. Cells (1 × 10⁵) were cultured in SITA medium in the presence or absence of LY294002 (10 μM). After 24 h, cells were washed twice in PBS and fixed in 70% ice-cold ethanol. Cellular DNA content was determined by staining with 50 μg/ml of propidium iodide and analyzed according to forward and side scatter properties using an EPICS Profile II (Coulter Electronics, Luton, United Kingdom). The proportion of cells in G₁, S, and G₂-M phases (minimum of 3000 cells analyzed per condition) was calculated using standard methods.

Materials. RPMI 1640, BSA agarose, IgG protein agarose, rapamycin, and wortmannin were purchased from Sigma Chemical Co. (Poole, United Kingdom). Monoclonal p85-SH3 antibody and the S40 peptide substrate were from TCS (Buckingham, United Kingdom); polyclonal p70^{SH} antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Adenosine 3',5'-cyclic monophosphate-dependent protein kinase inhibitor and LY294002 were from Calbiochem-Novabiochem Corp (Nottingham, United Kingdom). [γ-³²P]ATP (3000 μCi/mmol) and Hybond C nitrocellulose membranes were purchased from Amersham International (Amersham, United Kingdom), and horseradish peroxidase-conjugated antibodies were obtained from DAKO UK. PKBα antibody and Crosside substrate peptide were a kind gift from Dr. D. Alessi and Professor P. Cohen (University of Dundee, Dundee, United Kingdom). All other reagents were of the purest grade available.

RESULTS

SCLC Cells Display Constitutive PI 3-Kinase Activity, Which Is Inhibited by Wortmannin and LY294002. PI 3-kinase is known to be critically involved in cell proliferation and anchorage-independent growth (32). We therefore tested whether the highly aggressive phenotype of SCLC might, in part, result from constitutive PI 3-kinase activity. Western blot analysis of lysates from H69, H345, H510, LS274, and DMS79 SCLC cell lines showed expression of the p85α subunit of PI 3-kinase (Fig. 1A).

Fig. 1. A. PI 3-kinase expression and activity in SCLC cell lines. Upper panel, Western blots of SCLC cell lysates (DMS79, LS274, H69, H345, and H510) and Swiss 3T3 lysates were probed with a monoclonal anti-p85α-SH3 antibody and visualized using ECL. Lower panel, for activity assays, PI 3-kinase was immunoprecipitated from cell lysates using an anti-p85α-SH3 antibody. PI 3-kinase activity was assayed (as described in "Materials and Methods") using phosphatidylinositol as substrate. 3-Phosphorylated lipids were resolved using TLC, identified by autoradiography and quantified by liquid scintillation counting. Results are the means of two independent experiments; bars, SD. HBE, human bronchial epithelial cells; A549, human alveolar type II epithelial cells; 3T3, Swiss 3T3 fibroblasts. B, basal PI 3-kinase activity in SCLC cells. SCLC cells grown in serum-free SITA medium or quiescent medium (Q) were washed three times in PBS and then equilibrated in fresh PBS for 1 h. Ten % FCS indicates cells taken from quiescent medium and stimulated with 10% FCS for 10 min before lysis.



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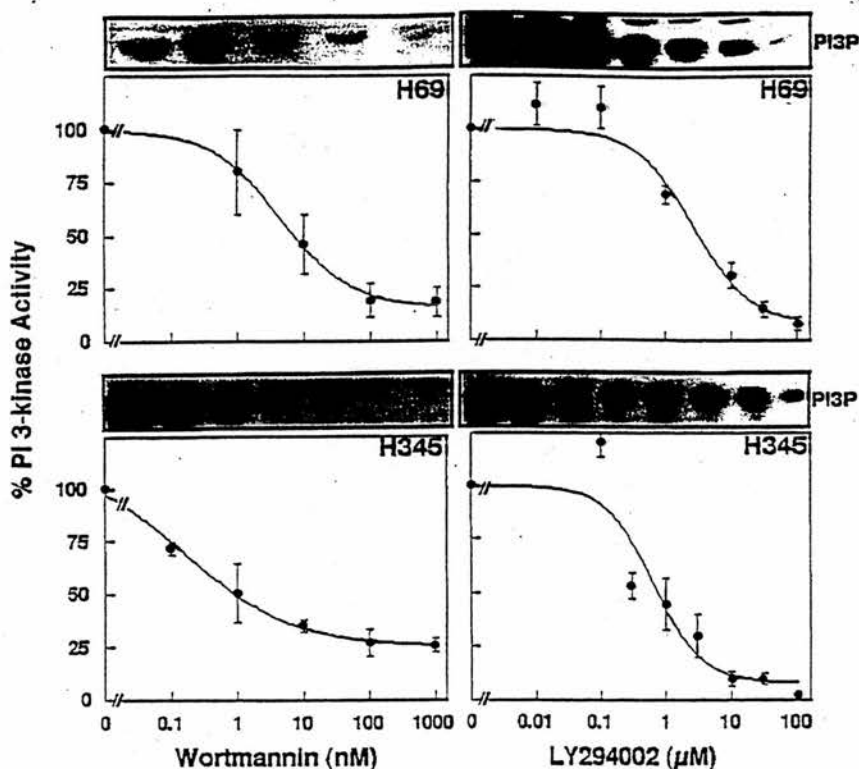


Fig. 2. Effect of PI 3-kinase inhibition on PI 3-kinase activity in SCLC cells. Concentration response of wortmannin (left) and LY294002 (right) on PI 3-kinase activity in H69 (upper) and H345 (lower) SCLC cells. Values shown are the means of two to three independent experiments performed in duplicate; bars, SE. Basal PI 3-kinase activity is taken as 100%. Representative autoradiographs showing the 3-phosphorylated reaction product (PI3P) are shown for each condition.

To examine basal PI 3-kinase activity, SCLC cells were cultured in serum-free SITA medium for 48 h and then maintained for an additional 24 h in growth factor-free quiescent medium. The cells were then washed extensively in PBS and allowed to equilibrate in PBS for 1 h to remove exogenous/autocrine growth factors. PI 3-kinase activity from p85 α immunoprecipitates was measured as described in "Materials and Methods." The identity of the PI (3)P was confirmed by monomethylamine deacylation and HPLC analysis using a SAX 5 column and (NH₄)₂HPO₄ gradient and authentic tritiated standards as markers (data not shown). Fig. 1A shows a markedly elevated basal PI 3-kinase activity in all of the SCLC cell lines tested (H69, H345, H510, DMS79, and LS274) compared with human bronchial epithelial cells (16HBE140), BTSM cells, human alveolar type II epithelial cells (A549), and Swiss 3T3 cells. Fig. 1B shows that SCLC cells grown in either serum-free SITA medium or growth factor-free quiescent medium have equally high basal PI 3-kinase activity. Thus, under conditions where every effort was made to remove all exogenous/autocrine growth factors, high basal PI 3-kinase activity was seen in SCLC cells. Furthermore, 10% FCS could induce only a modest additional increase in PI 3-kinase activity (151 \pm 19.4%; Fig. 1B). We therefore concluded that SCLC cells possess a constitutively active PI 3-kinase. This was further supported by data using the PI (3,4,5)P₃ mass assay (38), which showed that SCLC cells in a basal state have elevated levels of PI (3,4,5)P₃ (4.5 \pm 2.1 pmol/mg) compared with a number of other cell types including Swiss 3T3 and 1321N1 astrocytoma cells (2.16 \pm 1.0 pmol/mg).⁵

Wortmannin and LY294002 (at concentrations up to 100 nM and 100 μ M, respectively) are specific PI 3-kinase inhibitors and have

emerged as useful tools to elucidate the cellular function and signal transduction pathways of PI 3-kinase (10, 11). Treatment of SCLC cells with wortmannin for 20 min (Fig. 2, left panels) or the addition of LY294002 directly to immunoprecipitates 20 min before assaying for PI 3-kinase activity (Fig. 2, right panels) caused a concentration-dependent inhibition of basal PI 3-kinase activity in three SCLC lines examined. The IC₅₀ of PI 3-kinase activity by wortmannin in H69 and H345 cells was 8.0 \pm 2.1 nM and 0.27 \pm 1.1 nM, respectively, and for LY294002, 3.4 \pm 1.4 and 0.85 \pm 0.41 μ M, respectively (mean \pm SE; n = 2-4). Similar results were also seen in H510 cells (IC₅₀, 11.1 \pm 4.1 nM and 1.2 \pm 0.4 μ M for wortmannin and LY294002, respectively).

p70^{src} Activity Is PI 3-Kinase Dependent. The molecular targets of PI 3-kinase are being defined by studies using PI 3-kinase inhibitors. Evidence is accumulating that PI 3-kinase is a crucial mediator of p70^{src} activation (15). Fig. 3A shows a Western blot analysis of p70^{src} expression in SCLC cell lines H69, H345, and H510. We therefore investigated the possibility that the constitutive phosphorylation of p70^{src} described previously in SCLC cells (19) causes high basal p70^{src} activity that is driven by constitutive PI 3-kinase activity. Immunoprecipitation of p70^{src} from SCLC cells maintained under conditions identical to those described by Seufferlein and Rozengurt (Ref. 19; see "Materials and Methods") showed a high level of basal p70^{src} activity. For H69 cells, basal activity was 4317 \pm 346 cpm, and in H345 cells, basal activity was 4417 \pm 897 cpm (mean \pm SE; n = 3-4). Preincubation of intact SCLC cells with the PI 3-kinase inhibitors wortmannin (Fig. 3B, left panels) and LY294002 (Fig. 3B, right panels) for 20 min caused a concentration-dependent inhibition of p70^{src} activity in H69 cells (IC₅₀, 1.3 \pm 0.73 nM and 0.9 \pm 0.68 μ M, respectively) and H345 cells (IC₅₀, 14.6 \pm 5.5 nM and 4.2 \pm 1.2

⁵ J. van der Kaay, personal communication.

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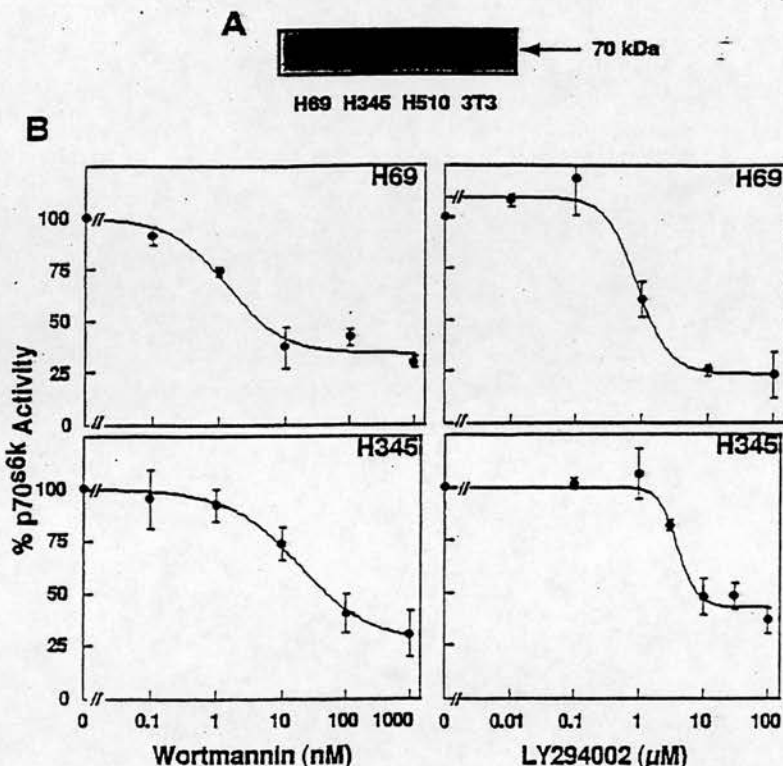


Fig. 3. A, expression of the p70 isoform of ribosomal s6 kinase in SCLC cell lines H69, H345, and H510 and Swiss 3T3. Cell lysates were Western blotted and probed using a rabbit polyclonal anti-p70^{s6k} antibody. Immunoreactive bands were visualized by ECL. B, p70^{s6k} activity in SCLC. Well-washed H69 (upper) and H345 (lower) SCLC cells were equilibrated in fresh PBS for 1 h and treated with wortmannin (left) or LY294002 (right), at concentrations indicated, for 20 min before lysis. p70^{s6k} was immunoprecipitated from cell lysates, and activity was assayed in an *in vitro* kinase reaction using S40 peptide as substrate (as described in "Materials and Methods"). Values shown are the means of three to four independent experiments performed in duplicate; bars, SE. Basal p70^{s6k} activity is expressed as 100%. For H69 experiments, basal activity was 4317 ± 346 cpm, and nonspecific background activity was 698 ± 158 cpm; in H345 experiments, basal activity was 4417 ± 897 cpm, and background activity was 392 ± 90 cpm.

μM, respectively; mean ± SEM; n = 3-4). Similar results were seen in H510 cells with wortmannin and LY294002 (IC₅₀, 4.1 nM and 1.0 μM, respectively). These results are similar to the IC₅₀s obtained for wortmannin and LY294002 inhibition of PI 3-kinase. The maximal inhibition of p70^{s6k} activity achieved using either 100 nM wortmannin or 10 μM LY294002 was 75%. The addition of rapamycin, a highly potent inhibitor of p70^{s6k}, to intact SCLC cells for 20 min caused a 100% inhibition of p70^{s6k} activity (data not shown). The addition of either 100 nM wortmannin or 10 μM LY294002 to the *in vitro* kinase reaction had no effect on the kinase activity of p70^{s6k} immunoprecipitates (data not shown). Thus, wortmannin and LY294002 do not affect p70^{s6k} activity directly but regulate p70^{s6k} activity via wortmannin/LY294002 sensitive PI 3-kinase activity.

Constitutive PKB Activity in SCLC Cells Is PI 3-Kinase Dependent. In integrin and growth factor signal transduction, PKB has been identified as a key downstream effector of PI 3-kinase (21). Western blot analysis of SCLC cell lines H510, H69, and H345 revealed expression of the α isoform of PKB (Fig. 4A). Hence, we examined basal PKB activity in SCLC cells. SCLC cells that had been maintained in growth factor-free quiescent medium for 24 h before experimentation were thoroughly washed, and PKB activity was determined using an *in vitro* kinase assay as described in "Materials and Methods." Fig. 4B shows that under basal conditions, SCLC H69 cells display constitutive PKB activity that could be completely inhibited by 100 nM wortmannin. The p70^{s6k} inhibitor rapamycin had no effect on PKB activity (Fig. 4B). Similar results were seen in H345 and H510 cells (results not shown). Under identical conditions, very low levels of basal PKB activity was seen in Swiss 3T3 cells despite a similar level of expression of PKBα as judged by Western blot analysis (Fig. 4, A and B). The addition of LY294002 (Fig. 4C, left)

or wortmannin (Fig. 4C, right) to H69 cells for 20 min caused a concentration-dependent inhibition of basal PKB activity. The IC₅₀ for LY294002 and wortmannin was 2.7 ± 0.1 μM and 2.0 ± 0.6 nM, respectively (mean ± SE; n = 3). These values correlate well with those seen for PI 3-kinase inhibition. When added directly to PKB immunoprecipitates, wortmannin and LY294002 had no effect on PKB activity. These results suggest that the elevated basal PKB activity in SCLC cells is a direct downstream consequence of constitutive PI 3-kinase activity in these cells.

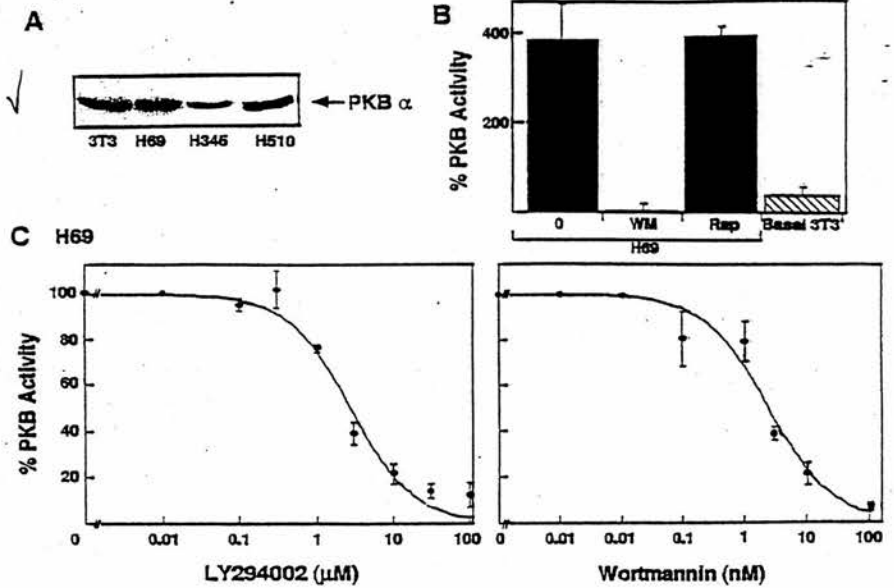
SCLC Cell Growth in Liquid Culture Is PI 3-Kinase Dependent. We went on to examine the biological consequences of blocking constitutive PI 3-kinase signaling. SCLC cells were grown in SITA in the presence of increasing concentrations of LY294002, and the cell number was counted at day 9. LY294002 caused a marked concentration-dependent reduction in SCLC cell number in liquid culture (IC₅₀s, 10.0 ± 0.8, 2.9 ± 0.2, and 13.3 ± 0.8 μM for H69, H345, and H510 cells, respectively; mean ± SE; n = 3-5; Fig. 5). These results are in good agreement with data obtained for inhibition of PI 3-kinase, PKB, and p70^{s6k} activity by LY294002. Thus, constitutive PI 3-kinase activity appears to play an important role in the proliferation of this aggressive cancer.

To determine the relative contribution of the p70^{s6k} pathway to PI 3-kinase-mediated growth in SCLC, SCLC cells were grown in the presence of the specific inhibitors LY294002 and rapamycin both alone and in combination, as described in "Materials and Methods." Fig. 6 shows that the addition of a maximally effective concentration of LY294002 (100 μM) was able to inhibit H69 and H345 SCLC growth by 92% over a 9-day period, whereas a maximally effective concentration of rapamycin (20 nM; Ref. 19) caused a 6.5 and 33% reduction in growth over the same time period in H69 and H345 cells,

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Fig. 4. A. PKB α expression in SCLC cell lines H69, H345, H510, and Swiss 3T3. Western blots of cell lysates were probed with an anti-PKB α antibody and visualized by ECL. B. PKB activity in SCLC cells. SCLC cells grown in quiescent medium were well washed and then equilibrated in fresh PBS for 1 h. PKB was immunoprecipitated from cell lysates, and activity was assayed in an *in vitro* kinase reaction using Crosslide as substrate (as described in "Materials and Methods"). Basal PKB activity was measured in H69 SCLC cells preincubated with diluent (O), 100 nM wortmannin (WM), or 20 nM rapamycin (Rap). Basal 3T3 shows PKB activity in Swiss 3T3 cells under identical conditions as described above. Results expressed as percentage of PKB activity above nonspecific background are the means of two independent experiments in duplicate; bars, SD. C. Concentration-dependent inhibition of basal PKB activity by PI 3-kinase inhibitors in SCLC cells. SCLC cells were treated with LY294002 (left) or wortmannin (right) for 20 min before lysis. Each point represents the mean of three independent experiments performed in duplicate; bars, SE. Basal PKB activity is expressed as 100% (1824 \pm 241 cpm; nonspecific background, 596 \pm 87 cpm).



respectively. Incubation of H69 and H345 cells with a combination of 10 μ M LY294002 (a value close to the IC₅₀) and 20 nM rapamycin (a maximally effective concentration) caused an ~61–68% inhibition of SCLC cell growth over 9 days (Fig. 6). Similar results were seen in H510 cells. These results suggest that PI 3-kinase exerts its growth effects by both p70^{s6k}-dependent and independent pathways in SCLC. However, it appears that the majority of the PI 3-kinase effect is mediated via a p70^{s6k}-independent pathway, potentially signaling through PKB.

SCLC Cell Survival Is PI 3-Kinase Dependent. Activation of PI 3-kinase and PKB activity has been implicated in the protection of cells from apoptosis. The effect of PI 3-kinase inhibitors on the rate of SCLC cell apoptosis was determined using acridine orange/ethidium bromide staining under fluorescent microscopy. The background level of SCLC cell apoptosis in these experiments was 8%. H345 cells treated with 10 μ M LY294002 or 100 nM wortmannin for 24 h showed a 41 \pm 12 and 31 \pm 13% increase in the percentage of cells undergoing apoptosis, respectively. The figures for H69 cells were 82 \pm 30% and 95 \pm 22%, respectively (mean \pm SE; n = 3; Fig. 7A). These data were corroborated using an immunoassay that detected

cytoplasmic histone-associated DNA fragments (data not shown). During this time course, trypan blue positivity remained consistently <5%. Thus, although inhibition of PI 3-kinase activity in SCLC cells caused a marked inhibition of cell growth in liquid culture, this did not occur as a result of increased cell necrosis but was due, in part, to an increase in apoptosis. Because the proapoptotic effect due to PI 3-kinase inhibition was relatively modest, we examined the effect of PI 3-kinase inhibitors on cell cycle kinetics. Fig. 7B shows that 10 μ M LY294002 causes a cell cycle delay in G₁ and a decrease in the number of cells entering mitosis. Therefore, the growth-inhibitory effects induced by LY294002 are due to a combination of an increase in apoptosis and a delay in the cell cycle.

LY294002 Inhibits SCLC Cell Tumorigenicity and Neuropeptide-stimulated Colony Growth. The ability to form colonies in agarose semisolid medium is a marker of anchorage-independent growth that is characteristic of the transformed phenotype. There is a positive correlation between the cloning efficiency of cells and the histological involvement and invasiveness of the tumor in specimens taken from SCLC (39). We therefore examined the effect of 10 μ M LY294002 on SCLC colony formation in agarose semisolid medium.

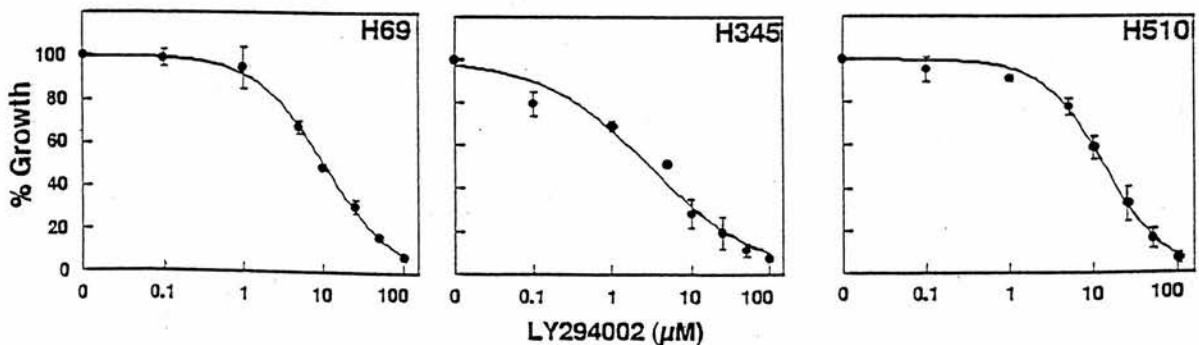


Fig. 5. Concentration-dependent effect of LY294002 on H69, H345, or H510 SCLC growth. SCLC cells (1×10^3) were washed and incubated in fresh SITA medium in the presence of increasing concentrations of LY294002 as shown. Cell number was determined on day 9, and results were expressed as a percentage of cell growth in the presence of diluent alone. Each point represents the mean of three to five independent experiments performed in triplicate; bars, SE.

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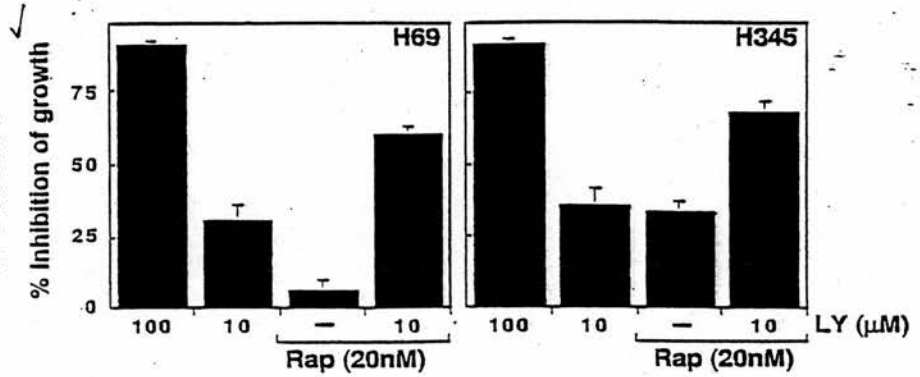


Fig. 6. Effect of LY294002 and rapamycin on H69 (left) and H345 (right) SCLC cell growth. Cells (1×10^5) were washed and incubated in fresh SITA medium in the presence of LY294002 (LY) and rapamycin (Rap) alone or in combination, at the concentrations indicated. On day 9, cell number was measured. Results expressed as percentage inhibition of growth compared with control cell growth are means of three to five independent experiments performed in triplicate; bars, SE.

Fig. 8 shows that $10 \mu\text{M}$ LY294002 inhibited basal colony formation of all three SCLC cell lines by up to 80%. Our previous work has suggested that SCLC cell growth is sustained by multiple autocrine and paracrine growth loops involving calcium mobilizing peptides. As shown previously, the neuropeptides vasopressin (50 nM), gastrin (100 nM), and bombesin (50 nM) caused a marked stimulation of colony formation in H69, H510, and H345 cells, respectively (30). However, the addition of vasopressin, gastrin, or bombesin failed to rescue the LY294002-induced inhibition of colony formation in these cell lines (Fig. 8). Thus, PI 3-kinase appears to play a critical role in sustaining the anchorage-independent growth of SCLC.

DISCUSSION

SCLC has the highest metastatic potential of any of the solid tumors with >90% of patients having widespread metastases at presentation. A characteristic feature of cancer cells is their ability to grow in the absence of cell adhesion to extracellular matrix. The development of secondary metastatic deposits at sites remote from the primary tumor is one of the main causes of death in patients with cancer. Therefore, the ability of tumor cells to survive and proliferate in inappropriate environments is central to cancer-related death. Thus, anchorage-independent growth is pivotal to the highly proliferative and metastatic nature of this cancer.

The novel findings of this study are that in all five SCLC cell lines examined, constitutive PI 3-kinase activity was found that results in high levels of basal PKB and $p70^{\text{S6k}}$ activity. Inhibition of PI 3-kinase activity blocks SCLC cell growth in liquid culture and colony formation in semisolid medium. We show that this occurs due to a combination of a stimulation of apoptosis and a delay in the cell cycle in G_1

with a consequent decrease in the number of cells entering mitosis. Our results also show that SCLC growth and survival is mediated both by $p70^{\text{S6k}}$ -dependent and -independent pathways, the latter potentially acting through PKB. Thus, downstream pathway(s) driven by a constitutively active PI 3-kinase appear to play an important role in promoting the growth and anchorage independence of SCLC. Recently, PI 3-kinase activation has been implicated in anchorage-independent growth, metastasis, and cell invasion (13, 14). To our knowledge, this is the first description of constitutively activated PI 3-kinase/PKB in any human cancer. Constitutive activation of these integrin-dependent signaling events may provide a molecular explanation for anchorage-independent growth and account for the highly metastatic nature of SCLC. We propose that anchorage independence mediated by constitutive PI 3-kinase activity, in concert with serum independence mediated by multiple autocrine/paracrine-growth loops driven by calcium-mobilizing neuropeptides, is responsible for the very aggressive nature of SCLC.

The origin of this constitutive PI 3-kinase activity in SCLC cells is unclear. Before analysis of PI 3-kinase activity, every effort was made to remove exogenous growth factors by extensive cell washing. Hence, we feel that it is unlikely that exogenous growth factor stimulation is mediating this high PI 3-kinase activity, and furthermore, under identical conditions PI 3-kinase activity is low in control cells. PI 3-kinase can be activated by intracellular second messengers such as *ras*, FAK, and *src*, which are also able to promote anchorage-independent growth (24, 32, 40, 41). However, several studies have failed to show any evidence of activating *ras* mutations in SCLC (33, 34). In addition, we have shown previously that under the basal conditions described above, FAK phosphorylation is low (2). Further-

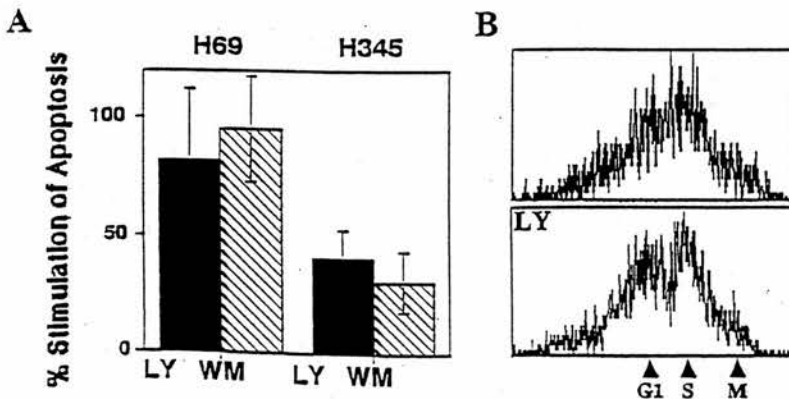


Fig. 7. A, effect of the PI-3 kinase inhibitors LY294002 and wortmannin on H69 (left) and H345 (right) SCLC cell apoptosis. Cells (1×10^5) were washed and incubated in fresh SITA for 24 h in the presence of either diluent or $10 \mu\text{M}$ LY294002 (LY) or 100 nM wortmannin (WM). Apoptotic cells were identified using acridine orange/ethidium bromide under fluorescence microscopy. The results that are expressed as a percentage of stimulation of apoptosis over control cells are the means of six independent experiments performed in triplicate; bars, SE. B, effect of LY294002 on SCLC cell cycle. H69 cells that had incubated for 24 h in the presence or absence of $10 \mu\text{M}$ LY294002 (LY) were washed in PBS and fixed with 70% ice-cold ethanol. Cellular DNA content was determined by staining with propidium iodide, and cell cycle phase was analyzed using an EPICS Profile II. G_1 , S, and M refer to stages of the cell cycle.

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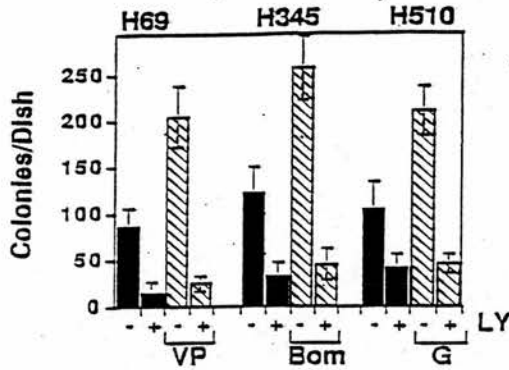


Fig. 8. Effect of LY294002 on SCLC clonal growth in semisolid medium. H69, H345, and H510 SCLC cells, 3-5 days after passage, were washed, and 1×10^6 viable cells/ml were plated in SITA medium containing 0.3% agarose on top of a base of 0.5% agarose in culture medium as described in "Materials and Methods." Both layers contained either no neuropeptide additions (■) or 50 nM vasopressin (VP), 50 nM bombesin (Bom), or 100 nM gastrin (G; ▨) in the presence (+) or absence (-) of $10 \mu\text{M}$ LY294002 (LY). After 21 days, colonies of $>120 \mu\text{M}$ (16 cells) were counted. Results are expressed as colonies/dish, means of triplicates of two independent experiments; bars, SD.

more, pp60 src activity is low in SCLC cells (35). This suggests the possibility of a novel mechanism of PI 3-kinase activation or a mutationally activated PI 3-kinase in SCLC cells.

The molecular targets of PI 3-kinase are being defined by studies using PI 3-kinase inhibitors. In this study, we used the PI 3-kinase inhibitors wortmannin and LY294002. To ensure the specificity of effects seen with pharmacological agents, it is important that inhibitors with differing mechanisms of action produce similar effects within the concentration range described for other cell systems. Wortmannin (IC_{50} , 3 nM) is noncompetitive with respect to ATP, binding irreversibly to the p110 catalytic subunit of PI 3-kinase (10), whereas LY294002 (IC_{50} , 1.4 μM) behaves as a competitive inhibitor for the ATP binding site of PI 3-kinase (11).

PKB and p70^{src} have been identified as downstream effectors of PI 3-kinase (15, 21). Our results may underlie the constitutive basal phosphorylation of p70^{src} noted previously in SCLC cell lines H510, H69, and H345 (19). This constitutive basal phosphorylation is reflected in the high basal activity of p70^{src}. Wortmannin and LY294002 inhibit p70^{src} activity by 70-80% with IC_{50} s similar to those seen for both PI 3-kinase and p70^{src} inhibition. Thus, the constitutive phosphorylation and kinase activity of p70^{src} is, in part, driven by constitutive PI 3-kinase activity. However, even when high concentrations of wortmannin (100 nM) and LY294002 (100 μM) were used, residual p70^{src} activity was noted. This may be due to activation of p70^{src} by other pathways, e.g., the PKC pathway as described in other cell systems (17). This may also explain why H345 cell p70^{src} was more resistant to the effects of PI 3-kinase inhibitors than in H69 cells with an IC_{50} shifted to the right and higher residual p70^{src} activity, despite maximal PI 3-kinase inhibition. Constitutive activation of the ϵ isoform of PKC has been described in a SCLC cell line (42).

PKB overexpression in human ovarian, breast, and pancreatic cancers has been shown to be associated with a poor prognosis and increased tumorigenicity (22, 23). However, these studies did not examine PKB activity. All three SCLC lines that we examined expressed the α isoform of PKB and had elevated basal PKB activity that could be completely inhibited by wortmannin and LY294002 in a concentration-dependent manner similar to PI 3-kinase. Therefore, the high basal PKB activity in SCLC cells occurs as a consequence of constitutive PI 3-kinase activity. The doublet band for PKB α was only

seen in H510 cells and was a constant finding ($n = 3$). The reason is obscure but may be due to a posttranslational modification or an alternatively spliced form of the protein.

To examine the functional importance of this up-regulated pathway, we used the PI 3-kinase inhibitor LY294002. The addition of LY294002 to SCLC cells in liquid culture resulted in a marked concentration-dependent reduction in cell numbers. The IC_{50} s are very similar to the IC_{50} s for inhibition of PI 3-kinase, PKB, and p70^{src} activity by LY294002. PI 3-kinase inhibition can almost totally block SCLC cell proliferation. Thus, constitutive PI 3-kinase-dependent signaling is playing a critical role in SCLC growth.

The addition of PI 3-kinase inhibitors, wortmannin, and LY294002 to SCLC cells showed a modest but consistent increase in the percentage of cells undergoing apoptosis. In addition, PI 3-kinase inhibition causes a cell cycle delay, and the combination of cell cycle delay and stimulation of apoptosis appear to be responsible for the growth inhibition seen. Studies using trypan blue and acridine orange excluded a toxic effect being responsible. PI 3-kinase inhibition may arrest growth by preventing normal growth factor signaling. Recent evidence suggests that growth factors do not induce DNA synthesis in 3T3 cells in the absence of integrin activation (43). This appears to result from the inability of growth factors to activate the MAP kinase pathway in the absence of integrin-mediated second messenger signals. We propose that constitutive activation of PI 3-kinase in SCLC cells may mimic integrin-dependent signal transduction and facilitate growth factor-mediated activation of the MAP kinase pathway. When this second messenger pathway is blocked, growth factors can no longer activate MAP kinase, leading to SCLC cell growth arrest.

PI 3-kinase acting through PKB has been shown to promote anchorage-independent growth (32). This may be central to the survival and growth of tumor cells in inappropriate environments. The ability of cells to grow in soft agar is a feature of anchorage independence and pathognomonic of the transformed phenotype, correlating with tumorigenicity and invasiveness of the tumor (39). SCLC basal colony growth can be stimulated by neuropeptide growth factors (30, 44). These observations, along with the finding that SCLC cells produce a variety of neuropeptides and hormones (45), gave rise to the autocrine/paracrine theory of SCLC growth. LY294002 markedly inhibited basal colony formation of SCLC cells in agarose semisolid medium. This block could not be overcome by the addition of vasopressin, gastrin, or bombesin in H69, H510, and H345 cells, respectively. This shows that PI 3-kinase activity is crucial for anchorage-independent growth in SCLC cells. Furthermore, these results suggest that PI 3-kinase activity is required for neuropeptide mitogenic signaling. In SCLC cells, neuropeptides have been shown to activate MAP kinase through G-protein-coupled receptors (46). PI 3-kinase activity may be necessary for neuropeptide activation of MAP kinase in SCLC. It remains to be established whether MAP kinase activation can be potentiated by growth factor/integrin-dependent PI 3-kinase activation and whether this underlies our observations of the effects of PI 3-kinase inhibition on SCLC growth.

Although both PKB and p70^{src} are known to be physiological targets of PI 3-kinase, it is unclear whether these enzymes lie on the same signaling pathway or on parallel pathways. Observations that constitutively activated forms of PKB led to the activation of p70^{src} implied that PKB may mediate mitogenic signaling through p70^{src} activation (47). Recently, PDK1, the enzyme that phosphorylates Thr308 of PKB, has been shown to directly phosphorylate and activate p70^{src}, resulting in a PI 3-kinase-dependent mechanism of activation that may circumvent PKB in the proliferative pathway (48). Differential activation of PKB and p70^{src} can be achieved under distinct cellular calcium levels; this may be important for integrating signals from multiple signaling inputs (49). Our results support these

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latter findings. PI 3-kinase regulation of SCLC cell growth is mediated both by p70^{src}-dependent and -independent pathways. It appears that p70^{src} activation makes approximately a 20-30% contribution to the PI 3-kinase effect, with a 70-80% contribution from another pathway, potentially signaling through PKB. H69 cells were noted to be less sensitive to rapamycin than H345 cells, and this may be due to different cell line growth rates. Similar effects were seen by Seufferlein and Rozengurt (19). Because the principle mechanism of action of rapamycin is G₁ cell cycle delay rather than apoptosis or necrosis, we propose that the faster growth rate and cell cycling of H69 cells diminishes the growth-inhibitory effect of rapamycin at longer time points compared with H345 cells. This is supported by the observation that at shorter time points the effect of rapamycin on H69 and H345 cell growth is similar (38 ± 1.3% versus 29 ± 2.7% growth inhibition, respectively).

We have shown that constitutive PI 3-kinase activity in SCLC regulates proliferation, anchorage-independent growth; and apoptosis. Recent studies have suggested that PI 3-kinase activation induces integrin-mediated cell motility and invasiveness (13, 14). In addition, PI 3-kinase/PKB activation can promote adhesion-independent growth, protecting cells in suspension from undergoing apoptosis (32). The pathways driven by constitutive PI 3-kinase in SCLC cells may therefore be responsible for the highly metastatic and nonadherent phenotype of SCLC cells. We predict that constitutive PI 3-kinase activation may be a late event in the development of SCLC, allowing transformed cells to become locally invasive and metastatic. Given the likely importance of growth and survival in suspension for maintenance of the transformed phenotype, and particularly for metastatic spread of cancer cells within the body, an understanding of the mechanisms that result in constitutive activation of PI 3-kinase and of its downstream effectors could be of benefit in designing novel therapies for SCLC.

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Platelet-Derived Growth Factor-BB and Thrombin Activate Phosphoinositide 3-Kinase and Protein Kinase B: Role in Mediating Airway Smooth Muscle Proliferation

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ABSTRACT

Proliferation of airway smooth muscle results from persistent inflammatory cytokine and growth factor stimulation and is a critical component of airway luminal narrowing in chronic asthma. Using primary cultures of bovine tracheal smooth muscle (BTSM) cells to examine the signaling basis of cell proliferation, platelet-derived growth factor (PDGF)-BB and thrombin (which act through distinct receptor types) were found to induce DNA synthesis in BTSM cells. Mitogen-induced DNA synthesis could be completely inhibited by LY294002, a selective phosphoinositide 3-kinase (PtdIns 3-kinase) inhibitor. Exposure of BTSM cells to PDGF-BB or thrombin resulted in rapid activation of PtdIns 3-kinase and accumulation of phosphoinositide-3,4,5-trisphosphate. Protein kinase B, a novel signaling protein kinase, was identified in BTSM cells and was activated by PDGF-BB and thrombin in a PtdIns 3-kinase-dependent manner; this may underlie mitogen-stimulated activation of

p70^{S6k}. PD98059, a mitogen-activated protein kinase kinase 1 inhibitor, also partially inhibited PDGF-BB- and thrombin-stimulated DNA synthesis, indicating a modulatory role for mitogen-activated protein kinase in proliferation. GF109203X, Ro 31-8220, calphostin C, and chelerythrine (selective protein kinase C inhibitors) had no effect on PDGF-BB- or thrombin-stimulated DNA synthesis, suggesting that, despite abolishment of mitogen-stimulated protein kinase C activity, cell proliferation stimulated by PDGF-BB and thrombin is protein kinase C-independent. These data demonstrate that the PtdIns 3-kinase/protein kinase B pathway represents a key signaling route in airway smooth muscle proliferation, with the mitogen-activated protein kinase kinase 1/mitogen-activated protein kinase cascade providing a complementary signal required for the full mitogenic response.

Hypertrophy and hyperplasia of airway smooth muscle are major components of the structural changes that result in airway luminal narrowing in chronic asthma. This response is thought to occur as a consequence of inflammation and the subsequent release of cytokines and mitogens. Because airway resistance relates to the fourth power of the luminal radius, minor increases in the cell size and the number of airway smooth muscle cells in the bronchial walls of asthmatic patients have pronounced effects on airway resistance. In addition, mathematical modeling has demonstrated that

an increase in smooth muscle mass is the most important component in reducing airflow in patients with chronic asthma (James *et al.*, 1989). Although a number of candidate pathways have been proposed, the signaling events that mediate airway smooth muscle proliferation have not been fully elucidated. Activation of the serine/threonine protein kinase p70 ribosomal S6 kinase family (p70^{S6k}) has been shown to be involved in protein and DNA synthesis in these cells (Scott *et al.*, 1996) and may explain in part the mitogenic effects of certain growth factors. In addition, growth factor-induced activation of PtdIns 3-kinase, which results in phosphorylation of phosphoinositide-4,5-bisphosphate to PtdIns(3,4,5)P₃, has been proposed to play an important role in DNA synthe-

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ABBREVIATIONS: PtdIns 3-kinase, phosphoinositide 3-kinase; BTSM, bovine tracheal smooth muscle; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); EGTA, ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; MAP kinase, mitogen-activated protein kinase; MEK1 or -2, mitogen-activated protein kinase kinase 1 or 2; PDK-1, 3-phosphoinositide-dependent protein kinase-1; PtdIns(3,4,5)P₃, phosphoinositide-3,4,5-trisphosphate; ERK, extracellular signal-regulated kinase; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle medium; PDGF, platelet-derived growth factor; EGF, epidermal growth factor.

sis. Furthermore, because wortmannin, a PtdIns 3-kinase inhibitor, inhibits p70^{s6k} activation by growth factors, PtdIns 3-kinase has been proposed to lie upstream of p70^{s6k} in the proliferative pathway (Scott *et al.*, 1996).

More recently, protein kinase B has been identified as a novel signaling protein kinase that is activated in response to insulin and growth factors (Marte and Downward, 1997). Overexpression of a constitutively active form of protein kinase B was found to activate p70^{s6k}, and this response was sensitive to inhibition by rapamycin (Burgering and Coffey, 1995). Because protein kinase B activity can be inhibited by wortmannin, this again suggests that protein kinase B operates upstream of p70^{s6k} and downstream of PtdIns 3-kinase.

Another pathway shown to be associated with growth in a variety of cell types is the MAP kinase cascade; activation of this pathway through Ras/Raf-1 stimulates phosphorylation of substrates such as p90^{s6k} and *c-jun* and initiates protein synthesis (Malarkey *et al.*, 1995b). The MAP kinases, also termed ERKs, are 42- and 44-kDa serine/threonine kinases that are activated after phosphorylation by the dual-specificity protein kinase MEK1 (MAP or ERK kinase). Activation of MEK1, and a second isoform termed MEK2, occurs after phosphorylation on Ser218 and Ser222 by Raf-1 (Zheng and Guan, 1994).

A role for protein kinase C in airway smooth muscle proliferation has also been proposed, on the basis that selective inhibitors of this enzyme family could attenuate mitogen-induced proliferation of rabbit tracheal smooth muscle cells (Hirst *et al.*, 1995). Recently, a number of protein kinase C isoforms, namely protein kinase C δ , ϵ , η , and ζ , have been demonstrated to be activated by 3-phosphorylated phosphoinositides generated by the action of PtdIns 3-kinase (Nakanishi *et al.*, 1993; Toker *et al.*, 1994). A study in canine airway smooth muscle has identified the presence of protein kinase C β I, β II, δ , ϵ , θ , and ζ isoforms (Donnelly *et al.*, 1995). Most protein kinase C inhibitors do not, however, show substantial selectivity among protein kinase C isoforms; therefore, the possibility that these novel isoforms represent downstream targets of PtdIns 3-kinase remains to be addressed.

In this study, we have investigated the role of PtdIns 3-kinase in mitogen-induced BTSM cell proliferation and demonstrated that PDGF-BB and thrombin activate PtdIns 3-kinase, stimulate PtdIns(3,4,5)P₃ accumulation, and activate protein kinase B. We have also shown that the degree of activation of PtdIns 3-kinase achieved by PDGF-BB and thrombin correlates closely with their mitogenic efficacy and that 10-min exposure to these stimuli is sufficient to commit cells to DNA synthesis. Finally, inhibitors of PtdIns 3-kinase, in contrast to inhibitors of protein kinase C or MEK1, cause complete inhibition of proliferation. These data suggest that agonist-stimulated activation of the PtdIns 3-kinase/PtdIns(3,4,5)P₃/protein kinase B pathway represents a key route for initiating cell division in airway smooth muscle cells.

Materials and Methods

Cell culture. Bovine trachealis was obtained from the local abattoir. Small strips of trachealis muscle, dissected free of epithelium and connective tissue, were washed in DMEM containing penicillin/streptomycin (5 units/ml and 5 μ g/ml, respectively) and amphotericin B (2.5 μ g/ml) and were incubated in this medium with collage-

nase type IV (1 mg/ml) for 1 hr at 37°, with intermittent shaking. After addition of fetal calf serum, the muscle digest was filtered through gauze, and isolated tracheal smooth muscle cells were collected by centrifugation at 250 \times g for 4 min. Cells were plated and cultured in supplemented DMEM containing fetal calf serum (10%, v/v). Cells from passages 3–9 were used for all experiments. Cells were made quiescent in DMEM containing fetal calf serum (0.5%, v/v) for 48 hr before experiments. The identity of the tracheal smooth muscle cells was confirmed by immunocytochemistry using a smooth muscle-specific, anti- α -actin, mouse monoclonal antibody (data not shown).

PCR amplification of PDGF- α and - β receptor mRNA. Total RNA was isolated by lysis of adherent cells with Trizol reagent and extraction, according to the instructions provided by the manufacturer (Life Technologies, Paisley, UK). A total of 2 μ g of RNA was reverse-transcribed in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 10 units of RNase inhibitor, 0.1 mM deoxynucleotide mixture, 50 μ g/ml oligo(dT)_{11–18}, with 200 units of murine leukemia virus reverse transcriptase, at 35° for 60 min. After first-strand cDNA synthesis, PCR amplifications were carried out in 25- μ l volumes containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 1 μ M primers, 200 μ M deoxynucleotide mixture, and 1 unit of *Thermus aquaticus* DNA polymerase and were performed as follows: 94° for 30 sec, 55° for 60 sec, and 72° for 30 sec for 35 cycles and then 72° for 5 min. The PDGF- α primers were 5'-AATAAGATCAAGAGTGGGTACAG-3' and 5'-TATGCCGATGTCGCCATCATGTC-3', PDGF- β primers were 5'-TGACCACCCAGCCATCTTTC-3' and 5'-GAGGAGGTGTTGACTTCATTC-3', and glyceraldehyde-3-phosphate dehydrogenase primers were 5'-CCACCATGGCAAATTCATGGCA-3' and 5'-TCTAGACGCGAGGTCCAGTCCACC-3'. The amplified products were subjected to electrophoresis on 1.5% agarose gels in 45 mM Tris-borate, 1 mM EDTA, were identified with a UV transilluminator after staining with ethidium bromide (0.5 μ g/ml), and were photographed using a Polaroid DS-5 system.

[³H]Thymidine incorporation. Confluent and quiescent cells were washed twice in serum-free DMEM, inhibitors and mitogens were added as indicated, and cells were incubated for an additional 24 hr. [³H]Thymidine (0.1 μ Ci/ml) was added for the final 4 hr of the incubation. Cells were washed twice with phosphate-buffered saline, twice with trichloroacetic acid (5%, w/v), and twice with ethanol and were finally solubilized with NaOH (0.3 M). [³H]Thymidine incorporation was determined by liquid scintillation counting.

Cell stimulation, immunoprecipitation, and PtdIns 3-kinase activity assay. Cells were incubated in serum-free DMEM before the addition of inhibitors and agonists, as detailed in the figure legends. Reactions were terminated by rapid aspiration of the medium, followed by two washes with phosphate-buffered saline and the addition of ice-cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10%, v/v, glycerol, 1%, v/v, Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 200 μ M Na₃VO₄, 10 mM sodium pyrophosphate, 100 mM NaF). Insoluble material was removed from cell lysates by centrifugation, and PtdIns 3-kinase was immunoprecipitated using anti-p85 or anti-phosphotyrosine antibody complexed to Pansorbin (Calbiochem, Nottingham, UK) (Scott *et al.*, 1996). PtdIns 3-kinase activity in immunoprecipitates was assayed as previously described (Carter and Downes, 1993). Immunoprecipitates were suspended in 50 μ l of assay buffer (100 mM HEPES, pH 7.4, 200 mM NaCl, 1 mM EGTA) and mixed with 20 μ l of sonicated phosphatidylinositol and phosphatidylserine (3:1, 0.2 mg/ml). Reactions were initiated by the addition of [γ -³²P]ATP (10 μ Ci) and were incubated for 30 min at 30°. Reactions were terminated by the addition of 750 μ l of chloroform/methanol/HCl (40:80:1, v/v/v) and phase partitioning achieved with the subsequent addition of 250 μ l of chloroform and 250 μ l of HCl (0.1 M). Phospholipids were washed with chloroform/methanol/HCl (47:48:3, v/v/v). ³²P-labeled phosphoinositide 3-phosphate was then quantified by thin layer chromatography using a solvent system

containing chloroform/methanol/ammonia/water (20:15:3:5, v/v/v/v); ^{32}P incorporation was determined by liquid scintillation counting.

PtdIns(3,4,5)P₃ accumulation. Cells grown to confluence in 100-mm cell culture dishes were made quiescent, washed twice with phosphate-free HEPES-buffered saline (20 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM MgSO₄, 1 mM CaCl₂, 5 mM KCl), and labeled in the same buffer containing 2 mCi/ml [^{32}P]orthophosphate, for 3 hr at 37°. Cells were then washed twice with HEPES-buffered saline before addition of agonists, as detailed in the figure legends. Reactions were terminated by the addition of trichloroacetic acid (0.5 M), and lipids were extracted with chloroform/methanol/0.1 M HCl (4:8:3, v/v/v) in the presence of Folch fraction I phosphoinositides (0.5 mg/extraction). Dried lipids were deacylated with monomethylamine at 53° for 30 min, with intermittent shaking, and the resulting deacylated lipids were analyzed by strong anion exchange high performance liquid chromatography using a two-step (NH₄)₂PO₄ (0.5 M)/water gradient, essentially as described by Carter and Downes (1993). Deacylated lipids were identified by comparison of retention times with those of ^3H -labeled phosphoinositide standards and relevant nucleotides; ^{32}P incorporation was determined by liquid scintillation counting.

Assay of protein kinase B α activity. BTSM cells were lysed in buffer containing 50 mM Tris-HCl, pH 7.5, 0.1% (w/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM sodium glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.1% (v/v) 2-mercaptoethanol, 0.27 M sucrose, 1 μM microcystin-LR, and protease inhibitors (protease inhibitor cocktail; Boehringer Mannheim, Lewes, UK). Insoluble material was removed from cell lysates by centrifugation, and protein kinase B α was immunoprecipitated by incubation at 4° with 2 μg of anti-protein kinase B α antibody conjugated to 5 μl of Protein G-Sepharose. Immunoprecipitates were washed three times with 1 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM EGTA, 0.1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, and 0.5 M NaCl and twice in the same buffer without NaCl. Kinase activity was assayed by incubation with peptide substrate (GRPRTSSFAEG, termed 'Crosstide') and [γ - ^{32}P]ATP (0.5 μCi) for 10 min at 30° in a shaking water bath; the reaction was terminated by transferring 40 μl of the assay mixture to phosphocellulose P81 paper. The phosphocellulose paper, which binds the peptide substrate but not ATP, was washed five times with orthophosphoric acid (0.5%, v/v) and once with acetone and dried; ^{32}P incorporation was determined by liquid scintillation counting.

Assay of MAP kinase activity. After pretreatment with inhibitors and addition of mitogens as described in the figure legends, BTSM cells were lysed under conditions identical to those used for protein kinase B activity measurements; insoluble material was removed by centrifugation, and activity was immunoprecipitated using anti-p42 MAP kinase antibodies. After conjugation with Protein G-Sepharose, enzymatic activity was assayed using a specific MAP kinase substrate peptide derived from the EGF receptor (5 μg /incubation) and [γ - ^{32}P]ATP (1 μCi), in a buffer containing 75 mM HEPES, pH 7.4, and 1.2 mM MgCl₂, for 30 min at 37°. Reactions were terminated and phosphorylated peptide was isolated as detailed for assays of protein kinase B activity.

Assay of cytosolic protein kinase C activity. BTSM cells were lysed in buffer containing 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.5 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 0.02% (v/v) Triton X-100, and protease inhibitors (protease inhibitor cocktail; Boehringer Mannheim), after pretreatment with inhibitors and mitogens. After centrifugation at 30,000 $\times g$ for 30 min at 4°, supernatants were removed, Nonidet P-40 (1%, v/v, final concentration) was added, and the mixture was shaken for 30 min at 4° to form the cytosolic fraction. Protein kinase C activity in the cytosolic fraction was partially purified on DE52 diethylaminoethyl cellulose matrix, with activity being eluted with lysis buffer containing 120 mM NaCl. Enzymatic activity was assayed in a buffer containing 10 mM MgCl₂, 1.2 mM CaCl₂, phospholipids (96 $\mu\text{g}/\text{ml}$ phosphatidylserine and 6.4 $\mu\text{g}/\text{ml}$ diolein), 1 μCi of [γ - ^{32}P]ATP, and 1 mg/ml histone III_s as

substrate. Reactions were carried out at 30° for 30 min and were terminated by addition of buffer containing 10% (w/v) trichloroacetic acid, 10 mM sodium pyrophosphate, and 1 mM ATP plus bovine serum albumin (500 $\mu\text{g}/\text{reaction}$, added before vortex-mixing). Samples were filtered through phosphocellulose P81 paper and washed four times with buffer containing 5% (w/v) trichloroacetic acid and 10 mM sodium pyrophosphate; ^{32}P incorporation was determined by liquid scintillation counting.

Materials. Antibody to the p85 regulatory subunit of PtdIns 3-kinase (monoclonal anti-amino-terminal SRC homology 3 domain of p85) was purchased from TCS (Botolph Claydon, UK), and antibodies to phosphotyrosine (PY20) and p42 MAP kinase (ERK2) were purchased from Insight Biotechnology (Wembley, UK). [γ - ^{32}P]ATP, [^{32}P]orthophosphate, [^3H]thymidine, and enhanced chemiluminescence reagents were purchased from Amersham International (Amersham, UK), secondary antibodies were obtained from SAPU (Carlisle, UK), and antibody to smooth muscle α -actin was purchased from DAKO (Ely, UK). Trizol reagent, murine leukemia virus reverse transcriptase, and *T. aquaticus* DNA polymerase were purchased from Life Technologies (Paisley, UK), and the deoxynucleotide mixture was purchased from Pharmacia (St. Albans, UK). Protein kinase B α peptide substrate (Crosstide) and antibodies to protein kinase B α were generous gifts from Dr. D. Alessi and Prof. P. Cohen (University of Dundee, UK). Folch fraction I phosphoinositides were obtained from Sigma (Poole, UK). Protease inhibitor cocktail tablets were purchased from Boehringer Mannheim. All other reagents were of the highest purity commercially available.

Results

PDGF receptor expression in primary cultures of BTSM cells. PDGF is a dimer composed of A chains and B chains connected by disulfide bonds, and it exists in three isoforms, namely PDGF-AA, PDGF-AB, and PDGF-BB. PDGF receptors are composed of two subunits, namely α and β , that form dimeric receptors as $\alpha\alpha$, $\alpha\beta$, or $\beta\beta$ forms; ligand binding studies have demonstrated that $\alpha\alpha$ receptors bind all three PDGF isoforms, $\alpha\beta$ receptors bind PDGF-AB and -BB, and $\beta\beta$ receptors bind only PDGF-BB (Seifert *et al.*, 1989). Using reverse transcription-PCR of RNA derived from primary cultures of BTSM cells, we demonstrated the presence of both α and β chains of the PDGF receptor (Fig. 1). In subsequent experiments, PDGF-BB was used as an agonist with activity at all three PDGF receptor subtypes.

Induction of BTSM cell proliferation by PDGF-BB and thrombin. Confluent primary cultures of BTSM cells that had been made quiescent for 48 hr in DMEM containing 0.5% fetal calf serum were used to assess cell proliferation by measurement of [^3H]thymidine incorporation after agonist treatment. PDGF-BB and thrombin were used as mitogens, to allow comparison of the effects of growth factor receptor stimulation (which involves autophosphorylation of specific tyrosine residues) and G protein-coupled receptor stimulation (where activation occurs through a 'tethered-ligand' mechanism of action) (Vu *et al.*, 1991). As demonstrated in Fig. 2a, PDGF-BB (20 ng/ml) induced a 51.3 ± 1.0 -fold increase and thrombin (1 unit/ml) induced a 13.3 ± 0.3 -fold increase in [^3H]thymidine incorporation, compared with control values, after a 24-hr exposure. The potential role of PtdIns 3-kinase in mediating this response was studied initially by using the selective inhibitor LY294002 (Vlahos *et al.*, 1994). PDGF-BB- and thrombin-induced [^3H]thymidine incorporation was inhibited to control levels, in a concentration-dependent manner, by LY294002 (IC₅₀, 2.4 and 2.2 μM , respectively) (Fig. 2b).

Time course of mitogen-induced proliferation. A 'washout' protocol was used to assess the minimal period of agonist stimulation required to induce [³H]thymidine incorporation; at each time point, PDGF-BB or thrombin was added to quiescent BTSM cells for varying times, the mitogen-containing medium was removed, and the cells were washed extensively with DMEM containing 0.5% fetal calf serum (quiescent medium) and then incubated for an additional 20 hr in fresh quiescent medium before assessment of [³H]thymidine incorporation. The data presented in Fig. 3 demonstrate a rapid time-dependent increase in [³H]thymidine incorporation and indicate that a 10-min exposure to PDGF-BB or thrombin is sufficient to induce proliferation equivalent to that observed after a 24-hr exposure to agonist. These data suggest that these cells are fully committed to undergo cell division after only a brief period of agonist exposure; hence, a rapid signaling mechanism underlies the proliferative response induced by these mitogens.

Time course of PtdIns 3-kinase activation. Because mitogen-induced BTSM cell proliferation was sensitive to inhibition by PtdIns 3-kinase inhibitors and BTSM cells could be committed to transition into the S-phase of the cell cycle by a rapid signaling mechanism, we examined the time course of PtdIns 3-kinase activation. In anti-p85 immunoprecipitates, PDGF-BB (20 ng/ml) and thrombin (1 unit/ml) induced time-dependent activation of PtdIns 3-kinase, which was maximal by 5 min for thrombin and by 10 min for PDGF-BB, with increases of 9 ± 1.2 - and 3.5 ± 0.7 -fold over control values, respectively (Fig. 4a). PDGF-BB- and thrombin-induced activation of PtdIns 3-kinase was sensitive to inhibition by LY294002 over a concentration range similar to that used for inhibition of [³H]thymidine incorporation (IC_{50} , 1.7 and 1.9 μ M, respectively) (Fig. 4b).

Time course of PtdIns(3,4,5)P₃ generation. Using [³²P]orthophosphate-labeled BTSM cells, we examined the generation of PtdIns(3,4,5)P₃, the lipid product of PtdIns 3-kinase activation, after cell stimulation with PDGF-BB or thrombin. Phosphoinositides were extracted from control and agonist-stimulated cells, deacylated to generate glycerol derivatives of phosphoinositide lipids, and analyzed by high performance liquid chromatography using a SAX 5 column (Whatman, Maidstone, UK) and an (NH₄)H₂PO₄/water two-step gradient. As shown in Fig. 4c, PDGF-BB (20 ng/ml) induced rapid generation of glycerol-PtdIns(3,4,5)P₃ over 10 min, achieving an 8.8 ± 1.5 -fold increase over control levels.

Thrombin (1 unit/ml) induced a 4.1 ± 1.7 -fold increase in glycerol-PtdIns(3,4,5)P₃ levels, with maximal stimulation being observed at 5 min (Fig. 5).

Activation of protein kinase B α in BTSM cells. Mitogen-induced BTSM cell proliferation was previously shown to occur through activation of p70^{s6k} in a PtdIns 3-kinase-dependent manner (Scott *et al.*, 1996). Recently, protein kinase B α has been proposed to act as a signaling intermediate between PtdIns 3-kinase and the activation of p70^{s6k} after stimulation of cells with growth factors (Marte and Downward, 1997). Protein kinase B α was identified by Western blotting in BTSM cells (data not shown) and was found to be 4.5 ± 0.7 - and 3.7 ± 0.2 -fold activated by PDGF-BB and thrombin, respectively, by 10 min (Fig. 5a). In addition, protein kinase B α activity was found to be inhibited after pretreatment of BTSM cells with the PtdIns 3-kinase inhibitor LY294002, confirming that protein kinase B α lies downstream from PtdIns 3-kinase in the proliferative pathway (Fig. 5b).

Role of MAP kinase in BTSM cell proliferation. To investigate a potential role for the MAP kinase pathway in mitogen-induced proliferation, we used the specific MEK1 inhibitor PD98059. The mechanism of action of PD98059 involves interaction with and inhibition of the inactive form of MEK1, the upstream activator of MAP kinase (Dudley *et al.*, 1995). Pretreatment of BTSM cells with 100 μ M PD98059 inhibited PDGF-BB- and thrombin-induced [³H]thymidine incorporation by 79.4 ± 4.5 and $82.9 \pm 4.5\%$, respectively (Fig. 6a); however, PD98059-mediated inhibition of PDGF-BB-stimulated MAP kinase activity in these cells was maximal at a concentration of 10 μ M (Fig. 6b), suggesting that PD98059 may have nonspecific effects on DNA synthesis and cell integrity at concentrations above 10 μ M. Of note, pretreatment of BTSM cells with a maximally effective concentration of LY294002 (10 μ M) had no significant effect on MAP kinase activation induced by PDGF-BB or thrombin (97.9 ± 1.2 and $98.3 \pm 1.8\%$ of control values, respectively) (Fig. 6b, *inset*), suggesting that the MAP kinase pathway is not a downstream target of PtdIns 3-kinase. In addition, PD98059 (10 μ M) had no significant effect on PtdIns 3-kinase activation induced by PDGF-BB or thrombin (95.0 ± 6.8 and $98.2 \pm 4.4\%$ of control values, respectively) (Fig. 4b). Taken together, these data suggest that the PtdIns 3-kinase pathway and MAP kinase pathway act in a parallel manner.

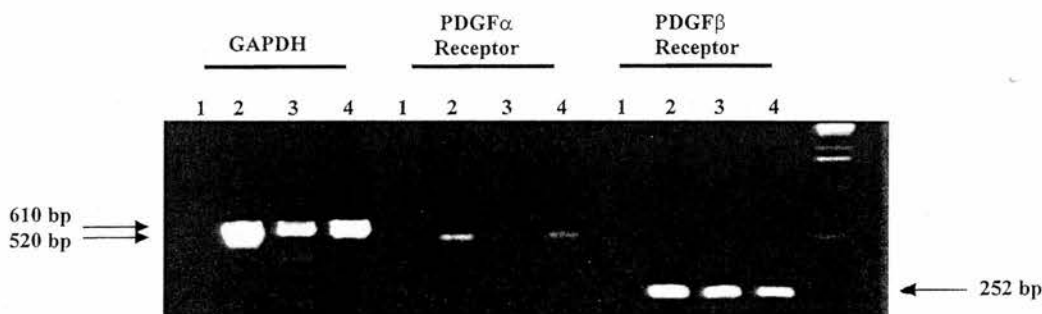


Fig. 1. Expression of PDGF- α and - β receptor subunits in BTSM cells. After reverse transcription-PCR with appropriate primers, amplified products were separated by electrophoresis on 1.5% (w/v) agarose gels, stained with ethidium bromide, and visualized using a UV transilluminator. Primers specific for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (610-base pair product) were used to amplify this 'housekeeping' gene product; the PDGF- α receptor (520-base pair product) and PDGF- β receptor (252-base pair product) were identified as indicated. Amplification was performed using cDNA originally derived from normal human articular chondrocytes (lane 2), LL24 fetal lung fibroblasts (lane 3), and primary cultures of BTSM cells (lane 4); negative controls containing no cDNA were included in each PCR amplification (lane 1).

Role of protein kinase C in BTSM cell proliferation.

In a number of cell types, including airway smooth muscle cells, protein kinase C has been proposed to play a role in cell proliferation. Despite the identification of a number of isoforms of protein kinase C, with differing requirements for Ca^{2+} and phosphatidylserine, no inhibitors exist that are entirely selective for distinct protein kinase C isoforms, to enable the precise cellular roles for these isoforms to be established. Using purified baculovirus-expressed kinases, activation of certain protein kinase C isoforms has been demonstrated to occur in the presence of 3-phosphorylated phosphoinositides generated as a result of PtdIns 3-kinase activation. GF109203X, a bisindolylmaleimide that is reported to be a potent, selective, broad-spectrum, protein kinase C inhibitor whose action is mediated by competitive inhibition for ATP (Toullec *et al.*, 1991), was found to have no effect on either PDGF-BB- or thrombin-induced [^3H]thymidine incorporation, even at a maximally effective concentra-

tion of $1 \mu\text{M}$ (Fig. 7). Use of GF109203X at concentrations above $3 \mu\text{M}$ was found to induce cell detachment and necrosis (data not shown). Similar results were obtained with the protein kinase C inhibitor Ro 31-8220, which acts by a similar mechanism (data not shown). In addition, calphostin C (100 nM), an inhibitor of protein kinase C that acts at the regulatory domain, and chelerythrine ($1 \mu\text{M}$), which inhibits protein kinase C in a noncompetitive manner with respect to ATP, were both found to have no significant effect on PDGF-BB- and thrombin-induced [^3H]thymidine incorporation (97.3 ± 2.7 and $99.3 \pm 1.4\%$ of PDGF-BB control values and 98.1 ± 2.5 and $97.6 \pm 3.4\%$ of thrombin control values, respectively). Both PDGF-BB and thrombin were found to activate protein kinase C, as assessed in cytosolic extracts of BTSM cells using histone III_s as substrate, and this activity was completely inhibited by GF109203X ($1 \mu\text{M}$) (Fig. 7b). These data suggest that protein kinase C does not play a major role in mediating or modulating mitogen-induced cell proliferation in primary cultures of BTSM cells.

Discussion

In this study, we have demonstrated that PDGF-BB and thrombin are potent mitogens for primary cultures of BTSM cells and that a relatively short exposure to these agonists (<10 min) is sufficient to initiate a full mitogenic response. PDGF-BB and thrombin were used as agonists because both are known to act through a distinct receptor type; this would possibly allow us to determine whether a common key signaling pathway operates to mediate cell proliferation.

The ability of LY294002 to completely abolish PDGF-BB- and thrombin-induced [^3H]thymidine incorporation by BTSM cells suggested that PtdIns 3-kinase is a key regulator of this response. This finding was supported by the demonstration that these agonists caused rapid activation of PtdIns 3-kinase and generation of the second messenger PtdIns(3,4,5) P_3 , the extent of which correlated closely with the magnitude of [^3H]thymidine incorporation. Importantly, this is one of the first direct observations that stimulation of a G protein-coupled receptor can activate a p85/p110 PtdIns 3-kinase in a nonhematopoietic cell type. Other studies examining G protein-coupled receptors demonstrated activa-

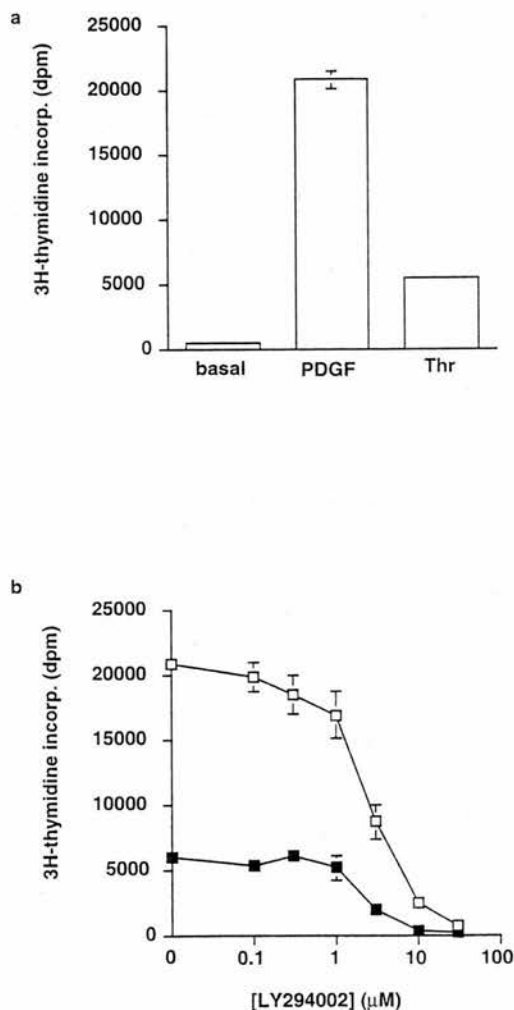


Fig. 2. DNA synthesis induced by PDGF-BB and thrombin in BTSM cells. a, [^3H]Thymidine incorporation was assessed in quiescent BTSM cells after exposure to PDGF-BB (20 ng/ml) (PDGF) or thrombin (1 unit/ml) (Thr) for 24 hr, as detailed in Materials and Methods. b, BTSM cells were treated with LY294002 for 20 min before addition of PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (■). [^3H]Thymidine incorporation was assessed after 24 hr, as detailed in Materials and Methods; results are expressed as mean \pm standard error of incorporation from an experiment that was performed in triplicate and was representative of three other experiments with similar results.

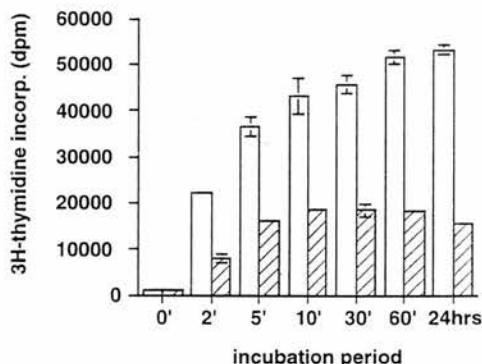


Fig. 3. Time course of mitogen-induced DNA synthesis in BTSM cells. BTSM cells were exposed to PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (▨) for the times indicated and were then removed by washing the cells with quiescent DMEM containing 0.5% (v/v) fetal calf serum; [^3H]thymidine incorporation was assessed after an additional 24 hr. Results are expressed as mean \pm standard error of incorporation from an experiment that was performed in triplicate and was representative of four other experiments with similar results.

tion of a novel $\beta\gamma$ -sensitive form of PtdIns 3-kinase; in particular, thrombin was shown to activate this form of PtdIns 3-kinase in platelets, as was formyl-methionyl-leucyl-phenyl-alanine in neutrophils (Zhang *et al.*, 1995; Stephens *et al.*, 1997). A recent study by Belham *et al.* (1997) showed that thrombin-induced activation of p70^{s6k} in pulmonary artery fibroblasts was inhibited by wortmannin but PtdIns 3-kinase activity was undetectable in antiphosphotyrosine immunoprecipitates. These observations suggested the involvement of a PtdIns 3-kinase isoform that was distinct from the activity found in antiphosphotyrosine immunoprecipitates from PDGF-treated cells. Wilson *et al.* (1996) demonstrated that lysophosphatidic acid activated p70^{s6k} via a G protein-coupled receptor in Rat-1 fibroblasts and this activation could be abrogated by pretreatment with wortmannin; therefore, it was speculated that a $\beta\gamma$ -sensitive form of PtdIns 3-kinase might mediate this activity.

A previous study with BTSM cells demonstrated that cell growth was mediated through activation of p70^{s6k} in a PtdIns 3-kinase-dependent manner (Scott *et al.*, 1996). In addition, that study showed that rapamycin, a selective inhibitor of p70^{s6k} activation, could completely inhibit BTSM cell proliferation, thus supporting the proposal that activation of p70^{s6k} is a key step in the growth pathway.

Protein kinase B was originally identified as a product of the *v-akt* oncogene, from the acutely transforming retrovirus AKT-8 found in rodent T cell lymphoma cells (Bellacosa *et al.*, 1991). Reports that protein kinase B lies downstream from PtdIns 3-kinase and can associate with 3-phosphorylated phosphoinositides (James *et al.*, 1996) led to the characterization of two phosphorylation sites on protein kinase B,

namely Thr308 and Ser473, which must both be phosphorylated for full activation (Alessi *et al.*, 1997a).

Burgering and Coffey (1995) reported that a constitutively active form of protein kinase B induced p70^{s6k} activation but had no effect on MAP kinase. In addition, because both protein kinase B and p70^{s6k} are known to lie downstream from PtdIns 3-kinase, this suggests that p70^{s6k} activation may be mediated by protein kinase B. We demonstrated in this study that protein kinase B is activated in BTSM cells after stimulation with PDGF-BB and thrombin, both of which were shown to be potent mitogens and were able to induce rapid activation of PtdIns 3-kinase. More recently, PDK-1 was demonstrated to directly phosphorylate and activate p70^{s6k} both *in vivo* and *in vitro* (Alessi *et al.*, 1997b). Phosphorylation of p70^{s6k} *in vitro* was found to be independent of PtdIns(3,4,5)P₃, which contrasts with the phosphoinositide-dependent phosphorylation of protein kinase B by PDK-1. These observations suggest that PDK-1 is involved in a cooperative role to phosphorylate and mediate p70^{s6k} activation.

Activation of the MAP kinase cascade is known to be involved in mitogenesis. Using PD98059, a specific MEK1 inhibitor, a role for the MAP kinase pathway in BTSM cell proliferation was investigated. At concentrations of PD98059 that completely inhibit MAP kinase activity, DNA synthesis was only partially inhibited. This suggests that MAP kinase is necessary, but not sufficient, for a full mitogenic response in these cells. These data are in accord with those reported by Malarkey *et al.* (1995a), who showed marked differences in the mitogenic potency of PDGF-BB and endothelin-1 in BTSM cells despite identical degrees of MAP kinase activa-

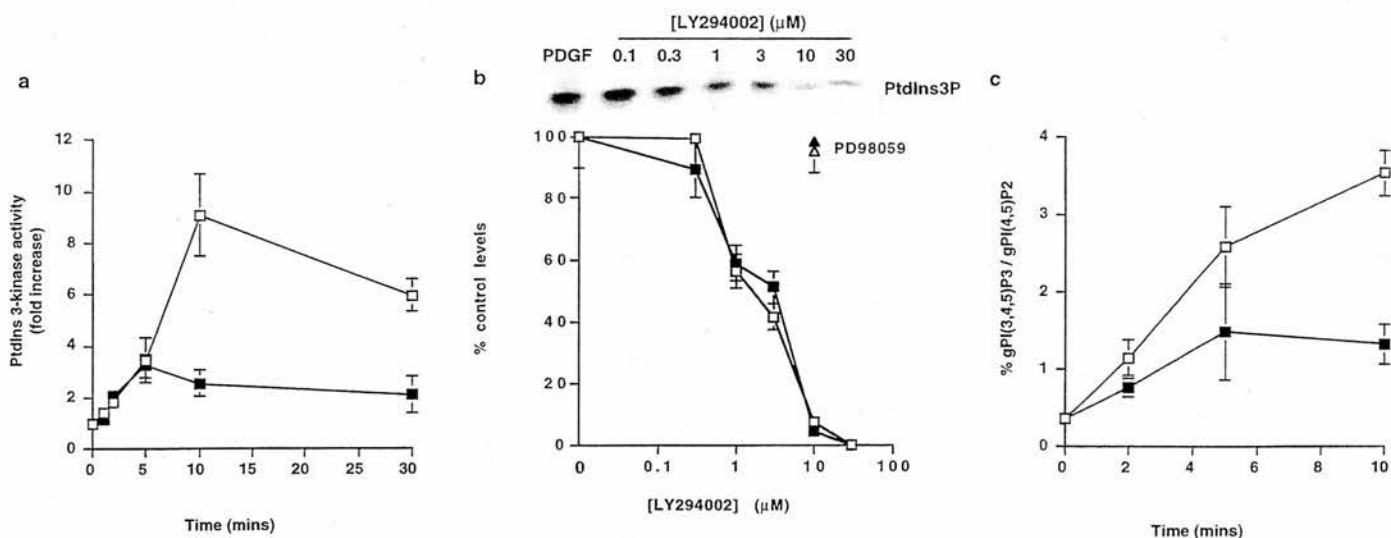


Fig. 4. PtdIns 3-kinase activation and PtdIns(3,4,5)P₃ generation in mitogen-stimulated BTSM cells. **a**, BTSM cells were stimulated with PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (■) for the times indicated. Cells were lysed and PtdIns 3-kinase was immunoprecipitated using an anti-p85 antibody; PtdIns 3-kinase activity was assayed using phosphoinositide as substrate. Results are expressed as mean \pm standard error of the fold increase from three independent experiments performed in duplicate. **b**, BTSM cells were pretreated with LY294002 at varying concentrations, as indicated, for 20 min before addition of PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (■) for 10 min. Alternatively, BTSM cells were pretreated with PD98059 (10 μ M, 30 min) before addition of PDGF-BB (20 ng/ml) (△) or thrombin (1 unit/ml) (▲) for 10 min; these experiments were carried out in the absence of LY294002. Cells were assayed for PtdIns 3-kinase activity as described above; results are expressed as mean percentage of control values (PDGF-BB- or thrombin-treated cells, as appropriate) from a single experiment that was performed in triplicate and was representative of two others with similar results. An autoradiograph of phosphoinositide-3-phosphate (PtdIns3P), separated by thin layer chromatography, from a representative experiment is also shown (top). **c**, BTSM cells were labeled with [³²P]orthophosphate as detailed in Materials and Methods and were stimulated with PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (■) for varying times, as indicated. Phosphoinositide lipids were extracted, deacylated, and analyzed by strong anion exchange high performance liquid chromatography. Results are expressed as mean \pm standard error of the percentage of deacylated PtdIns(3,4,5)P₃/deacylated phosphoinositide-4,5-bisphosphate [gPI(3,4,5)P₃/gPI(4,5)P₂] from three individual experiments; mean deacylated phosphoinositide-4,5-bisphosphate values were 386,809 cpm.

tion. In contrast, a report by Karpova *et al.* (1997) concluded that activation of MEK1 and the MAP kinase pathway was essential for PDGF-induced DNA synthesis. However, in this study even supramaximal concentrations of PD98059 did not attenuate PDGF-induced [³H]thymidine synthesis to control levels; the pathway mediating this resistant component has not yet been elucidated. Mitogen-induced MAP kinase activation was unaffected by pretreatment with a PtdIns 3-kinase inhibitor, and PD98059 did not inhibit PtdIns 3-kinase activity; this suggests that in BTSM cells the MAP kinase and PtdIns 3-kinase pathways act in a parallel manner to induce proliferation. However, it is evident from our study that PtdIns 3-kinase is the principal regulatory pathway, with MAP kinase activation being required for a full mitogenic response. Interactions with PtdIns 3-kinase seem to vary among cell types and within cell lines, depending on the strength of the mitogenic signal. Duckworth and Cantley

(1997) demonstrated that PDGF-induced MAP kinase activity was inhibited by wortmannin at low PDGF concentrations but wortmannin had no effect at maximal concentrations of PDGF, suggesting that PtdIns 3-kinase activates MAP kinase when small numbers of PDGF receptors are activated but a parallel pathway activates MAP kinase when most PDGF receptors are activated. Our study has focused on cell proliferation and its underlying signaling events induced by maximally effective concentrations of mitogens; the possibility remains, however, that a more complex interaction be-

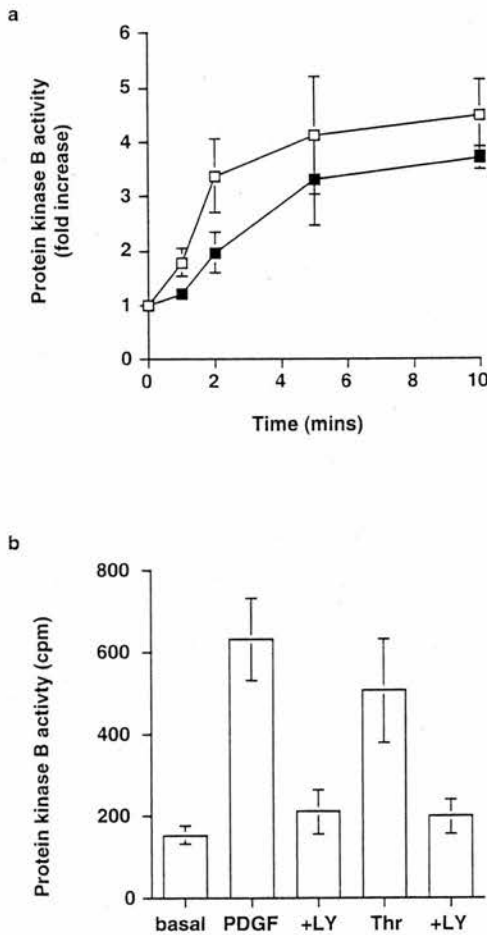


Fig. 5. Time course of protein kinase B activation in mitogen-stimulated BTSM cells. a, BTSM cells were stimulated with PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (■) for varying times, as indicated. Cells were lysed, protein kinase B was immunoprecipitated, and activity was assayed using Crosstide as the substrate, as detailed in Materials and Methods. Results are expressed as the mean ± standard error of the fold increase above control values (untreated cells) from four experiments, each performed in duplicate. b, BTSM cells were pretreated with LY294002 (10 μM) (+LY) for 20 min before addition of PDGF-BB (20 ng/ml) (PDGF) or thrombin (1 unit/ml) (Thr) as indicated. Protein kinase B activity was assessed in immunoprecipitates as detailed above; results are expressed as mean ± standard error of activity from a single experiment that was performed in triplicate and was representative of two others with similar results.

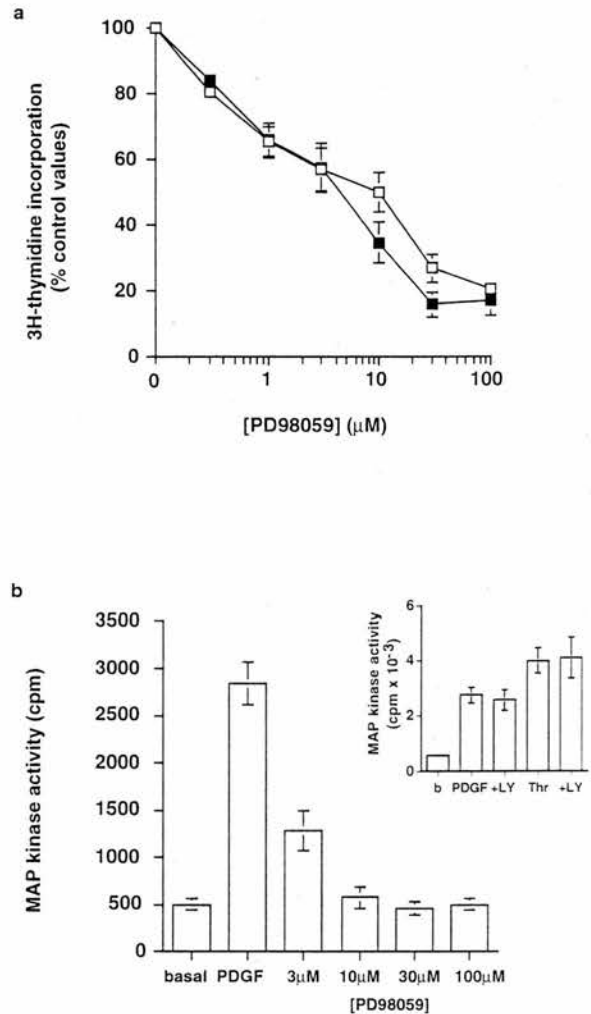


Fig. 6. Role of MAP kinase in mediating DNA synthesis in BTSM cells. a, BTSM cells were incubated with varying concentrations of PD98059 for 30 min before the addition of PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (■), as indicated. [³H]Thymidine incorporation was assessed after 24 hr, as detailed in Materials and Methods; results are expressed as mean ± standard error of the incorporation from five independent experiments, each performed in triplicate. b, BTSM cells were stimulated with PDGF-BB (20 ng/ml, 10 min) (PDGF) after preincubation with PD98059 at varying concentrations, as indicated. Cells were lysed, MAP kinase was immunoprecipitated using an anti-p42 MAP kinase antibody, and enzyme activity was assayed using an EGF receptor fragment as the substrate, as detailed in Materials and Methods. Results are expressed as mean ± standard error of the activity from a single experiment that was performed in triplicate and was representative of two others with similar results. *Inset*, BTSM cells were incubated with LY294002 (10 μM) (+LY) for 20 min before addition of PDGF-BB (20 ng/ml) (PDGF) or thrombin (1 unit/ml) (Thr), as indicated, and assessment of MAP kinase activity; results are expressed as mean ± standard error from three experiments performed in triplicate. b, basal.

tween signaling pathways may occur at submaximal mitogen concentrations.

We have demonstrated that DNA synthesis is unaffected by a maximally effective concentration of GF109203X, suggesting that mitogen-induced proliferation of BTSM cells is independent of protein kinase C. Similar results were also obtained with calphostin C and chelerythrine, which have different mechanisms of action, either targeting the regulatory domain of protein kinase C or acting as a noncompetitive inhibitor for ATP. Furthermore, it has been shown that pretreatment of BTSM cells with phorbol-12-myristate-13-acetate to down-regulate protein kinase C does not inhibit PDGF-induced [3 H]thymidine incorporation (Scott *et al.*, 1996), and it remains unlikely that protein kinase C is significantly involved in DNA synthesis in this tissue. Recently, preliminary data were presented indicating that transfection

of human airway smooth muscle cells with a dominant negative protein kinase C ζ inhibited PDGF-induced proliferation by approximately 80% (Black *et al.*, 1998). Hirst *et al.* (1995) also reported inhibition of serum-stimulated proliferation of rabbit tracheal smooth muscle cells by protein kinase C inhibitors. Donnelly *et al.* (1995) demonstrated the presence of conventional protein kinase C isoforms β I and β II, novel isoforms δ , ϵ , and θ , and the atypical isoform ζ in canine airway smooth muscle but did not address whether selective activation occurs after spasmogenic or mitogenic stimulation. The differences in results in these studies may reflect differential expression of protein kinase C isoforms or may indicate that there is differential regulation of these isoforms among species.

The ability of thrombin, acting through a G protein-coupled receptor, to activate PtdIns 3-kinase has been observed in only a limited number of cell types. In neutrophils, a novel form of PtdIns 3-kinase that is composed of a p101 regulatory subunit and a p120 catalytic subunit and is sensitive to activation by $\beta\gamma$ subunits derived from heterotrimeric G proteins has been identified (Stephens *et al.*, 1997). However, the finding that thrombin induced activation of PtdIns 3-kinase in anti-p85 immunoprecipitates suggests an alternative mechanism of activation. Such an effect could be explained by *trans*-activation of a growth factor receptor, as has been described by Daub *et al.* (1996) for Rat-1 fibroblasts transfected with EGF receptors. Agonists such as endothelin-1, lysophosphatidic acid, and thrombin induced tyrosine phosphorylation of EGF receptors in these cells in a manner similar to that observed after stimulation with EGF. Therefore, thrombin may act through tyrosine kinases to induce phosphorylation of growth factor receptors in BTSM cells and thus induce activation of a p85/p110 form of PtdIns 3-kinase. Chen *et al.* (1994) showed that thrombin, acting through its G protein-coupled receptor, induces activation of *src* and *fyn* in a lung fibroblast cell line, suggesting a possible mechanism of activation to initiate proliferation.

In conclusion, this study has demonstrated that BTSM cell DNA synthesis induced by two distinct mitogens is initiated by the rapid activation of PtdIns 3-kinase. The MAP kinase pathway contributes significantly to the proliferative response and is necessary for a full mitogenic response. This pathway, however, does not seem to be the key regulatory pathway that drives growth but seems to act in a parallel manner. Because proliferation in BTSM cells is known to be dependent on PtdIns 3-kinase-mediated p70^{s6k} activation (Scott *et al.*, 1996), the precise role of mitogen-induced protein kinase B activation in this response remains to be determined. The predominant involvement of receptor-mediated PtdIns 3-kinase activation in airway smooth muscle proliferation and the major consequences of this cellular response *in vivo* make this pathway an attractive therapeutic goal that could be targeted by the inhalation route.

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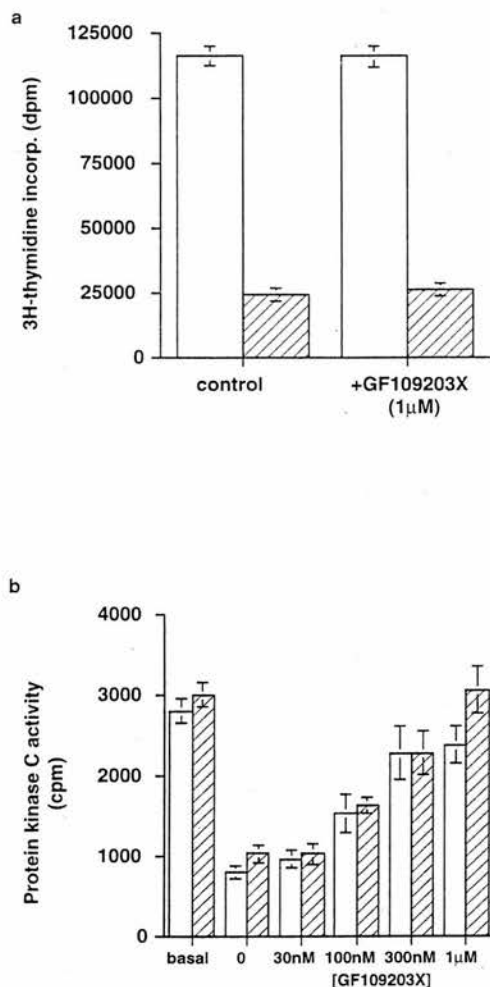


Fig. 7. Role of protein kinase C in mediating DNA synthesis in BTSM cells. a, BTSM cells were incubated with GF109203X (1 μ M), as indicated, for 5 min before the addition of PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (▨) as indicated. [3 H]Thymidine incorporation was assessed after 24 hr, as detailed in Materials and Methods; results are expressed as mean \pm standard error of the incorporation from four independent experiments, each performed in triplicate. b, BTSM cells were stimulated with PDGF-BB (20 ng/ml) (□) or thrombin (1 unit/ml) (▨) after preincubation with GF109203X at the concentrations indicated. Cells were lysed and cytosolic protein kinase C activity was assayed as detailed in Materials and Methods. Results are expressed as mean activity from a single experiment that was performed in triplicate and was representative of two others with similar results.

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