

**THE MOLECULAR MECHANISMS
REGULATING ANCHORAGE-DEPENDENCE,
DRUG RESISTANCE AND APOPTOSIS IN
SMALL CELL LUNG CANCER**

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DECLARATION

The work presented in this thesis was performed solely by myself, unless otherwise stated, under the supervision of Dr Tariq Sethi of the Rayne Laboratory, Department of Respiratory Medicine, Medical School, University of Edinburgh and Professor Peter Downes of the Department of Biochemistry, University of Dundee.

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ABSTRACT

Small cell lung cancer (SCLC) accounts for 25% of all new bronchial tumours and is characterised by an extremely aggressive clinical course resulting in a five-year survival rate of only 5%. The early dissemination of metastases and the development of resistance to chemotherapy are the two fundamental processes responsible for the highly malignant phenotype of this tumour. This thesis examines the mechanisms that regulate SCLC anchorage-dependence, drug resistance and apoptosis.

Phosphoinositide 3-kinase (PI 3-kinase), a regulator of multiple cellular processes including mitogenesis and invasion, is shown to be constitutively active in SCLC cell lines resulting in elevated levels of phosphatidylinositol 3,4,5 trisphosphate and protein kinase B (PKB). Inhibition of PI 3-kinase abrogated SCLC cell proliferation and colony formation and stimulated apoptosis. These data represent the first description of constitutively activated PI 3-kinase/PKB in any human cancer. Constitutive activation of PI 3-kinase may mimic integrin-dependent signal transduction thereby promoting cellular proliferation, anchorage-independence and tumourigenicity in SCLC.

Tumour recurrence following chemotherapy remains a major obstacle to the cure of SCLC. We hypothesised that a factor(s) within the local environment of SCLC cells might provide a 'survival' signal or block a 'death' signal, thereby accounting for the protection of SCLC cells from chemotherapy-induced apoptosis. Integrin-dependent adhesion of SCLC cells to ECM proteins, which occur in profusion in SCLC tumours *in vivo*, conferred resistance to chemotherapy-induced apoptosis. ECM protein-induced tyrosine phosphorylation was found to block chemotherapy-induced activation of the 'death' protease caspase-3 and hence, apoptosis. Survival of tumour cells attached to ECM proteins may explain the local recurrence of SCLC often seen clinically after chemotherapy.

In vivo, cancer cells exist in a state of dynamic interplay between anchorage-dependence and independence. Alterations in cell adhesion and migration are

dependent upon rapid, controlled alterations of the affinity of integrins for their extracellular ligands. CD98 has recently been identified as a unique and highly specific regulator of integrin affinity but its mechanism of action is not known. CD98 is highly expressed on SCLC cells both *in vivo* and *in vitro*. PI 3-kinase and PKB could both be activated with an anti-CD98 antibody (4F2) but not with monovalent 4F2-Fab showing that cross-linking of CD98 is required for their activation. Cross-linking CD98 stimulated SCLC anchorage-independent growth in semi-solid agarose medium. This effect could be blocked by a PI 3-kinase inhibitor. These data supports the hypothesis that CD98 promotes integrin-like intracellular signalling and is able to regulate anchorage-dependence and independence.

In conclusion, one can hypothesise that SCLC cells, *in vivo*, exist in a state of dynamic flux between anchorage-dependence and independence. The regulation of integrin adhesion is governed by complex signalling pathways and feedback loops involving PI 3-kinase and CD98. At any one time a proportion of cells are anchorage-independent and therefore can potentially migrate and metastasise, while others, are adhered to ECM proteins and are protected from chemotherapy-induced apoptosis. This would appear to be a good model to explain the aggressive nature of SCLC thereby accounting for this tumour's propensity to metastasise early and relapse following chemotherapy.

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ABBREVIATIONS

ATP	Adenosine 5' triphosphate
Bad	Bcl-X _L /Bcl-2 associated death factor
Bax	pro-apoptotic Bcl-2 homology protein
BCA	Bicinchoninic acid
Bcl-2	B cell leukaemia oncogene 2
BSA	Bovine serum albumin
BTSM	Bovine tracheal smooth muscle
Ca ²⁺	Calcium
CaCl ₂	Calcium chloride
CD	Cluster of differentiation
CHCl ₃	Chloroform
CHO	Chinese Hamster Ovary
CMF-PBS	Calcium and magnesium-free phosphate buffered saline
CPM	Counts per minute
CSF	Colony stimulating factor
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagles medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis (β -aminoethyl ether) N, N, N', N'- tetraacetic acid
ELISA	Enzyme linked immunosorbant assay
eNOS	endothelial nitric oxide synthase
Fab	Fragment for antigen binding
FAK	Focal adhesion kinase
FBS	Foetal bovine serum

FGF	Fibroblast growth factor
FH	Forkhead
FITC	Fluorescein isothiocyanate
FKBP12	FK506 binding protein 12
FN	Fibronectin
Grb2	Growth-factor-receptor-bound protein 2
GRP	Gastric releasing peptide
GSK3	Glycogen synthase kinase-3
GST	Glutathione-s transferase
GTP	Guanosine triphosphate
HBSS	Hanks' buffered salt solution
HCl	Hydrochloric acid
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HPLC	High pressure liquid chromatography
HRP	Horse radish peroxidase
ICE	Interleukin 1- β converting enzyme
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IGF-R	Insulin-like growth factor receptor
I κ B	NF- κ B inhibitory proteins
IL	Interleukin
IP ₃	Inositol 1,4,5-trisphosphate
IU	International unit
kDa	Kilodalton
LiCl	Lithium chloride
LN	Laminin
LY294002	2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
M	Molar
MAPK	Mitogen activated protein kinase
MCP	Monocyte chemotactic protein
MDCK	Mabry Darby canine kidney
MeOH	Methanol

MgAc	Magnesium Acetate
MgCl ₂	Magnesium chloride
MMP	Matrix metalloprotein
MRP	Multi-drug resistance protein
mTOR	Mammalian target of rapamycin
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NaF	Sodium fluoride
NFκB	Nuclear factor-κB
NGF	Nerve growth factor
(NH ₄) ₂ HPO ₄	Ammonium phosphate
NSCLC	Non small cell lung cancer
p70 ^{s6k}	Ribosomal protein S6 kinase
P-gp	P-glycoprotein
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDGF-R	Platelet derived growth factor receptor
PDK	PI (3,4,5)P ₃ -dependent kinase
PH	Pleckstrin homology domain
PI 3-kinase	Phosphoinositide 3-kinase
PIF	PDK-1 interacting fragment
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PLL	Poly-L-lysine
PMSF	Phenylmethylsulfonyl fluoride
PS	Phosphatidylserine
PtdIns	Phosphatidylinositol
Rac	Ras-related C3 botulinum toxin substrate
Ras	Rat sarcoma virus
Rho	Ras homologous
RPMI	Roswell Park Memorial Institute medium

SCF	Stem cell factor
SCLC	Small cell lung cancer
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SH	Src homology
SITA	RPMI 1640 medium containing 25 mM HEPES supplemented with 30 nM selenium, 5 µg/ml insulin, 10 µg/ml transferrin and 0.25% (w/v) BSA.
Shc	SH2-domain-containing α 2-collagen related protein
Src	Rous sarcoma virus
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
VEGF	Vascular endothelial growth factor
z-VAD	z-Val-Ala-DL-Asp-fluoromethylketone

CHAPTER 1

INTRODUCTION

1.1 LUNG CANCER

1.1.1 Epidemiology of Lung Cancer

Each year nearly six million people are diagnosed with cancer worldwide. Of these, approximately two thirds will die as a consequence of their disease. This represents about 10% of all deaths (WHO, 2000). In most countries lung cancer is the commonest cause of cancer death. It has been calculated recently that lung cancer is the cause of 12.8% of cancer cases and 17.8% of cancer deaths worldwide (Parkin et al., 1999). In the UK in 1995 there were 36,600 deaths from lung cancer (WHO, 2000).

The incidence of lung cancer varies widely between different countries being highest in the more developed countries of North America and northern Europe. For men, Scotland has the highest national incidence in the world at 135/100,000 compared with 70/100,000 for the United States. The incidence is lower in Scandinavia at 44/100,000 and only 7/100,000 in India (WHO, 2000). For women, the overall pattern is similar but the incidence is generally about a third of that in men. In recent years there has been evidence from western countries of a fall in the incidence of lung cancer in men (Peto et al., 2000). However, rather worryingly, there continues to be a rise in the incidence in women (Osann, 1998). Furthermore, in developing nations where smoking rates are high, mortality from lung cancer has been rising steadily and this rise is expected to continue (Liu et al., 1998).

1.1.2 Classification of Lung Cancer

Bronchogenic carcinomas arise from the mucosa of the tracheobronchial tree. The most extensively used classification currently is the World Health Organisation's histological classification published in 1999 (WHO, 1999). This classification subdivides the main types of lung cancer into four categories:

- a. Squamous cell carcinoma
- b. Adenocarcinoma
- c. Large cell carcinoma
- d. Small cell carcinoma

Squamous cell carcinoma, adenocarcinoma and large cell carcinoma are collectively known as non-small cell lung cancer (NSCLC).

1.1.2a Squamous cell carcinoma

Squamous cell carcinoma arises from metaplastic squamous epithelial cells. These tumours are endobronchial and arise centrally within the lung. They tend to grow relatively slowly and produce symptoms and signs such as lobar collapse and obstructive pneumonia due to local 'mass' effects. Squamous cell carcinoma tends to metastasise relatively late and therefore is the subtype most amenable to surgical resection. In recent years in North America the proportion of squamous cell carcinoma has decreased from about 40% of all lung cancers to 20-25%. However, this trend is not so apparent in European countries and squamous cell carcinoma remains the commonest cell-type (Hoffman et al., 2000).

1.1.2b Adenocarcinoma

Adenocarcinomas appear to arise from either surface epithelial cells (ciliated and mucus producing cells) which give rise to mucin-containing and mucin-secreting tumours or the progenitor cells of the peripheral airways (Clara cells and type II pneumocytes) which give rise to peripheral airway cell tumours (Gazdar and McDowell, 1988).

Until recently adenocarcinoma accounted for about 20-25% of lung cancers in Europe and North America. Recently this figure has risen to 40% in North America and is also increasing in Europe. The reasons for this are not fully understood but may, in part, reflect the increasing numbers of women who are smoking, as this subtype is more common in women. It has also been suggested that these changes

are the result of alteration in cigarette design (Thun et al., 1997). The smoke in filter tip cigarettes tends to be inhaled more deeply than that of the older unfiltered types and as a result tobacco-specific carcinogens are transported more distally into the lung toward the bronchoalveolar junction where adenocarcinomas often arise. Although cigarette smoking causes the majority of adenocarcinomas, this is the subtype that occurs most commonly in non-smokers. Not infrequently patients present with symptoms due to metastatic disease because of the propensity of this tumour to metastasise early. The primary tumour may be a relatively symptomless peripheral nodule. The principal subgroup of adenocarcinoma is bronchoalveolar carcinoma. These may be unifocal or multifocal. This subgroup of adenocarcinomas metastasise less commonly than the other cell types.

1.1.2c Large cell carcinoma

These represent a group of primary lung carcinomas arising from bronchial epithelium that have not differentiated sufficiently to allow them to be placed in either the squamous or adenocarcinoma groups. Clinically these tumours are often large peripheral masses, sometimes with cavitation and they account for 10% of all lung cancers.

1.1.2d Small cell lung cancer

Small cell lung cancer (SCLC) accounts for 20-25% of all new bronchial carcinomas. The precise origin of small cell lung cancer is open to debate. These tumours were originally thought to arise from cells of the diffuse neuroendocrine system which occurs throughout the bronchial tree (Gazdar et al., 1985a). However, it is now thought that they may arise from a common stem cell (see below). SCLC is now considered to be a systemic disease and almost invariably has metastasised by the time of presentation. Unlike other bronchial tumours, SCLC is extremely sensitive to chemotherapy and radiotherapy.

1.1.3 Aetiology of lung cancer

The main risk factor for lung cancer is cigarette smoking. This risk was first appreciated in 1950 in a series of case control studies (Wynder and Graham, 1950;

Levin et al., 1950; Mills and Porter, 1950; Schrek et al., 1950). Sir Richard Doll established a cohort of 40,637 British doctors in 1951 and this group has been followed for 40 years (Doll and Peto, 1976; Doll et al., 1994). The mortality rate ratio for lung cancers in smokers versus non-smokers was 14.9 and this dropped to 4.1 in ex-smokers. The loss of expectation of life for all cigarette smokers in this study was 8.0 years. A similar study by the American Cancer Society found that the lung cancer mortality rate ratio for smokers versus non-smokers was 23.9 for men and 14 for women (Thun et al., 1995). Overall, it is estimated that 85-90% of lung cancers can be linked to active smoking.

Besides smoking, other environmental and occupational exposures, such as to aromatic hydrocarbons, radon, asbestos, nickel, arsenic and chromium are important determinants of lung cancer risk. These account for 5% of female and 15% of male lung cancer deaths (WHO, 1982). Of these, the most important aetiology in terms of number of cancers caused is probably asbestos exposure. Asbestos exposure (particularly to the crocidolite type) significantly increases the risk of developing lung cancer quite apart from the risk of developing malignant mesothelioma. Hammond et al, (1979) reported on a cohort of 17,800 asbestos insulators in the United States and Canada. Compared with non-smoking controls with no history of asbestos exposure, asbestos workers with a history of smoking had a 53-fold increased mortality rate from lung cancer. This was greater than the sum of the increases for lung cancer from asbestos exposure alone (5-fold) or cigarette smoking alone (11-fold).

Although lung cancer is largely preventable by elimination of these exposures, this disease will be a major public health problem for the foreseeable future because ex-smokers continue to have a significantly increased risk of developing lung cancer.

1.1.4 A model for lung cancer development

Each of the four main types of lung cancer reflect phenotypic features of the cell types that make up the normal bronchial epithelium. One hypothesis that is becoming increasingly accepted is that the four types of lung cancer are related

through a common differentiation pathway in the bronchial epithelium (reviewed in Mabry et al., (1991)). This proposal is supported by clinical evidence that demonstrates potential for transitions between the different tumours (i.e. that tumours can be admixtures of the different phenotypes). For instance, neuroendocrine features can be found in 25% of bronchial adenocarcinomas and this confers an initial sensitivity to chemotherapy and radiotherapy. Furthermore, individual cells have been observed to manifest features of SCLC and NSCLC simultaneously. It is proposed that these transitions mimic normal cellular transitions in bronchial mucosa and are mediated by the consistent genetic abnormalities now being described for lung cancer.

Mabry et al., (1991) described an *in vitro* model system which examined these transitions. They utilised a non-tumourigenic cell line derived from bronchial epithelium (BEAS-2B) which had been transfected with the simian virus (SV40), allowing continuous growth (Gruenert et al., 1988; Bonfil et al., 1989). Using this model it was shown that overexpression and point mutations of different oncogenes produced different histological types of tumours. Overexpression of *c-myc* and *c-raf-1* resulted in the development of a large cell carcinoma (Pfeifer et al., 1989). Transfection with *H-ras* and *HER-2/neu* led to the development of adenocarcinomas, whereas transfection of mutant *p53* led to the development of squamous cell carcinomas. Furthermore, insertion of a mutated *H-ras* gene into SCLC cells that overexpressed either an endogenously amplified *c-myc* or *N-myc* gene, or a transfected human *c-myc* gene caused transition to a large-cell undifferentiated phenotype (Mabry et al., 1988).

1.1.5 Molecular events in lung cancer development

The current hypothesis of lung carcinogenesis is that it is a multi-stage process involving mutations in several genes involved in DNA repair, cell growth, signal transduction and cell cycle control. These mutations can cause activation of oncogenes (e.g. members of the *myc* and *ras* families, *c-raf-1* and *bcl-2*) or deletion of tumour suppressor genes (e.g. *rb* gene, *p53*, *3p*, *9q* and *5q*). In combination with environmental exposures and inherited polymorphisms in a variety of genes,

particularly the carcinogen metabolism genes, these mutations can affect the susceptibility of an individual to develop lung cancer. Such genetic changes result in pathological changes, which initially at least, may be reversible. However, as progressive chromosomal changes become more complex, irreversible damage occurs and lung cancer develops (Figure 1.1). The multi-step model proposes that two or more genetic mutations are required to transform a cell. Inheritance of one or more of these mutations allows tumours to develop earlier than if several spontaneous mutations have to accumulate in a cell. Many studies have been undertaken to assess the role of genetics in the development of lung cancer after exposure to long-term carcinogens such as cigarette smoke (Heighway et al., 1986). The evidence that susceptibility to lung cancer has a genetic component is suggested by studies, which show that only 10% of individuals who smoke develop lung cancer and that some individuals who do not smoke develop lung cancer. Furthermore, there is a significantly increased risk of lung cancer, as well as of other tumours, among blood relatives of lung cancer patients compared with controls (Ooi et al., 1986; Lynch et al., 1986). There are reports linking inherited mutations in the retinoblastoma gene on chromosome 13 (Sanders et al., 1989), the *p53* gene on chromosome 17 (Malkin et al., 1992) and an *H-ras* allele on chromosome 11 (Heighway et al., 1986) with an increased risk for lung cancer. The molecular changes which lead to an accumulation of genetic changes resulting in activation of oncogenes and inactivation of tumour suppresser genes are not fully understood, but a mutator phenotype leading to genomic instability may be an early step in the development of some cancers. Work on a mutator or replication error phenotype has demonstrated a high frequency of microsatellite instability in SCLC compared to NSCLC (Merlo et al., 1994; Mao et al., 1994; Fong et al., 1995; Fong et al., 1995). In both SCLC and NSCLC with associated microsatellite instability, tumours are characterised by widespread allelic loss (loss of heterozygosity) as a result of loss of chromosomes or portions of chromosomes. Sequential accumulation of loss of heterozygosity is a frequent event in lung cancer and occurs during progression from primary tumours to metastatic sites (Shiseki et al., 1994). Dominant oncogenes require only one mutation for their tumourigenic potential to be activated, whereas the inactivation of recessive oncogenes usually requires a genetic event, such as a

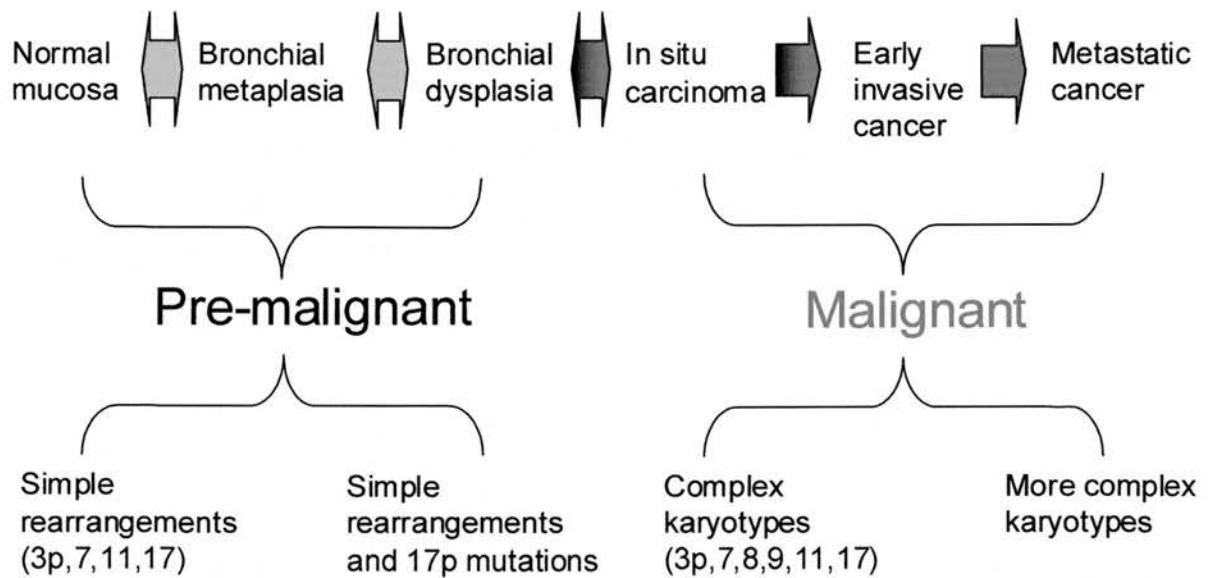


Figure 1.1

Molecular pathogenesis of lung cancer. Lung cancer is believed to be a multi-stage process involving mutations in up to 20 genes involved in DNA repair, cell growth and signal transduction. Such mutations can cause activation of oncogenes or deletion of tumour suppressor genes. The resulting genetic changes result in pathological changes, which initially, may be reversible. However, as the chromosomal changes become more complex irreversible damage occurs and lung cancer develops.

deletion or mutation, in both alleles. SCLC has been shown to exhibit frequent losses of 3p, 5q, 13q and 17p (Whang-Peng et al., 1982a; Yokota et al., 1987; Whang-Peng et al., 1982b). NSCLC show consistent loss of heterozygosity at 3p, 9p, 11p, 13q and 17p (Shiseki et al., 1994).

1.2 SMALL CELL LUNG CANCER

1.2.1 The clinical picture

SCLC tumours usually arise centrally and are smoking-related. Histologically SCLC cells may be relatively small and round with little cytoplasm (lymphocyte-like) or somewhat larger and fusiform (classical 'oat' cell type) or of relatively nondescript or 'polygonal' cell type. As well as these true small cell tumours, a variant known as the 'intermediate' type can occur which consists of larger cells although they maintain the histological characteristics of the smaller cell variants. The cytoplasm is characterised by small dense-core granules which are a manifestation of the peptide and hormone secretion that is the hall mark of the 'neuroendocrine' properties observed in SCLC, but relatively infrequently in other types of lung cancer.

SCLC accounts for 20-25% of all new bronchial tumours and is characterised by an extremely aggressive clinical course. Untreated the median survival from diagnosis is only 5 to 12 weeks depending upon the stage at diagnosis. Clinically there are no specific features to distinguish SCLC from other primary lung tumours. The primary site is usually within the central bronchial tree and cough, dyspnoea, haemoptysis, wheeze and post obstructive pneumonia are all common symptoms. Chest pain can occur with parietal pleural involvement and mediastinal invasion. Superior sulcus tumours may produce shoulder pain, arm pain, brachial plexopathy or a Horner's syndrome. Mediastinal invasion can lead to recurrent laryngeal nerve palsy, superior vena cava obstruction and phrenic nerve palsy. Oesophageal symptoms and pericardial invasion are recognised but are rarer.

SCLC is associated with a variety of paraneoplastic syndromes. These syndromes are due to the synthesis and release of a variety of hormones and peptides by the

tumour. Adrenocorticotrophic hormone and anti-diuretic hormone secretion cause Cushing's syndrome and the syndrome of inappropriate anti-diuretic hormone respectively (Bliss et al., 1990). Immune reactions to tumour antigens can result in the Eaton-Lambert myasthenic syndrome, in which the presence of circulating antibodies react with voltage-gated calcium channels (De Aizpurua et al., 1988).

Two thirds of patients will have evidence of distant metastases, most often to liver, bone, bone marrow, the central nervous system and the adrenals, at the time of initial diagnosis. This excludes the possibility of surgical resection for the majority of cases. SCLC is extremely sensitive to chemotherapy and radiotherapy and this remains the mainstay of treatment. However, although most patients will benefit from treatment with an improvement in symptoms and increase in survival, the tumour invariably recurs and proves fatal. Survival to 5 years is rare.

1.2.2 The management of small cell lung cancer

The management of SCLC is, in part, dependent upon the stage of the disease at presentation. The traditional TNM staging system is not appropriate for SCLC, as the tumour is almost always systemic at the time of diagnosis. A two-stage classification for SCLC staging has been proposed which divides patients into those with either limited or extensive disease. Limited disease describes tumour confined to one hemithorax (including pleural effusion), ipsilateral mediastinal and supraclavicular lymph nodes. Extensive disease is defined as the presence of tumour beyond these sites. About 70% of patients fall into the latter category (Elias, 1997).

1.2.2a Chemotherapy for small cell lung cancer

The first study on the use of chemotherapy for the treatment of SCLC was published in 1969 by the Veterans' Administration lung cancer study group (Green et al., 1969). The use of single-agent cyclophosphamide produced a doubling of median survival compared with a placebo-controlled group. Subsequent studies comparing radiotherapy alone with radiotherapy and chemotherapy showed significant improvements in survival for the patients who had received chemotherapy. Phase II studies have now shown that a number of cytotoxic drugs are active against SCLC.

However, historical data suggests that the use of combination chemotherapy gives better results than monotherapy. The use of combination chemotherapy has improved survival by four to five fold compared with untreated patients (Evans et al., 1985; Hong et al., 1989; Jett et al., 1990). With aggressive combined modality therapy or combination chemotherapy, response rates of 80 to 100 percent (50 to 70 percent complete response) in limited disease and 60 to 80 percent (15 to 40 percent complete response) in extensive disease have been achieved (Seifter and Ihde, 1988). However, responses tend to be short-lived with a median duration of approximately six to eight months. From the time of diagnosis, the median ranges of survival for limited and extensive disease are 14 to 20 months and 8 to 13 months, respectively (Osterlind et al., 1986). Once recurrent, median survival is about four months. Approximately 20 to 40 percent of limited stage and less than 5 percent of extensive stage patients survive two years. Survival beyond five years occurs in only 3 to 8 percent of patients.

At least 15 to 20 different chemotherapeutic agents have major activity against SCLC in both untreated and relapsed patients. The major agents are etoposide, cisplatin, carboplatin, ifosfamide, cyclophosphamide, vincristine and doxorubicin. Despite a large number of regimens almost all produce similar outcomes. However, one conclusion from these studies is that etoposide containing regimens are well tolerated and highly active (Hong et al., 1989; Jett et al., 1990). The etoposide and cisplatin combination is most frequently used and appears to be superior to cyclophosphamide, adriamycin and vincristine (Evans et al., 1985).

The inability to destroy residual cells despite initial chemosensitivity suggests the existence of a small number of tumour stem cells resistant to cytotoxic therapy. In order to try and overcome the problem of drug resistance alternating or sequential combination protocols were designed to expose the tumour to a large number of active cytotoxic agents. However, a number of randomised trials have failed to show clinically significant improvements in disease-free or overall survival with these alternating regimens (Evans et al., 1987; Roth et al., 1992; Fukuoka et al., 1991).

Maintenance chemotherapy beyond four to six cycles produces minor prolongation of response without survival benefit. Furthermore, cumulative toxicity is encountered.

1.2.2b Consolidation radiotherapy

For patients with limited-stage disease who have achieved a complete clinical response to chemotherapy, it is usual to give a course of radiotherapy to the mediastinum and the site of primary disease. A large randomised study showed that the combined-modality group had a median survival of 15 months, and 2 year survival of 28% compared with 11.6 months and 12% for the chemotherapy-only group (Bunn, Jr. et al., 1987). A meta-analysis of 2103 patients in 13 trials confirmed a 14% reduction in mortality and 5% increase in 3-year survival (Pignon et al., 1992).

There has been a considerable amount of work performed looking at the benefits of giving radiotherapy in combination with initial chemotherapy for patients with limited disease. Although there are theoretical advantages, such as reducing the development of drug resistant clones, there is an increased risk of serious toxicity and this approach is still restricted to trials (Arriagada et al., 1995).

1.2.2c Prophylactic cranial irradiation

For patients who achieve a complete remission after chemotherapy there is a 20% risk of subsequently developing brain metastases. For some, this may be the first and only site of metastasis. Therefore, patients with limited-stage disease who achieve a complete remission should be offered prophylactic cranial irradiation. This reduces the risk of brain metastases to about 8% but does not affect overall survival (Abner, 1993; Bunn, Jr. and Kelly, 1995).

1.2.2d Surgery for Small cell lung cancer

When it was appreciated in the early 1970s that SCLC is a systemic disease the use of surgery to resect the primary tumour was largely abandoned. However, for a small group of patients surgery may still play a role. Patients who present with

small, apparently localised, pulmonary nodules may benefit from resection with adjuvant chemotherapy. One study by Meyer (1987) combined surgery with adjuvant chemotherapy for stage I and II carcinomas and reported an 80% survival rate at 30 months. However, the overall number of patients who will fall into this category are very small (about 1%).

1.2.2e Management of relapsed SCLC

Most patients with SCLC who have been treated with chemotherapy will relapse within two years of initial treatment. When this occurs the results of treating them again with chemotherapy are generally poor with response rates of only 20-25% and a median survival of only three to four months. In general, response to second line chemotherapy is better if there has been a disease-free interval. It may be possible to induce a period of remission with the same drugs used for the initial treatment or with a different regimen in the hope that there is not cross-resistance.

1.2.2f New agents

At the time of writing a number of new agents are being evaluated for activity against SCLC. These include altretamine, paclitaxel, docetaxel, vinorelbine, gemcitabine and topoisomerase I inhibitors such as topotecan and irinotecan. In a recent phase III study topotecan was compared with the standard cyclophosphamide, adriamycin and vincristine regimen. Response rates, median time to progression and survival were similar in the two groups of patients, but topotecan resulted in an improved control of several symptoms (von Pawel et al., 1999). In another study, taxol and topotecan are being evaluated as a first line treatment in previously untreated patients with extensive disease. Early reports have been encouraging with complete remission in 19 percent and partial remission in 29 percent (Jacobs et al., 1999).

1.2.3 An 'in vitro' model for small cell lung cancer

In 1985 Carney et al., described the growth and characterisation of 50 SCLC cell lines established from patients between 1977 and 1984. Much of the subsequent work on SCLC has utilised either these cell lines, or others, established in a similar

fashion. Biochemical characterisation of these lines for the expression of L-dopa decarboxylase, bombesin-like immunoreactivity, neurone-specific enolase and the brain isoenzyme of creatine kinase showed that SCLC cell lines could be subdivided into two types, classical and variant. Classical SCLC lines express elevated levels of all four biomarkers while variant lines have undetectable levels of L-dopa decarboxylase and bombesin-like immunoreactivity but continue to express neurone-specific enolase and the brain isoenzyme of creatine kinase.

Based on their appearance, SCLC cell lines can be grouped into 4 major categories called types 1, 2, 3 and 4 (Carney et al., 1985). Type 1 lines grow as tightly packed spherical aggregates of floating cells, which frequently demonstrate areas of central necrosis in larger spheroids. Type 2 lines grow as relatively densely packed floating aggregates, amorphous and irregular in outline and Type 3 cells grow as very loosely adherent floating aggregates in small clumps and intertwined cords. Central necrosis does not occur in either of these lines. Type 4 cells grow attached to substrate and consist of large overlapping polygonal cells lacking the epithelioid appearance of adherent NSCLC cell cultures. In addition, SCLC cells are able to form colonies in soft agarose and are tumourigenic in athymic nude mice (Carney et al., 1980).

Doubling times in culture range from 32-72 hours. *In vitro* studies have shown that variant cell lines have a more rapid growth rate and a higher cloning efficiency in soft agarose and nude mice than classical cell lines (Gazdar et al., 1985b). It has been proposed that variant cell lines represent the *in vitro* counterpart of SCLC tumours that have relapsed and become chemoresistant. However, when biomarker expression of cell lines established from untreated patients were compared with those who had relapsed from intensive combination chemotherapy considerable overlap occurred and no significant differences could be observed (Carney et al., 1985).

1.2.4 Growth factors for SCLC

1.2.4a Neuropeptides

One of the most striking characteristics of SCLC cells is their ability to produce a large number of hormones and peptides *in vivo* and *in vitro* including bombesin, neurotensin, cholecystokinin and vasopressin (Gazdar et al., 1985b). Early work

concentrated on the hypothesis that these peptides might regulate the growth and perhaps degree of differentiation of SCLC in an autocrine or paracrine fashion. The hypothesis of autocrine growth inhibition by gastrin releasing peptide (GRP) in SCLC was first tested by Cuttitta et al., (1985) using a monoclonal antibody (2A11) to [Lys³] bombesin. Under serum-free conditions this antibody inhibited the clonal growth of two SCLC cell lines and retarded the growth of SCLC xenografts in nude mice. Furthermore the specific bombesin/GRP receptor antagonist, [Leu¹³- ϕ (CH₂NH)-Leu¹⁴] bombesin was shown to inhibit basal colony growth of H345 cells in semi-solid medium. The use of exogenously added bombesin to stimulate human SCLC growth *in vivo* was extended by Alexander and colleagues (1988) who found that intra-peritoneal administration of bombesin to nude mice bearing human SCLC implants significantly stimulated tumour growth compared with controls.

A number of studies examining the molecular pathways by which neuropeptide mitogens elicit cellular growth have exploited a murine fibroblast model system. Several neuropeptides including bombesin (Rozenfurt and Sinnott-Smith, 1983), vasopressin (Rozenfurt et al., 1979), bradykinin (Woll and Rozenfurt, 1988) and vasoactive intestinal peptide (Zurier et al., 1988) are able to act as growth factors for cultured Swiss 3T3 cells. Cloning and sequencing of the cDNA for several mitogenic peptide receptors including bombesin (Battey et al., 1991), neuromedin-B (Corjay et al., 1991), bradykinin (McEachern et al., 1991) and vasoactive intestinal peptide (Sreedharan et al., 1991) revealed that these receptors are seven transmembrane domain spanning G-protein-coupled receptors. Binding of neuropeptides and vasoactive peptides causes phospholipase C (PLC) induced hydrolysis of phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P₂) generating the second messengers inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), which mobilise Ca²⁺ from intracellular stores and activate protein kinase C (PKC) respectively (Rozenfurt, 1992). Studies using SCLC cell lines have demonstrated a similar set of signalling events to those in murine 3T3 fibroblasts. Woll and Rozenfurt (1989) demonstrated that bradykinin, cholecystokinin, galanin, neurotensin, and vasopressin induce a rapid and transient increase in intracellular calcium in several SCLC cell lines (Woll and Rozenfurt, 1989). There is, however,

considerable heterogeneity of response to individual peptides among SCLC cell lines. Such variation is attributed to differences in receptor expression. The Ca^{2+} - mobilising effects of each neuropeptide are mediated by distinct receptors as shown by the use of specific peptide antagonists and by the induction of homologous desensitisation.

Sethi and Rozengurt (1991) subsequently showed that a variety of neuropeptides could, over the same concentration range as required for Ca^{2+} mobilisation, stimulate the clonal growth of SCLC cell lines in semi-solid agarose. These findings showed that the previously identified autocrine growth loop involving bombesin-like peptide is only one of an extensive network of paracrine and autocrine circuits that sustain the proliferation of SCLC.

1.2.4b Polypeptide hormones

Insulin-like growth factor (IGF-I) is a mitogen for many cell types both *in vitro* and *in vivo* (Schoenle et al., 1982; Froesch et al., 1985). Several groups have shown that IGF-I or IGF-I-like peptides are produced and released by cultured human tumour cell lines including lung cancer (Minuto et al., 1986; Macaulay et al., 1990). Subsequent studies confirmed the presence of an autocrine proliferation loop. Exogenous addition of IGF-I, IGF-II or insulin resulted in marked proliferation of human SCLC cells as measured by *in vitro* growth assays (Nakanishi et al., 1988). More recently the type I insulin-like growth factor receptor (IGF-R) has been identified as the receptor for these ligands (Sell et al., 1994; Resnicoff et al., 1994). The growth and tumourigenicity of transformed cells can be inhibited by disruption of IGF-R function using a monoclonal antibody (α -IR3) which inhibits the interaction of the IGF-R with its ligands (Zia et al., 1996). Historically IGF-I was believed to be the biologically relevant ligand for the IGF-1/IGF-R autocrine loop but recent work has identified IGF-II as the predominant IGF involved. Despite expression of IGF-I, IGF-II and IGF-R mRNA in the majority of lung and breast cancer cell lines examined, only IGF-II peptide was identified (Quinn et al., 1996). A similar role for IGF-II has recently been reported in prostate cancer (Angelloz-Nicoud and Binoux, 1995) and cervical cancer cell lines (Steller et al., 1995).

A number of other polypeptide hormones have been proposed as being regulators of growth in lung cancer. Epidermal growth factor (EGF) is a polypeptide hormone that has both growth stimulatory and growth inhibitory effects on normal and tumour cells *in vitro* (Kamata et al., 1986). Both NSCLC and SCLC have receptors for EGF and various studies have shown that EGF can have both stimulatory (Haeder et al., 1988; Veale et al., 1989) and inhibitory effects (Rabiasz et al., 1992) on tumour cell growth. Similarly, the data available for nerve growth factor (NGF) in lung cancer is conflicting. One group has reported that NGF can stimulate clonal growth of human SCLC cell lines (Oelmann et al., 1995) while another group has reported that NGF abrogates the tumorigenicity of SCLC cell lines (Missale et al., 1998). These differences may be attributable to the use of different cell lines expressing different receptor populations but more work is required to elucidate the role of NGF in lung cancer cell proliferation.

1.2.4c Stem cell factor and c-kit

Over 70% of SCLC cell lines and tumour specimens co-express the c-kit proto-oncogene and its ligand stem cell factor (SCF) (Hibi et al., 1991; Plummer, III et al., 1993; Rygaard et al., 1993). The *c-kit* gene encodes a tyrosine kinase growth factor receptor in the same group as those for platelet-derived growth factor (PDGF) and colony stimulating factor-1 (CSF-1) (Yarden et al., 1987). SCF, also known as mast cell growth factor, kit ligand or steel factor is a haemopoietic growth factor that can support the proliferation and differentiation of multiple haemopoietic cell lineages from early precursors (Martin et al., 1990; Anderson et al., 1990; Huang et al., 1990). Growth stimulation by exogenous SCF has been demonstrated in selected SCLC cell lines (Sekido et al., 1993). However, most SCLC cell lines that co-express receptor and ligand fail to respond to soluble SCF (Turner et al., 1992). In order to examine this further Krystal et al., (1996) transfected a SCLC cell line that expresses only SCF, with a *c-kit* expression vector. The transfected cells grew more vigorously in serum-free medium compared with control-transfected cells. This growth advantage could be blocked by a selective tyrosine kinase inhibitor. Furthermore, a cell line which naturally co-expresses SCF and *c-kit* was transfected with a kinase-defective

c-kit gene. These cells showed a marked decrease in their ability to grow under growth factor-free conditions compared with cells transfected with the empty expression vector. Taken together these experiments showed that SCF and *c-kit* do contribute to SCLC growth.

1.3 THE TUMOUR CELL MICROENVIRONMENT

Under normal physiological conditions, tissue architecture is a complex and highly ordered arrangement. Far from existing as independent units, cells are critically dependent upon interactions with their immediate environment. The mammalian organism is divided into a series of tissue compartments separated by the extracellular matrix unit consisting of the basement membrane and its underlying interstitial stroma. Both the interstitial matrix and the basement membranes contain collagens, glycoproteins and proteoglycans. The interstitial matrix, which is produced by mesenchymal cells, contains fibrillar collagens, fibronectin, hyaluronic acid and fibril-associated proteoglycans. The basement membrane is produced by parenchymal cells. Cells are polarised to it such that their basal surfaces abut it. Basement membranes are composed of collagen type IV, laminin and proteoglycans.

Although tumour stroma is composed of the same components as normal connective tissues it is recognised that the amounts of extracellular matrix (ECM) can vary markedly between tumours. At one end of the spectrum lie tumours such as scirrhous carcinomas where almost all of the tumour mass is formed by dense, hard collagenous connective tissue and at the other end are, for example, medullary carcinomas with only minimal connective tissue between clusters of tumour cells.

1.3.1 Extracellular matrix proteins

1.3.1a Fibronectin

The fibronectin family consists of a large family of heterodimeric and polymeric glycoproteins. In humans, fibronectin is encoded for by a single gene and is notable for its multiple forms of alternative splicing (Kornblihtt and Gutman, 1988). Splicing of the IIIICS region can be combined with splices of the ED-A and ED-B regions to form the 20 variants constituting the fibronectin family. The final product

is a dimer of approximately 250 kDa. While the IIICS region has adhesive functions the roles of the ED-A and ED-B regions have been unknown until recently. Recent studies show that they are expressed during tissue remodelling including embryogenesis, injury and wound healing and in malignancy (Vartio et al., 1987; Peters et al., 1988).

Fibronectin is the prototype cell attachment protein found in the matrix surrounding normal cells. Fibronectin mediates cell adhesion and anchorage through a number of fibronectin-binding integrins including $\alpha 5\beta 1$, $\alpha 3\beta 1$ and $\alpha 4\beta 1$. Secreted fibronectin does not form matrix spontaneously. Cells use $\alpha 5\beta 1$ integrin to capture secreted fibronectin and convert it into fibrils, which are then deposited in the matrix.

1.3.1b Laminin

Laminin is the major glycoprotein found in the basement membrane. It is able to bind to other matrix components such as type IV collagen, heparan sulphate proteoglycan and entactin and to itself. Laminin is composed of three chains designated A, B1 and B2 which are held together in a cruciform-like structure by disulphide bonds. The complete structure has now been determined through cDNA cloning and sequencing (reviewed in Sasaki and Yamada, 1987).

Laminin has a variety of biological activities including promotion of cell adhesion, polarity, growth, migration, differentiation, neurite outgrowth and formation of tumour metastases.

Using proteolytic cleavage and specific antibodies recent progress has been made in identifying, at the amino acid level, some of the biologically active sites on laminin. A unique sequence of five amino acids, YIGSR, from one of the EGF-like repeats in the $\beta 1$ chain, has been identified as important in promoting cell adhesion and migration. An RGD sequence in the A chain can also promote cell attachment. It is this site which is important in binding to integrins, in particular, $\alpha 3\beta 1$, $\alpha 4\beta 1$ and $\alpha 6\beta 1$.

1.3.1c Collagen IV

Collagen is the most abundant protein in mammals constituting about one quarter of the total. It is the major fibrous element of skin, bone, tendon, cartilage, blood vessels and teeth. It is present to some extent in nearly all organs and serves to hold cells together in discrete units. Five forms have now been described and the basic structural motif is modified to meet the specialised needs of particular tissues. Collagen IV and collagen V are the only types to be found in basement membrane, and in the lung are localised to the basement membrane of the alveolar walls of capillaries and bronchioles. Within the basement membrane collagen IV co-localises with laminin and provides a scaffold on which other components of the basement membrane are formed.

1.3.1d Tenascin

The tenascin family comprises four members, C-, R-, X- and Y-tenascin (Chiquet-Ehrismann et al., 1994). Tenascin-C was identified initially and is the best characterised form. It is a high molecular weight glycoprotein consisting of 6 similar subunits linked to their amino terminal by disulphide bonds. Tenascin is widely expressed in the developing brain, cartilage and mesenchyme and is re-expressed in tumours, wound healing and sites of inflammation. This apparently regulated and specific expression implies a crucial role in embryonic development and restructuring. Although various roles for tenascin have been proposed as a result of *in vitro* work, recent experiments using a tenascin-C gene knockout revealed that tenascin deficient mice develop normally and show no apparent defects (Saga et al., 1992). Therefore, at present the role for tenascin remains elusive.

1.3.2 *Proteoglycans*

In addition to proteins and glycoproteins, tumour stroma contains significant amounts of proteoglycans, including versican, decorin and various chondroitin sulphate-based proteoglycans. These molecules have recently been implicated in the regulation of cytokine concentration and function in tumours (Iozzo and Cohen, 1994). Several tumours are reported to contain elevated levels of decorin and chondroitin sulphate. It is speculated that some cytokines such as PDGF, EGF,

fibroblast growth factor and transforming growth factor α and β may be presented by binding to proteoglycans (Yeo et al., 1991).

1.3.3 Inflammatory cells and cytokines

The presence of leucocytes in tumour tissues has been recognised for many years. Their role is still unclear. However, it has been demonstrated that macrophages, when appropriately stimulated, are capable of killing transformed cells *in vitro* whereas untransformed cells are normally fairly resistant to killing by activated macrophages (Adams and Snyderman, 1979). Although *in vivo* studies are difficult to perform, one group has recently reported that the number of macrophages in human tumours inoculated in nude mice was negatively correlated with tumour size (Zhang et al., 1997). Whether this finding is a true representation of the *in vivo* situation is uncertain. Novel immunohistochemical techniques suggest that the number of inflammatory cells present in a tumour is possibly much higher than previously appreciated. Such results raise the question of whether tumours are able to regulate the recruitment of inflammatory cells. Human tumours are reported to release a range of factors chemotactic for monocytes and macrophages including monocyte chemotactic protein-1 (MCP-1) (Graves et al., 1989; Yoshimura et al., 1989), MCP-2 and MCP-3 (Van Damme et al., 1992), tumour necrosis factor (TNF), vascular endothelial growth factor (VEGF) and macrophage colony stimulating growth factor (Clauss et al., 1990; Graves and Valente, 1991). Furthermore, an inhibitory polypeptide known as macrophage deactivating factor has been reported (Ding et al., 1990). This suggests that a balance between chemotactic and inhibitory cytokines may regulate extravasation of monocytes in neoplasms. A further degree of complexity resulted from the identification that macrophages could enhance the proliferation of tumour cells. The macrophage products interleukin-6 (IL-6), IL-1 and TNF have been reported to promote ovarian carcinoma proliferation (Naylor et al., 1993). Such data suggest the existence of a paracrine loop.

1.3.4 Proteases

Tumours, like all actively growing tissues, are constantly turning over. New blood vessels, ECM and basement membrane are constantly being laid down and

subsequently remodelled. ECM is degraded by proteases of which several types have been identified in tumours including plasmin, cathepsins and matrix metalloproteinases (MMP). MMPs are believed to promote tumour progression by initiating carcinogenesis, enhancing tumour angiogenesis, disrupting local tissue architecture and by breaking down basement membrane barriers for metastatic spread. Although some MMPs are expressed by tumour cells themselves (MMP-2, 7 and 13), most are produced by surrounding host stromal and inflammatory cells in response to factors released by tumours (Guo et al., 1997). As well as proteases, inhibitors of proteases exist in tumour tissues. Overexpression of a tissue inhibitor of metalloproteinases (TIMP) led to suppression of tumour growth in an *in vivo* model of breast carcinoma (Anand-Apte et al., 1996). However, a number of studies correlating the levels of matrix metalloproteinases and TIMPS with outcome from cancer have shown a mixed picture. Sier et al. (1996) measured MMP-2 and MMP-9 in gastric carcinomas and found that high levels were of prognostic significance for a poor overall survival. Similar studies in breast carcinoma failed to show any correlation between MMP levels and outcome (Remacle et al., 2000). In lung cancer, recent surveys of MMPs and TIMPs have shown that they are widely expressed in both NSCLC and SCLC but again a correlation between expression levels and prognosis is unclear (Michael et al., 1999; Thomas et al., 2000).

1.4 INTEGRINS

The term integrin is used to describe cell surface receptors that mediate cellular adhesion to ECM proteins and to other cells (Tamkun et al., 1986; Ruoslahti and Pierschbacher, 1987). Integrins are composed of non-covalently associated α and β chains which form heterodimeric receptor complexes. Each subunit contains a large extracellular domain, a short transmembrane domain and a cytoplasmic carboxy terminal domain of variable length. The extracellular domains of the α and β chains are responsible for ligand binding. To date, the integrin family is composed of 17 α and 8 β subunits that form heterodimers to produce some 22 different $\alpha\beta$ cell surface receptors. Whilst some subunits ($\beta 4$) only bind with one other subunit ($\alpha 6$), others such as $\beta 1$, $\beta 2$ and αv are far more promiscuous. The extracellular ligand-binding specificity of an integrin is generated jointly by both subunits. As well as binding to

various ECM components such as fibronectin, collagen and laminin, certain integrins can bind soluble ligands such as fibrinogen or counter receptors on adjacent cells such as intracellular adhesion molecules. Integrins display various degrees of specificity. While some integrins are widely expressed, others are only expressed in one cell type. Examples of such cell type specific integrins are α IIb β 3 in platelets and α 6 β 4 in epithelial cells. Although some integrins selectively recognise a single ECM protein ligand, others can bind two or more ligands (Hynes, 1992). Several integrins recognise the tripeptide Arg-Gly-Asp (RGD), whereas others recognise alternative short peptide sequences. Nevertheless, they are capable of distinguishing different RGD sequences such that some bind primarily to fibronectin and others to vitronectin (Ruoslahti and Pierschbacher, 1986).

1.4.1 Integrins in cancer

It has been known for a long time that tumour cells, unlike normal cells, have the ability to grow in suspension and are not dependent upon adhesion to an ECM for survival, so called anchorage-independent growth. Anchorage-independence correlates with tumourigenesis *in vivo*. Not surprisingly, the role of integrins in tumour growth has received considerable attention.

Fibronectin mediates cell adhesion and anchorage through a number of integrins, in particular α 5 β 1. The fibronectin matrix appears to play an important role in the behaviour of cells. When Chinese hamster ovary cells were transfected with the α 5 and β 1 integrin subunit genes, a 30-fold increase in expression of α 5 β 1 was achieved and the cells showed a loss of tumourigenicity and a decrease in proliferation rate (Giancotti and Ruoslahti, 1990). Conversely, loss of α 5 β 1 expression led to enhanced tumourigenicity (Schreiner et al., 1991). While some of the α 5 β 1 integrin effect may be related to the signalling role of the integrin (Varner et al., 1995), the increase in fibronectin matrix assembly that accompanies increased α 5 β 1 expression or activity appears to be responsible for most of the effect (Giancotti and Ruoslahti, 1990; Wu et al., 1996). Loss of expression of α 2 β 1 in breast epithelial cells has been correlated with the transformed phenotype and ectopic expression of α 2 β 1 in the

same cell type has been shown to suppress growth and induce differentiation (Zutter et al., 1995).

By contrast, expression of some integrins positively regulates tumour cell proliferation. The integrin $\alpha v\beta 3$ is seen in malignant melanomas but not in benign forms (Albelda et al., 1990). When melanoma cells were selected for loss of the αv integrin subunit, the cells exhibited significantly reduced proliferation and tumourigenicity and this could be restored by re-expression of the integrin (Felding-Habermann et al., 1992; Sanders et al., 1992). Expression of $\alpha 3$ and $\alpha 6$ integrin subunits is also associated with transformation and tumour progression. Integrin $\alpha 3\beta 1$ is expressed in a high percentage of metastatic tumours (Bartolazzi et al., 1994) and $\alpha 6$ is expressed in head and neck, bladder and lung cancers (Costantini et al., 1990; Van Waes and Carey, 1992; Liebert et al., 1993).

1.4.2 Anchorage independence and anoikis

The fundamental difference between a transformed cell and an untransformed cell is that the former has the potential to metastasise and invade. In order to achieve this the cancer cell must become independent of anchorage. This phenomenon has been recognised for many years but it is only relatively recently that it has been recognised to be an integrin-mediated event, and even more recently that the molecular mechanisms underlying it have begun to be elucidated (Frisch and Francis, 1994). If an untransformed endothelial or epithelial cell becomes detached from its matrix it undergoes apoptosis. This has been termed anoikis (Frisch and Francis, 1994). The likely biological purpose of this response is that it causes detached cells to die before they can reattach in a new location and disturb normal tissue architecture. However, transformed cells are able to overcome these regulatory control mechanisms, migrate in the bloodstream or lymph and invade new tissues thus spreading the cancer. Anchorage-independent transformed cells are able to circumvent the necessity of integrin-mediated signalling. The mechanisms by which this can occur seem to be varied but probably focus around focal adhesions. Focal adhesions are specialised areas of cell-matrix contact in which integrin clusters link actin filaments to ECM. As a result of the integrin and cytoskeletal clustering, various signalling molecules

including a number of protein tyrosine kinases become concentrated in focal adhesions at the cytoplasmic surface of the cell membrane. One such kinase is focal adhesion kinase (FAK) which can bind directly to integrin cytoplasmic domains. Activated FAK is able to activate the Ras/Raf/mitogen activated protein kinase (MAPK) pathway through the adapter protein Grb-2 as well as the phosphoinositide 3-kinase (PI 3-kinase) pathway (Chen and Guan, 1994a; Schlaepfer et al., 1994). Recent work by Khwaja et al., (1997) suggests that the PI 3-kinase/protein kinase B pathway plays a more important role in preventing anoikis than does the MAPK pathway. The importance of FAK in regulating anoikis is further supported by the observation that microinjection of a peptide-mimicking the FAK binding site of the $\beta 1$ integrin cytoplasmic domain inactivates endogenous FAK and induces anoikis (Hungerford et al., 1996). The role of FAK in integrin signalling is discussed in more detail in the section on integrin signalling below.

1.4.3 Invasion and motility

Cell migration is a result of a dynamic interplay between the migrating cell, the ECM and the cellular cytoskeleton. The $\alpha 5\beta 1$, $\alpha v\beta 3$ and other αv integrins appear to play an important role in cell migration. The $\alpha v\beta 3$ integrin has a central role in melanoma survival and metastasis. Negative selection of $\alpha v\beta 3$ expressing melanoma cells results in cells that cannot form tumours or invade. Restoration of $\alpha v\beta 3$ expression by transfection leads to tumour formation and the ability to invade the dermis (Albelda et al., 1990; Natali et al., 1997). In wound healing keratinocytes express the αv integrin subunit at the leading edge of the epidermis migrating into the wound (Gailit et al., 1994; Zambruno et al., 1995; Sheppard, 1996). The $\alpha 5\beta 1$ integrin is also expressed in migrating but not resting keratinocytes (Zambruno et al., 1995). Cell migration is dependent upon rapid controlled alterations of the affinity of integrins for their extracellular ligands. Thus, the repeated activation and inactivation of integrins regulates cell migration through co-ordinated adhesion at the leading edge of the cell and de-adhesion events at the trailing edge. The NPXY motif, which regulates the activation of integrin β subunits, may play a critical role during tumour cell migration. Transfection of melanoma cells with a mutated NPXY sequence inhibits tumour cell migration *in vitro* and metastasis formation *in vivo*

(Filardo et al., 1995). Recent work suggests that cell migration and invasion is likely to be regulated by complex interactions between 'outside-in' signalling and 'inside-out' signalling. It seems likely that members of the small GTP-binding protein family such as R-ras and H-ras play an important role here. R-ras activates integrins (Zhang et al., 1996), whereas H-ras blocks integrin activation (Hughes et al., 1997). The modulation of integrin affinity will be considered in more detail in Chapter 5.

In addition to the mechanisms discussed above, cell migration and invasion is also dependent upon proteases to facilitate the breakdown of tissues and allow tumour cells to spread. This is covered in the section on proteases (see above).

1.4.4 Angiogenesis

Tumours up to 1 mm³ are able to obtain nutrients and oxygen by diffusion. However, further growth requires the development of a blood supply. Certain integrins have been shown to play a crucial role in the process of angiogenesis. The integrin $\alpha v\beta 3$ is minimally expressed on resting or normal blood vessels but is significantly upregulated on vascular cells within tumours (Brooks et al., 1994a; Brooks et al., 1994b; Brooks et al., 1994b) and in response to growth factors (Sepp et al., 1994). The induction of tumour angiogenesis is mediated by the release of a variety of angiogenic molecules including basic fibroblast growth factor (bFGF), transforming growth factor α (TGF α), VEGF and TNF α from tumour cells and host cells (Fidler and Ellis, 1994). These growth factors promote increased expression of the *Hox D3* homeobox gene, which, in turn, controls expression of $\alpha v\beta 3$ (Boudreau et al., 1997). Evidence to support a role for $\alpha v\beta 3$ integrin in angiogenesis comes from experiments using a monoclonal antibody to $\alpha v\beta 3$ which was able to inhibit angiogenesis both *in vitro* and *in vivo* (Friedlander et al., 1995). Recently the existence of a second angiogenic pathway has been demonstrated. Whereas bFGF mediates $\alpha v\beta 3$ mediated angiogenesis, VEGF appears to mediate an $\alpha v\beta 5$ -dependent mechanism (Friedlander et al., 1995).

1.5 INTEGRIN SIGNALLING

Despite the vast amount of work that has been done on integrin signalling no specific integrin mediators have been identified. Instead, the majority of signalling molecules implicated in ECM-integrin interactions are the rather ubiquitous mediators of signal transduction. At least twenty different proteins including Rho GTPases, Raf, Ras, FAK and MAPKs, PI 4-kinase and PI 3-kinase can be recruited to the ECM ligand/integrin-binding site.

1.5.1 FAK

The binding of ligands to integrin receptors leads to integrin cross-linking and clustering (Kornberg et al., 1991). This promotes the formation of focal adhesions at the cell membrane. A large number of structural and signalling proteins including integrins, cytoskeletal proteins and protein kinases have been shown to be concentrated at these sites. Actin binding proteins that co-localise with integrins at focal adhesion plaques include α -actinin, talin, tensin, paxillin and vinculin (Richardson and Parsons, 1995). Early work demonstrated that tyrosine kinase inhibitors could block the formation of focal adhesions (Burrige et al., 1992). Integrins, however, lack intrinsic tyrosine kinase activity. This led to the identification of FAK, a 120 kDa non-receptor tyrosine kinase. FAK undergoes rapid tyrosine kinase phosphorylation following integrin ligation and clustering (Schaller et al., 1992; Hanks et al., 1992). The mechanism by which integrins activate FAK is incompletely understood. Although FAK is unable to phosphorylate other substrates directly, integrin-dependent autophosphorylation allows it to interact with docking or adapter proteins including paxillin, tensin and Grb-2 (Vuori et al., 1996; Schlaepfer and Hunter, 1997; Schlaepfer et al., 1997). These, in turn, are able to activate downstream signalling mediators including the Ras/Raf/MAPK pathway and the PI 3-kinase/protein kinase B pathway (Chen et al., 1996). Evidence for a role for FAK in integrin-mediated signalling comes from experiments in which tyrosine residues critical for FAK autophosphorylation are mutated (Schlaepfer et al., 1994). In this case integrin-mediated proliferation is blocked. Furthermore, oncogenic transformation of cells, which removes the requirement for anchorage-dependent growth, activates FAK (Guan and Shalloway, 1992). Consistent with

these findings is the observation that constitutively active FAK leads to cell transformation, anchorage-independent growth and the suppression of apoptosis (Frisch et al., 1996).

In addition to activation by integrins, FAK can be activated by certain soluble growth factors. Recent work has shown that focal adhesions also contain receptors for bFGF, EGF and PDGF (Plopper et al., 1995; Miyamoto et al., 1995; Miyamoto et al., 1996). Recruitment of growth-factor receptors to focal adhesions has been shown to result in enhanced phosphorylation and activation of the growth-factor receptor, accumulation of downstream substrates and an enhanced growth response to exogenous mitogens.

1.5.2 Phosphoinositide 3-kinase

The role of PI 3-kinase in integrin signalling is discussed in the section on PI 3-kinase (see below).

1.5.3 MAPK

Activation of the MAPK pathway leads to transcriptional regulation of genes that are crucial for cell growth and differentiation. Early studies showed that the MAPK pathway could be activated by soluble mitogens. Adhesion of cells to ECM proteins including fibronectin, vitronectin, collagen, tenascin and laminin can also activate the MAPK pathway probably through FAK as described above. *In vivo*, it is likely that integrins and growth factor receptors may synergise to enhance Ras-MAPK activation. However, activation of MAPK independent of FAK and Ras can occur implying the use of alternative or parallel pathways. In certain cases FAK related proteins such as CAK β and PYK2 may compensate for FAK thereby linking integrins to MAPK by alternative effectors (Lev et al., 1995)

1.6 PHOSPHOINOSITIDE 3-KINASE SIGNALLING

1.6.1 The canonical phosphoinositide pathway

For many years our knowledge of phosphoinositide metabolism was confined to the canonical phosphoinositide turnover pathway. In this pathway phosphatidylinositol

is sequentially phosphorylated by PI 4-kinase and PI(4)P 5-kinase to generate phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂). PtdIns(4,5)P₂ is the major substrate of phospholipase C (PLC). Activation of PLC in response to ligand-receptor interaction results in the hydrolysis of PtdIns(4,5)P₂ by PLC and the generation of two second messengers, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ promotes release of Ca²⁺ from intracellular stores and DAG activates PKC (Figure 1.2). Both the rises in intracellular Ca²⁺ and PKC activity are thought to be important steps in signalling from the cell surface to the nucleus.

1.6.2 Phosphoinositide 3-kinase

PI 3-kinase was identified following characterisation of the different PI kinase activities present in fibroblasts. Two biochemically distinct PI kinases termed type I and type II had previously been recognised. Type I PI kinase activity could be distinguished from type II by its preference for substrate presented as vesicles rather than micelles, by its K_m for ATP and by its resistance to inhibition by adenosine (Whitman et al., 1987). Using immunoprecipitates of pp60v-src and middle T/pp60c-src complexes derived from transformed cells, Whitman et al., (1988) showed that type I PI kinase specifically phosphorylated the D3 position of the inositol ring to generate a novel phospholipid, PtdIns(3)P. In contrast, the main PI kinase in fibroblasts (Type II) specifically phosphorylated the D4 position to produce PtdIns(4)P. At about the time that PI 3-kinase was first described, PtdIns(3,4,5)P₃, a lipid not previously known to exist, was detected in activated neutrophils (Traynor-Kaplan et al., 1988). Subsequently, PI 3-kinase was shown to phosphorylate PtdIns(4)P and PtdIns(4,5)P₂ to produce PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ respectively.

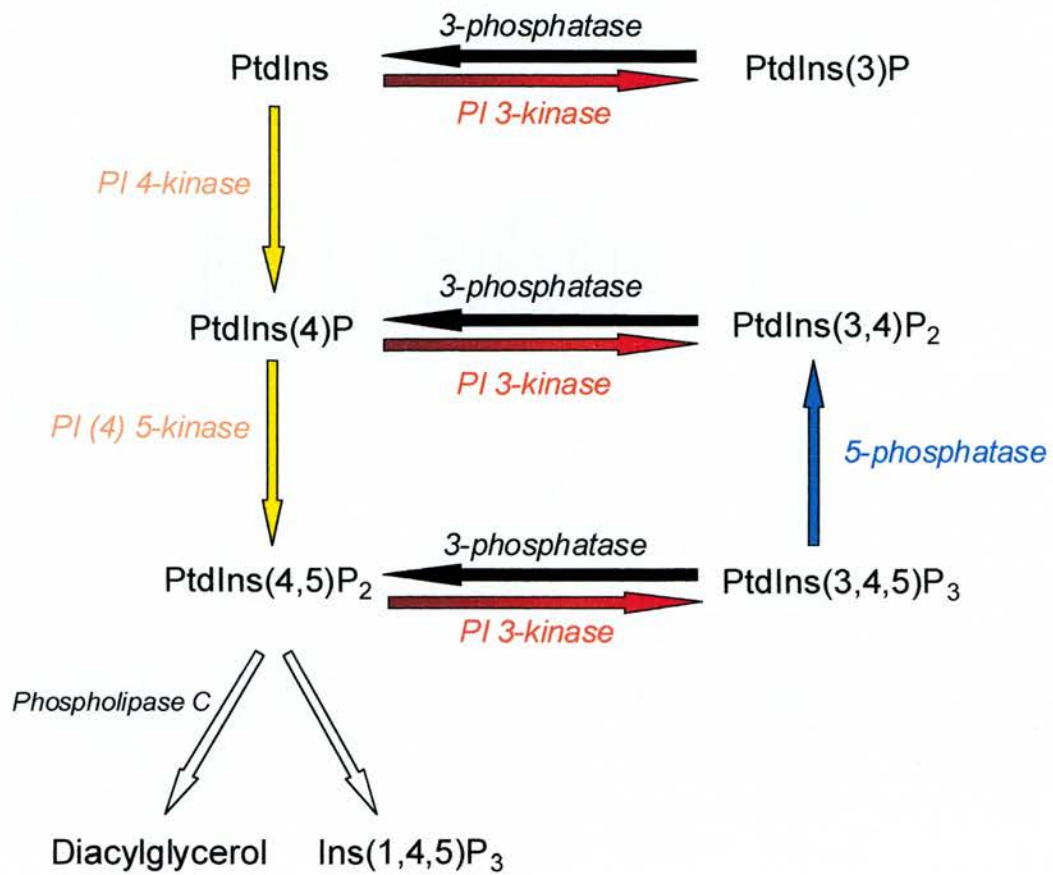


Figure 1.2
Phosphoinositide pathways.

Three classes of PI 3-kinase have now been described on the basis of their primary structure, regulation and *in vitro* lipid substrate specificity.

Class I PI 3-kinases phosphorylate PtdIns, PtdIns(4)P and PtdIns(4,5)P₂ to PtdIns(3)P, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ respectively. However, PtdIns(4,5)P₂ is believed to be their preferred substrate. Class I PI 3-kinases are heterodimeric complexes comprised of a catalytic subunit (p110) and a regulatory subunit (p50, p55, p85 or p101). Class I PI 3-kinase catalytic subunits can be divided into two subclasses according to the type of regulatory subunit with which they associate. Class Ia PI 3-kinases comprise either an α , β or δ p110 catalytic subunit plus one of a family of regulatory subunits (p85 α , p85 β , p55 γ and their splice variants). The regulatory units bind phosphorylated tyrosine residues, thereby linking class Ia PI 3-kinase subunits to tyrosine kinase signalling pathways. Each regulatory subunit protein contains two SH2 domains linked by an inter-SH2 region to which the catalytic subunit binds. The SH2 domains bind phosphorylated tyrosine residues specifically within a P-Tyr-x-x-Met motif. The 85 kDa adapters also contain an SH3 domain and a breakpoint cluster region homology domain whose precise role is unclear.

Class Ib PI 3-kinases are stimulated by G-protein $\beta\gamma$ subunits and do not interact with the SH2 domain containing adapters that bind to class Ia PI 3-kinases. The mammalian form of the catalytic subunit in this class is p110 γ and this has been shown to bind to a p101 regulatory subunit (Stephens et al., 1994). To date, p110 γ has only been identified in haemopoietic cell types and osteosarcoma cell lines (Stephens et al., 1994; Morris et al., 1995).

Class II PI 3-kinases phosphorylate PtdIns and PtdIns(4)P, *in vitro*, but not PtdIns(4,5)P₂. Presently it is not known whether class II PI 3-kinase activity is regulated by extracellular stimuli. Class III PI 3-kinases have a substrate specificity restricted to PtdIns. These PI 3-kinases are homologous to Vps34p, the only PI 3-kinase present in yeast.

1.6.3 Mechanism of activation of PI 3-kinase

Class I PI 3-kinases can be activated by the majority of receptors with intrinsic or associated tyrosine kinase activity and by receptors linked to heterotrimeric G proteins. Phosphorylated tyrosine residues, generated on receptors (e.g. PDGF-R) or associated substrate molecules (e.g. IRS-1), form the docking sites for the SH2 domains of the PI 3-kinase adapter subunits. This adapter-mediated translocation of PI 3-kinases to receptor tyrosine kinases and their substrates is likely to help position the catalytic subunits close to membranes that contain their lipid substrates. At present the mechanism of activation of PI 3-kinase via G-protein coupled receptors is not fully understood.

All class I PI 3-kinases can be activated by the small GTP binding protein Ras (Rodriguez-Viciana et al., 1994; Kodaki et al., 1994; Rodriguez-Viciana et al., 1996). Evidence from experiments using PDGF-R mutants also suggests that accumulation of GTP-bound Ras is required for full activation of class 1a PI 3-kinases by PDGF. However, at present it is not known to what extent PI 3-kinase activation by receptor tyrosine kinases also occurs independently of Ras.

1.6.4 Integrin Activation

Although not fully understood, integrin activation of PI 3-kinase is believed to occur through a number of mechanisms. The best characterised mode of recruitment of PI 3-kinase is via the association of the SH2 domain of the p85 subunit with the tyrosine phosphorylated sequence P-Tyr-x-x-Met in the cytoplasmic domain of a cell surface receptor. A number of integrin regulators possess this motif, in particular CD28, CD7 and CD19 (Weng et al., 1994; Ward et al., 1995; Rudd, 1996). Other molecules that function as integrin regulators, such as CD2, CD3 and surface immunoglobulins are also coupled to PI 3-kinase signalling pathways although the mode of association of PI 3-kinase does not appear to involve a P-Tyr-x-x-Met motif (Shimizu et al., 1995; Cambier, 1995). Another mechanism of integrin activation of PI 3-kinase is through FAK. Phosphorylation of FAK occurs upon clustering of β 1 integrins and PI 3-kinase has been shown to associate with phosphorylated tyrosine residues of FAK (Kornberg et al., 1992; Chen and Guan, 1994b; Chen et al., 1996).

Alternatively, as PI 3-kinase is a major target of Ras, the activation of PI 3-kinase by integrins could be mediated by the ability of the SH2-SH3 adapter protein Shc to activate Ras. Shc is known to lie in close proximity to focal adhesion complexes which are formed on the integrin binding of specific heterodimers such as $\alpha 1\beta 1$, $\alpha v\beta 3$ and $\alpha 5\beta 1$ (Wary et al., 1996; Giancotti, 1997).

1.6.5 Tools for studying the role of PI 3-kinase

The majority of work examining the roles and downstream effectors of PI 3-kinase has made use of either pharmacological inhibitors or constitutively active or dominant negative mutants of PI 3-kinase. The two principal pharmacological inhibitors of PI 3-kinase that have been widely used are wortmannin and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). Wortmannin, originally isolated from soil bacteria, is a non-competitive inhibitor of PI 3-kinase with respect to ATP. It binds irreversibly to the p110 catalytic subunit and alkylates lysine 802 in the ATP-binding site (Powis et al., 1994; Wymann et al., 1996). LY294002 is a morpholino derivative of the broad-spectrum kinase inhibitor quercetin. It acts as a competitive inhibitor at the ATP binding site of PI 3-kinase (Vlahos et al., 1994a). The IC_{50} values for wortmannin and LY294002 are 3 nM and 1.4 μ M respectively. Neither compound shows any significant selectivity within the PI 3-kinase family. Most isoforms are inhibited at comparable concentrations to those required to inhibit p85 α /p110 α . Recently some doubt has been cast on the specificity of wortmannin and LY294002 as both compounds lose specificity for PI 3-kinase at higher concentrations. Protein kinases related to PI 3-kinase such as ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK) are also inhibited by LY294002 and wortmannin at concentrations 50-100 fold higher than those that inhibit p85 α /p110 α (Stein and Waterfield, 2000). LY294002 has also been found to inhibit casein kinase 2 at similar concentrations to those which inhibit PI 3-kinases (Davies et al., 2000). This finding may have implications for the interpretation of data obtained using LY294002, as casein kinase 2 is a ubiquitous, constitutively active kinase that is involved in transcription, signalling and proliferation. However, awareness of the shortcomings of each compound

coupled with their use at appropriate concentrations should allow them to continue to be used in the investigation of PI 3-kinase and its effectors.

Using these tools, PI 3-kinase has been implicated in a wide range of cellular functions including secretion and trafficking, morphological change, proliferation, resistance to apoptosis, transformation, anchorage independence and migration.

1.7 EFFECTORS OF PI 3-KINASE

1.7.1 Protein Kinase B

Protein kinase B (PKB), also known as Akt and RAC, is a 57 kDa serine/threonine kinase which was first identified in 1991 by three independent groups. Two groups identified the kinase as a result of its homology to both protein kinase A and protein kinase C. A third group identified the kinase as the product of the oncogene *v-akt* of the acutely transforming retrovirus AKT8 found in a rodent T-cell lymphoma (Bellacosa et al., 1991; Coffey and Woodgett, 1991; Jones et al., 1991a).

Mammals have three closely related PKB genes that encode the isoforms PKB α , PKB β and PKB γ . PKB β and PKB γ share 81% and 83% homology with PKB α respectively. PKB has both a serine/threonine kinase domain and a pleckstrin homology (PH) domain at its amino-terminal end (amino acids 1-106), which makes up the major part of the amino-terminal regulatory domain (residues 1-147). The kinase domain stretches from amino acid 148-411, with the carboxy-terminal tail region (amino acids 412-480) accounting for the remainder of the protein.

All PKB isoforms show a broad tissue distribution. Highest levels have been shown to exist in the brain, thymus and lung (Coffey and Woodgett, 1991; Jones et al., 1991b). Expression of PKB γ appears to be more selective with the highest levels found in the brain and testes. Currently, little is known about the regulation of expression of PKB; however, it appears to be upregulated as cells become terminally differentiated (Altomare et al., 1995; Kohn et al., 1996).

PKB can be activated by a wide variety of growth stimuli including insulin, thrombin, NGF, PDGF and EGF. It is now well established that PKB is a downstream effector of PI 3-kinase. Evidence to support this concept has come from several avenues. Growth factor-induced activation of PKB is inhibited by the PI 3-kinase inhibitor wortmannin and also by the expression of a dominant negative form of PI 3-kinase, $\Delta p85$, derived from its regulatory subunit (Franke et al., 1995). In addition, the ability of mutants of the PDGF receptor to activate PKB correlates with their ability to activate PI 3-kinase. PDGF receptors in which Tyr⁷⁴⁰ and Tyr⁷⁵¹ have been mutated to phenylalanine fail to bind to the SH2 domains of p85 and fail to stimulate both PI 3-kinase and PKB activity (Franke et al., 1995). Furthermore, the expression of constitutively active forms of PI 3-kinase result in stimulation of PKB (Klippel et al., 1996; Marte and Downward, 1997; Franke et al., 1997).

1.7.1a Mechanism of activation of PKB

Full activation of PKB α by insulin and growth factors requires phosphorylation of both Thr³⁰⁸ in the kinase domain and Ser⁴⁷³ in the C-terminal regulatory domain (Alessi et al., 1996). The PKB β and PKB γ isoforms are also activated by phosphorylation of residues equivalent to Thr³⁰⁸ and Ser⁴⁷³ (Walker et al., 1998). Thr³⁰⁸ is phosphorylated by 3'-phosphoinositide-dependent kinase-1 (PDK1) (Alessi et al., 1997). PDK1 is a 63 kDa serine/threonine kinase which is ubiquitously expressed in human tissues. It consists of an N-terminal kinase domain and a C-terminal PH domain. *In vitro*, its PH domain binds PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ with higher affinity than other PIs such as PtdIns(4,5)P₂. Initially it was thought that phosphorylation of Ser⁴⁷³ would be catalysed by a distinct protein kinase and this was provisionally named PDK2 (Alessi et al., 1997). The true identity of this kinase is still unknown. It has recently been claimed that integrin-linked kinase is capable of phosphorylating Ser⁴⁷³ of PKB α *in vitro* (Delcommenne et al., 1998). A more recent study suggests that integrin-linked kinase may not directly phosphorylate PKB at Ser⁴⁷³ but rather promote phosphorylation by an indirect mechanism (Lynch et al., 1999). PDK1 has also been shown to interact with a fragment of the C-terminus of PKC-related kinase 2 which has been named PDK1-interacting fragment (PIF) (Balendran et al., 1999).

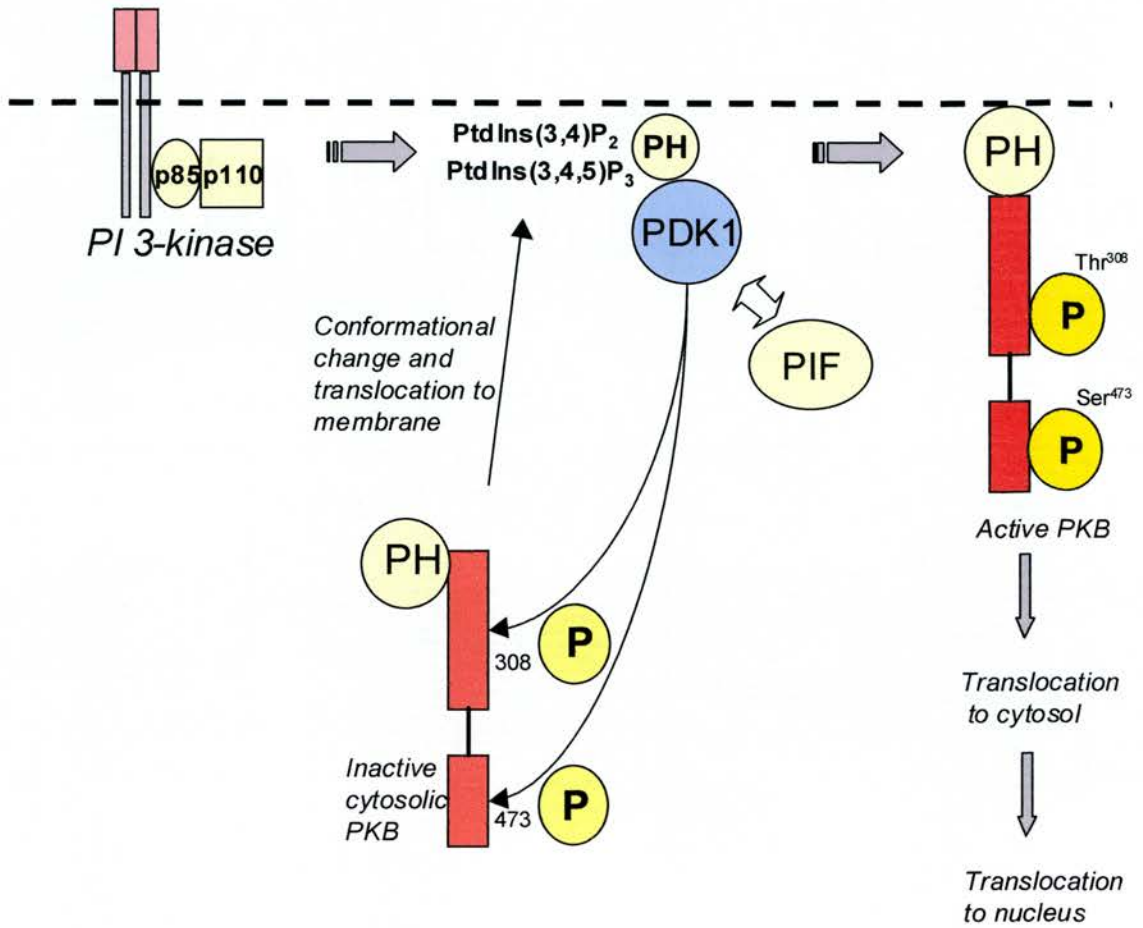


Figure 1.3

Proposed mechanism of activation of PKB by PI 3-kinase and PDK1. See text for details.

PIF converts PDK1 from an enzyme that phosphorylates PKB α only on Thr³⁰⁸ to one that can phosphorylate PKB α at both Thr³⁰⁸ and Ser⁴⁷³. This area needs further work but it does suggest that PDK1, in complex with another protein, may mediate the phosphorylation of PKB α on Ser⁴⁷³ rather than it being phosphorylated by a distinct enzyme. Very recently this version of events has been challenged by Toker and Newton, (2000). They suggest that phosphorylation of Thr³⁰⁸ triggers autophosphorylation of Ser⁴⁷³ both *in vitro* and *in vivo*, indicating that PDK2 or 'PDK2-like' activity is not required for PKB activation.

The current model of activation for PKB suggests that upon activation of PI 3-kinase, PKB translocates from the cytosol to the plasma membrane and interacts with PtdIns(3,4,5)P₃/PtdIns(3,4)P₂ via its PH domain (Figure 1.3). This interaction induces a conformational change in PKB, which converts it into a substrate for PDK1, possibly by exposing Thr³⁰⁸ and Ser⁴⁷³ phosphorylation sites. Membrane localised PDK1 complexes with PIF allowing it to phosphorylate PKB at Thr³⁰⁸ and Ser⁴⁷³. Activated PKB then translocates to the cytosol and nucleus.

1.7.1b Biological functions of PKB

Since PKB activity is stimulated by a variety of growth factors and insulin, much effort has focussed on investigating the metabolic effects associated with its activation. Currently there are no pharmacological inhibitors of PKB and as a result the approaches used to assess the role of PKB have involved expression of activated alleles or dominant negative mutants.

The majority of PKB substrates described to date are either involved in the regulation of insulin metabolism or in cell survival pathways. Figure 1.4 shows the currently known targets of PKB. The first direct *in vivo* substrate to be identified was glycogen synthase kinase-3 (GSK3). PKB mediated phosphorylation of GSK3 leads to GSK3 inactivation and the consequent activation of glycogen synthesis (Shaw et al., 1997a). PKB has also been found to stimulate glucose uptake and GLUT4, a glucose transporter (Cross et al., 1995).

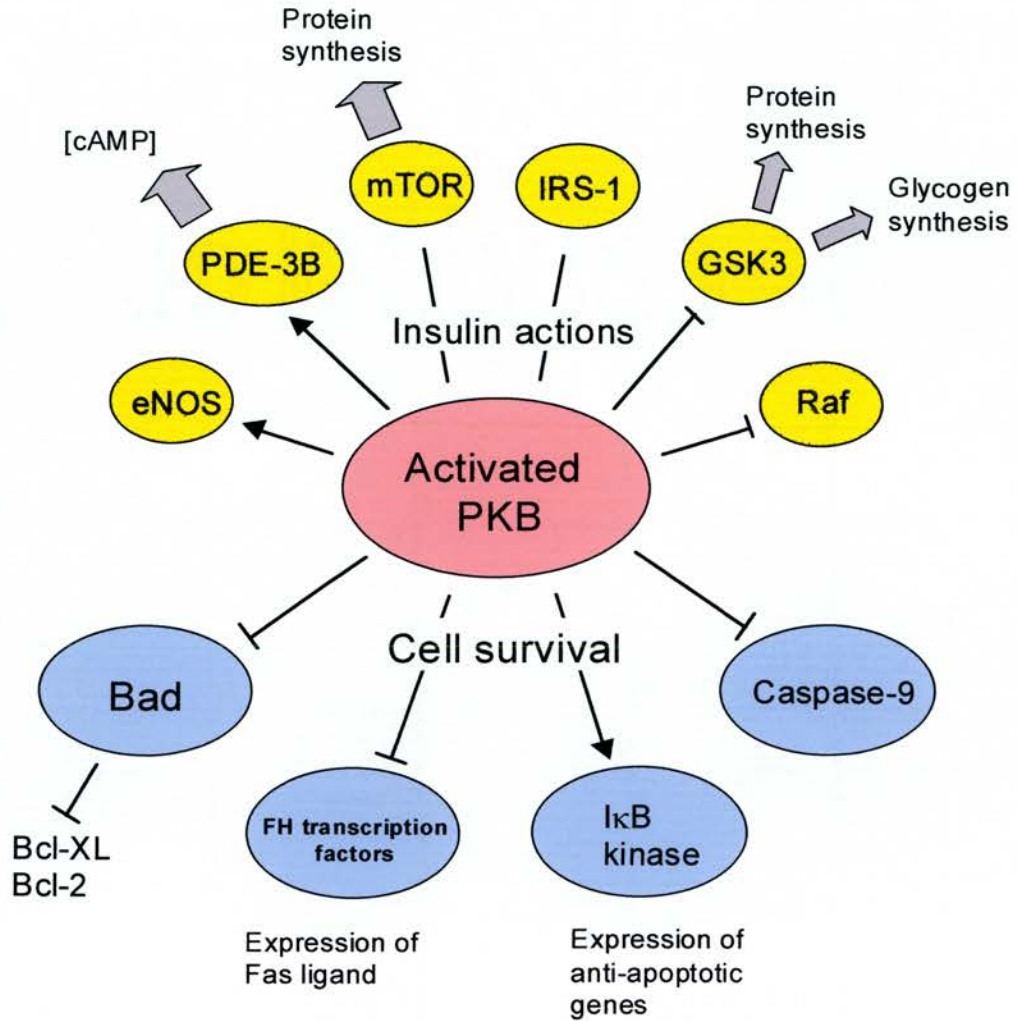


Figure 1.4

Targets of PKB phosphorylation. A horizontal bar or an arrowhead indicate an inhibitory or stimulatory effect of PKB-mediated phosphorylation respectively. The absence of a symbol indicates that the effect of PKB-mediated phosphorylation is unclear at present. FH transcription factors, Forkhead transcription factors; PDE-3B, phosphodiesterase-3B; mTOR, mammalian target of rapamycin; IRS-1, insulin receptor-1; eNOS, endothelial nitric oxide synthase; GSK3, glycogen synthase kinase-3.

1.7.1c Anti-apoptotic signalling mediated by PKB

It has been demonstrated previously that inhibition of PI 3-kinase blocks the ability of survival factors to protect various cell types from programmed cell death (Yao and Cooper, 1995). Transfection of constitutively active forms of PI 3-kinase and PKB can protect Cos-7 epithelial cells from apoptosis induced by ultraviolet irradiation and neuronal cells from cell death induced by withdrawal of IGF-1 or NGF (Kulik et al., 1997). Activated PI 3-kinase and PKB have also been shown to protect MDCK cells from apoptosis induced by detachment of adherent cells from their extracellular matrix (Khwaja et al., 1997).

The mechanism by which PKB protects cells from apoptosis has received intensive study. PKB is able to phosphorylate the pro-apoptotic Bcl-2 family member BAD both *in vitro* and *in vivo* (del Peso et al., 1997; Datta et al., 1997). Phosphorylation of BAD creates a binding site for 14-3-3, an adapter protein. Binding of 14-3-3 prevents BAD from undergoing heterodimerisation with, and inhibiting the activity of, Bcl-2 and Bcl-X_L (Zha et al., 1996). However, BAD is only expressed in a limited range of tissues and cell lines and most of the work reported to date on BAD has made use of overexpressed protein often in cell lines that do not normally express it. This suggests that in cells that do not express BAD, another mechanism must be at work to explain the ability of PKB to protect cells from apoptosis. Recently the cysteine protease caspase-9 and the forkhead transcription factor FKHL1, both of which are potent inducers of apoptosis, have been found to be inhibited by PKB-mediated phosphorylation (Cardone et al., 1998; Brunet et al., 1999).

1.7.2 P70^{s6k}

P70^{s6k} is a serine/threonine protein kinase that phosphorylates the 40S ribosomal protein S6. Translation of several hundred mRNAs with 5' oligopyrimidine tracts is controlled by p70^{s6k}. These mRNAs largely encode for proteins necessary for the assembly of the translational machinery including ribosomes and elongation factors. Work on p70^{s6k} has been expedited by the discovery of a specific inhibitor, rapamycin (Price et al., 1992). Rapamycin is an immunosuppressive macrolide,

which forms a complex with a specific 12 kDa protein called FK506 binding protein-12 (FKBP12). The rapamycin-FKBP12 complex binds and inhibits the autophosphorylation of the mammalian target of rapamycin (mTOR).

p70^{s6k} was the first effector of PI 3-kinase to be described. This work utilised mutants of the PDGF β receptor as well as PI 3-kinase inhibitors to show that inhibition of PI 3-kinase activation also impaired p70^{s6k} activation. However, activation and regulation of p70^{s6k} appears to be far more complex than originally thought requiring phosphorylation of up to eight sites by at least three signalling pathways.

The initial step in activating p70^{s6k} seems to be mediated by the MAPK pathway. This involves a phosphorylation-induced conformational change in the C-terminus of the kinase domain, revealing additional phosphorylation sites (Dufner and Thomas, 1999). Subsequent phosphorylation is dependent upon the PI 3-kinase/PDK1 (Alessi et al., 1998; Pullen et al., 1998), TOR and PKC ζ pathways (Romanelli et al., 1999). However, the precise mechanism of activation of p70^{s6k} remains elusive and will no doubt become clearer in the near future.

1.7.3 PI 3-kinase mediated PKC activation

The PKC superfamily which comprises 12 protein kinases subdivided into conventional PKCs, novel PKCs and atypical PKCs according to their activation profiles and requirement for DAG/phorbol esters, phosphatidylserine and Ca²⁺ has been studied extensively (Nishizuka, 1992).

More recently PI 3-kinase has been found to activate members of the PKC family via PDK1 (Chou et al., 1998). The principal PKC family member involved appears to be PKC ζ . PKC ζ has been shown to activate an NF- κ B-like activity *in vivo* and to phosphorylate a novel I κ B kinase leading to gene transcription (L'Allemain et al., 1989). PKC ζ may exert its transcriptional activity via activation of the MAPK pathway. There have now been several reports of the requirement of functional PI 3-kinase and PKC ζ for MAPK activation (Cai et al., 1997; Takeda et al., 1999).

Therefore, evidence is now mounting that activation of PKC ζ by PI 3-kinase is required for many cellular processes including mitogenesis, insulin signalling and protein synthesis.

1.7.4 Regulation of MAPK by PI 3-kinase

It is now well established that PI 3-kinase can, in part, activate the MAPK pathway. This may occur by more than one mechanism and involve both PI 3-kinase, lipid kinase activity, (via PKC ζ as described above) and PI 3-kinase, protein kinase activity. In addition to being a lipid kinase, PI 3-kinase possesses an intrinsic Ser/Thr kinase activity. Bondeva et al., (1998) showed that a PI 3-kinase mutant which had protein kinase activity but no lipid kinase activity could activate MAPK but not PKB. This suggested that PtdIns(3,4,5)P₃ is not required for MAPK activation. However, the precise target of PI 3-kinase protein kinase is not known currently.

1.8 APOPTOSIS

Cells die either by necrosis or apoptosis. Unlike apoptosis or programmed cell death, necrotic cell death is usually the result of an environmental insult. Necrosis is characterised by damage to the plasma membrane such that the cell becomes abnormally permeable to water and ions, swells and disintegrates, resulting in cellular contents spilling into the extracellular space and causing an inflammatory response (Wyllie, 1980). By contrast, in apoptosis, the plasma membrane remains intact so that the cellular contents do not leak into the extracellular space and as a result, there is no inflammation (Wyllie et al., 1980). This is extremely important to minimise damage to healthy bystander cells. Macrophages and neighbouring phagocytic cells rapidly phagocytose the apoptotic cell and its fragments creating apoptotic bodies.

Apoptotic cells were initially identified by their morphological appearance (Wyllie et al., 1980). Cells undergoing apoptosis shrink and lose up to one third of their cytoplasmic and nuclear volume and the plasma membrane becomes ruffled and blebbed. The nucleus condenses and its chromatin becomes very dense forming

'pyknotic' spheres. These changes are accompanied by chromatolysis, which involves the activation of endonuclease activity (Wyllie, 1980).

Apoptosis is essential for correct development and tissue homeostasis. It acts as a defence mechanism protecting the organism against cells which have undergone malignant transformation or which have been infected by viruses (Vaux et al., 1994). Furthermore, cells that migrate to the wrong site during development undergo apoptosis thereby protecting the correct architecture of tissues (Williams, 1991).

Much of the work elucidating the molecular mechanisms underlying apoptosis has come from study of the nematode *Caenorhabditis elegans*. Of the 1090 cells formed during development of this worm, 131 die by apoptosis (Ellis et al., 1991). Genetic analysis has identified two genes, *ced-3* and *ced-4*, that must function for these cells to die (Ellis and Horvitz, 1986). If either gene is inactivated by mutation, death fails to occur. A third gene *ced-9* antagonises the function of *ced-3* and *ced-4* by protecting cells from death (Hengartner et al., 1992). Cloning of *ced-3* has shown that its product is similar to the mammalian cysteine protease interleukin-1 β -converting enzyme (ICE) (Yuan et al., 1993). This observation led to the identification of a growing family of cysteine proteases with homology to CED-3 that have been designated caspases (Alnemri et al., 1996). To date more than 14 caspases have been cloned and partially characterised in mammals although not all have been implicated in the apoptotic process. Two main caspase cascades have been identified in mammalian cells (Figure 1.5). One pathway is activated by a variety of either intra or extracellular death stimuli whilst the other links caspase-8 to death receptors expressed at the cell surface including Fas, TNF-related proteins and DR3. Activated caspase 8 and 9 can cleave and activate the 'effector caspases' including caspases 3, 6 and 7 which result in increased expression of protease activity and cell death.

The caspase cascade can be modified by various other factors. In the 1980s, two major regulators of apoptosis were identified.

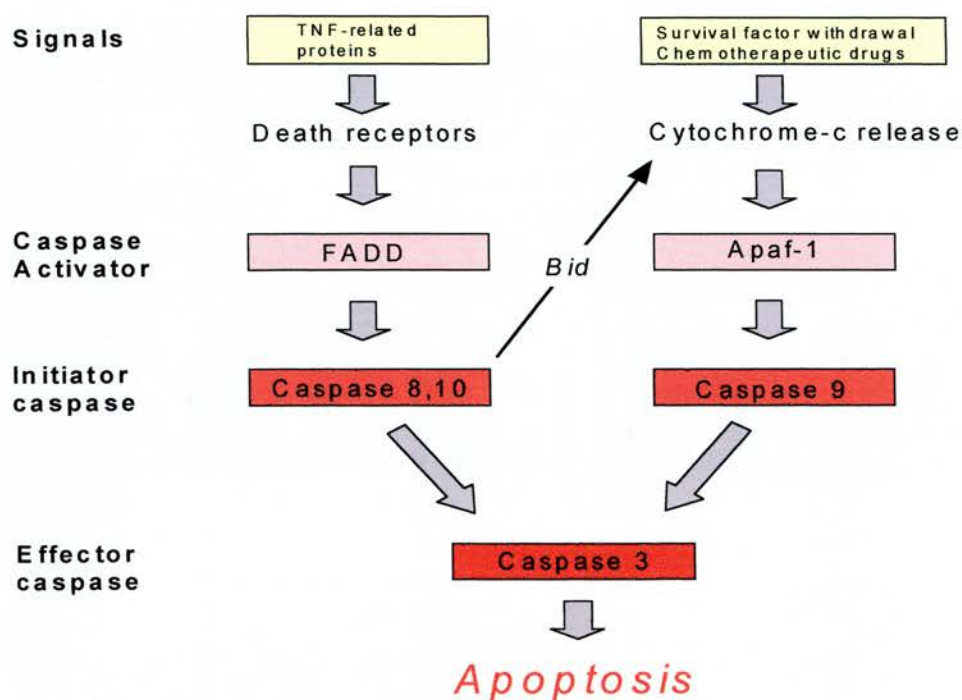


Figure 1.5 The caspase cascade. Apoptosis can be triggered by activation of the caspase cascade through two linked pathways. Ligation of ‘death receptors’ such as Fas, TNFR1 and DR3 activates caspase 8 or 10 via the adapter molecule FADD. Alternatively, various intra- or extracellular ‘death stimuli’ trigger the release of cytochrome c from mitochondria. It is believed that chemotherapeutic agents may induce opening of the mitochondrial ‘megachannel’ (also called the permeability transition pore). The megachannel is regulated by numerous endogenous effectors including members of the Bcl-2/Bax family, the redox status of the cell, cytosolic Ca^{2+} levels, ceramide and amphipathic peptides. Chemotherapeutic agents may induce opening of the megachannel by modulating some of these endogenous effectors. Subsequent disruption of mitochondrial membrane integrity involves loss of metabolic function and the liberation of intermembrane proteins into the cytosol including cytochrome c. In the cytosol, cytochrome c can bind Apaf-1, which enhances its ability to associate with procaspase 9. Activated caspase 8 and 9 activate effector caspases such as caspase 3. These caspases promote cleavage of apoptosis regulators and house-keeping proteins resulting in DNA fragmentation.

Wild type *p53* appears to act as an anti-oncogene, whereas mutant *p53* may function as a dominant oncogene. Of the known tumour suppressor genes, *p53* is the gene most frequently mutated in human malignancies. The highest number of *p53* mutations in lung cancer occur in SCLC in which 70% of tumours have mutations, followed by 65% of squamous cell, 60% of large cell tumours and 33% of adenocarcinomas (Greenblatt et al., 1994). The gene product of *p53* is a nuclear phosphoprotein that is important in cell cycle control. It arrests cells in the checkpoint for the G1/S cell cycle which controls genetic stability, growth arrest and cell death when there has been DNA damage. Through mechanisms which are not fully understood at present mutant *p53* appears to be able to prevent activation of the apoptotic pathway in response to DNA damage. This can lead to unchecked progression of the cell cycle before appropriate DNA repair can occur so that the DNA mutation is passed on to progeny. This may eventually lead to malignant transformation.

Conversely, work on the *Bcl-2* oncogene led to the identification of an important anti-apoptotic function for this protein. *Bcl-2* belongs to a family of related and interacting family members some of which are anti-apoptotic and some of which are pro-apoptotic (Chinnaiyan et al., 1996; Shimizu et al., 1996). Pro-caspase-9 activation requires CED-4 oligomerisation. *Bcl-2* family members may function by preventing oligomerisation of CED-4 (Srinivasula et al., 1998; Hu et al., 1998). Likewise, pro-apoptotic *Bcl-2* members can promote apoptosis and to date it is thought that this group of proteins acts by interacting and antagonising pro-survival *Bcl-2* family members (Adams and Cory, 1998). *Bcl-2* family members can, in turn, be regulated by extracellular factors. Growth factors acting via PKB can phosphorylate the pro-apoptotic *Bcl-2* family protein Bad thereby preventing it from antagonising *Bcl-2* and consequently inhibiting apoptosis (del Peso et al., 1997; Datta et al., 1997).

1.9 AIMS

The principal aim of this thesis was to examine the molecular mechanisms regulating growth, anchorage-dependence, apoptosis and drug resistance in SCLC.

- 1 Initial studies examined the role of PI 3-kinase and PKB in the regulation of growth of SCLC. Having established that PI 3-kinase is constitutively active in SCLC cells, the functional consequences of this upregulation were investigated.
- 2 The role of SCLC cell adhesion to ECM proteins on chemotherapy-induced apoptosis was investigated. Having discovered that integrin-dependent adhesion protects SCLC cells from chemotherapy-induced apoptosis, the mechanism underlying this phenomenon was examined.
- 3 Following the identification of CD98 as a highly specific regulator of integrin affinity, the role of CD98 in SCLC was determined. In particular, the intracellular signalling and functional consequences of cross-linking CD98 in SCLC were examined.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

The following reagents were obtained from Gibco Life Technologies (Paisley, UK): Dulbecco's Modified Eagles Medium (DMEM); Hanks' buffered salt solution (HBSS); Penicillin; Streptomycin; dialysed foetal bovine serum (FBS); L-glutamine; 30% (w/v) Acrylamide/bis solution.

The following reagents were obtained from Calbiochem-Novabiochem Corporation (Nottingham, UK): Wortmannin; Rapamycin; LY294002, Tyrphostin A25; Fibronectin; Fura-2-tetraacetoxymethylester AME; z-Val-Ala-DL-Asp-fluoromethylketone (z-VAD).

The following products were purchased from Amersham Life Science Ltd (Cheshire, UK): [$\gamma^{32}\text{P}$] ATP and Hybond C nitrocellulose membrane.

Antibodies (monoclonals unless stated otherwise) were obtained from the following sources: Anti-human collagen type IV (COL-94), anti-human fibronectin (FN-3E2), anti-human laminin (LAM-89), anti-human tenascin (BC-24) and anti-human CD29 (P4C10) were from Sigma Chemical Company (Poole, UK). Phospho-Akt (Ser 473) was from New England BioLabs (Beverly, USA). Anti-PI 3-kinase p85 N-SH3 antibody and anti-human PKB-PH domain antibodies were purchased from Upstate Biotechnology (Lake Placid, USA) and anti-human CD29 (4B4) was from Coulter Immunology (Hialeah, USA). Anti-procaspase-3 antibody (CPP-32) was obtained from Transduction Laboratories (Lexington, USA). Anti-human topoisomerase II α was purchased from TopoGEN (Columbus, USA). Anti-PKB α , β and γ antibodies were kind gifts from Dr D Alessi (Department of Biochemistry, University of Dundee). Goat polyclonal anti-CD98 antibody (SC-7095) was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Horseradish peroxidase-conjugated goat-

polyclonal anti-mouse secondary antibody and FITC-conjugated goat anti-mouse Ig polyclonal antibody were obtained from DAKO (Ely, UK).

Crosstide (substrate peptide for PKB) was obtained from Upstate Biotechnology (Lake Placid, USA). Adenosine 3', 5'-cyclic monophosphate-dependent protein kinase inhibitor and microcystin-LR were obtained from Alexis Corporation (San Diego, USA). CIS-4-Sulfoethylthio-cyclophosphamide was from Asta Pharmaceuticals (Frankfurt, Germany).

All other reagents were obtained from Sigma Chemical Company (Poole, UK).

2.2 METHODS

2.2.1 SCLC cell lines

SCLC cell lines NCI-H69, NCI-H345 and NCI-H510 were purchased from the American Type Tissue Culture Collection (Rockville, Maryland, USA). All of these lines have been well characterised (Carney et al., 1985) – see table 2.1.

Table 2.1 Characteristics of SCLC cell lines.

Cell Line	H69	H345	H510
Sex	M	M	M
Prior Treatment	Chemotherapy	Chemotherapy	Radiotherapy
Source	PE	BM	Adrenal
Class	C	C	C
DDC u/mg	240	98	214
CK-BB μ /mg	2.2	5.8	2.7
NSE ng/mg	817	4075	491
BLI pmol/mg	1.7	4.7	7.4

Abbreviations: PE, pleural effusion; BM, bone marrow; C, classical; DDC, L-dopa decarboxylase (elevated > 1.0 u/mg; CK-BB, creatine kinase brain isoenzyme

(elevated > 0.4 µg/mg); NSE, neurone specific enolase (elevated > 100 ng/mg); BLI, bombesin-like immunoreactivity (elevated > 0.1 pmol/mg).

SCLC cell lines LS274 and LS310 were a kind gift from Dr J Plumb (Cancer Research Campaign, Beatson Institute, Glasgow, UK). SCLC cell lines DMS79 and WX330 were a kind gift from Professor J Smyth (Imperial Cancer Research Fund, University of Edinburgh, UK). SCLC cell line GLC19 was a kind gift from Dr de Leij (Groningen, Holland).

2.2.2 Cell culture

All SCLC cell lines were cultured in Roswell Park Memorial Institute Medium 1640 (RPMI 1640) containing 25 mM HEPES supplemented with 10% (v/v) FBS (heat-inactivated at 57°C for 1 hour). Cells, which were grown in 25 mls medium to a density of 1×10^6 /ml in 75 cm² flasks (Costar; Cambridge, USA), were passaged (1:3) into fresh medium every five days. When the passage number reached forty, fresh stocks were utilised.

Prior to experimentation SCLC cells were removed from culture medium, washed twice and subsequently cultured for 24 hours in, either serum-free medium, or in quiescent medium. Serum-free medium comprised RPMI 1640 medium containing 25 mM HEPES supplemented with 30 nM selenium, 5 µg/ml insulin, 10 µg/ml transferrin and 0.25% (w/v) BSA (SITA). Quiescent medium comprised RPMI 1640 medium containing 25 mM HEPES supplemented with 0.25% (w/v) BSA.

Swiss 3T3 cells were obtained from the European Cell Culture Collection (Porton Down, UK) and grown in 162 cm² flasks (Costar; Cambridge, USA) in DMEM supplemented with 10% (v/v) heat-inactivated FBS. 3T3 cells were allowed to grow to confluence and were then quiesced for 24 hours before use in DMEM containing 0.5% FBS.

Bovine tracheal smooth muscle cells (BTSM) were established in primary culture in our laboratory by Dr T Walker and grown in DMEM containing 10% (v/v) FBS. BTSM cells were quiesced for 48 hours before use in DMEM containing 0.5% FBS.

Human alveolar type II epithelial cells (A549) were purchased from the American Type Tissue Culture Collection and grown in DMEM containing 10% FBS. A549 cells were quiesced in serum-free DMEM for 24 hours before use.

All media contained 50 IU/ml penicillin, 50 IU/ml streptomycin and 5 µg/ml L-glutamine and all cell lines were maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C. Culture media for BTSM cells was also supplemented with amphotericin B to a final concentration of 2.5 µg/ml. All cell lines were regularly screened for mycoplasma infection and were consistently negative.

2.2.3 Production, isolation and purification of mAb 4F2 and 4F2-Fab.

The hybridoma cell line 4F2 (C13) was purchased from the American Type Tissue Culture Collection and cultured in DMEM containing 15% FBS, 50 IU/ml penicillin, 50 IU/ml streptomycin and 5 µg/ml L-glutamine and OPI media supplement. Secreted antibody was purified using protein-A affinity chromatography (Amersham Pharmacia; Uppsala, Sweden). F(ab)₂ fragments were prepared by pepsin digestion of purified 4F2 IgG (2 mg/ml) for 6 hours at 37°C. Digestion was terminated by adding 1.5 M Tris pH 8.8 to achieve a final pH 7.5. F(ab)₂ fragments were dialysed against 20 mM Tris/0.14 M NaCl pH 7.5 and Fab fragments were produced by addition of L-cysteine to a final concentration of 10 mM. Digestion was terminated by the addition of iodoacetamide. Fab fragments were purified on Protein-A sepharose columns. Fab fragments were characterised by SDS-PAGE and exhibited characteristic mobilities.

2.2.4 PI 3-kinase activity assay

Cultures of SCLC cells 3-5 days post-passage were washed twice, resuspended in quiescent medium and incubated at 37°C for 24 hours. The following day cells were washed twice in pre-warmed HBSS and disaggregated by gentle passage through a

21-gauge needle prior to aliquoting into 6 well plates (Costar; Cambridge, USA). Cells were allowed to equilibrate at 37°C for 1 hour prior to the addition of agonist or antagonist. The plates were then transferred to a water/ice bath at 4°C and the cells aspirated and centrifuged at 3000 x g for 30 seconds. The supernatant was aspirated and the cells lysed by addition of 250 µl of ice-cold lysis buffer containing 50 mM HEPES (pH 7.4), 15 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, 50 µM 4-(2-aminoethyl-benzenesulfonyl fluoride, 5 µg/ml leupeptin, 20 µg/ml aprotinin, and 10 µg/ml soybean trypsin inhibitor. After 30 minutes lysates were clarified by centrifugation at 13,000 x g for 10 minutes. For each assay point, 0.5 mg of total protein from the supernatant was incubated with 1.0 µg of p85-SH3 PI 3-kinase antibody overnight at 4°C. 30 µl of goat anti-mouse Ig G agarose beads in lysis buffer were added for an additional 2 hours. The beads were then pelleted and washed twice with lysis buffer, once with a high salt buffer containing 100 mM Tris-HCl and 500 mM LiCl (pH 7.6) and twice with a solution containing 200 mM HEPES, 40 mM MgCl₂ and 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, [³²P] ATP (5 µCi, 5000 Ci/mmol), 50 µM ATP, and 0.5 mg/ml sonicated phosphatidylinositol:phosphatidylserine (3:1, w/w). Reactions were carried out for 20 minutes 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 (methanol:1 M HCl:chloroform, 48:47:3, v/v/v), dried and the lipids were separated by thin layer chromatography (TLC). TLC plates were obtained from Whatman, Maidstone, UK.. The running buffer for the TLC consisted of methanol:chloroform:ammonia soln: water, in the proportions 100/70/15/25 (v/v/v/v). 3'-phosphorylated lipids were identified by autoradiography using Kodak 'X-OMAT' film and radioactivity was quantified by

liquid scintillation counting using 'Flo-Scint IV' (Packard, Biosciences; Groningen, Netherlands) and a Packard 1900 TR liquid scintillation analyser.

2.2.5 HPLC analysis of phosphorylated lipids

The identity of the 3'-phosphorylated products obtained by TLC was confirmed by high performance liquid chromatography (HPLC). Labelled lipids were excised from the TLC plate and subjected to deacylation by adding the dried silica to 3 ml of 25% methylamine in water:methanol:butanol (4:4:1, v/v/v). The resulting mixture was tightly capped and incubated at 53°C for 30 minutes with 30 second vortexing every 10 minutes. Following centrifugation at 1000 x g for 3 minutes the deacylated lipids were dried overnight at room temperature before addition of 1 ml of water and 1.2 mls of butanol:petrol ether 40-60 bp:ethyl formate (20:4:1, v/v/v). Following phase partition the upper phase, containing transacylated lipids, was discarded and the lower phase was washed with 1.2 ml of butanol:petrol ether 40-60 bp:ethyl formate (20:4:1, v/v/v). The resultant lower phase containing cleaved lipid head groups was separated using an HPLC strong anion exchange 5 column (Whatman; Maidstone, UK) with an $(\text{NH}_4)_2\text{HPO}_4$ gradient running from 0-10 minutes at 0% salt, 10-70 minutes at 0-20% salt and from 70-130 minutes at 20-100% salt at a flow rate of 1 ml/minute. Samples were spiked with adenine and guanine nucleotides and 1 ml fractions collected. Samples were compared with tritiated standards originally derived from 1321N astrocytoma cells and validated by Dr I Batty (Dept. of Biochemistry, University of Dundee).

2.2.6 Assessment of protein concentration

Protein concentrations were quantified using a BCA protein assay (Pierce; IL, USA). This assay relies upon the ability of protein present in the test sample being able to cause a reduction of Cu^{2+} to Cu^+ . Bicinchoninic acid (BCA) chelates Cu^+ forming a purple compound, which can be measured using spectrophotometry (562nm) (Smith et al., 1985). Samples were diluted 1 in 10 in dH_2O and 10 μl incubated with 200 μl of test solution (30 minutes, 37°C) in 96 well plates prior to analysis using an automated plate reader (MRX microplate reader: Dynatech; Chantilly, USA).

Samples were assayed in triplicate and standard curves formed using pre-made standard solutions.

2.2.7 Protein kinase B activity assay

Cultures of SCLC cells 3-5 days post-passage were washed twice and resuspended in quiescent medium and incubated for a further 24 hours. The following day, cells were washed twice in HBSS at 37°C and disaggregated by gentle passage through a 21-gauge needle prior to aliquoting into 6 well plates. Cells were allowed to equilibrate at 37°C for 1 hour prior to the addition of agonist or antagonist and then aspirated, centrifuged at 3000 x g for 30 seconds and lysed using 250 µl of ice-cold lysis buffer. Lysis buffer contained 50 mM Tris/HCl (pH 7.5), 0.1% (v/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.27 mM sucrose, 50 mM sodium fluoride, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 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 x g for 10 min, and PKB was immunoprecipitated by incubating the lysates (containing 150 µg of protein) for 90 minutes at 4°C with 1 µg of anti-PKBα antibody pre-conjugated to 5 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 minutes in a thermomixer in 30 µl of kinase assay buffer (50 µM Crosstide, 50 mM Tris/HCl, 0.1 mM EGTA, 20 µM adenosine 3', 5'-cyclic monophosphate-dependent protein kinase inhibitor, 20 µM ATP, 1 µM microcystin and [γ -³²P] ATP (1.0 µCi 5000 Ci/mmol). The assays were terminated by placing 40 µl of the assay mixture onto P81 chromatography paper (Whatman; Maidstone, UK) and washing four times with 0.5% (v/v) phosphoric acid and once with acetone. Radioactive incorporation was quantified by liquid scintillation counting.

2.2.8 Radioligand displacement assay for mass measurement of PtdIns(3,4,5)P₃.

The assay is based on radioligand displacement of radiolabelled Ins(1,3,4,5)P₄ from binding sites present in platelets that display high affinity and specificity for Ins(1,3,4,5)P₄. Following a Folch extraction of cellular phospholipids, Ins(1,3,4,5)P₄, the polar headgroup of PtdIns(3,4,5)P₃, is released by alkaline cleavage and its mass can be measured by radioligand displacement using a calibration curve obtained using unlabelled Ins(1,3,4,5)P₄ standards (see below). The sensitivity limit of the assay is approximately 0.3 pmol and in general lipid extracts from sample containing 1 mg of cellular protein are sufficient for determination of the basal level of PtdIns(3,4,5)P₃. The full method has been described by van der Kaay et al., (1999).

The Ins(1,3,4,5)P₄ assay requires:

- a. Samples
- b. Ins(1,3,4,5)P₄ standards
- c. [³²P]Ins(1,3,4,5)P₄ label
- d. GAP1^{IP4BP}
- e. Wash buffer
- f. Assay buffer

Ins(1,3,4,5)P₄ stock, [³²P]Ins(1,3,4,5)P₄ and GAP1^{IP4BP} were kindly prepared by Dr J van der Kaay as described (van der Kaay et al., 1999).

2.2.8a Sample preparation

For adherent cells, such as Swiss 3T3, the medium was aspirated and the cells, which had been grown in 6 well plates (Costar; Cambridge, USA), were quenched with 1 ml of 10% trichloroacetic acid. After 15 minutes on ice, the wells were scraped and washed once with 0.5 ml of 10% trichloroacetic acid. The cell lysates were centrifuged for 5 minutes at 13,000 x g, and the resulting pellet was washed twice with 0.5 ml of 5% trichloroacetic acid, 1 mM EDTA. Lipids were extracted in 0.75 ml of CHCl₃/MeOH/HCl (40:80:1, by volume) for 20 minutes, and phases were then split by the addition of 0.25 ml of CHCl₃ and 0.45 ml of 0.1 M HCl. The lower

phase, obtained after a 1 minute centrifugation at 13,000 x g, was transferred to a screw cap tube and the upper phase was re-extracted once with 0.45 ml of the synthetic lower phase. The lower phases were pooled and dried down. Alkaline hydrolysis of dried lipids was carried out by vortexing in 50 μ l of 1 M KOH and boiling for 30 minutes. Following neutralisation with 50 μ l of 1 M acetic acid, the fatty acids were removed by 2 extractions with 0.5 ml of water-saturated butan-1-ol/petroleum ether (40-60°C)/ethyl acetate (20:4:1 by volume). Finally, the resulting water-soluble fractions were dried down by centrifugation and stored at -20°C. Prior to assaying, samples were resuspended in 110 μ l of dilute acetic acid to a final pH of 5.0 measured using accurate pH indicator strips. This step is important because the sample, which now contains approximately 0.5 M KOH/acetic acid, dictates the pH of the subsequent Ins(1,3,4,5)P₄ binding assay. Cells grown in suspension (such as SCLC cells) were transferred to eppendorf tubes, pelleted by centrifugation at 3000 x g for 2 minutes and killed by the addition of 0.75 ml of CHCl₃/MeOH/HCl (40:80:1, by volume) for 20 minutes. A phase split was then obtained as above and further sample processing was carried out as above.

2.2.8b Preparation of standards.

Ins(1,3,4,5)P₄ standards containing 0.5 M KOH/acetic acid were prepared at the following concentrations: 8 x 10⁻⁶ M, 24 x 10⁻⁷ M, 8 x 10⁻⁷ M, 24 x 10⁻⁸ M, 8 x 10⁻⁸ M, 24 x 10⁻⁹ M, 8 x 10⁻⁹ M, 24 x 10⁻¹⁰ M, 8 x 10⁻¹⁰ M, 24 x 10⁻¹¹ M, 8 x 10⁻¹¹ M.

2.2.8c [³²P]Ins(1,3,4,5)P₄ label

0.05 μ Ci [³²P]Ins(1,3,4,5)P₄ label was used per assay point.

2.2.8d GAP1^{IP4BP}

GAP1^{IP4BP} coupled to agarose beads is stored in high salt buffer with 50% glycerol. Just before the start of the assay, an appropriate amount of the beads was washed twice in Ins(1,3,4,5)P₄ wash buffer and resuspended in this buffer. The amount of GAP1^{IP4BP} required for each assay was determined for each new preparation of GAP1^{IP4BP}. Maximal and non-specific binding was determined in 0.5 M KOH/acetic acid pH 5.0 in the absence or presence of 1 mM Ins(1,3,4,5)P₄ respectively. A

maximal binding of approximately 10% of the total input was aimed for when using [³²P]Ins(1,3,4,5)P₄. See van der Kaay et al., (1999) for method.

2.2.8e Wash buffer

25 mM sodium acetate, 25 mM KH₂PO₄, 1 mM EDTA, 5 mM NaHCO₃, pH 5.0 with acetic acid.

2.2.8f Assay buffer

0.1 M sodium acetate, 0.1 M KH₂PO₄, 4 mM EDTA, pH 5.0 with acetic acid.

Each assay sample contained 400 µl final volume and comprised:

100 µl of sample or Ins(1,3,4,5)P₄ standard.

100 µl assay buffer.

100 µl [³²P] Ins(1,3,4,5)P₄ (0.05 µCi in H₂O).

100 µl GAP1^{IP4BP}.

The incubation was started by addition of the binding protein. The samples were vortexed and incubated on ice for 30 minutes with occasional vortexing as the agarose beads settled out fairly quickly. Filtration of bound [³²P]Ins(1,3,4,5)P₄ from unbound was achieved by filtration through GF/C filters (Whatman; Maidstone, UK). The samples were filtered onto pre-wetted filters and immediately rinsed twice with 10 ml of ice-cold wash buffer. The filters were then allowed to air-dry, transferred to scintillation vials containing 4 mls of 'Floscint V' (Packard Biosciences; Groningen, Netherlands) and the radioactivity counted in a liquid scintillation counter. A typical calibration curve obtained using the standards is shown in Figure 2.1. The *K_d* for Ins(1,3,4,5)P₄ is approximately 3 nM (van der Kaay et al., 1997). When PtdIns(3,4,5)P₃ is subjected to alkaline hydrolysis, the yield of Ins(1,3,4,5)P₄ is 62%. Therefore, for absolute amounts of PtdIns(3,4,5)P₃ to be calculated, the final values read from the calibration curve need to be corrected by this factor. Figure 2.2 shows the relative ratios of the side products obtained by alkaline hydrolysis of PtdIns(3,4,5)P₃.

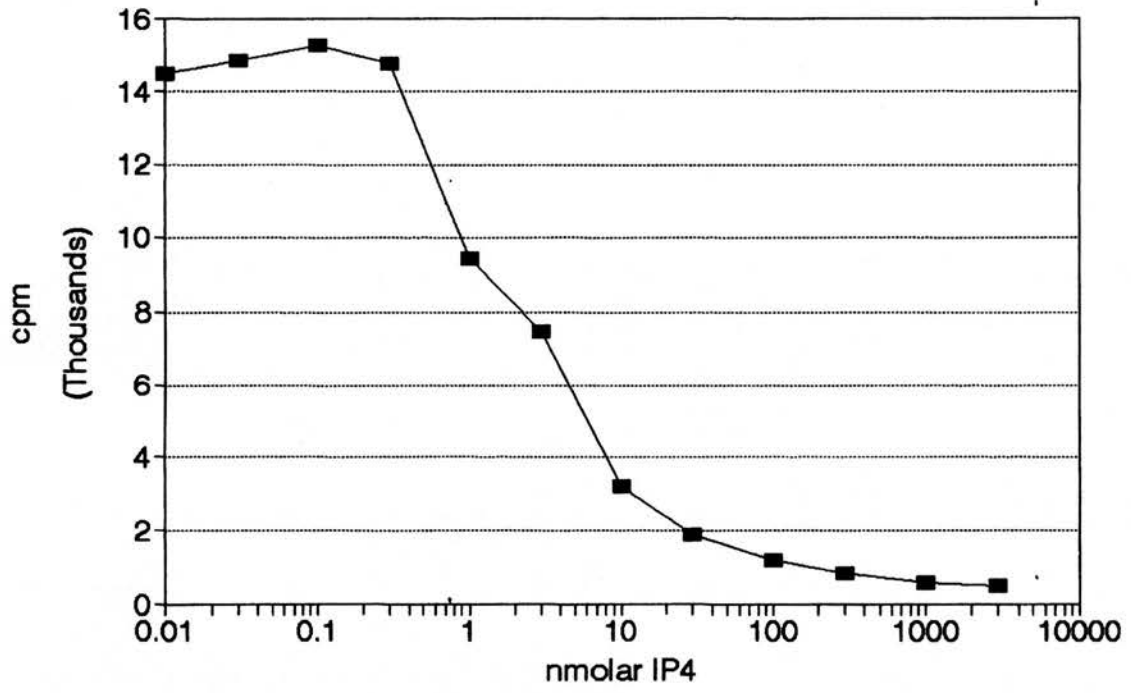


Figure 2.1
Displacement of [^{32}P]Ins(1,3,4,5)P₄ by Ins(1,3,4,5)P₄ using recombinant GAP1^{IP4BP}.

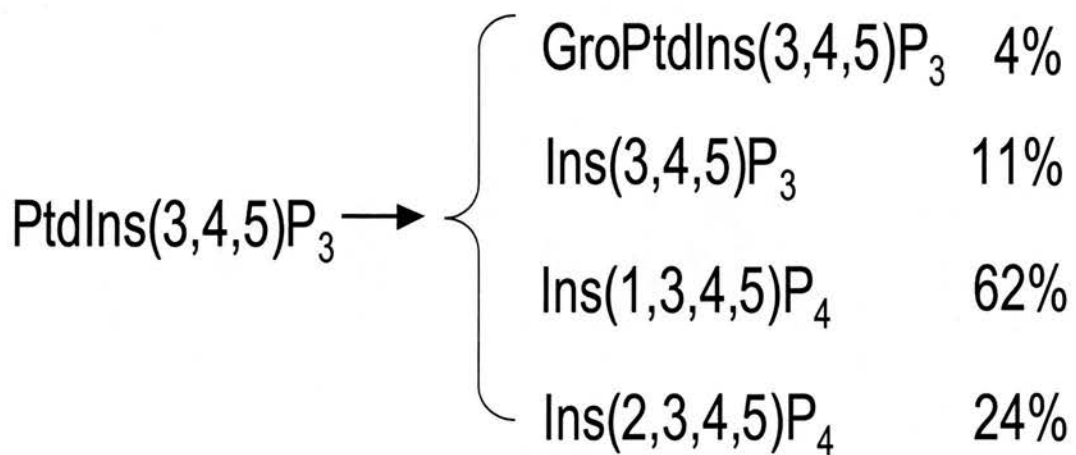


Figure 2.2

Schematic representation of the products from $\text{PtdIns}(3,4,5)\text{P}_3$ subjected to alkaline hydrolysis.

Van der Kaay et al., (1997) have shown that these products do not interfere with or contribute to the measured signal. A very important feature of the assay is its selectivity for Ins(1,3,4,5)P₄ over Ins(1,4,5)P₃. Ins(1,4,5)P₃ is formed by alkaline cleavage of PtdIns(4,5)P₂ which is usually present at 1000-fold higher levels in resting cells than PtdIns(3,4,5)P₃. The *K_d* for Ins(1,3,4,5)P₄ is approximately 5000-fold lower than the *K_d* for Ins(1,4,5)P₃ so there should be no noise arising from PtdIns(4,5)P₂ also present in lipid extracts from the cells.

2.2.9 Immunoblotting

Whole cell lysates were solubilised in 5 x sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at 100°C for 5 minutes. Samples were cooled and undissolved material pelleted at 13,000 x g for 1 minute before equal amounts of protein (50-100 µg) were resolved on SDS polyacrylamide gels for 2-3 hours at 150 volts using tris-glycine gel electrophoresis buffer and an electrophoresis gel tank (ATTO Corp., Japan). For optimal separation, 8% gels were used for 60-120 kDa proteins, 10% gels for 40-70 kDa proteins and 12% gels for 10-40 kDa proteins. The resolved proteins were 'Western blotted' onto Hybond C nitrocellulose membranes in ice-cold blotting buffer at 100 volts for 1 hour using a Mini Protean II blotting tank (Biorad Co., UK). Equal protein loading was confirmed by incubating blots for 5 minutes in Ponceau S which allows visualisation of protein bands. The membranes were then incubated overnight at 4°C in TBS-Tween 20 wash buffer in order to prevent subsequent non-specific binding of primary antibodies prior to addition of the primary antibody (diluted in washing buffer) for 2 hours. Next, membranes were washed in TBS-Tween 20 (3 x 30 second vigorous washes and 3 x 10 minute washes) before species-appropriate horseradish peroxidase (HRP) conjugated secondary antibody (diluted in washing buffer) was added for 1 hour. Finally, membranes were rewashed as above and immunoreactive bands were identified using enhanced chemiluminescence (ECL) (Amersham Life Sciences; Cheshire, UK) as per manufacturer's instructions. ECL is a high resolution, non-radioactive method of detecting specific immobilised antigens that are conjugated (directly or indirectly) to HRP labelled antibodies.

2.2.9a 5 x SDS-PAGE sample buffer

625 mM Tris HCl (pH 6.8), 10% SDS (v/v), 25% glycerol (v/v), 0.015% bromophenol blue (v/v) and 5% 2-mercaptoethanol (v/v).

2.2.9b SDS polyacrylamide gels

Stacking gel:

Stacking gel buffer (1 M Tris base (pH 6.8), 10% SDS)	2.50 ml
30% Acrylamide solution	1.50 ml
Distilled H ₂ O	5.70 ml
25% ammonium persulphate	0.05 ml
TEMED	0.02 ml

Resolving gel:

Resolving gel buffer (1.5 M Tris base (pH 8.8), 10% SDS)	6.25 ml
30% Acrylamide solution	8.33 ml
Distilled H ₂ O	10.38 ml
25% ammonium persulphate	0.10 ml
TEMED	0.02 ml

2.2.9c Gel electrophoresis buffer

25 mM Tris base (pH 8.3), 250 mM glycine and 0.1% SDS.

2.2.9d Blotting buffer

210 mM glycine, 24.7 mM Tris base and 20% methanol.

2.2.9e Wash buffer

20 mM Tris/HCl (pH 7.4), 150 mM NaCl and 0.2% (v/v) Tween-20.

2.2.10 Determination of intracellular calcium concentration

Aliquots of 4×10^6 SCLC cells cultured in SITA for two days were washed and incubated for 2 hours at 37°C in 10 ml of fresh SITA medium. Cells were then resuspended in HBSS without Ca^{2+} and $1 \mu\text{M}$ fura-2-tetraacetoxymethylester AME, from a stock of 1 mM in DMSO, was added and the cells were incubated for a further 10 minutes at 37°C . The cell suspension was centrifuged at $2000 \times g$ for 15 seconds and the cells were resuspended in 2 ml of electrolyte solution (140 mM NaCl, 5 mM KCl, 0.9 mM MgCl_2 , 1.8 mM CaCl_2 , 25 mM glucose, 16 mM HEPES, 16 mM Tris and a mixture of amino acids at pH 7.2), transferred to a quartz cuvette and stirred continuously at 37°C . Fluorescence was recorded continuously in a Model F2000 fluorescence spectrophotometer (Hitachi Corp., Japan) with an excitation wavelength of 340 nm and an emission wavelength of 380 nm. $[\text{Ca}^{2+}]_i$ was calculated using the formula:

$$[\text{Ca}^{2+}]_i = Kd(r-r_{\min}) / (r_{\max}-r)B$$

where r is the ratio 340/380, r_{\min} is $F_{\min 340}/F_{\max 380}$, r_{\max} is $F_{\max 340}/F_{\max 380}$ and B is $F_{\min 380}/F_{\max 380}$. F is the fluorescence at the unknown $[\text{Ca}^{2+}]_i$, F_{\max} is the fluorescence after the trapped fluorescence is released by the addition of 0.02% Triton-X 100 and F_{\min} is the fluorescence remaining after the Ca^{2+} in the solution is chelated with 10 mM EGTA. The Kd for fura-2 is 224 nM (Tsien, Pozzan et al., 1982).

2.2.11 Decatenation assay

Cells were washed twice with ice-cold CMF-PBS and spun at 1,200 rpm for 10 minutes at 4°C . The cell pellet was washed again with 1 ml PBS and spun for 15 seconds at 14,000 rpm at 4°C before being washed in 400 μl of a buffer containing 10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl_2 , 1 mM dithiothreitol, 0.1 mM EDTA, 0.4 mM PMSF, 0.2 mM sodium fluoride, 0.2 mM sodium orthovanadate and 0.3 mg/ml leupeptin. Following a 15 minute incubation on ice the mixture was vortexed for 15 seconds with 25 μl of 10% Nonidet P-40. Following centrifugation for 30 seconds at 14,000 rpm at 4°C the supernatant was removed by pipetting and

the pelleted nuclei resuspended and mixed for 20 minutes in 50 μ l of a buffer containing 50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% (w/v) glycerol, 0.2 mM sodium fluoride and 0.2 mM sodium orthovanadate. The lysed nuclei were spun for 5 minutes at 14,000 rpm at 4°C and the resulting supernatant collected and used in the topoisomerase II assay.

Topoisomerase II activity was measured by the ATP-dependent decatenation of kinetoplast DNA (kDNA) in which an interlocking kDNA network is decatenated to individual DNA minicircles and small catenanes. Reaction buffer, consisting of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 mM KCl, 0.5 mM EDTA, 30 μ g/ml BSA, 1 mM ATP, 0.5 mM dithiothreitol and 100 μ g/ml kDNA was mixed with each sample in a total volume of 45 μ L. Reactions were incubated for 30 minutes at 37°C and then terminated by the addition of 5 μ l of stop buffer consisting of 50% sucrose, 50 mM EDTA and 0.1% bromophenol blue. Reaction products were analysed by 1% agarose gel electrophoresis using a buffer consisting of 89 mM Tris-borate (pH 8.3) and 2 mM EDTA. Following electrophoresis gels were stained with 1 μ g/ml ethidium bromide for 30 minutes, destained with several washings of distilled water and photographed under UV light.

2.2.12 Adhesion Assay

96 well plates (Costar; Cambridge, USA) were coated with 100 μ l of fibronectin, laminin or collagen IV (see figure legends for details of concentrations) overnight at 4°C and blocked with 1 mg/ml BSA (1h at 25°C). SCLC cells (5×10^5) that had been washed twice in RPMI 1640 were added to the wells in the presence or absence of test compounds (see results section and figure legends for details). After 1 hour of incubation at 37°C, non-adherent cells were removed by gentle washing with PBS and adherent cells were fixed with 3% formaldehyde and stained with 1% methylene blue. After several washes with distilled water, 100 μ l of 0.1 M HCl was added to each well and the absorbance of the resulting solution was measured by spectrophotometry at 630 nm on an MRX microplate reader plate reader (Dynatech; Chantilly, USA). Each condition was assayed in triplicate.

2.2.13 Flow cytometric analysis of antigen expression

Aliquots of 5×10^5 cells were washed and resuspended in 100 μ l PBS containing 0.2% (w/v) BSA and 0.1% sodium azide (EPICS buffer) and incubated with 10 μ l AB serum for 10 min at 4°C. Primary antibody was then added for a further 30 minutes followed by two washes with EPICS buffer. Samples were then incubated with FITC-conjugated goat anti-mouse Ig polyclonal secondary antibody (1:40) for 30 minutes at 4°C and again washed twice in EPICS buffer. Samples were finally resuspended in EPICS buffer and analysed by flow cytometry using an EPICS profile 2 (Coulter Electronics; Luton, UK).

2.2.14 Liquid growth assay

Cultures of SCLC cell lines H69, H345 and H510, 5 days post-passage, were washed and subsequently incubated in serum-free SITA medium for 24 hours. 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 haemocytometer. Cell number was determined using a Coulter Counter (Coulter Electronics, Luton, UK) and cells were diluted using SITA medium to a density of 1×10^5 /ml. Cells were seeded into 24 well plates and incubated for 4 hours in a humidified atmosphere of 5% CO₂/95% air at 37°C before the addition of agonists or antagonists. Cells were then further incubated under the same conditions before being removed at various time points and disaggregated into a single cell suspension for counting using a Coulter Counter.

2.2.15 Clonogenic assay

SCLC cells, 5 days post-passage were washed and subsequently incubated in serum-free SITA medium for 24 hours. 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 haemocytometer. Cells (1×10^4) were mixed with SITA containing 0.3% (w/v) agarose and agonist/antagonist (see figure legends for details) and layered over a

solid base of 0.5% (w/v) agarose in SITA containing the agonist/antagonist at the same concentration. The cultures were incubated in a humidified atmosphere of 5% CO₂/95% air at 37°C for 21 days and then stained with the vital stain nitro-blue tetrazolium. Colonies of >16 cells were counted with a microscope.

2.2.16 Assessment of SCLC cell apoptosis

2.2.16a Ethidium bromide/acridine orange staining.

1 µl of an ethidium bromide (100 µg/ml) /acridine orange (100 µg/ml) (1/1 v/v) mix was added to 200 µl SCLC cells and the percentage of cells undergoing apoptosis was assessed using fluorescent microscopy using an Olympus BH2 fluorescence microscope (Olympus Optical Co., Japan). This assay is based upon the loss of plasma membrane integrity when cells die. Acridine orange and ethidium bromide excite green and orange fluorescences respectively, when they are intercalated into DNA. Whilst acridine orange can cross the plasma membrane of all cells, ethidium bromide is excluded from viable and early apoptotic cells which have intact plasma membranes. Where cells have taken up both dyes (late apoptotic and necrotic cells), the fluorescence due to ethidium bromide is the stronger and therefore these cells have an overall orange appearance. Normal healthy cells appear as having a bright green nucleus with an intact nuclear structure. Early apoptotic cells have a bright green nucleus but show condensed chromatin as dense areas within the nucleus. Late apoptotic cells have an orange nucleus and condensed chromatin appears as dense orange areas. Necrotic cells have an orange nucleus with an intact structure.

2.2.16b Morphological assessment.

Cells were cytocentrifuged at 300 x g for 3 minutes onto glass slides using a Cytospin 3 (Shandon; Pittsburgh, USA). Resulting cell preparations were air dried, fixed in methanol and stained with May-Grunwald-Giemsa stain. Cell morphology was examined using an Olympus BH2 microscope.

2.2.16c ELISA cell death detection assay.

This commercial assay (Cell Death Detection ELISA from Boehringer Mannheim, Mannheim, Germany) is based upon a quantitative sandwich-enzyme-immunoassay

principle using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates.

The assay was performed according to the manufacturer's instructions. Briefly, cell lysates were placed into a streptavidin-coated microtitre plate. Subsequently, a mixture of anti-histone-biotin and anti-DNA peroxidase antibodies were added and incubated for 2 hours. During the incubation period, the anti-histone antibody binds to the histone component of the nucleosomes and simultaneously fixes the immunocomplex to the streptavidin coated microtitre plate via its biotinylation. Additionally, the anti-DNA peroxidase antibody reacts with the DNA component of the nucleosomes. After removal of unbound antibodies by a washing step, the amount of nucleosomes is quantified by the peroxidase retained in the immunocomplex. Peroxidase was determined photometrically with 2, 2' Azino-di [3-ethylbenzthiazolin-sulfonat] as substrate. Plates were read by spectrophotometry at 405 nM using an MRX microplate reader (Dynatech; Chantilly, USA) and results expressed as the increase in absorption of treated cells in comparison to untreated cells.

2.2.17 Tissue immunohistochemistry

Formalin fixed, paraffin embedded SCLC resection specimens were 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 or microwave-oven boiling for 15 minutes in 0.1 M citrate (pH 6.0). Sections were processed using a Techmate 500 (DAKO; Ely, UK). Antibody detection was performed using a multi-link streptavidin biotin complex method and visualisation was achieved using a diaminobenzidine chromagen method as per the manufacturer's instructions. Slides were counterstained using Harris' haematoxylin. Negative controls were incubated in primary antibody diluent only. All slides were reviewed independently by two people.

2.2.18 Statistical Analysis

All data are expressed as the mean \pm SEM of the indicated number of experiments or on occasion as the mean \pm SD of 2 experiments each performed in duplicate or triplicate. Data were analysed by one way analysis of variance with comparison between groups made using the Newman-Kuels procedure. P values less than 0.05 were considered to be significant.

CHAPTER 3

CONSTITUTIVE PHOSPHOINOSITIDE 3-KINASE ACTIVITY REGULATES ANCHORAGE-INDEPENDENT GROWTH IN SMALL CELL LUNG CANCER

3.1 INTRODUCTION

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

Transformed cells are characterised by their ability to proliferate, a) in the absence of serum and, b) in the absence of contact with extracellular matrix, so called anchorage-independence (Schwartz, 1997). Constitutive stimulation of growth factor pathways alone is mitogenic rather than oncogenic resulting in benign hyperplasia in animal models (Chang et al., 1989; Sandgren et al., 1990). For full malignant transformation to occur, tumour cells also require to be anchorage-independent. In SCLC cells proliferation is driven by multiple autocrine and paracrine growth loops involving a variety of hormonal and peptide mitogens including calcium-mobilising neuropeptides, IGF-1 and NGF (Oelmann et al., 1995; Macaulay et al., 1990; Nakanishi et al., 1988; Sethi and Rozengurt, 1991).

In vitro, SCLC cells grow as non-adherent, free-floating aggregates. However, the mechanisms resulting in anchorage-independent growth of SCLC cells are unclear. 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* (Frisch and Francis, 1994). 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 the PI 3-kinase/PKB pathway (Khwaja et al., 1997).

PI 3-kinase was initially discovered as a PI kinase activity that co-purified with the oncoproteins pp60v-*src* and polyoma middle T antigen (Sugimoto et al., 1984; Whitman et al., 1985). Interest in PI 3-kinase increased with the finding that the transforming ability of several oncoproteins correlated with their ability to associate with PI 3-kinase and elevate specific lipid products of the enzyme. PI 3-kinase is involved in the regulation of multiple cellular processes. Early studies using chemical PI 3-kinase inhibitors and dominant negative or constitutively active mutations of PI 3-kinase suggested several distinct functions including mitogenesis, inhibition of apoptosis, intracellular vesicle trafficking and secretion, and regulation of actin and integrin functions (reviewed in Carpenter and Cantley, 1996). More recent studies have begun to explore potential roles for PI 3-kinase in transformed cells. Shaw et al., (1997b) recently identified a specific integrin-mediated pathway involving PI 3-kinase that promotes carcinoma invasion while Keely et al., (1997) have demonstrated that activation of Cdc42 and Rac1 in mammary epithelial cells promotes motility and invasion in a PI 3-kinase-dependent manner. In view of these important physiological and pathological functions much interest has focussed on the downstream effectors of PI 3-kinase.

In 1991 the serine/threonine kinase PKB, was identified as the product of the oncogene *v-akt* of the acutely transforming retrovirus AKT 8 found in a rodent T-cell lymphoma (Bellacosa et al., 1991). PKB can be activated by growth factors, oncogenes and integrins signalling through PI 3-kinase and has been shown to provide a survival signal that protects cells from apoptosis induced by a variety of stresses (reviewed in Hemmings, 1997).

These findings suggest that the PI 3-kinase/PKB 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.

3.2 RESULTS

3.2.1 Expression of p85 α , the regulatory subunit of PI 3-kinase, in SCLC cell lines

Whole cell lysates prepared from cells that had been incubated in serum-free 'quiescent' medium for 24 hours were resolved on 10% SDS polyacrylamide gels and transferred to nitrocellulose membrane by 'Western' blotting. P85 expression in SCLC cells was compared with that in Swiss 3T3 cells. Equal protein loading was confirmed by incubation of membranes in Ponceau S and p85 expression was examined using an antibody to the α isoform of the p85 subunit of PI 3-kinase. Figure 3.1 shows p85 expression as a single band at 85 kDa. The levels of expression of PI 3-kinase in SCLC cells were very similar to that in the Swiss 3T3 cells.

3.2.2 SCLC cells exhibit constitutive PI 3-kinase activity

PI 3-kinase activity was examined in a panel of SCLC cell lines using an *in vitro* kinase assay as described in 'Materials and Methods'. In order to examine basal PI 3-kinase activity, SCLC cells 5-7 days post-passage, were taken from growth medium, washed twice in RPMI 1640 containing 0.25% (v/v) BSA and incubated for a further 24 hours in the same medium. The following day cells were washed twice in HBSS, aliquoted and allowed to equilibrate at 37°C for 1 hour prior to lysis.

Initial experiments suggested that basal PI 3-kinase activity in SCLC cells was not reduced following incubation of cells in quiescent medium. In order to examine this further, PI 3-kinase activity was measured in several SCLC cell lines and compared with activity in a panel of untransformed cell lines including human bronchial epithelial cells, bovine tracheal smooth muscle cells and Swiss 3T3 cells. PI 3-kinase activity was also measured in a NSCLC cell line (A549).

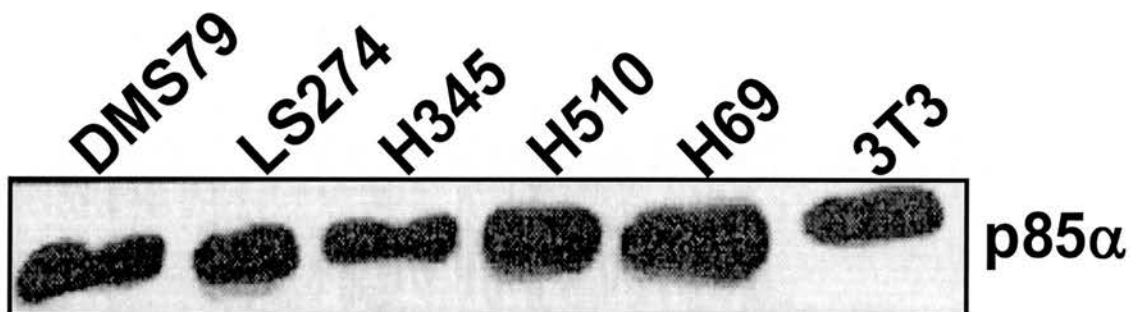


Figure 3.1

P85α expression in SCLC cell lines DMS79, LS274, H345, H510, H69 and Swiss 3T3 cells. The blot shown is representative of three independent experiments.

One of the potential problems of measuring PI 3-kinase activity in a variety of cell types is that activity levels may vary depending on phase of growth, cell number or cell density. In order to minimise these potential differences all cell types were quiesced in serum-free media for 24 hours prior to experimentation. This was performed to ensure that all growth factors were removed so that basal PI 3-kinase activity was being measured. Adherent cells were grown to confluence prior to incubation in serum-free media for 24 hours. Cell lysates were also equilibrated for protein so that in each experiment identical amounts of protein were being assayed for PI 3-kinase activity.

Figure 3.2 shows that basal PI 3-kinase activity in all the SCLC cell lines tested (H69, H345, H510, DMS79 and LS274) was markedly higher than that in human bronchial epithelial cells, bovine tracheal smooth muscle cells, Swiss 3T3 cells and A549 adenocarcinoma cells. The addition of either 10% FBS or IGF-1, both potent growth factors for SCLC, could induce only a modest additional increase in PI 3-kinase activity (1.51 ± 0.19 and 1.82 ± 0.28 fold respectively) (Figure 3.3). Thus, under conditions where every effort was made to remove all exogenous/autocrine growth factors, elevated basal PI 3-kinase activity was seen in SCLC cells.

3.2.3 Elevated basal PI 3-kinase activity in SCLC cells can be abrogated by PI 3-kinase inhibitors

Wortmannin and LY294002 are specific PI 3-kinase inhibitors. We hypothesised that if SCLC cells display elevated basal PI 3-kinase activity, PI 3-kinase inhibitors should be able to abrogate this enhanced activity. Therefore, H69 SCLC cells were treated with increasing concentrations of wortmannin for 20 minutes prior to cell lysis. Figure 3.4 shows that wortmannin caused a concentration-dependent inhibition of basal PI 3-kinase activity in H69 cells with an IC_{50} value of 8.0 ± 2.1 nM (Table 3.1).

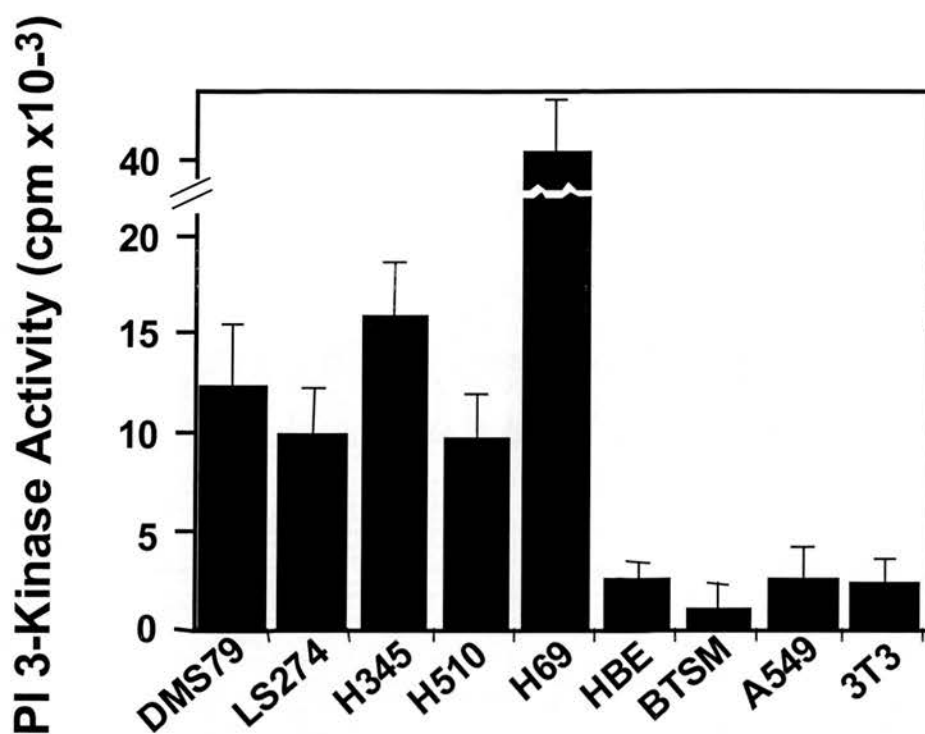


Figure 3.2

Comparison of PI 3-kinase activities in SCLC cell lines DMS79, LS274, H345, H510 and H69, human bronchoepithelial cells (HBE), bovine tracheal smooth muscle cells (BTSM), NSCLC cell line A549 and Swiss 3T3 cells. The results shown are the mean \pm SD of two independent experiments carried out in duplicate.

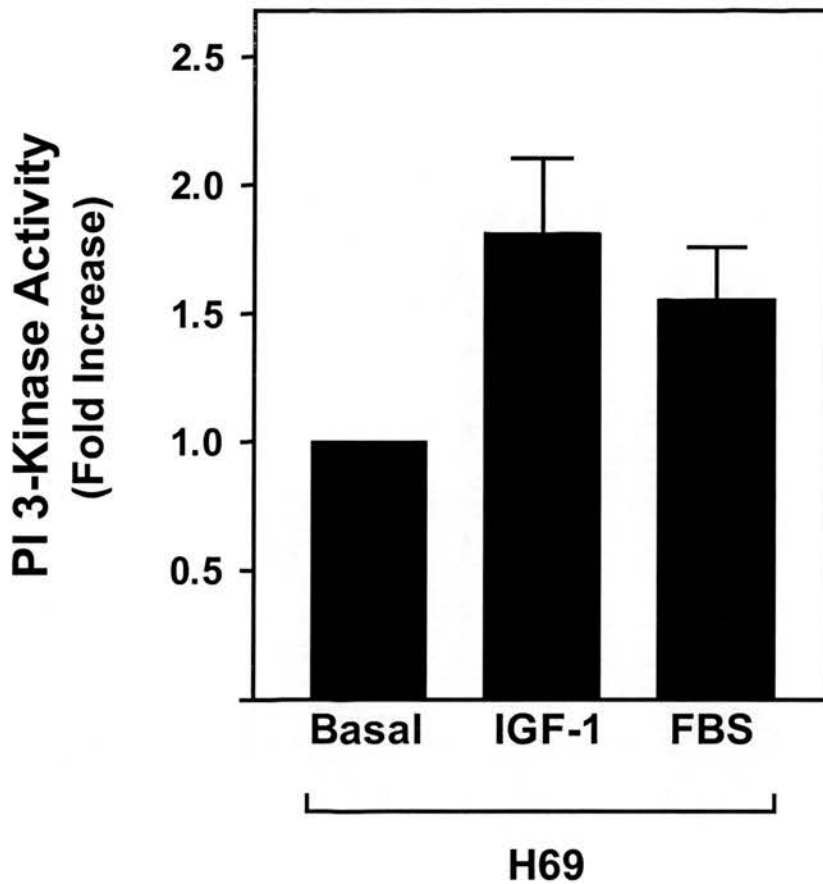


Figure 3.3

The effect of insulin growth factor 1 (IGF-1) and foetal bovine serum (FBS) on PI 3-kinase activity in H69 SCLC cells. H69 cells were taken from serum-containing medium, ‘quiesced’ in serum-free medium for 24 hours prior to experimentation and extensively washed before being stimulated with either IGF-1 (100 ng/ml) or 10% FBS for 5 minutes. PI 3-kinase was assayed as described in ‘Materials and Methods’. The results, which are expressed as fold increase over basal PI 3-kinase levels, are the mean \pm SD of two independent experiments carried out in duplicate.

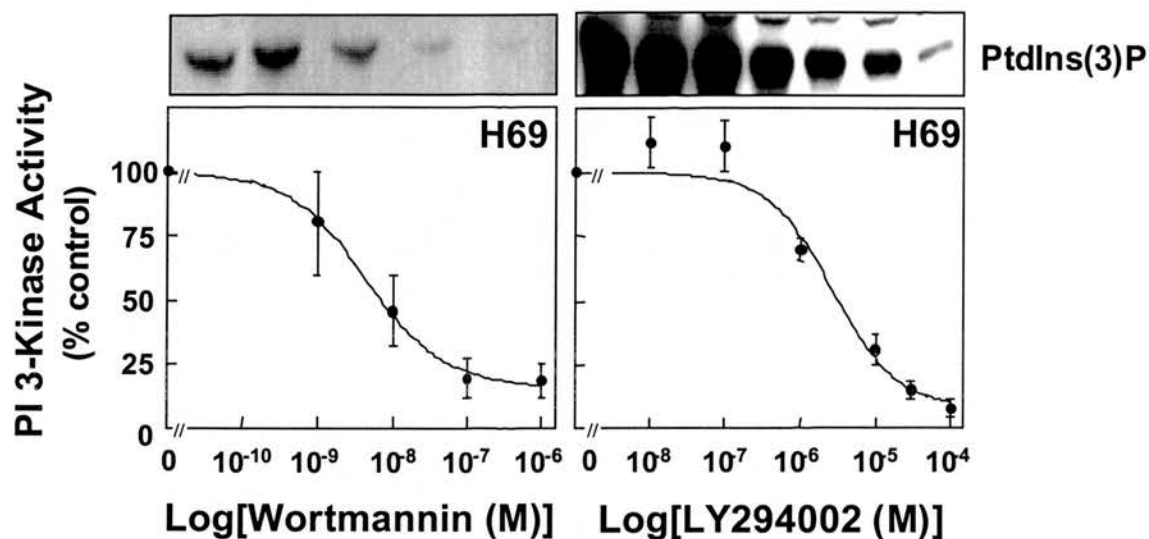


Figure 3.4

The effect of PI 3-kinase inhibitors on PI 3-kinase activity in SCLC cells.

Concentration response of wortmannin (left) and LY294002 (right) on PI 3-kinase activity in H69 SCLC cells. Basal PI 3-kinase activity is expressed as 100%. The results shown are the means \pm SD of two independent experiments performed in duplicate for experiments using wortmannin and the means \pm SEM of three independent experiments performed in duplicate for experiments involving LY294002. Representative autoradiographs showing the 3-phosphorylated reaction product (PtdIns(3)P) are shown for each condition. Experiments involving LY294002 were performed by Dr S Moore.

Assay	LY294002 (μM)	Wortmannin (nM)
PI 3-Kinase	3.4 \pm 1.4	8.0 \pm 2.1
Protein Kinase B	2.7 \pm 0.1	2.0 \pm 0.6
Proliferation	10.0 \pm 0.8	NP

Table 3.1

Comparative IC₅₀ values for LY294002 and wortmannin for PI 3-kinase, protein kinase B and cell proliferation in H69 SCLC cells. The values shown are means \pm SEM of at least three independent experiments except for PI 3-kinase assays involving wortmannin which are means \pm SD of two independent experiments. NP, not performed.

Because LY294002 is a competitive inhibitor for the ATP binding site of PI 3-kinase and can be competed off during immunoprecipitation, the inhibitor was added directly to the enzyme assay. LY294002 inhibited PI 3-kinase activity in PI 3-kinase immunoprecipitates from H69 cells with an IC_{50} value of $3.4 \pm 1.4 \mu\text{M}$ (Figure 3.4 and Table 3.1). The IC_{50} values for LY294002 and wortmannin in this system are very similar to those quoted in the published literature; $1.4 \mu\text{M}$ for LY294002 and 3 nM for wortmannin (Vlahos et al., 1994b; Powis et al., 1994). Therefore, SCLC cells appear to have constitutively active PI 3-kinase, which can be activated by only 1.8-fold above control at maximally effective concentrations of IGF-1 (Figure 3.3).

3.2.4 Basal phosphatidylinositol 3,4,5 trisphosphate levels are elevated in SCLC cells

Agonist stimulated forms of PI 3-kinase can phosphorylate PtdIns, PtdIns(4)P and PtdIns(4,5)P₂ as substrates *in vitro*, but several lines of evidence suggest that PtdIns(4,5)P₂ is the preferred substrate *in vivo*, generating PtdIns(3,4,5)P₃. PtdIns(3,4,5)P₃ has been shown to increase rapidly and transiently in many cell types in response to a wide variety of stimuli and since there are no known phospholipases that can metabolise PtdIns(3,4,5)P₃, it has been suggested that PtdIns(3,4,5)P₃ is itself a second messenger.

To date, measuring PtdIns(3,4,5)P₃ levels has been done by labelling cells with [³H] inositol or [³²P] orthophosphate. Labelling cells with [³H] inositol is often relatively inefficient, expensive and time consuming and labelling with [³²P]-orthophosphate involves the use of high levels of radioactivity. Both labelling procedures also require time-consuming HPLC analysis of deacylated lipid extracts that are required to resolve the glycerol derivative, GroPtdIns(3,4,5)P₃, from other ³²P labelled lipids.

Van der Kaay et al., (1997) have recently described an isotope dilution assay that detects PtdIns(3,4,5)P₃ at sub-picomole levels and can be used for measurements of PtdIns(3,4,5)P₃ from both control and stimulated cells. The full method is described in 'Materials and Methods' but, in brief, total lipid extracts containing PtdIns(3,4,5)P₃ are subjected to alkaline hydrolysis which results in the release of the

polar head group Ins(1,3,4,5)P₄. Ins(1,3,4,5)P₄ is measured by its ability to displace [³²P]Ins(1,3,4,5)P₄ from a highly specific binding protein. Using this method basal PtdIns(3,4,5)P₃ levels in H69 and H345 SCLC cell lines were measured and compared with basal levels of PtdIns(3,4,5)P₃ in Swiss 3T3 and Rat-2 fibroblasts. Figure 3.5 shows PtdIns(3,4,5)P₃ levels in basal H69 and H345 SCLC cells and Swiss 3T3 and Rat-2 fibroblasts. The basal levels of PtdIns(3,4,5)P₃ were markedly higher in the SCLC cell lines compared with the fibroblast cell lines.

In another set of experiments H69 SCLC cells were stimulated with IGF-1. This resulted in a 1.6 fold increase in PtdIns(3,4,5)P₃ levels (Figure 3.6). This figure is similar to the fold activation of PI 3-kinase seen with IGF-1 (Figure 3.3).

Taken together these data show that basal PtdIns(3,4,5)P₃ levels are elevated in SCLC cells and support the hypothesis that PI 3-kinase is constitutively active in SCLC cells.

3.2.5 PKB activity in SCLC cells

PKB can be activated by a wide variety of growth stimuli including PDGF, EGF, insulin, thrombin and NGF. A number of studies have shown that PI 3-kinase is involved in the regulation of PKB (reviewed in Coffey et al., 1998). Growth factor-induced activation of PKB is inhibited by PI 3-kinase inhibitors and by the expression of a dominant negative form of PI 3-kinase (Franke et al., 1995; Burgering and Coffey, 1995).

It was hypothesised that constitutive PI 3-kinase activity in SCLC cells might lead to sustained PKB activation. Using monoclonal antibodies to the α , β and γ isoforms of PKB it was found that all three isoforms were expressed in H69 SCLC cells at similar levels (Figure 3.7). PKB activity was examined using an *in vitro* kinase assay as described in 'Materials and Methods'. PKB was immunoprecipitated from cell lysates using an antibody to the PH domain of PKB and then assayed by incubating washed immunoprecipitates with [γ -³²P] ATP and the peptide substrate 'Crosstide'.

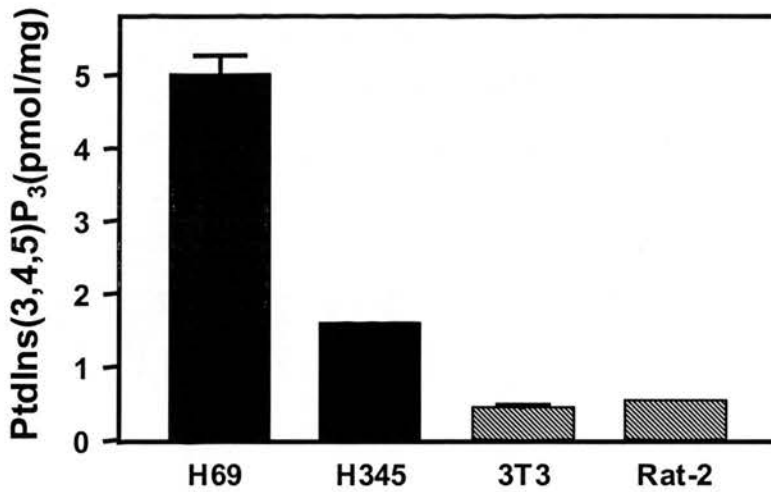


Figure 3.5

PtdIns(3,4,5)P₃ levels in basal H69 and H345 SCLC cells, Swiss 3T3 fibroblasts and Rat-2 fibroblasts. In order to measure basal PtdIns(3,4,5)P₃ levels cells were 'quiesced' in serum-free media for 24 hours and thoroughly washed prior to experimentation. PtdIns(3,4,5)P₃ levels were measured as described in 'Materials and Methods'. Results shown are either the mean \pm SD of two experiments performed in triplicate (H69 and Swiss 3T3) or the mean of a single experiment performed in triplicate (H345 and Rat-2).

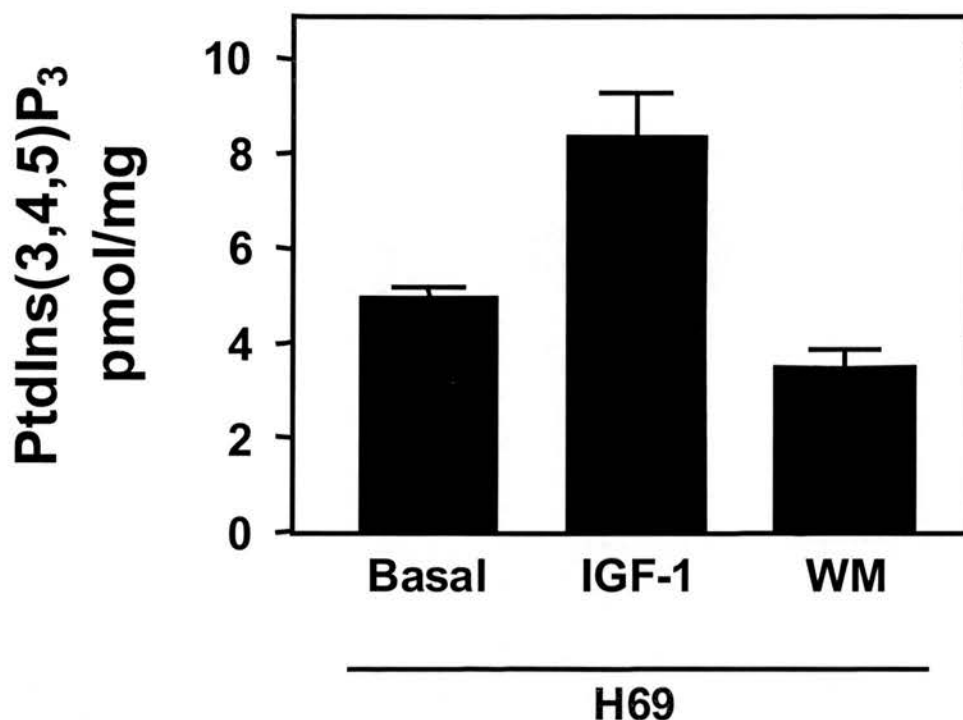


Figure 3.6

The effect of IGF-1 and wortmannin on basal PtdIns(3,4,5)P₃ levels in H69 SCLC cells. H69 cells were taken from serum-containing medium, ‘quiesced’ in serum-free medium for 24 hours prior to experimentation and extensively washed before being treated with either IGF-1 (100 ng/ml for 5 minutes) (IGF-1), or wortmannin (100nM for 20 minutes) followed by IGF-1 (100 ng/ml for 5 minutes) (WM) or diluent alone (Basal). PtdIns(3,4,5)P₃ levels were measured using an isotope dilution assay as described in ‘Materials and Methods’. The results shown are the mean ± SD of two experiments performed in triplicate.

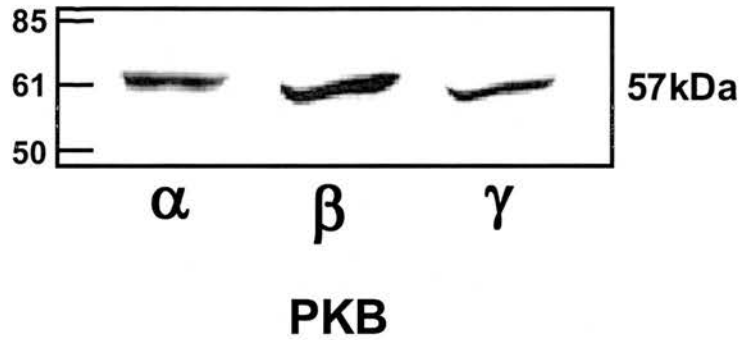


Figure 3.7

PKB expression in SCLC cells. Whole cell lysates of H69 SCLC cells were resolved on 10% SDS polyacrylamide gels and transferred onto nitrocellulose membrane by 'Western' blotting. PKB expression was examined using monoclonal antibodies to the α , β and γ isoforms of PKB. PKB is expressed at 57 kDa. The blot shown is representative of three independent experiments. Molecular weight markers in kDa are shown at the left hand margin.

Initially, basal PKB activity in SCLC cells was compared with basal activity in Swiss 3T3 cells. Prior to experimentation both sets of cells had been maintained in growth factor-free quiescent medium for 24 hours and thoroughly washed using the same conditions as described for PI 3-kinase activity measurements. Under basal conditions PKB activity in H69 SCLC cells was 3.75 fold higher than that in Swiss 3T3 cells despite similar levels of expression of PKB as determined by 'Western' blotting (Figure 3.8). These results were confirmed by 'Western' blotting using an anti-phospho-PKB antibody that recognises phosphorylation of PKB at serine 473. Figure 3.8 shows markedly increased phospho-PKB expression in H69 SCLC cells compared with Swiss 3T3 cells. Figure 3.9 shows that IGF-1 could only induce a modest additional increase in PKB activity (1.76 fold). This figure is very similar to the increase in PI 3-kinase activity seen when H69 cells were treated with IGF-1 (Figure 3.3).

The addition of the PI 3-kinase inhibitors LY294002 and wortmannin to H69 SCLC cells, for 20 minutes prior to cell lysis, caused a concentration-dependent inhibition of basal PKB activity (Figure 3.10). The IC_{50} for LY294002 and wortmannin was $2.7 \pm 0.1 \mu\text{M}$ and $2.0 \pm 0.6 \text{ nM}$ respectively. These values correlate well with those seen for PI 3-kinase inhibition (Table 3.1). It should also be noted that when wortmannin and LY294002 were added directly to PKB immunoprecipitates no inhibition of PKB activity was seen. This confirmed that these inhibitors were not acting on PKB activity directly, but rather through PI 3-kinase. Furthermore, PKB activity was unaffected by the p70^{s6k} inhibitor rapamycin confirming that PKB lies upstream of p70^{s6k} (Figure 3.9).

These data indicated that basal PKB is elevated in SCLC cells and that this activity is a direct consequence of constitutive PI 3-kinase activity.

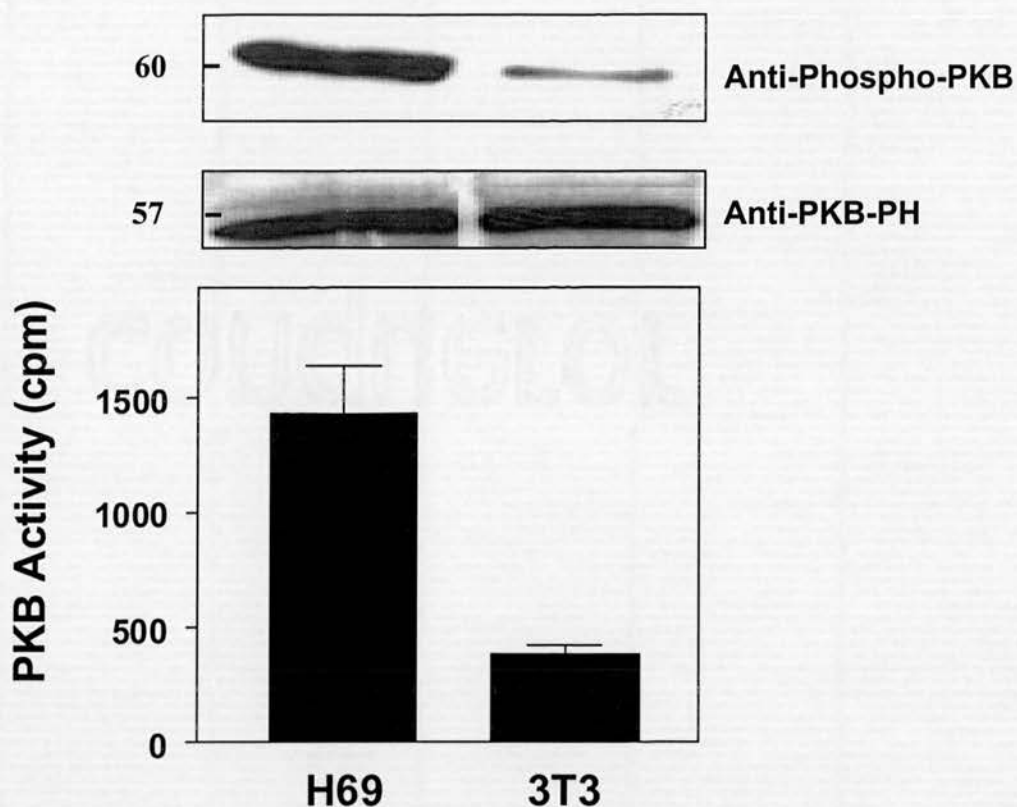


Figure 3.8

Comparative PKB expression and activity in H69 SCLC cells and Swiss 3T3 cells. H69 SCLC cells and Swiss 3T3 cells which had been ‘quiesced’ for 24 hours prior to experimentation were lysed in PKB lysis buffer and the lysates split. The bottom panel shows comparative PKB activity. PKB was immunoprecipitated from cell lysates using an anti-PKB-PH domain antibody and PKB activity was assayed in an *in vitro* kinase reaction using ‘Crosstide’ as substrate as described in ‘Materials and Methods’. PKB activities are expressed in cpm and are the means \pm SD of two independent experiments performed in duplicate. The upper panels show PKB expression using either an anti-PKB-PH domain antibody (middle panel) or an anti-phospho-PKB antibody that recognises phosphorylation of PKB at serine 473 (top panel). Blots representative of three independent experiments are shown and molecular weight markers in kDa are indicated at the left hand margin.

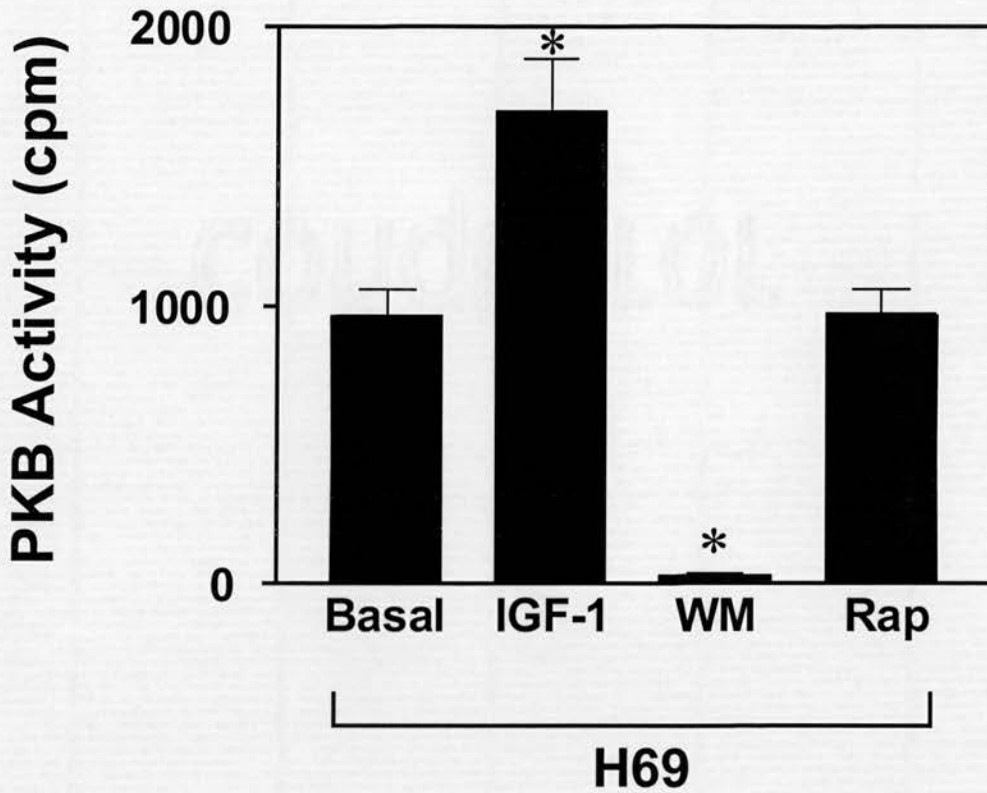


Figure 3.9

PKB activity in SCLC cells. H69 SCLC cells which had been ‘quiesced’ for 24 hours prior to experimentation were thoroughly washed and then equilibrated in PBS for 1 hour before being treated with IGF-1 (100 ng/ml for 5 minutes), wortmannin (WM) (100 nM for 20 minutes) or rapamycin (Rap) (20 nM for 20 minutes) or diluent alone (basal). PKB was immunoprecipitated from cell lysates using an anti-PKB-PH domain antibody and PKB activity was assayed in an *in vitro* kinase reaction using ‘Crosstide’ as substrate as described in ‘Materials and Methods’. Results are expressed in cpm and are the means \pm SEM of three independent experiments performed in duplicate. * $p < 0.01$ compared with basal values.

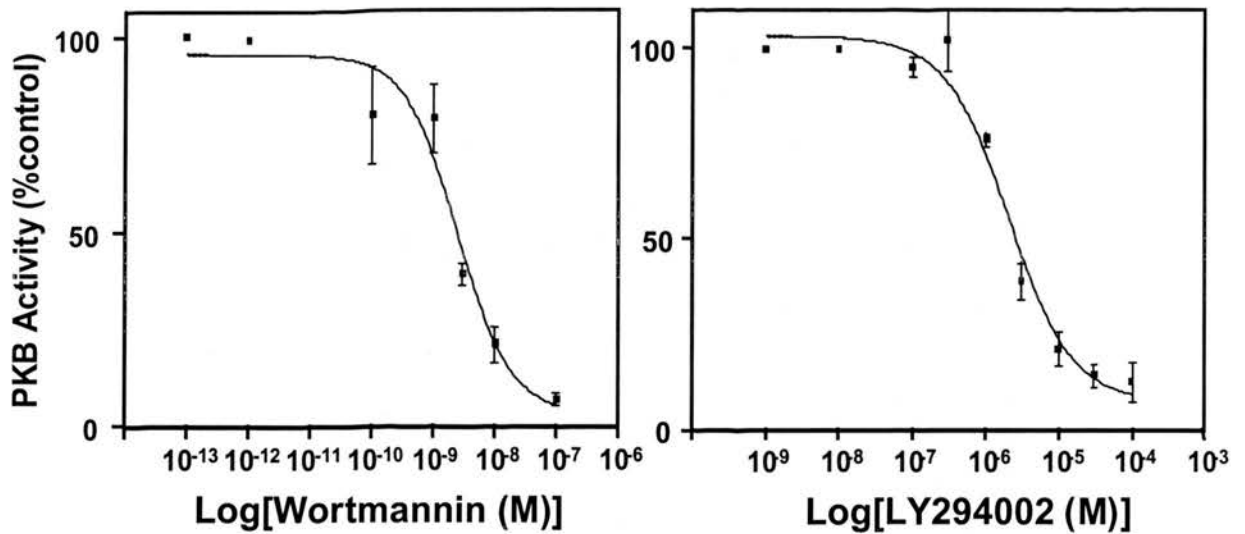


Figure 3.10

Concentration dependent inhibition of basal PKB activity by PI 3-kinase

inhibitors in SCLC cells. SCLC cells which had been ‘quiesced’ for 24 hours prior to experimentation were thoroughly washed and then equilibrated in PBS for 1 hour before being treated with wortmannin (left) or LY294002 (right) for 20 minutes before lysis. PKB was immunoprecipitated from cell lysates using an anti-PKB-PH domain antibody and PKB activity was assayed in an *in vitro* kinase reaction using ‘Crosstide’ as substrate as described in ‘Materials and Methods’. Each point represents the mean \pm SEM of three independent experiments performed in duplicate.

3.2.6 Functional consequences of constitutive PI 3-kinase activity

Having established that PI 3-kinase is constitutively active in SCLC cells, the biological consequences of blocking this upregulated pathway were examined. It has been known for several years that PI 3-kinase is involved in cell growth. Studies of the mutants of the polyoma virus middle T antigen that were defective in transforming ability initially identified PI 3-kinase as necessary for transformation by middle T (Whitman et al., 1985; Kaplan et al., 1986). Subsequent work involving mutations of *v-src* and the PDGF receptor correlated the stimulation of PI 3-kinase activity with transformation and mitogenesis respectively (Cantley et al., 1991). There is also evidence for PI 3-kinase regulating pathways controlling apoptosis. Inhibition of PI 3-kinase can block the ability of survival factors to protect various cell types from programmed cell death (Yao and Cooper, 1995; Yao and Cooper, 1996). Recent reports have revealed that PKB is also involved in regulating cell survival. Activated forms of PKB have been shown to protect Cos-7 epithelial cells from apoptosis induced by ultraviolet irradiation (Kulik et al., 1997) and to protect neuronal cells from cell death induced by the withdrawal of the survival factor IGF-1 (Dudek et al., 1997).

3.2.7 SCLC cell growth is PI 3-kinase-dependent

SCLC cells were grown in serum-free SITA medium in the presence of increasing concentrations of LY294002 and cell number was counted at day 9. LY294002 caused a marked concentration-dependent reduction in SCLC cell number in liquid growth with IC₅₀ values of 10.0 ± 0.8 μM, 2.9 ± 0.2 μM and 13.3 ± 0.8 μM for H69, H345 and H510 cell respectively (Figure 3.11) compared with control cells that received DMSO only (final concentration 0.01%). These figures are similar to the values obtained for PI 3-kinase and PKB activity inhibition by LY294002 in SCLC cells (Table 3.1). Similar experiments using wortmannin did not show any inhibition of growth between treated and control cells. Previous studies have shown that wortmannin has a relatively short half-life (Powis et al., 1994; Brown et al., 1995). A recent study has shown that although 100 nM wortmannin can completely inhibit PI 3-kinase activity for the first few minutes following addition, PI 3-kinase activity can be detected after one hour.

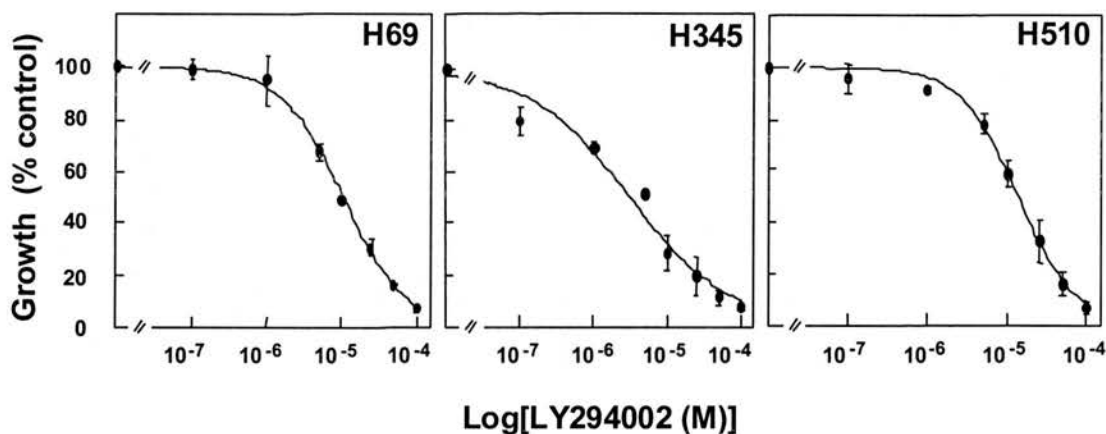


Figure 3.11

The effect of LY294002 on SCLC cell growth. H69, H345 and H510 SCLC cells (1×10^5) were washed and then incubated in fresh SITA medium in the presence of increasing concentrations of LY294002 as shown. Cell number was determined on day 9 using a Coulter counter. The results are expressed as a percentage of cell growth in the presence of diluent alone (DMSO, final concentration 0.01%). 100% cell growth represents approximately 1.5×10^6 cells in the H69 cell line and 1.0×10^6 cells in the H345 and H510 cell lines. Each point represents the mean \pm SEM of three to five independent experiments performed in triplicate.

After 2-3 hours wortmannin is no longer able to inhibit PI 3-kinase activity (Jones et al., 1999). These findings explain why a single addition of wortmannin at the beginning of the experiment was insufficient to inhibit growth over a nine day period.

Seufferlein and Rozengurt (1996) initially showed that rapamycin was able to inhibit SCLC cell growth. In order 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 LY294002 and rapamycin both alone and in combination. Figure 3.12 shows that the addition of a maximally effective concentration of LY294002 (100 μ M) was able to inhibit H69 and H345 SCLC cell growth by 92% over a nine day period. However, a maximally effective concentration of rapamycin (20 nM) caused only a 6.5% and 33% reduction in growth over the same time period in H69 and H345 cells respectively. Incubation of H69 and H345 cells with a combination of 10 μ M LY294002 and 20 nM rapamycin caused a 62% and 68% inhibition of growth respectively over 9 days (Figure 3.12). These results suggest that PI 3-kinase exerts its growth effects by both p70^{s6k}-dependent and independent pathways in SCLC.

3.2.8 SCLC cell survival is PI 3-kinase-dependent

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 apoptosis was 8%. Figure 3.13 shows the dose-dependent increase in apoptosis with increasing concentrations of LY294002.

3.2.9 LY294002 inhibits SCLC cell tumorigenicity and neuropeptide-stimulated colony growth

The ability to form colonies in agarose semi-solid 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 tumour in specimens taken from SCLC (Carney et al., 1980). A number of neuropeptides have been shown to stimulate SCLC cell

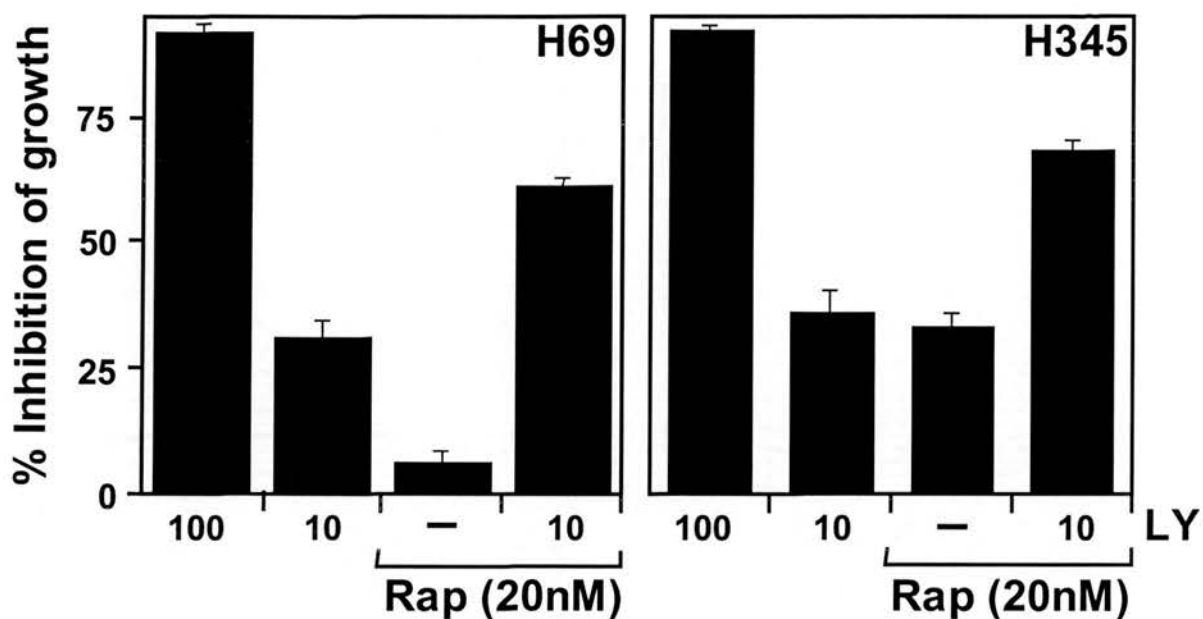


Figure 3.12

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) or rapamycin (Rap) alone or in combination, at the concentrations indicated. On day 9 cell number was measured using a Coulter Counter. Results expressed as percentage inhibition of growth compared with control cell growth are the means \pm SEM of three to five independent experiments performed in triplicate.

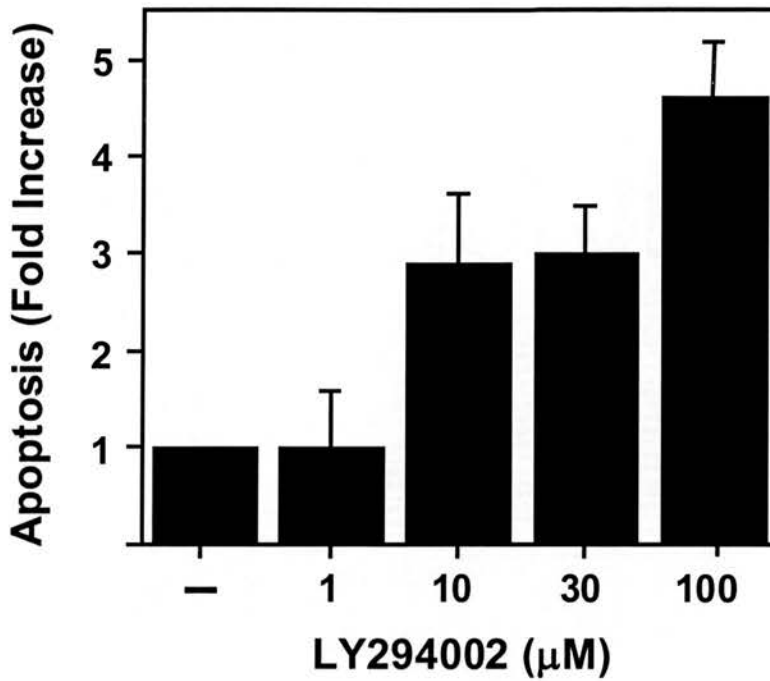


Figure 3.13

The effect of increasing concentrations of LY294002 on apoptosis in SCLC cells. H69 SCLC cells (5×10^4 /ml), 3-5 post-passage were incubated in 'quiescent' medium overnight before being treated with increasing concentrations of LY294002. After 24 hours cells were removed and apoptosis was determined using ethidium bromide and acridine orange as described in 'Materials and Methods'. The basal level of apoptosis was 8%. The results shown are the mean \pm SD for two independent experiments performed in triplicate.

growth *in vivo* giving rise to the autocrine and paracrine growth loop theory of SCLC proliferation (Sethi and Rozengurt, 1991). The effect of LY294002 on SCLC colony formation in agarose semi-solid medium was studied. LY294002 inhibited basal colony formation of all three SCLC cell lines by up to 80% (Figure 3.14). As shown previously, the neuropeptides vasopressin, gastrin and bombesin caused a marked stimulation of colony formation in H69, H510 and H345 cells respectively. However, the addition of vasopressin, gastrin or bombesin failed to rescue the LY294002-induced inhibition of colony formation in these cell lines (Figure 3.14). Thus, PI 3-kinase appears to play a critical role in sustaining the anchorage-independent growth of SCLC.

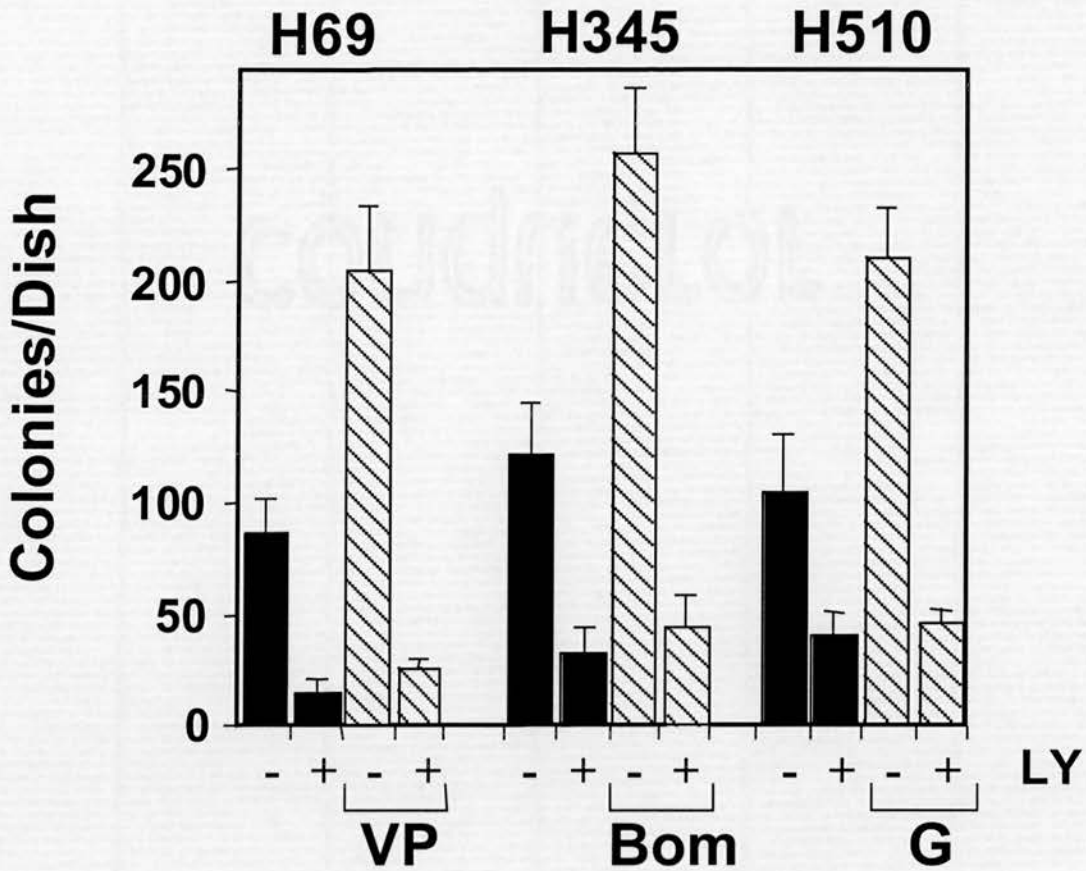


Figure 3.14

Effect of LY294002 on SCLC cell growth in semi-solid medium. H69, H345 and H510 SCLC cells, 3-5 days post passage, were washed and 1×10^4 viable cells/ml were plated in SITA medium containing 0.3% agarose on top of a base of 0.5% agarose in SITA medium as described in 'Materials and Methods'. Both layers contained either no neuro peptide additions (filled bars) or 50 nM vasopressin (VP), 50 nM bombesin (Bom) or 100 nM gastrin (G) (hatched bars) 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 and are the means \pm SD from two independent experiments.

3.3 DISCUSSION

In this chapter the role of PI 3-kinase and PKB in the regulation of SCLC cell proliferation, anchorage-independence and apoptosis have been examined.

Basal PI 3-kinase activity levels in SCLC cell lines are high compared with basal PI 3-kinase activity levels in a panel of untransformed cells types. Furthermore, basal PtdIns(3,4,5)P₃ levels in SCLC cell lines are elevated compared to basal PtdIns(3,4,5)P₃ levels in untransformed cells. Similarly, it has been shown that PKB, a key effector of PI 3-kinase, is partially activated in H69 SCLC cells under control conditions. Taking these data together it is proposed that PI 3-kinase is constitutively active in SCLC cells and that as a consequence, PKB has elevated basal activity. We have also demonstrated that inhibition of PI 3-kinase activity blocked SCLC cell growth in liquid culture and colony formation in soft agarose as well as stimulating apoptosis.

Although PI 3-kinase activation has been implicated in anchorage-independent growth, metastasis and cell invasion (Shaw et al., 1997b; Keely et al., 1997) there has, until recently, been limited evidence for dysregulation of the PI 3-kinase/PKB pathway in human tumours.

Following the identification of the AKT8 retrovirus from a rodent T-cell lymphoma, the cellular homologue of *v-akt* was cloned and found to be a 57 kDa protein serine/threonine kinase (Coffer and Woodgett, 1991; Jones et al., 1991a; Bellacosa et al., 1991). The human homologues *AKT1* and *AKT2* were subsequently identified and cloned (Staal, 1987) and the human *AKT1* locus has been mapped to chromosome 14q32 (Staal et al., 1988), a region frequently affected by translocations and inversions in human T-cell leukaemia/lymphoma, mixed-lineage childhood leukaemia and clonal T-cell proliferations in ataxia telangiectasia (Bertness et al., 1990). Analysis of a panel of human primary gastric adenocarcinomas showed a 20-fold amplification of *AKT1*. *AKT2* has been mapped to the chromosome region 19q13.1-q13.2 and has been shown to be amplified and overexpressed in several ovarian carcinoma and pancreatic cell lines (Cheng et al., 1992; Miwa et al., 1996).

A large scale study of *AKT2* alterations in ovarian and breast tumours showed amplification in 12.1% of ovarian and 2.8% of breast carcinomas. Amplification of *AKT2* was more frequent in undifferentiated tumours suggesting that *AKT2* alterations may be associated with tumour aggressiveness (Bellacosa et al., 1995). Although these workers studied *AKT* expression they did not examine AKT activity status.

To date, there has been only one other report of increased PI 3-kinase activity in a human solid tumour. Phillips et al. (1998) examined PI 3-kinase activity in a panel of 37 human colorectal tumours and compared it to PI 3-kinase activity in normal-appearing colonic mucosa from the same patients. They found that 86% of tumours demonstrated increased PI 3-kinase activity with an overall mean increase of 3.8 fold. However, the frequency and extent of increased PI 3-kinase activity in the tumours did not correlate with the degree of differentiation or tumour stage (Phillips et al., 1998).

The cause of the elevated PI 3-kinase activity in SCLC cells is unclear. Protein expression in the SCLC cell lines was similar to that in the untransformed control cell lines confirming that it was not simply due to an increase in PI 3-kinase expression.

PI 3-kinase can be activated by a wide variety of growth stimuli including PDGF, EGF, insulin, thrombin and NGF. Prior to assaying for PI 3-kinase activity every effort was made to remove all exogenous growth factors by extensive washing prior to experimentation. Hence, it seems unlikely that exogenous growth factors were stimulating PI 3-kinase under control conditions. The control cell lines used were washed and incubated in an identical fashion and under these conditions basal PI 3-kinase activity levels were very low.

A number of oncogenes including *ras* and *src* are able to activate PI 3-kinase. Transformation of many adherent cell types, including fibroblasts and epithelial cells, by cytoplasmic oncogenes such as *ras* and *src* is accompanied by the ability to

grow in suspension. SCLC cells, *in vitro*, grow in an anchorage-independent fashion. However, several studies have failed to show any evidence of activating *ras* mutations in SCLC (Mitsudomi et al., 1991; Suzuki et al., 1990). Pp60^{src} activity has also been reported as being low in SCLC cells (Budde et al., 1994). Therefore, it seems unlikely that the elevated basal PI 3-kinase activity in SCLC is due to *ras* or *src* transformation. Interestingly in the study by Phillips et al., (1998) there was no association between elevated PI 3-kinase activity in colorectal carcinoma and the frequency of *ras* mutations, again suggesting that *ras* mutations are not responsible for increased PI 3-kinase activity in colorectal cancers.

It is possible that elevated PI 3-kinase activity in SCLC may be due to a gene mutation. Such a mutation might either encode for a receptor protein that is continuously switched on, leading to constitutive PI 3-kinase activation, or for one of the PI 3-kinase subunits. Examples of both types of mutation have been described recently. Two groups have published data showing oncogenic mutation of PI 3-kinase. Jimenez et al., (1998) found that CMN-5 cells from a murine thymic lymphoma failed to express the p85 regulatory subunit of PI 3-kinase. However, a 65 kDa protein was identified that was not found in untransformed thymocytes. Analysis of p110 α immunoprecipitates from CMN-5 cells indicated that p65 associated with p110 in place of p85. Once cloned, expression of p65-PI 3-kinase in an unrelated cell line showed that p65-PI 3-kinase induced constitutive activation of PI 3-kinase and contributed to cellular transformation. Shayesteh et al., (1999) examined *PIK3CA*, which encodes the p110 α catalytic subunit of PI 3-kinase. They showed that *PIK3CA* is frequently increased in copy number in ovarian carcinomas and that the increased copy number is associated with increased *PIK3CA* transcription, p110 α protein expression and PI 3-kinase activity. In contrast, Moscatello et al., (1998) transfected NIH 3T3 cells with a mutated epidermal growth factor receptor and showed that PI 3-kinase was constitutively activated as a result. Whether one or more mutations of the types alluded to above are responsible for constitutive PI 3-kinase activity in SCLC cells remains to be established.

In addition to examining PI 3-kinase activity in SCLC cells we also measured basal PtdIns(3,4,5)P₃ levels in two SCLC cell lines and compared them to those found in Swiss 3T3 fibroblasts and Rat-2 fibroblasts. Levels of PtdIns(3,4,5)P₃ in the SCLC cell lines investigated were several fold higher than those in the fibroblast cell lines. This data supports the hypothesis that PI 3-kinase is constitutively active in SCLC.

Phospholipid levels are regulated on the one hand by kinases and on the other by phosphatases. PI 3-kinase phosphorylates the 3' position of the inositol head group of PtdIns(4,5)P₂ to generate PtdIns(3,4,5)P₃. PtdIns(3,4,5)P₃ can be dephosphorylated at the 5' position by an SH2-containing 5-phosphatase called SHIP to generate PtdIns(3,4)P₂ (Erneux et al., 1998). Recently a novel 3-phosphatase called PTEN (phosphatase and tensin homologue deleted on chromosome ten) has been described which can dephosphorylate the 3' position of both PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ to reverse the reactions catalysed by PI 3-kinase. PTEN was identified by two groups in 1997 as a candidate tumour suppressor gene located at 10q23 (Li et al., 1997; Steck et al., 1997). Initially the *PTEN* cDNA sequence suggested that it was a member of the protein-tyrosine phosphatase gene superfamily and PTEN was thought to be a dual-specificity phosphatase (Li et al., 1997; Steck et al., 1997). However, Maehama et al., (1998) have shown that PTEN can dephosphorylate the 3' position of PtdIns phosphates both *in vitro* and *in vivo*. This finding has led to a model for how PTEN acts as a tumour suppressor. Normally PtdIns(3,4,5)P₃ and PKB levels are low in the absence of growth factor stimulation. However, PTEN-deficient tumour cell lines and tumours derived from PTEN-deficient mice, exhibit high basal PtdIns(3,4,5)P₃ and PKB activity levels (Myers et al., 1998; Li and Sun, 1998; Haas-Kogan et al., 1998). PTEN mutations have now been identified in a number of cancers including glioblastoma multiforme, melanoma, advanced prostate cancers and endometrial carcinoma (Rasheed et al., 1997; Wang et al., 1997; Guldborg et al., 1997; Cairns et al., 1997; Risinger et al., 1997). Several groups have sought PTEN mutations in both NSCLC and SCLC. Although deletions in 10q22-25 are common in SCLC, PTEN mutations appear to be rather less common. PTEN mutations have been reported as occurring in between 8 and 18% of SCLC cell lines (Forgacs et al., 1998; Kim et al., 1998; Yokomizo et al.,

1998). Interestingly, neither the H69 nor the H345 cell lines have been shown to contain PTEN mutations (Yokomizo et al., 1998). Therefore, it would appear that the high PtdIns(3,4,5)P₃ and increased PKB activity seen in our SCLC cell lines are a result of constitutive PI 3-kinase activity rather than due to a PTEN mutation.

Recent work has shown that high levels of PKB, either as a result of PTEN mutation or constitutive PI 3-kinase activity, may have significant implications for cell survival both within and outwith their normal environments. Inhibition of PI 3-kinase is known to block the ability of survival factors to protect various cell types from apoptosis. Expression of constitutively active PI 3-kinase or PKB protects Cos-7 epithelial cells from apoptosis induced by ultraviolet irradiation (Kulik et al., 1997) and can protect neuronal cells from cell death induced by withdrawal of IGF-1 (Dudek et al., 1997). Khwaja et al., (1997) have recently shown that that activated PI 3-kinase and PKB can protect MDCK cells from apoptosis induced by detachment of adherent cells from their extracellular matrix. Detachment induced apoptosis, which has been christened 'anoikis', is the mechanism whereby cells are prevented from surviving outwith their normal environment. In MDCK cells, detachment from extracellular matrix leads to a rapid decrease in the levels of PI 3-kinase and PKB and vice versa on re-attachment. Transfection of constitutively active PI 3-kinase or PKB protects MDCK cells from apoptosis in suspension, while inhibition of PI 3-kinase caused enhanced apoptosis in adhered cells (Khwaja et al., 1997). The finding of constitutively active PI 3-kinase and PKB in SCLC cells provides a mechanism by which SCLC cells are able to grow in the absence of contact with extracellular matrix. We propose that constitutive activation of PI 3-kinase/PKB in SCLC cells may mimic integrin-dependent signal transduction and promote cell proliferation and protection from apoptosis in the absence of contact with extracellular matrix.

The addition of the PI 3-kinase inhibitor LY294002 to SCLC cells in liquid culture resulted in a marked concentration-dependent reduction in cell number. The addition of LY294002 to SCLC cells showed a modest but consistent increase in the percentage of cells undergoing apoptosis. Furthermore, LY294002 markedly

inhibited basal colony formation of SCLC cells in agarose semi-solid medium. The ability of cells to grow in soft agarose is a feature of anchorage-independence and is pathognomonic of the transformed phenotype, correlating with tumorigenicity and invasiveness of the tumour (Carney et al., 1980). Interestingly, the inhibition of colony formation induced by LY294002 could not be overcome by the addition of neuropeptides. Neuropeptides are known to be potent growth factors for SCLC, activating the MAP kinase pathway through G-protein coupled receptors (Seufferlein and Rozengurt, 1996a). These results suggest that PI 3-kinase may be required for neuropeptide mitogenic signalling in SCLC. It remains to be established whether MAP kinase activation can be potentiated by growth factor/integrin-dependent PI 3-kinase activation. Recent evidence suggests that growth factors do not induce DNA synthesis in 3T3 cells in the absence of integrin activation (Renshaw et al., 1997). This appears to result from the inability of growth factors to activate the MAP kinase pathway in the absence of integrin activation. However, 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.

In addition to using PI 3-kinase inhibitors to study the functional role of the PI 3-kinase/PKB pathway in SCLC, rapamycin, a specific inhibitor of p70^{s6k} was also used. P70^{s6k} is implicated in a wide range of cellular processes including protein synthesis, translation of specific mRNA species and progression from G₁ to S phase of the cell cycle (Chou and Blenis, 1995; Ferrari and Thomas, 1994; de Groot et al., 1994). P70^{s6k} is known to be an effector of PI 3-kinase and p70^{s6k} has been shown to be constitutively phosphorylated in SCLC cells (Seufferlein and Rozengurt, 1996b). Our group has recently shown that p70^{s6k} is also constitutively active in SCLC and that this is PI 3-kinase-dependent (Moore et al., 1998). Although PKB and p70^{s6k} are known to be physiological targets of PI 3-kinase, it is unclear whether these enzymes lie on the same signalling pathway or parallel pathways. The observation that constitutively active forms of PKB led to the activation of p70^{s6k} implied that PKB may mediate mitogenic signalling through p70^{s6k} (Burgering and Coffey, 1995). However, the situation is likely to be more complicated since PDK1 has recently been shown to be capable of inducing p70^{s6k} activation independently of PKB (Alessi

et al., 1998; Pullen et al., 1998). Thus PI 3-kinase mediated p70^{s6k} activation may well occur via alternative pathways. In support of this, certain agonists can differentially induce PKB and p70^{s6k} (Conus et al., 1998). The data presented here supports the latter view. It was found that PI 3-kinase regulation of SCLC cell growth was mediated by both p70^{s6k}-dependent and independent pathways. The latter pathway may signal through PKB.

In summary it has been 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. In addition, PI 3-kinase/PKB activation can promote anchorage-independent growth, protecting cells in suspension from undergoing apoptosis. The pathways driven by constitutive PI 3-kinase/PKB may therefore be responsible for the ability of SCLC cells to survive and proliferate in an anchorage-independent state leading to the development of a particularly aggressive metastatic phenotype. These results agree with an increasing cohort of data that points towards the PI 3-kinase/PKB pathway being upregulated in a number of tumour types and playing a key role in carcinoma proliferation and metastasis.

CHAPTER 4

EXTRACELLULAR MATRIX REGULATION OF SMALL CELL LUNG CANCER CELL APOPTOSIS

4.1 INTRODUCTION

SCLC is characterised by an extremely aggressive clinical course. Untreated, the median survival from diagnosis is only 5 to 12 weeks depending upon the stage at diagnosis. Two-thirds of patients will have evidence of distant metastases at the time of diagnosis thereby excluding the possibility of surgical resection for the majority of cases. In the 1970s SCLC was a candidate to be the first 'curable' solid malignancy because of its apparent marked sensitivity to chemotherapy. However, despite initial response rates of up to 80%, these tend to be short-lived with a median duration of 6-8 months. Following treatment, the median survival for patients with limited disease and extensive disease are 14-20 and 8-13 months respectively (Osterlind et al., 1986).

In the clinic, tumours often shrink dramatically in size following chemotherapy and may, at the macroscopic level, disappear entirely on chest radiographs and on conventional cross-sectional scans. However, the recurrence of tumour after chemotherapy implies the existence of a number of tumour 'stem' cells resistant to cytotoxic therapy. Apoptosis is now recognised as the predominant mechanism by which chemotherapeutic agents bring about cell death (Hannun, 1997). An initial study examining the mechanism of action of etoposide showed that etoposide induced internucleosomal DNA fragmentation (Kaufmann, 1989). Since then, the spectrum of chemotherapeutic agents causing apoptosis has expanded progressively. Vincristine, cis-platinum, cyclophosphamide, adriamycin, paclitaxel and 5'-fluorouracil have all been shown to exert their effects through apoptosis (reviewed in Hannun 1997).

In cancer biology it is becoming increasingly apparent that many tumour cells circumvent the normal apoptotic mechanisms in order to prevent destruction by chemotherapeutic drugs. One might hypothesise that, *in vivo*, some factor(s) in the local environment is able to provide a survival signal thereby accounting for the protection of SCLC cells from chemotherapy-induced apoptosis. One possible candidate to provide such a signal is ECM. Many epithelial and endothelial cell types are dependent upon adhesion to ECM for their continued survival undergoing apoptosis on detachment from matrix. Although transformed cells are characterised by their ability to grow in the absence of contact with a solid ECM, solid tumour cells exist, *in vivo*, in a state of dynamic interplay between anchorage-dependence and independence. Of note is the observation that after chemotherapy recurrent SCLC frequently occurs at the same sites as the original tumour rather than at distant sites. This suggests that tumour cells that are adhered to matrix may be relatively protected from chemotherapy compared with cells that are anchorage-independent.

Tumour stroma varies greatly in amount between different tumours and may make up more than ninety percent of the total tumour mass in tumours such as scirrhous carcinomas of the breast and stomach. In such cases almost all of the tumour mass may be constituted by dense collagenous connective tissue and it may be difficult to find tumour cells. At the other end of the spectrum, medullary carcinomas may contain only minimal connective tissue between clusters of malignant cells. Up until now, the ECM composition of SCLC tumours has received little attention.

The aim of this part of the study was to examine the role of anchorage-dependence on chemotherapy-induced apoptosis in SCLC.

4.2 RESULTS

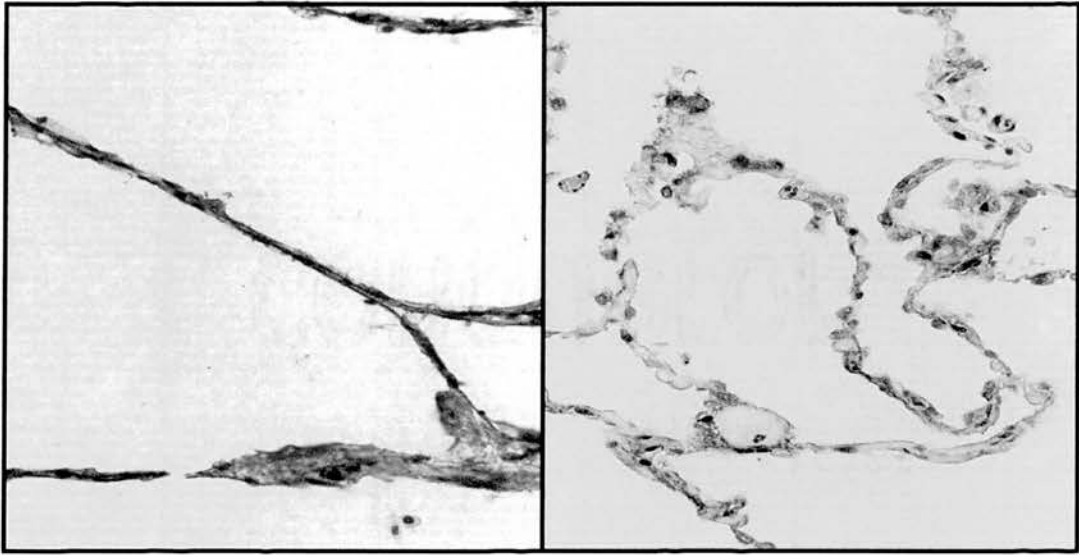
4.2.1 Extracellular matrix composition of SCLC tumours

The ECM composition of 23 SCLC surgical resection specimens from the University of Edinburgh pathology files was determined by immunohistochemical staining for fibronectin, laminin, collagen IV and tenascin.

Normal areas of the lung showed laminin and collagen IV localised to the basement membranes of alveoli, septae, blood vessels and bronchial glands (Figure 4.1), whereas fibronectin staining showed diffuse weak positivity throughout the pulmonary interstitium (Figure 4.1). No tenascin immunoreactivity was seen in normal lung. In SCLC sections extensive staining for fibronectin, collagen IV and tenascin was seen in 56%, 87% and 61% of tumours respectively (Table 4.1). Extensive staining was defined as staining covering more than 50% of the section area. Furthermore, focal areas of immunoreactivity for fibronectin, collagen IV and tenascin were seen in 35%, 9% and 39% of sections respectively (Table 4.1). Fibronectin, collagen IV and tenascin were expressed mainly in areas of reactive host connective tissue, which were present as extensive areas of scarring or as stromal bands delineating pockets of invasive tumour cells (Figure 4.2). Furthermore, it was noted that in SCLC sections the basement membranes of alveoli invaded by tumour were considerably thickened due to increased expression of fibronectin, laminin and collagen IV compared with adjacent uninvolved areas (Figure 4.2). In addition to ECM deposition it was also noted that in 26% and 16% of tumours respectively, fibronectin and laminin staining was visible within the cells themselves (Figure 4.2)

In three cases matched metastatic lymph nodes were also examined and in all cases fibronectin, collagen IV and tenascin immunoreactivity was as extensive as in the primary tumours.

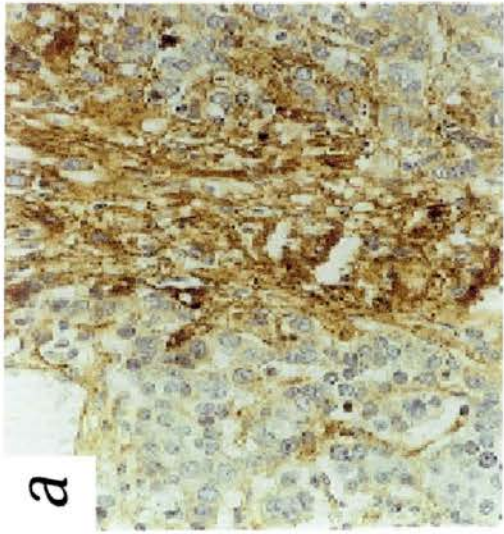
Thus, *in vivo*, SCLC cells exist in an ECM rich environment.



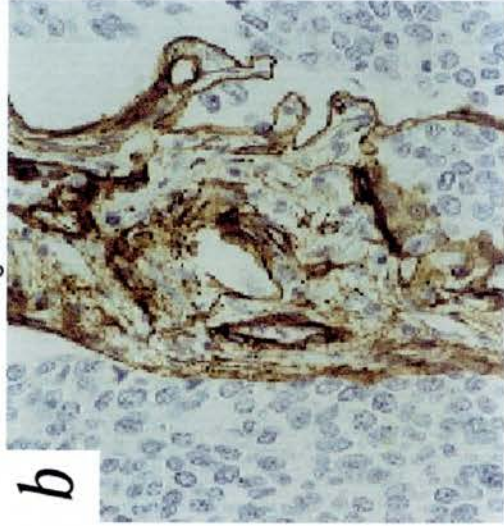
Collagen IV

Fibronectin

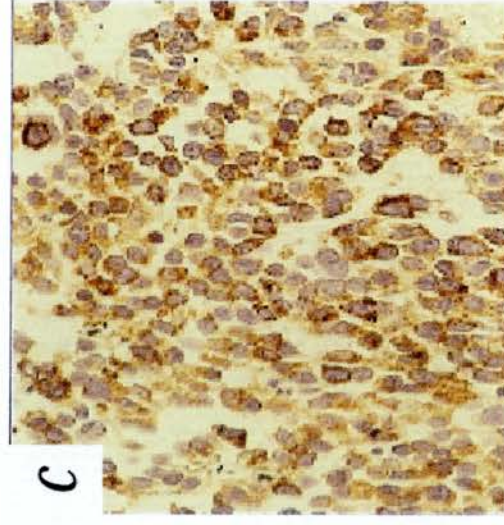
Figure 4.1
Immunohistochemical staining of ECM proteins in normal bronchial epithelium
Magnification X400.



Fibronectin



Collagen IV



Laminin

Figure 4.2

Immunohistochemical staining of ECM proteins around SCLC cells *in vivo*. Fibronectin (A) and collagen IV (B) immunoreactivity in SCLC. C, intra/pericellular immunoreactivity for laminin. Magnification X400.

ECM No of cases		STROMA						TUMOUR CELLS			
		Extensive		Focal		Negative		Positive		Negative	
		No	(%)	No	(%)	No	(%)	No	(%)	No	(%)
Fibronectin	23	13	(56)	8	(35)	2	(9)	6	(26)	17	(74)
Laminin	19	0	(0)	0	(0)	19	(100)	3	(16)	16	(84)
Tenascin	23	14	(61)	9	(39)	0	(0)	0	(0)	23	(100)
Collagen IV	23	20	(87)	2	(9)	1	(4)	0	(0)	23	(100)

Table 4.1

Expression of ECM proteins in primary lung SCLC resection specimens. After immunohistochemistry, ECM protein staining within areas of carcinoma stroma was recorded on a semi-quantitative scale: no staining, focal staining or extensive staining (>50% of section). Sections were also assessed for intracellular ECM protein expression. Data represent numbers of cases, with percentages in parentheses.

4.2.2 SCLC cells adhere to extracellular matrix proteins through $\beta 1$ integrins

In order to investigate the physiological relevance of ECM deposition in SCLC cell tumours we initially investigated whether SCLC cells are able to bind to ECM proteins. The principal family of cell surface receptors that are responsible for anchoring cells to ECM proteins are integrins. The integrin subunit profile of SCLC cells has been studied previously (Sethi et al., 1999; Hirasawa et al., 1994; Falconi et al., 1994). These studies showed that the predominant beta integrin expressed was $\beta 1$. The main alpha integrins expressed on SCLC cells are $\alpha 2$, $\alpha 3$, $\alpha 6$ and αv (Vogel et al., 1990; Elices et al., 1991; Hynes, 1992). Figure 4.3 shows that H69 SCLC cells adhered to fibronectin, laminin and collagen IV in a concentration-dependent manner. The EC_{50} values for H69 cell adhesion to fibronectin, laminin and collagen IV were 20 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$ and 12 $\mu\text{g/ml}$ respectively. Integrin function is known to be divalent cation dependent (Humphries, 1996). Therefore, if SCLC binding to ECM is integrin-mediated, divalent cations such as Mn^{2+} should potentiate binding to ECM components and EDTA, a cation chelator, should block binding. Figure 4.4 shows that when SCLC cells were allowed to adhere to fibronectin in culture medium (containing 0.65 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and 0.54 mM $\text{Mg}(\text{SO}_4) \cdot 7\text{H}_2\text{O}$) adhesion rates increased from $41 \pm 6\%$ in the absence of Mn^{2+} to $88 \pm 5\%$ in the presence of Mn^{2+} (1 mM) ($p < 0.05$). Co-incubation with EDTA (1.8 mM) decreased the adhesion level to $6 \pm 3\%$ ($p < 0.05$). Furthermore, when SCLC cells were incubated on a fibronectin matrix in the presence of a function-blocking $\beta 1$ antibody adhesion to fibronectin was reduced from $41 \pm 6\%$ to $8 \pm 2\%$ ($p < 0.05$). Similar results were obtained for laminin. These results show that SCLC cells adhere to ECM proteins in an integrin-dependent fashion and that $\beta 1$ integrin plays a significant role.

4.2.3 Extracellular matrix proteins protect SCLC cells from chemotherapy-induced apoptosis

In order to test the hypothesis that ECM proteins might protect SCLC cells from chemotherapy-induced apoptosis SCLC cells were treated with a variety of chemotherapeutic agents in the presence and absence of various ECM proteins.

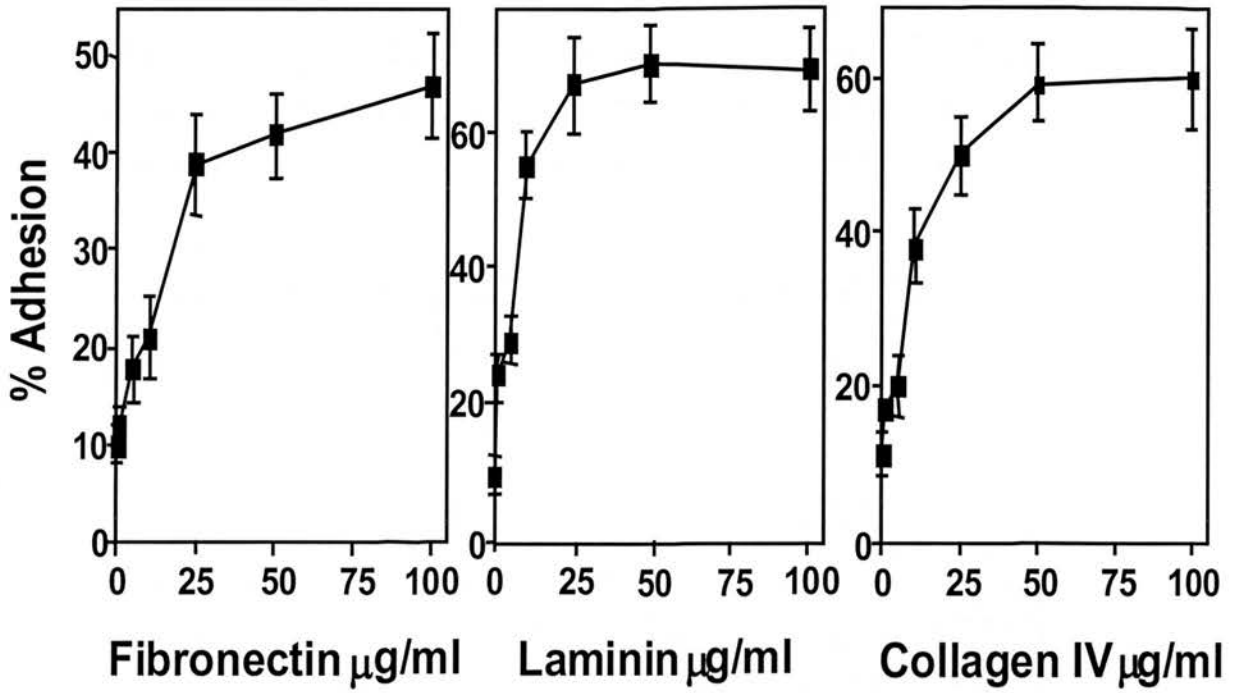


Figure 4.3

Adhesion of SCLC cells to ECM proteins. H69 SCLC cells (5×10^5) which had been washed twice in RPMI 1640 were added to wells which had been pre-coated overnight with increasing concentrations of fibronectin, laminin or collagen IV as shown and adhesion measured as described in 'Materials and Methods'. Adhesion is expressed as a percentage of maximal adhesion, which was defined as adhesion to poly-L-lysine (10 µg/ml). The results shown are the means \pm SEM of three experiments performed in triplicate.

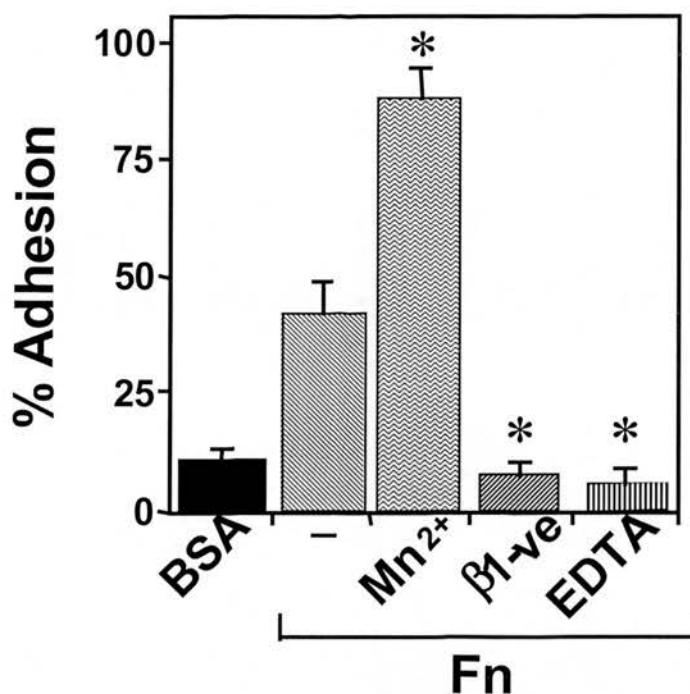


Figure 4.4

SCLC adhesion to fibronectin is $\beta 1$ integrin mediated. H69 SCLC cells (5×10^5), which had been washed twice in RPMI 1640, were added to wells that had been pre-coated overnight with either BSA 1% or fibronectin 20 $\mu\text{g/ml}$ (Fn) in the absence (-) or presence of Mn^{2+} (1mM), a $\beta 1$ function-blocking antibody, P4C10 (1:100) ($\beta 1\text{-ve}$) or EDTA (1.8 mM) and adhesion measured as described in 'Materials and Methods'. Adhesion is expressed as a percentage of maximal adhesion, which was defined as adhesion to poly-L-lysine (10 $\mu\text{g/ml}$). The results shown are the means \pm SEM of three experiments performed in triplicate. * $P < 0.05$ compared with basal fibronectin values.

Adhesion of SCLC cells to laminin, fibronectin and collagen IV markedly protected them against apoptosis induced by chemotherapeutic agents compared to those grown on plastic as measured using Giemsa staining (Figure 4.5). For example in H345 cells, in response to 50 $\mu\text{g/ml}$ etoposide, fibronectin reduced apoptosis from 75% to 14% (Figure 4.5). Similar results were seen when H510 cells were treated with doxorubicin in the presence and absence of fibronectin or laminin (Figure 4.5).

In order to corroborate these findings apoptosis was also assessed by two other methods. Figure 4.6 shows the effect of etoposide (25 $\mu\text{g/ml}$) on SCLC apoptosis in the presence and absence of fibronectin as measured by acridine orange/ethidium bromide staining (left hand panel) and by immunoassay of cytoplasmic histone-associated DNA (right hand panel). Using acridine orange/ethidium bromide staining the basal levels of apoptosis in H69 cells on plastic was $17 \pm 2\%$ and this increased to $43 \pm 3\%$ in the presence of etoposide. However, for cells plated on fibronectin the rates were $10 \pm 2\%$ and $13 \pm 1\%$ respectively. Similar results were obtained using the immunohistochemical method. This data supports our findings using Giemsa staining.

In order to determine whether ECM-mediated protection from chemotherapy-induced apoptosis was integrin-mediated or whether it was simply due to a non-specific adhesive effect, SCLC cells were plated onto plastic, poly-L-lysine or ECM and treated with etoposide. Cells adhere to poly-L-lysine in a non-specific fashion based solely on electrical charge. Figure 4.7 shows that when cells were plated onto plastic or poly-L-lysine and treated with etoposide (50 $\mu\text{g/ml}$) the level of apoptosis was $82 \pm 10\%$ and $89 \pm 8\%$ respectively compared with $20 \pm 4\%$ for untreated cells. However, when H69 cells were pre-plated on collagen IV and treated with the same concentration of etoposide the level of apoptosis fell to 23% ($p < 0.05$). This ECM-mediated protection from etoposide-induced apoptosis was abolished by the addition of a function blocking $\beta 1$ antibody.

Taken together, these data show that ECM-mediated protection from chemotherapy-induced apoptosis is integrin-dependent.

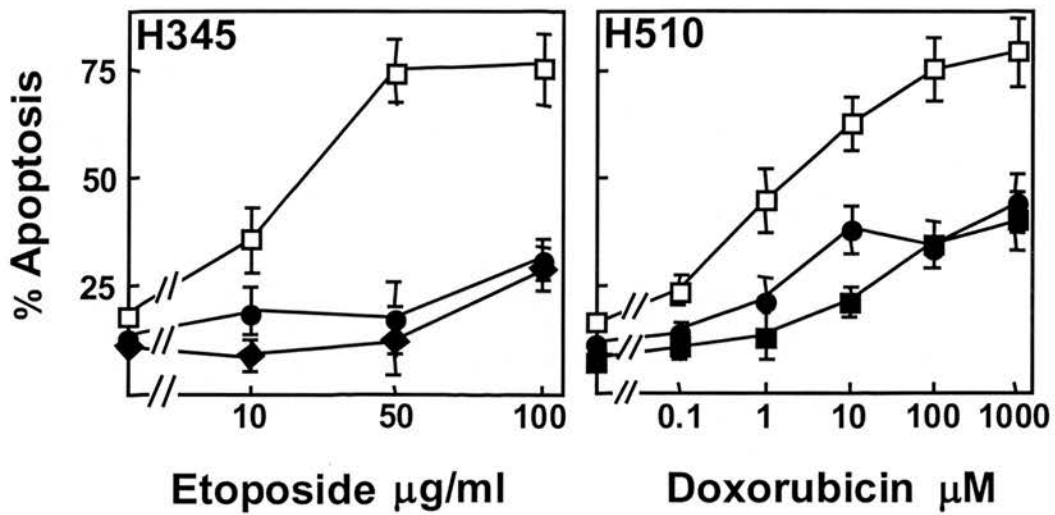


Figure 4.5

Effect of ECM proteins on chemotherapy-induced apoptosis in SCLC cells.

SCLC cells (1×10^5 /ml) that had been cultured in SITA medium were seeded into 96 well plates pre-coated with fibronectin 20 μ g/ml (●), laminin 10 μ g/ml (■) or collagen IV 10 μ g/ml (◆) or BSA 1% (□). After 1 hour increasing concentrations of etoposide or doxorubicin were added as shown and cells further incubated at 37°C. After 72 hours cells were detached from the plates, aspirated and cytospun onto glass slides. Apoptosis was detected by Giemsa staining as described in 'Materials and Methods'. Each point represents the mean \pm SEM of quadruplicates performed in duplicate plates. Initial experiments were performed by Dr T Sethi.

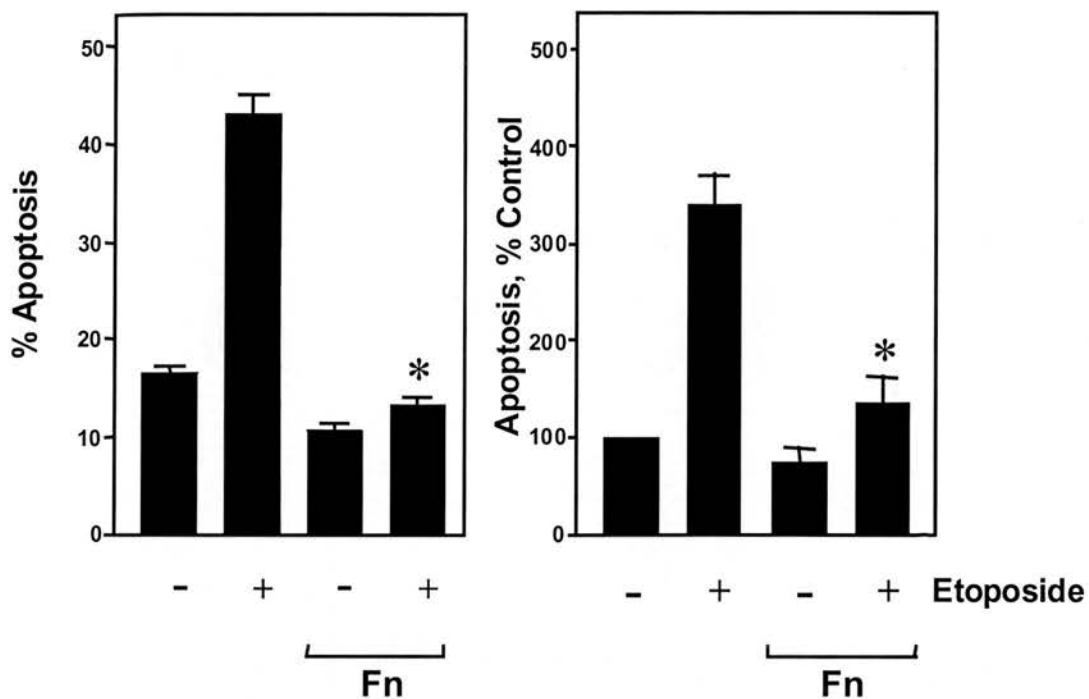


Figure 4.6

Effect of fibronectin on chemotherapy-induced apoptosis in H69 SCLC cells.

SCLC cells (1×10^5 /ml) that had been cultured in SITA medium were seeded into 96 well plates pre-coated with fibronectin (20 μ g/ml) or 1% BSA. After 1 hour etoposide (25 μ g/ml) was added and cells further incubated at 37°C. After 24 hours plates were spun at 200 x g for 4 minutes, supernatant aspirated and apoptosis detected by either acridine orange/ethidium bromide (left hand panel) or by a commercial cell death ELISA kit (right hand panel) as described in 'Materials and Methods'. Each point represents the mean \pm SEM of three independent experiments performed in triplicate. * $p < 0.05$ compared with cells on plastic treated with etoposide.

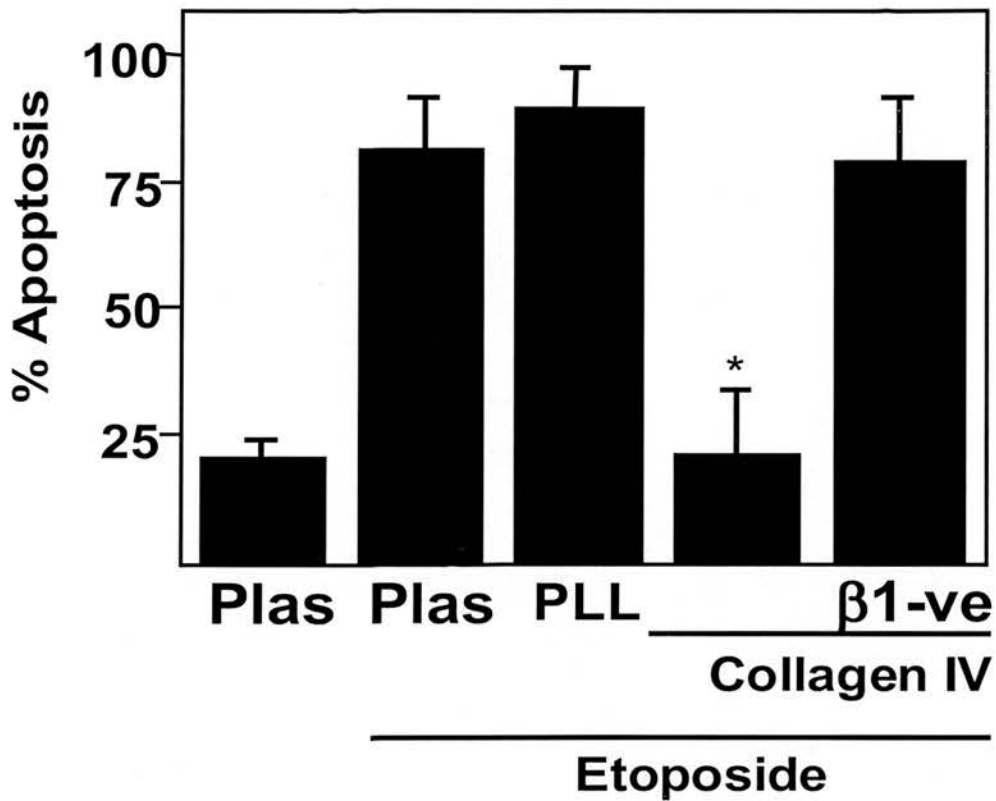


Figure 4.7

ECM-mediated protection from chemotherapy-induced apoptosis is mediated by $\beta 1$ integrins. H69 SCLC cells (1×10^5) were plated into 96 well plates which had been pre-coated with either no coating (PLAS), poly-L-Lysine (PLL) ($10 \mu\text{g/ml}$) or collagen IV ($25 \mu\text{g/ml}$) in the presence or absence of a function-blocking $\beta 1$ antibody (P5D2, 1:100). After 1 hour etoposide ($50 \mu\text{g/ml}$) was added and cells further incubated at 37°C for 24 hours. Apoptosis was assessed by Giemsa staining. Each point is the mean \pm SEM of three independent experiments performed in triplicate. * $p < 0.05$ compared with cells on plastic or PLL treated with etoposide.

4.2.4 Mechanism of ECM-mediated protection of SCLC from chemotherapy-induced apoptosis

Having made the observation that SCLC cells are protected from chemotherapy-induced apoptosis by adhesion to ECM proteins the mechanism by which this protection was occurring was investigated.

4.2.5 Effect of fibronectin on SCLC cell growth in liquid culture

We initially considered whether the protective effect conferred by ECM might be artefactual and simply due to an increase in total cell number when cells were grown on ECM proteins compared with plastic rather than due to an inhibition of apoptosis. In order to examine this we incubated SCLC cells in liquid culture on pre-plated fibronectin under identical conditions to those used for the chemotherapy assays. Figure 4.8 shows that over the first six days there was no significant difference in cell number between cells grown in the presence of ECM and control cells grown on plastic. Over a longer time period (9 days) fibronectin did stimulate a modest increase in cell number. However, the chemotherapy assays were performed between 24 and 96 hours. Therefore, it was thought that the survival effect was due to a true anti-apoptotic effect rather than simply due to an alteration in the growth rate of cells on matrix compared with those on plastic.

4.2.6 SCLC adhesion to ECM induces tyrosine phosphorylation

Early studies on integrin-dependent cell adhesion and signalling demonstrated that cell ligation to ECM proteins was accompanied by integrin aggregation and that this clustering could trigger increased tyrosine phosphorylation of a number of intracellular proteins (Guan et al., 1991; Kornberg et al., 1991; Hanks et al., 1992). Tallett et al. (1996) have previously shown that tyrosine phosphorylation is a mitogenic signal in SCLC cells and that the regulation of the level of protein tyrosine phosphorylation represents a critical determinant of whether SCLC cells survive and proliferate, or die by apoptosis. Therefore, the effect of fibronectin on tyrosine phosphorylation in SCLC cells was examined.

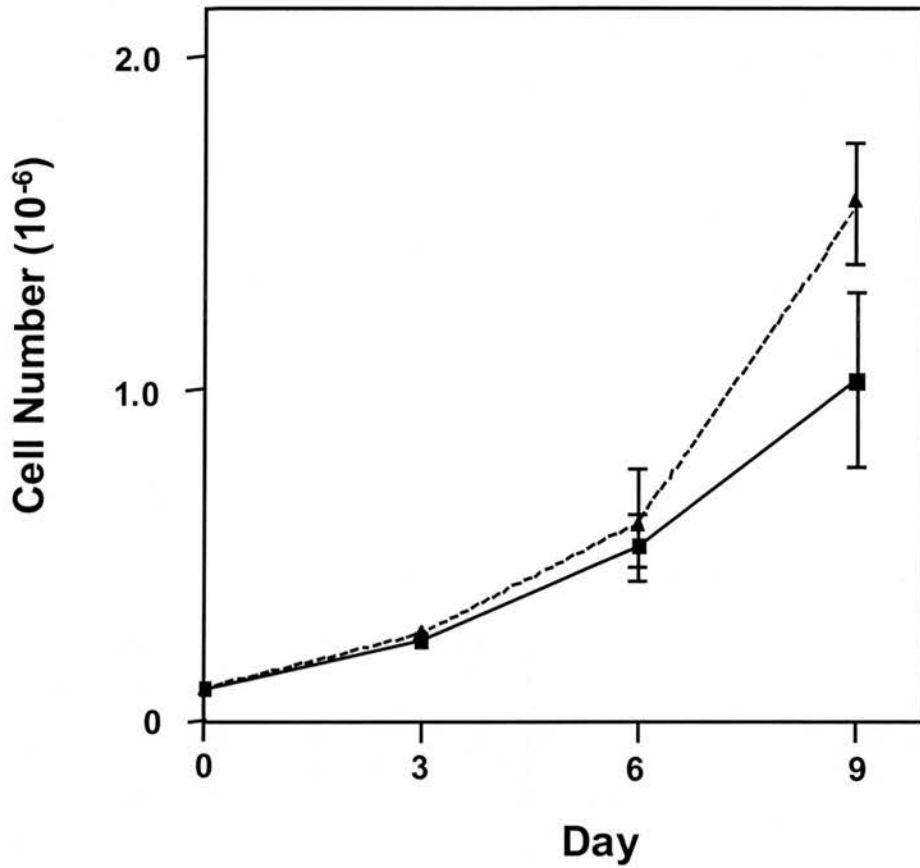


Figure 4.8

Effect of fibronectin on SCLC liquid growth. H69 SCLC cells (1×10^5), 3-5 days post-passage, were washed and then incubated in fresh SITA medium in 24 well plates which either had no coating (solid line) or had been pre-coated with fibronectin ($20 \mu\text{g/ml}$) (dashed line). Absolute cell number was determined at intervals as shown using a Coulter counter. Data from a representative experiment is shown.

Figure 4.9 shows a 'Western blot' using an anti-phosphotyrosine antibody to determine the effect of fibronectin on tyrosine phosphorylation in H69 SCLC cells. Figure 4.9 shows that when SCLC cells are adhered to fibronectin there is an increase in tyrosine phosphorylation of a number of proteins as indicated by an increase in the number and density of bands (lane 2) compared with those seen in cells plated on plastic (lane 1). The principal bands affected lay at 58, 60, 75, 85 and 120 kDa. This was unaffected by treatment of cells with etoposide (lane 3). However, this effect could be completely blocked by pre-treatment with the tyrosine kinase inhibitor tyrphostin-25 (lane 4) or by pre-treatment with a function-blocking β 1 antibody (lane 5).

The inhibition of phosphotyrosine kinase activity seen with tyrphostin-25 was not simply due to inhibition of SCLC cell adhesion to fibronectin because tyrphostin-25 has been shown not to have any effect on cell adhesion to fibronectin, laminin or collagen IV over a 24 hour period (Sethi et al., 1999).

These data show that SCLC cell resistance to chemotherapy can be mediated by a tyrosine phosphorylation-dependent mechanism.

4.2.7 Chemotherapy-induced caspase-3 activation is blocked by ECM-induced phosphorylation

A wide variety of chemotherapeutic agents have been shown to promote cell death through apoptosis. It is becoming increasingly clear that chemotherapeutic agents induce apoptosis via similar mechanisms. The final common pathway for the induction of apoptosis centres on caspase-3 (see Figure 1.5). Caspase-3 is activated by proteolytic cleavage of pro-caspase-3. Following activation, caspase-3 cleaves the chaperone inhibitor of caspase activated deoxyribonuclease releasing DNAase activity which causes DNA fragmentation in nuclei.

Pro-caspase-3 expression was measured as a marker of caspase-3 activation. When pro-caspase-3 expression is high, caspase-3 levels are low and vice-versa. Caspase-3 activation was also correlated with apoptosis in parallel samples.

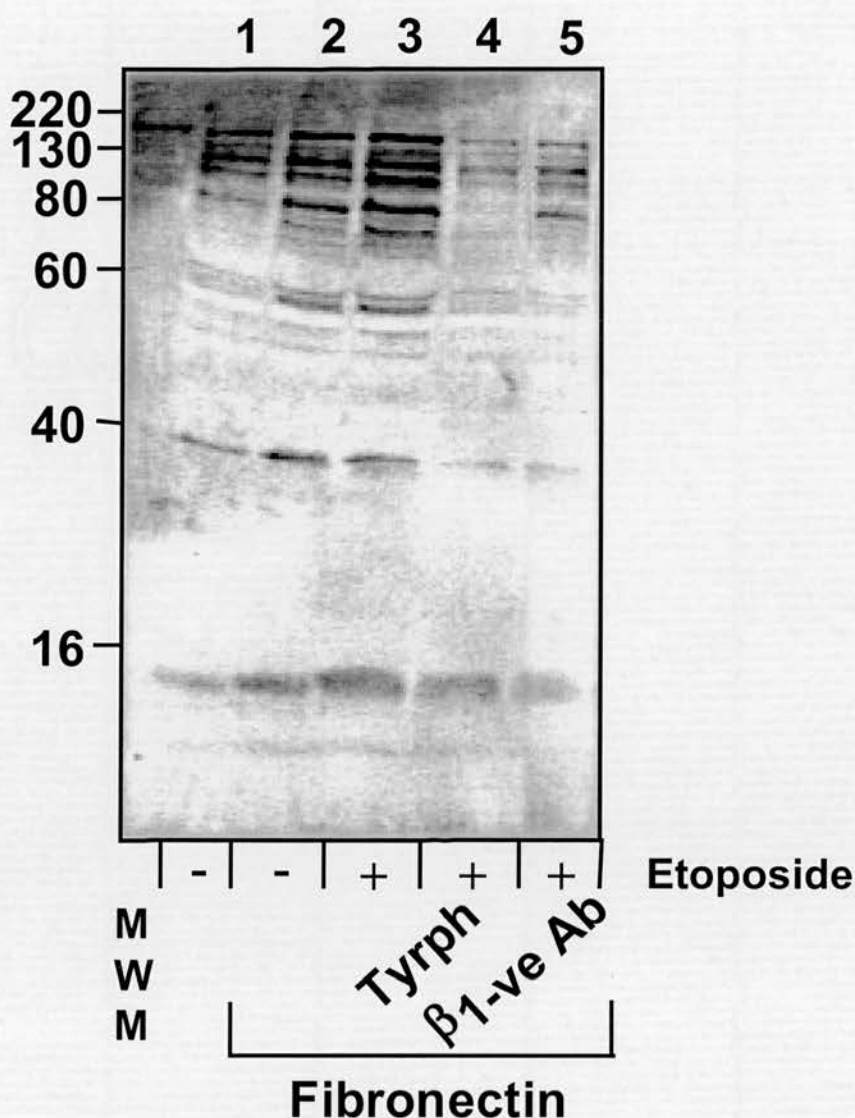


Figure 4.9

The effect of fibronectin on tyrosine phosphorylation in SCLC cells. H69 SCLC cells that had been washed and pre-incubated in SITA medium for 12 hours were seeded at a density of 5×10^6 /ml into 24 well plates in the presence or absence of pre-coated fibronectin (20 μ g/ml) with or without etoposide (25 μ g/ml), tyrphostin-25 (25 μ M) or a function blocking β 1 antibody, 4B4 (10 μ g/ml). After 72 hours cell lysates were assessed by 'Western' blot analysis as described in 'Materials and Methods' with antibodies against phosphotyrosine (50:50, PY20:4G10). The gel shown is representative of three independent experiments. MWM, molecular weight markers; Tyrph, tyrphostin-25; β 1-ve Ab, function blocking β 1 antibody.

Figure 4.10 shows that compared with basal untreated cells (column 1), etoposide markedly stimulated apoptosis and pro-caspase-3 cleavage in H69 SCLC cells grown on plastic or poly-L-lysine (column 2) increasing the apoptosis rate from $16 \pm 0.75\%$ to $43 \pm 1.8\%$. A similar effect was seen in cells grown in the presence of both etoposide and the tyrosine kinase inhibitor tyrphostin-25 (column 3). However, etoposide-induced pro-caspase-3 cleavage was completely blocked by co-incubation with either the broad-spectrum caspase inhibitor z-Val-Ala-DL-Asp-fluoromethylketone (z-VAD) (column 4) or the tyrosine phosphatase inhibitor, sodium orthovanadate (column 5). Sodium orthovanadate and z-VAD reduced the apoptosis rate from $43 \pm 1.8\%$ to $16 \pm 0.25\%$ and $15 \pm 0.35\%$ respectively, very similar to basal levels. When SCLC cells were grown on fibronectin, etoposide-induced pro-caspase-3 cleavage (column 7) was inhibited compared with that seen in cells grown on plastic (column 2). The apoptosis rate for etoposide treated cells on fibronectin was $13.4 \pm 0.6\%$ compared to $43 \pm 1.8\%$ for cells on plastic. However, this ECM-mediated protection could be completely abrogated either by a function blocking $\beta 1$ antibody (column 9) or by tyrphostin-25 (column 10). Treatment with the $\beta 1$ antibody or the tyrosine kinase inhibitor caused the apoptosis rates to rise to $42 \pm 0.65\%$ and $44 \pm 2.45\%$ respectively. Of note, tyrphostin-25 alone, had no significant effect on fibronectin plated cells (column 8). Furthermore, the function blocking $\beta 1$ antibody had no effect on etoposide-induced apoptosis in the absence of fibronectin. These results show that $\beta 1$ integrin-mediated adhesion to fibronectin protects SCLC cells from etoposide-induced caspase-3 activation by activating phosphotyrosine signalling.

4.2.8 Fibronectin protection of etoposide-induced apoptosis is blocked by a PtdIns 3-kinase inhibitor

Khwaja et al., (1997) demonstrated that PI 3-kinase mediates matrix-induced survival of normal epithelial cells. Inhibition of PI 3-kinase induced apoptosis in adherent epithelial cells while a constitutively active PI 3-kinase blocked anoikis. Given that PI 3-kinase requires tyrosine phosphorylation for full activation we reasoned that PI 3-kinase might be involved in mediating ECM protection from chemotherapy-induced apoptosis.

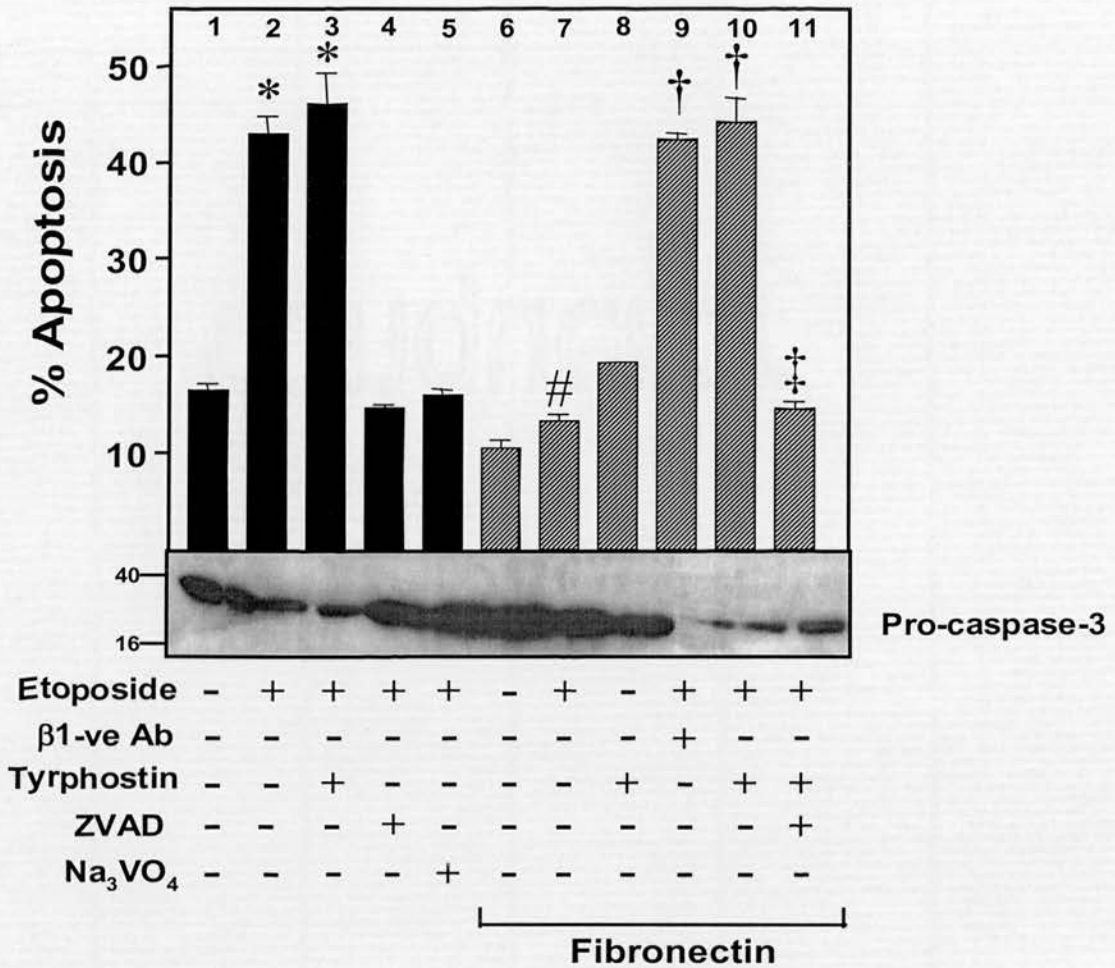


Figure 4.10

The effect of fibronectin on etoposide-induced apoptosis and caspase-3 activation.

H69 SCLC cells that had been washed and pre-incubated in SITA medium for 12 hours were seeded at a density of 5×10^6 /ml into 24 well plates in the presence or absence of pre-coated fibronectin (20 μ g/ml) with or without etoposide (25 μ g/ml), tyrphostin-25 (25 μ M), the function blocking β 1 antibody, (4B4, 10 μ g/ml), z-VAD (100 μ M) or sodium orthovanadate, (Na_3VO_4 , 200 μ M). After 72 hours apoptosis was determined by acridine orange/ethidium bromide staining. The data represent the mean \pm SEM of three independent experiments performed in triplicate. * $p < 0.05$ compared with Column 1. # $p < 0.05$ compared with Column 2. † $p < 0.05$ compared with Column 7. ‡ $p < 0.05$ compared with column 10. In a parallel experiment cell lysates were assessed by 'Western' blot analysis with antibodies against pro-caspase 3. A representative blot is shown. Figures in the left hand margin are molecular weights in kDa.

Using an ELISA based system to measure apoptosis it was found that, as before, etoposide-induced apoptosis was inhibited when cells were plated on fibronectin compared to those on plastic (Figure 4.11; column 6 versus column 2). The proapoptotic effects of etoposide, LY294002 and tyrphostin-25 alone on ECM plated cells were similar in magnitude (columns 6, 7 and 8 respectively). However, when apoptosis was measured in matrix-plated cells that had been co-incubated with either etoposide and tyrphostin-25 (column 9), or etoposide and LY294002 (column 10) the ECM-mediated protective effect was lost. These data suggest that PI 3-kinase is one of the tyrosine-phosphorylated proteins that signals integrin-dependent resistance to chemotherapy.

4.2.9 Effect of fibronectin on topoisomerase II α expression and activity

Etoposide initiates its cytotoxic action by acting as a specific inhibitor for DNA topoisomerase II. Topoisomerase II is a nuclear enzyme that effects unknotting and relaxation of supercoiled DNA molecules by a process of introducing transient double strand breaks through which the strands of an intact helix can pass. Topoisomerase poisoning results in the trapping of enzyme molecules on DNA as cleavable complexes and the generation of potentially lethal lesions.

Therefore the ability of ECM-induced tyrosine phosphorylation to block etoposide-induced topoisomerase II inhibition was investigated. Topoisomerase II activity was measured by the ATP-dependent decatenation of a high molecular weight DNA network. Figure 4.12 shows that while etoposide is able to inhibit topoisomerase II activity, tyrphostin-25 alone had no effect on either topoisomerase II expression or activity. Adhesion to fibronectin did not affect the ability of etoposide to inhibit topoisomerase II activity. Furthermore, adhesion to fibronectin did not alter topoisomerase II expression. These data suggest that ECM-induced protection from chemotherapy-induced apoptosis acts at a point downstream of DNA damage.

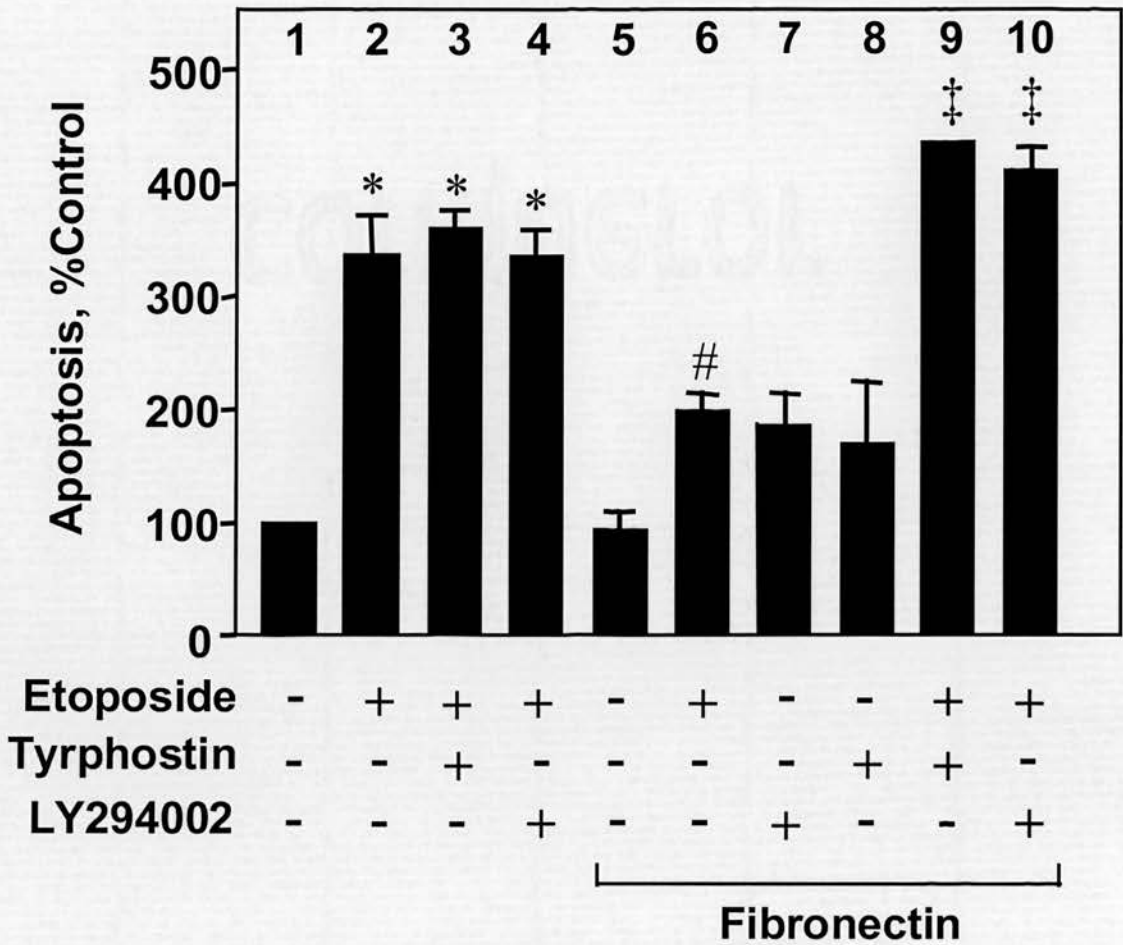


Figure 4.11

Fibronectin protection of etoposide-induced apoptosis is blocked by a PI 3-kinase inhibitor. H69 SCLC cells (5×10^4) which had been washed and incubated in SITA medium for 24 hours were seeded into 96 well plates in the presence or absence of pre-coated fibronectin (20 $\mu\text{g/ml}$). One hour after seeding LY294002 (30 μM) or tyrphostin-25 (25 $\mu\text{g/ml}$) were added and cells were further incubated at 37°C for 24 hours. Apoptosis was assessed using a commercial cell death ELISA kit as described in 'Materials and Methods'. The data shown are the means \pm SEM of three independent experiments performed in triplicate. * $p < 0.05$ compared with Column 1. # $p < 0.05$ compared with Column 2. ‡ $p < 0.05$ compared with Column 6.

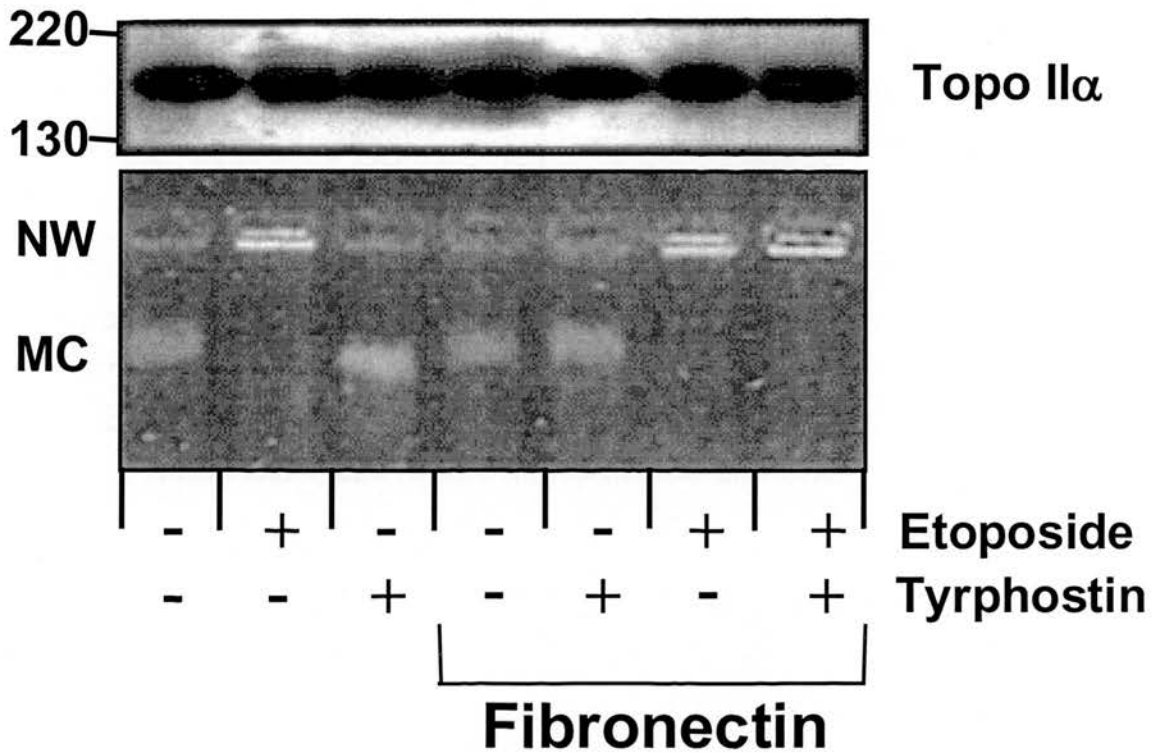


Figure 4.12

The effect of fibronectin on topoisomerase II α expression and activity. H69 SCLC cells that had been washed and pre-incubated in SITA medium for 12 hours were seeded at a density of 5×10^6 /ml into 24 well plates in the presence or absence of pre-coated fibronectin (20 μ g/ml) with or without etoposide (25 μ g/ml) or tyrphostin-25 (25 μ M). After 72 hours human topoisomerase II α expression was assessed by 'Western' blotting using antibody to human topoisomerase II α (upper panel). Topoisomerase II α activity was measured as described in 'Materials and Methods' (lower panel). NW, high molecular weight interlocking kDNA network; MC, decatenated individual DNA mini-circles. The gel shown is representative of two independent experiments performed in duplicate.

4.3 DISCUSSION

The results presented in this chapter show that, *in vivo*, SCLC cells are surrounded by an extensive stroma of fibronectin, laminin, collagen IV and tenascin at both primary and secondary metastatic sites. SCLC cells adhere to ECM proteins in a predominantly $\beta 1$ integrin-mediated fashion. When SCLC cells are adhered to ECM proteins they are protected from the pro-apoptotic effects of chemotherapeutic agents. The mechanism underlying this phenomenon is due to an ECM-induced increase in tyrosine phosphorylation, which blocks chemotherapy-induced caspase activation.

The extent of ECM deposition around SCLC cells, *in vivo*, has not been appreciated before. Two other groups have also reported laminin and collagen IV within basement membrane surrounding tumour cell nests in SCLC (Paakko et al., 1990; Wetzels et al., 1992). The extent of tenascin deposition in the SCLC sections examined is notable. Unlike laminin and collagen IV expression, which was confined to basement membranes, tenascin was expressed in areas of reactive host tissue scarring and within broad stromal bands around groups of tumour cells. Tenascin is transiently expressed in many developing organs but is generally absent in normal adult tissues. However, tenascin is re-expressed in adult tissues that are actively remodelling following denervation (Gatchalian et al., 1989) and in wound healing (Mackie et al., 1988) as well as in the stroma of a wide variety of tumours such as gliomas (Bourdon et al., 1983), breast carcinomas (Mackie et al., 1987) and basal cell carcinomas (Stamp, 1989). Similarly, neo-expression of some fibronectin isoforms, while absent in normal interstitium, occurs in tumour stroma (Carnemolla et al., 1989).

The origin of tumour associated extracellular matrix is unclear. In some sections stained for laminin and fibronectin, individual cells stained positively for ECM proteins suggesting that the cells themselves might be synthesising the proteins. Confirmation of intracellular production of ECM proteins would have to come from

in situ hybridisation studies or PCR. Mastroianni et al., (1993) noted that 15% of SCLC sections they examined showed positive cytoplasmic staining for laminin. Similarly tenascin production by melanoma cells and glioma cells has also been documented (Herlyn et al., 1991; Bouterfa et al., 1999). Although SCLC cells appear capable of producing ECM proteins the majority of tumour ECM probably arises from interstitial fibroblasts. Such a mechanism occurs in gliomas and malignant melanomas. Chiquet-Ehrismann et al., (1989) showed that the mammary carcinoma cell line MCF-7 stimulates tenascin production in normal interstitial fibroblasts by secreting TGF- β . In cultured fibroblasts TGF- β modulates the splicing of the primary fibronectin transcript leading to the production of other ECM proteins including laminin and tenascin (Borsi et al., 1990). Our group and others have found that SCLC cell lines are able to produce TGF- β *in vitro* (Fischer et al., 1994) and personal communication from Dr T Sethi).

The neo-expression of tenascin and fibronectin in the interstitium of tumours suggests that tumour cells may condition their stroma in order to support their growth and modulate their propensity to invasion. In addition to TGF- β , SCLC cells produce a variety of growth factors, cytokines and inflammatory mediators including IGF-1, gastrin-releasing peptide and IL-8, which may, through complex autocrine and paracrine interactions, be able to modulate the immediate environment of SCLC cells (Macaulay et al., 1990; Sethi and Rozengurt, 1991). IGF-1 and gastrin-releasing peptide are mitogenic for both SCLC cells and fibroblasts. IL-8 is an essential angiogenic factor for non-small cell lung cancer in nude mice (Arenberg et al., 1996). Therefore, *in vivo*, SCLC may create a specialised environment as a consequence of autocrine and paracrine effects that, using an analogy to inflammation, likens SCLC to 'a wounding reaction' with the laying down and remodelling of ECM, growth factor release and neo-vascularisation.

Integrins are the principal family of cell surface receptors that mediate the attachment of cells to extracellular matrices. The main beta integrin expressed by SCLC cells is $\beta 1$ and the principle alpha integrins expressed are $\alpha 2$, $\alpha 3$, $\alpha 6$ and αv (Falconi et al., 1994). Therefore, SCLC cells express the appropriate integrins for

binding to fibronectin ($\alpha3\beta1$ and $\alpha v\beta1$) and collagen and laminin ($\alpha2\beta1$, $\alpha3\beta1$ and $\alpha6\beta1$).

Although different tumour types display a great deal of heterogeneity in integrin expression it is now generally accepted that the up- or downregulation of some integrins in poorly differentiated tumours may represent a hallmark of invasive and metastatic behaviour (reviewed in Varner and Cheresch 1996). Decreased expression of integrin $\alpha2\beta1$ has been observed in poorly differentiated breast carcinomas (Zutter et al., 1995) and increased expression of $\alpha v\beta3$ is consistently observed in human metastatic melanomas (Albelda et al., 1990; Danen et al., 1995). Interestingly, while normal bronchial epithelial cells express $\alpha5$, SCLC cells do not (Koukoulis et al., 1997). Furthermore, the $\beta1$ subunit has been shown to be necessary for lung cancer cell migration towards fibronectin, laminin and collagen IV in an *in vitro* hapto- and chemotaxis assay system (Bredin et al., 1998). Although not conclusive, these findings do suggest that SCLC cells express an integrin profile that is in keeping with an aggressive phenotype.

Apoptosis is the process whereby cells are induced to activate their own death and it is crucial for maintaining appropriate cell number and tissue organisation.

Extracellular matrix is known to play a crucial role as a survival factor since the disruption of integrin-mediated cell-matrix interactions induces apoptosis (Frisch and Francis, 1994). In a mammary epithelial cell model, addition of an antibody to $\beta1$ integrin led to disruption of cell-ECM interactions and to an increase in apoptosis (Boudreau et al., 1995).

We have found that extracellular matrix proteins are able to protect SCLC cells from chemotherapy-induced apoptosis. There has been one other report of laminin-induced chemoresistance of SCLC cells (Fridman et al., 1990). These findings have now been extended to show that this is a general property of several ECM proteins found in increased amounts in SCLC tumours *in vivo*. The finding that ECM-mediated chemoresistance could be abrogated by a function-blocking $\beta1$ antibody showed that the effect is $\beta1$ integrin-mediated. One other group has also recently

reported that etoposide-induced DNA strand breakage in xenograft tumour-derived endothelial cells (TDECs) could be inhibited by culturing TDECs on gelatin, collagen IV, laminin, fibronectin or the integrin ligand hexapeptide GRGDSP but not on the integrin peptide GRADSP (Hoyt et al., 1996). DNA damage was also inhibited when TDECs were plated on surfaces coated with antibodies to $\alpha 5$, $\beta 1$ and $\beta 3$ integrin subunits and by clustering integrins with soluble antibodies.

Adhesion of SCLC cells to ECM proteins induced tyrosine phosphorylation and inhibition of tyrosine kinase activity abrogated ECM-induced chemoresistance and promoted apoptosis. Furthermore, etoposide-induced apoptosis could be blocked in the presence of the tyrosine phosphatase sodium orthovanadate. Protein tyrosine phosphorylation has been described previously in response to cell attachment to ECM proteins (Hanks et al., 1992) and on integrin clustering (Kornberg et al., 1991; Kornberg et al., 1992). Recently, protein tyrosine kinase activity has been reported to regulate apoptosis. Abl protein, a non-receptor type tyrosine kinase, inhibited cytokine withdrawal-mediated (Evans et al., 1993) or Fas-mediated apoptosis (McGahon et al., 1995). Inhibition of protein tyrosine kinase activity by its inhibitors such as erbstatin, genistein or tyrphostin-25 has been shown to induce apoptosis in SCLC cells (Tallett et al., 1996; Simizu et al., 1996).

With the developing understanding of mechanisms regulating apoptosis it is becoming increasingly clear that chemotherapeutic agents act through similar mechanisms. Kaufmann et al., (1993) identified proteolytic cleavage of poly (ADP) ribose polymerase (PARP) in response to etoposide. Subsequently this was shown to be due to activation of a specific protease and that it preceded endonuclease activation and DNA fragmentation (Lazebnik et al., 1994). It now appears that many inducers of cell death, including cytokines and chemotherapeutic agents, ultimately converge on the activation of PARP and related proteases, which then trigger the terminal and execution stages of apoptosis. The data presented in this chapter shows that etoposide-induced caspase-3 activation is modulated by ECM-induced tyrosine phosphorylation. ECM-mediated protection from etoposide-induced caspase-3 activation could be blocked by either a $\beta 1$ function-blocking antibody or by a

tyrosine kinase inhibitor. Boudreau et al., (1995) reported that apoptosis of CID-9 mammary epithelial cells could be induced by antibodies to $\beta 1$ integrins or by overexpression of stromelysin-1 which degrades ECM. Expression of interleukin-1 β converting enzyme (ICE) correlated with the loss of ECM and inhibitors of ICE activity prevented apoptosis. These results suggested that ECM regulates apoptosis in mammary epithelial cells through an integrin-dependent negative regulation of ICE expression. Similarly, Simizu et al., (1998) showed that induction of apoptosis by erbstatin in SCLC cells resulted in the activation of caspase-3 (-like) proteases. They too concluded that since erbstatin and herbimycin A do not inhibit other protein kinases, such as protein kinases A and C, tyrosine kinase activity or tyrosine phosphorylated proteins may be a negative regulator of caspase activity. The exact mechanism by which this occurs is not known. It would seem likely that there may be one or more tyrosine phosphorylated proteins involved. We studied PI 3-kinase as a possible candidate. All class Ia PI 3-kinases contain adapter subunits containing SH2 domains. These adapters bind phosphorylated tyrosine residues, thereby linking class Ia PI 3-kinase subunits to tyrosine kinase signalling pathways. Recently there have been a number of publications linking the PI 3-kinase/PKB pathway to cell survival and protection from apoptosis (Khwaja et al., 1997; Kulik et al., 1997; Farrelly et al., 1999). We found that the PI 3-kinase inhibitor LY294002 was able to block the ECM-mediated protective effect against chemotherapy-induced apoptosis in the same way that tyrphostin-25 did. This suggested that PI 3-kinase is one of the tyrosine-phosphorylated proteins that signals integrin-dependent resistance to chemotherapy. One could argue that rather than blocking the ECM-mediated protective pathway with LY294002 or tyrphostin-25, our results simply show the additive effect on apoptosis of etoposide and LY294002 or etoposide and tyrphostin. However, the same effect can be obtained by blocking the ECM-mediated pathway with a function blocking $\beta 1$ antibody. Furthermore, adding a tyrosine phosphatase inhibitor to cells on plastic mimicked the ECM effect. Therefore, we think it more likely that our results do show that blocking tyrosine kinases or PI 3-kinase abrogates the ECM protective effect against chemotherapy-induced apoptosis.

Interestingly, another group has reported that IGF-1 and the IGF-1 receptor can prevent etoposide-induced apoptosis in BALB/c 3T3 cells (Sell et al., 1995). Although they did not examine the mechanism underlying this effect it is likely to be mediated by the PI 3-kinase/PKB pathway, as IGF-1 is a major activator of this pathway. Taken together it would seem likely that PI 3-kinase and PKB are involved in mediating integrin-dependent resistance to chemotherapy. One might speculate that the final mediator inhibiting caspase-3 activation is one of the Bcl-2 family of proteins. PKB phosphorylates BAD thereby preventing BAD from inhibiting the anti-apoptotic proteins Bcl-2 and Bcl-X_L. Recently it has been demonstrated that Bcl-X_L can inhibit caspase-3 activation (Suzuki et al., 1998). Although our group has not been able to demonstrate any alteration in Bcl-2 expression in SCLC cells adhered to fibronectin (personal communication from Dr T Sethi), Simizu et al., (1998) demonstrated that Bax mediated erbstatin-induced caspase-3 activation. Bax, another member of the Bcl-2 family promotes apoptosis and antagonises the function of Bcl-2.

The mechanisms involved in the acquisition of drug resistance by SCLC cells are still unclear. Several drugs used in the treatment of SCLC such as etoposide, teniposide and doxorubicin act on the nuclear enzyme DNA topoisomerase II α by freezing an enzyme-DNA cleavable complex, thereby creating DNA breaks and eventually leading to cell death. Several cellular resistance mechanisms towards topoisomerase II poisons have now been identified. The first mechanism called the altered topoisomerase II multi-drug resistance phenotype, described either a reduction and/or mutation in the enzyme itself (Pommier et al., 1986; Danks et al., 1988). Such a mechanism has been described in an adriamycin-resistant SCLC cell line (de Jong et al., 1990). Two well characterised drug efflux pumps, P-glycoprotein (P-gp) (Borst et al., 1993) and the multi-drug resistance protein (MRP) (Cole et al., 1992) also occur in both SCLC and NSCLC and have been shown in transfection studies to be sufficient to confer resistance. However, their clinical importance is still unclear (Narasaki et al., 1996; Nooter et al., 1996). Of note, H69 SCLC cells, which were used for all experiments examining the mechanism of chemoresistance, do not express P-gp or MRP (Kreisholt et al., 1998).

Hannun (1997) proposed a three phase hypothesis to explain how many disparate chemotherapeutic agents can bring about apoptosis: In phase I each class of chemotherapeutic agent interacts with a specific target such as DNA or RNA, bringing about target injury or dysfunction. In phase II the cell, through as yet poorly defined mechanisms, is able to decipher and assess the specific injury to the chemotherapy target. In the final phase (III) a decision point may exist such that susceptible cells react to the signals generated in response to chemotherapy-induced injury as a go-ahead for the execution phase of apoptosis. Other cell types may preferentially enter programmes of cell cycle arrest or damage repair in response to the same signals.

Based on existing work and the data presented here, the following model for the development of chemoresistance in SCLC cells is proposed (see figure 4.13).

Etoposide acts by inhibiting the re-annealing action of topoisomerase II causing multiple DNA nicks. This persistent DNA damage results in cell cycle delay in S phase and G2/M with subsequent caspase-3 activation leading to apoptosis.

However, β 1-integrin-mediated cell adhesion to ECM proteins results in tyrosine phosphorylation, which, despite persistent chemotherapy-induced DNA damage, prevents caspase activation and apoptosis. In this way, tumour cells bound to ECM escape chemotherapy-induced cell death and then, with subsequent genetic damage, drug resistant clones may be selected. The mechanism described here may be responsible for the initial resistance of SCLC cells to chemotherapy.

The exact point at which the ECM-mediated tyrosine phosphorylation pathway impinges upon the chemotherapy-induced pro-apoptotic pathway is unclear currently. Increased tyrosine phosphorylation had no effect on etoposide-induced topoisomerase II inhibition indicating that it acts downstream of DNA damage. One possible mechanism is that ECM-induced tyrosine phosphorylation prevents cell cycle arrest by modulating cyclin-dependent kinase activity. Progression from G2 into M phase is controlled in part by a cyclin-dependent kinase (cyclin B/Cdk I) that is regulated by tyrosine phosphorylation (Kaufmann, 1998). This model fits well

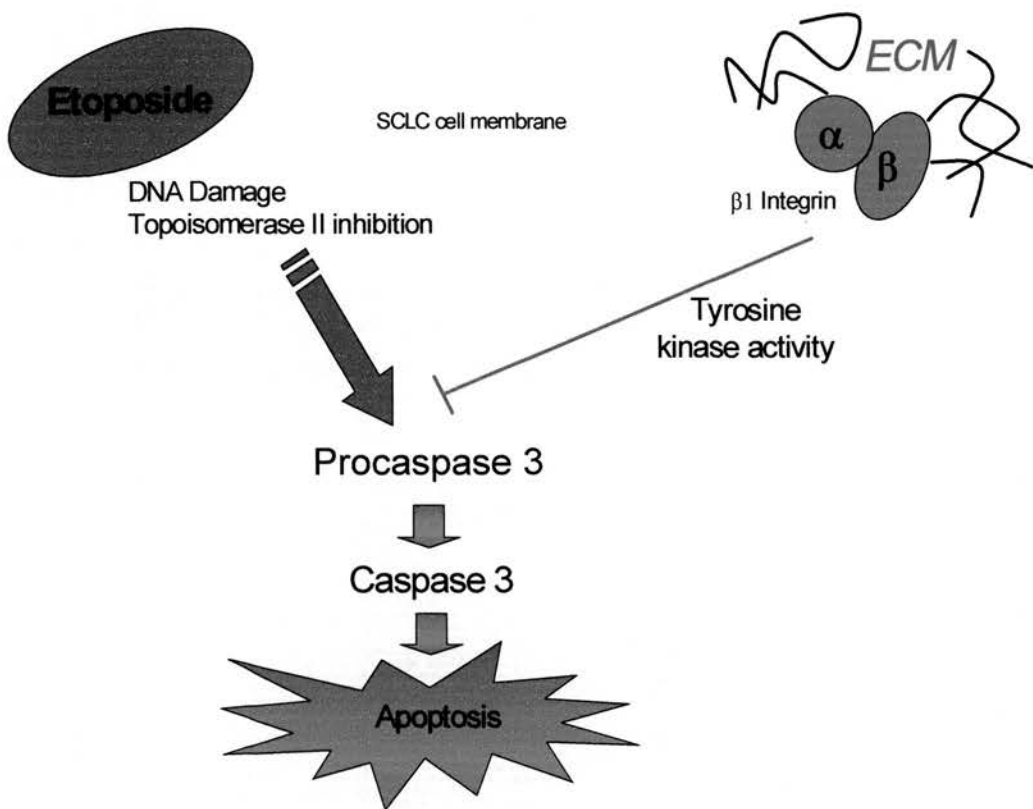


Figure 4.13

Proposed mechanism by which SCLC cell adhesion to ECM proteins can promote chemoresistance. See text for details.

with Hannun's hypothesis. In Hannun's hypothesis the 'phase III decision point' is akin to the presence or absence of the ECM-mediated tyrosine kinase pathway. In the absence of ECM, susceptible cells undergo chemotherapy-induced apoptosis but in the presence of the ECM-mediated 'rescue pathway' cells may enter a period of temporary cell cycle arrest or damage repair before continuing to cycle normally albeit having sustained some genetic damage.

In summary it has been shown that SCLC cells are surrounded by a stroma of ECM proteins, the origin of which are likely to be fibroblasts and other surrounding cells. SCLC cells secrete a variety of mitogenic growth factors and cytokines including IGF-1, neuropeptides, TGF- β and IL-8. TGF- β can modulate the primary fibronectin transcript leading to production of other ECM proteins while IL-8, an angiogenic factor, likely facilitates neovascularisation. Our working hypothesis is that, *in vivo*, SCLC creates a specialised environment that, using an analogy to inflammation likens SCLC to a 'wounding reaction' with the laying down and remodelling of ECM, growth factor release and neovascularisation. Thus, at primary and secondary metastatic sites, by the mechanisms described above there seems to be a permissive environment for SCLC cell proliferation, protection from apoptosis and resistance to chemotherapy. We believe that this is a good model to explain the partial responses and local recurrence of tumour often seen after chemotherapy.

CHAPTER 5

CD98: ROLE IN INTRACELLULAR SIGNALLING AND ANCHORAGE-INDEPENDENCE OF SCLC

5.1 INTRODUCTION

The processes of cell migration and adhesion are central, not only to tumour metastasis, but also to a range of physiological processes such as embryogenesis, haemostasis, the immune response and the maintenance of tissue integrity. The regulation of cell migration is dependent upon rapid, controlled alterations of the affinity of integrins for their extracellular ligands. Integrins are capable of mediating bi-directional transmembrane signalling from the outside to the inside of the cell ('outside-in' signalling) and from the inside to the outside of the cell ('inside-out' signalling) (Dedhar and Hannigan, 1996). The former is initiated by integrin clustering at focal sites of the plasma membrane and results in the propagation of signals into the cytoplasm. Inside-out signalling regulates the affinity state for a ligand through the propagation of conformational changes from the integrin cytoplasmic domain to the extracellular binding site. The classical example of this is platelet aggregation, a physiological process dependent upon integrin affinity modulation. Platelet aggregation relies upon fibrinogen cross-linking the platelet integrin $\alpha\text{IIb}\beta\text{3}$. Resting platelets are unable to aggregate because their $\alpha\text{IIb}\beta\text{3}$ is in a partially active conformation that has a low affinity for fibrinogen. Following activation by specific agonists such as thrombin, $\alpha\text{IIb}\beta\text{3}$ undergoes a conformational change that dramatically increases its binding affinity for fibrinogen resulting in platelet aggregation (Shattil et al., 1998). In addition to integrin affinity modulation, cells can use integrin clustering or avidity modulation to regulate ligand binding. Changes in both integrin affinity and avidity are not mutually exclusive and the relative contributions of each to ligand binding vary with integrin type.

At present the signalling pathways regulating integrin affinity are poorly understood. Recent studies indicate that the small GTP binding proteins H-ras and R-ras and their effectors play a role in regulating integrin affinity. Whereas activated H-ras and its effector Raf-1 inhibit integrin activation in CHO cells (Hughes et al., 1997), R-ras (which is highly homologous to H-ras) stimulates integrin binding affinity (Zhang et al., 1996). These proteins may act together to modulate integrin affinity.

In the last few years work in several laboratories has pointed to CD98 involvement in regulating adhesive interactions mediated by integrins. Initially, Ito and colleagues (1992) showed that antibodies to CD98 promote integrin-mediated virus-induced cell fusion. Subsequently, Fenczik et al., (1997) identified CD98 as a unique and highly specific regulator of integrin affinity. They showed that an anti-CD98 heavy chain mAb increased $\beta 1$ integrin-dependent adhesion of a SCLC cell line to the ECM proteins laminin and fibronectin.

CD98, a ubiquitously expressed cell surface antigen, is an early marker of T cell activation (Diaz, Jr. et al., 1997), which is highly conserved between species (Parmacek et al., 1989). CD98 is a cell surface heterodimer formed by the covalent linkage of CD98 heavy chain with up to five different light chains. (Quackenbush et al., 1987; Teixeira et al., 1987; Warren et al., 1996, Kanai et al., 1998; Mastroberardino et al., 1998; Nakamura et al., 1999). Sequence analysis has not suggested a function for the heavy chain, but the light chain has been demonstrated to function as an amino acid transporter.

CD98 is expressed at low levels on the surface of quiescent cells but is rapidly upregulated early in transition from G0 to G1 phase and remains at elevated levels until the cell cycle is complete (Suomalainen, 1986; Parmacek et al., 1989). CD98 is strongly expressed on adherent human embryonic and newborn fibroblasts but expression gradually diminishes on cells from older individuals. While 100% of embryonic and newborn fibroblasts express CD98, less than 1% of adherent human fibroblasts from normal adults do so. However, expression is reconstituted to high levels on sarcomatous cell lines and is present constitutively on the surface of

activated and proliferating cells (Azzarone et al., 1985). These expression patterns suggest that the function of CD98 is coupled to cellular activation.

A number of studies have shown that perturbation of CD98 function with monoclonal antibodies (mAbs) has profound effects on cellular proliferative responses, but none have been definitive. Anti-CD98 heavy chain mAbs block differentiation of murine foetal liver, bone marrow, peripheral blood, pluri-potent progenitor cells and erythroid lineages (Warren et al., 1996). In LIN haemopoietic progenitor cells (cells lacking lineage specific surface markers), anti-CD98 heavy chain mAbs trigger DNA fragmentation (Warren et al., 1996). The effects of anti-CD98 heavy chain mAbs on T-cell proliferative responses are controversial, with studies describing both inhibitory and stimulatory effects (Spagnoli et al., 1991; Diaz, Jr. et al., 1997; Warren et al., 2000).

Early studies suggested that an intracellular signalling pathway mediates the action of CD98 (Warren et al., 1996; Okamoto et al., 1997; Tabata et al., 1997). Tyrosine kinase inhibitors such as genistein and herbimycin inhibit CD98 activity in haematopoietic cells (Warren et al., 1996) suggesting that tyrosine kinase activation may be an early signal transduction pathway activated by CD98. CD98 may also regulate the intracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_i$ through the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in a cell type-specific manner (Michalak et al., 1986; Posillico et al., 1987; Freidman et al., 1994). Anti-CD98 heavy chain mAbs trigger a transient increase of $[\text{Ca}^{2+}]_i$ in human parathyroid adenoma cells resulting in a decrease in the basal level of PTH secretion (Posillico et al., 1987). In contrast, anti-CD98 heavy chain mAbs inhibit 90% of the Na^+ -dependent Ca^{2+} uptake by rabbit skeletal muscle or bovine cardiac sarcolemmal vesicles (Michalak et al., 1986). However, anti-CD98 heavy chain mAbs have no effect on $[\text{Ca}^{2+}]_i$ in peripheral blood lymphocytes or T cell lines (Freidman et al., 1994). CD98 has also been implicated in transport of amino acids and recently the light chain of CD98 has been isolated and identified as an E16/TA1 amino acid transporter (Mannion et al., 1998). Finally, overexpression of CD98 in the murine fibroblast cell line NIH3T3 has been shown to result in cellular transformation (Hara et al., 1999).

Therefore, CD98 appears to modulate several basic cellular signal transduction pathways and functions during activation. One could hypothesise that CD98 is a central regulator of cellular activation that governs passage through the G1 phase of the cell cycle. However, a definitive role for CD98, its mechanism of action in cellular activation and transformation and its relevance in human cancer has not been determined. The aim of this part of the study was to examine the expression of CD98 in SCLC cells *in vivo* and *in vitro* and to determine the intracellular signalling and functional consequences of cross-linking CD98 in SCLC.

5.2 RESULTS

5.2.1 CD98 expression in SCLC cells

CD98 expression on SCLC cells, *in vivo*, was examined by immunohistochemical staining of SCLC specimens from the University of Edinburgh pathology files. In all samples examined, high levels of CD98 expression were seen on SCLC cells.

Furthermore, CD98 expression on SCLC cells was markedly upregulated compared with that on normal bronchial epithelium (Figure 5.1).

CD98 expression was also determined on several SCLC cell lines by flow cytometric analysis using an indirect immunofluorescence technique with the monoclonal antibody 4F2 directed against the heavy chain of CD98. Figure 5.2A shows that CD98 is highly expressed on H69 SCLC cells. Mean fluorescence intensity was 79.4 arbitrary units (AU) compared to an IgG2a isotype matched control of 14.2 AU.

When H69 SCLC cells were incubated in the presence of increasing concentrations of 4F2, a dose-dependent increase in binding was seen with a half maximal value of 6.5 µg/ml (Figure 5.2B). Saturated binding occurred between 30-100 µg/ml (Figure 5.2B). Furthermore, high levels of expression of CD98 were seen in all SCLC cell lines examined including H345, H510, LS310, WX330 and GLC19 (Figure 5.3).

The expression level of CD98 on these SCLC cell lines was compared with that on untransformed cell lines including human bronchoepithelial cells, Swiss 3T3 cells and bovine tracheal smooth muscle cells. In all cases, the expression of CD98 on the SCLC cell lines was significantly higher than that on the untransformed cell types (Figure 5.3).

Thus high expression of CD98 on the surface of SCLC cells is a general phenomenon both *in vitro* and *in vivo*.

5.2.2 Mobilisation of intracellular calcium

Several groups have previously investigated the role of CD98 on $[Ca^{2+}]_i$ with variable results suggesting that this response may be cell type specific (Michalak et al., 1986; Posillico et al., 1987; Freidman et al., 1994). Figure 5.4 shows that 4F2, at

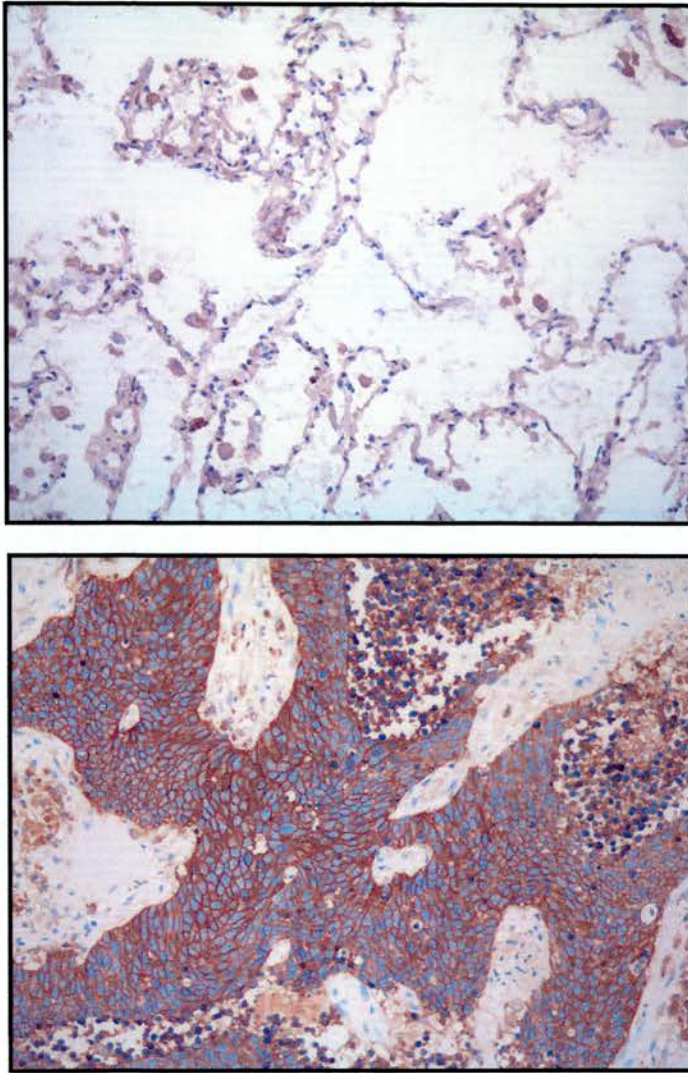


Figure 5.1

CD98 expression in SCLC. Immunohistochemical staining for CD98 in normal bronchial epithelium (upper panel) and SCLC cells *in vivo* (lower panel). Areas of positive staining appear brown. Slides were counterstained with Harris' haematoxylin. Magnification X200.

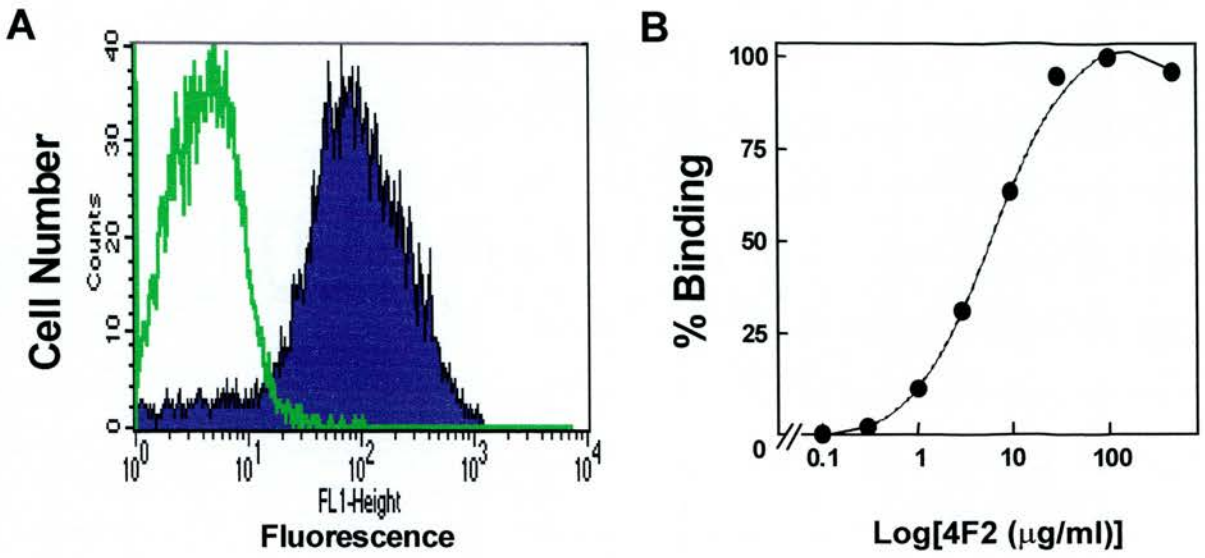


Figure 5.2

CD98 expression in H69 SCLC cells. (A) CD98 expression in H69 SCLC cells (in blue) was determined by flow cytometric analysis using an indirect immunofluorescence technique with the monoclonal antibody 4F2 to the heavy chain of CD98. Binding using a control IgG2a antibody is shown in green. (B) A dose-dependent increase in binding to H69 SCLC cells was seen with increasing concentrations of 4F2.

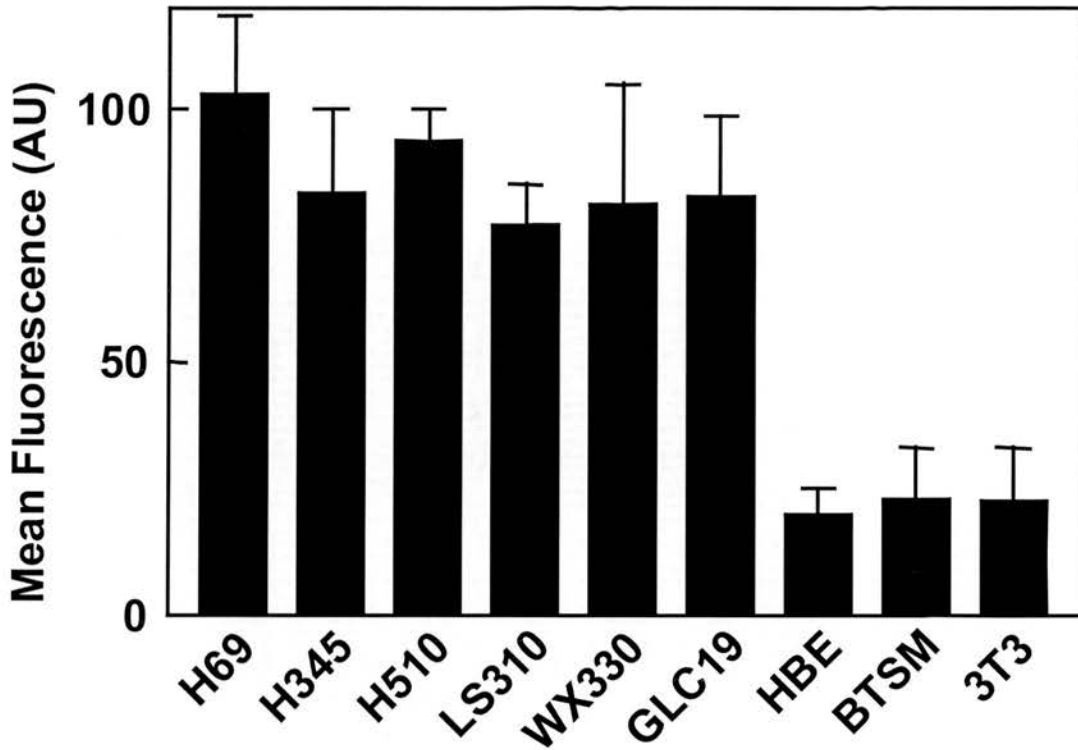


Figure 5.3

Comparison of CD98 expression on SCLC cell lines (H69, H345, H510, LS310, WX330, GLC19) with human bronchoepithelial cells (HBE), bovine tracheal smooth muscle fibroblasts (BTSM) and Swiss 3T3 fibroblasts (3T3). Each value represents the mean fluorescence intensity, arbitrary units (AU) \pm SEM of three independent experiments. Background control values were 2.4 ± 0.2 AU.

concentrations up to 20 µg/ml did not stimulate mobilisation of $[Ca^{2+}]_i$ in fura-2 loaded SCLC cells. However, $[Ca^{2+}]_i$ mobilisation was seen in response to FBS or neuropeptides as previously demonstrated.

5.2.3 Cross-linking CD98 stimulates PI 3-kinase activation

Previous studies have suggested that tyrosine kinase activation might be an early signal transduction pathway activated by CD98 (Warren et al., 1996). Class Ia PI 3-kinases interact with adapter subunits containing SH2 domains. These adapters bind phosphorylated tyrosine residues, thereby linking class Ia PI 3-kinase subunits to tyrosine kinase signalling pathways. Therefore, the effect of cross-linking CD98 on PI 3-kinase activity was examined using an *in vitro* kinase assay as described in 'Materials and Methods'.

The addition of 4F2 (20 µg/ml) caused a 2.5-fold increase in PI 3-kinase activity, first evident within 2 minutes, maximal at 5 minutes and returning to baseline by 50 minutes (Figure 5.5). Cross-linking of the cytoplasmic tails of CD98 is believed to be important to its mechanism of action (Fenczik et al., 1997). If this is the case, one would hypothesise that 4F2-Fab fragments, that are unable to cross-link CD98, would have no effect on PI 3-kinase activation. Indeed, monoclonal 4F2-Fab fragments did not have any effect on PI 3-kinase activity, confirming that cross-linking of CD98 was required for PI 3-kinase activation (Figure 5.6). Furthermore, pre-incubation with 4F2-Fab antibody blocked subsequent 4F2 stimulation of PI 3-kinase supporting this hypothesis. Importantly, isotype matched control IgG2a antibodies failed to activate PI 3-kinase indicating that this was not a non-specific effect (Figure 5.6). Thus cross-linking CD98 stimulates PI 3-kinase activation.

In chapter 3 it was shown that PI 3-kinase activity in SCLC cells is sensitive to the effect of the PI 3-kinase inhibitor wortmannin. If CD98 does activate PI 3-kinase, wortmannin should block this effect. Figure 5.6 shows that wortmannin did completely inhibit CD98-stimulated PI 3-kinase activity in PI 3-kinase immunoprecipitates from H69 cells.

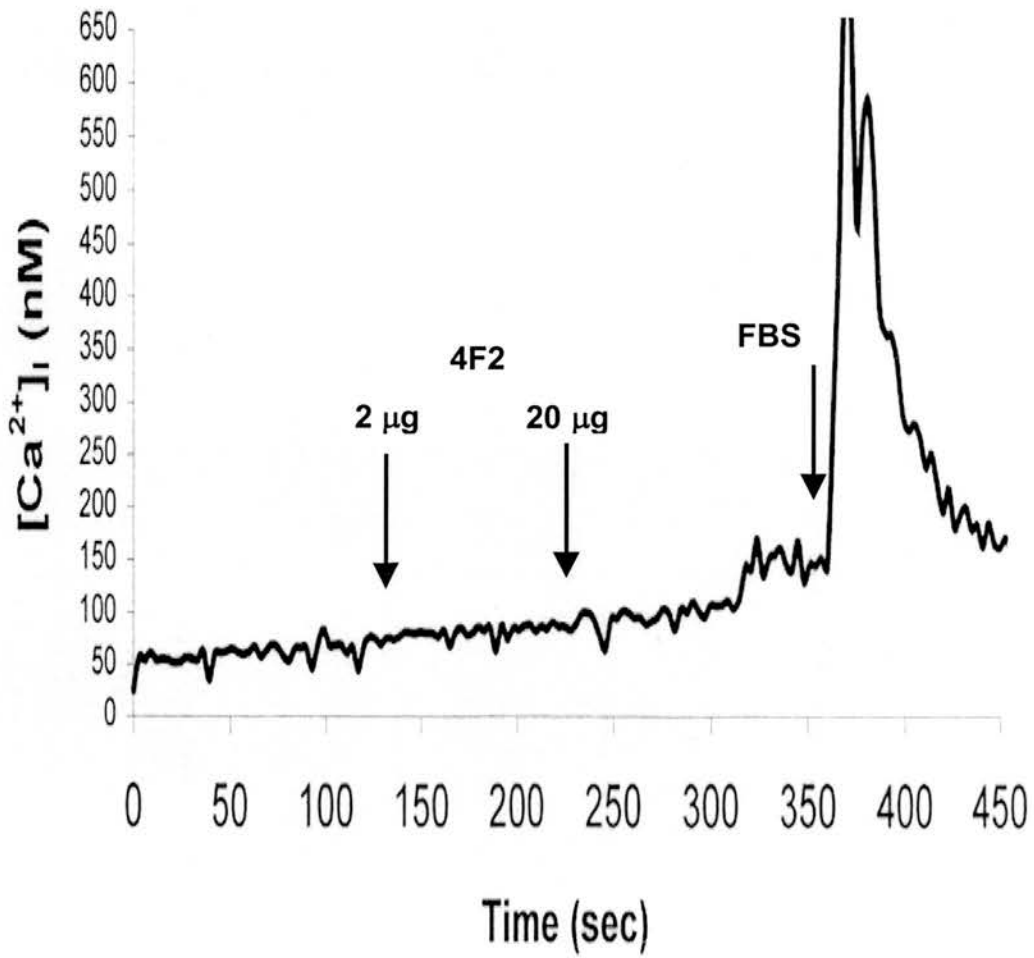


Figure 5.4

Effect of 4F2 on intracellular calcium concentration in SCLC cells. H69 SCLC cells, loaded with fura-2/AME, were resuspended in electrolyte solution and placed in a quartz cuvette. Fluorescence was monitored and basal and peak $[Ca^{2+}]_i$ calculated as described in 'Materials and Methods'. The concentrations of 4F2 ($\mu\text{g/ml}$) are shown. FBS was used at a final concentration of 1%.

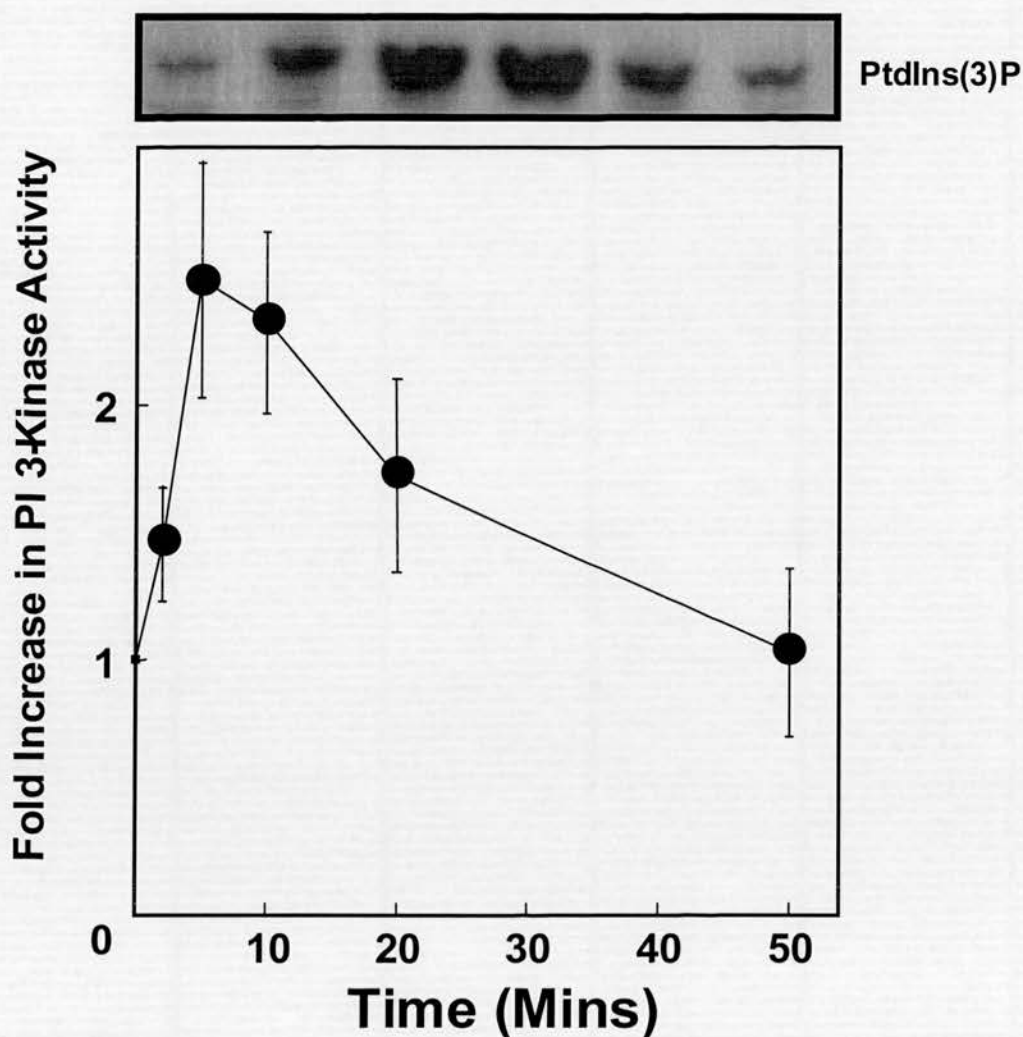


Figure 5.5

Effect of cross-linking CD98 on PI 3-kinase activity. H69 SCLC cells were 'quiesced' in serum-free media for 24 hours and thoroughly washed prior to experimentation. Cells were treated with 4F2 (20 $\mu\text{g/ml}$) for variable times as shown prior to lysis. PI 3-kinase activity was measured as described in 'Materials and Methods'. All data are the mean of four independent experiments performed in triplicate \pm SEM. An autoradiograph showing the 3-phosphorylated reaction product (PtdIns(3)P) is shown for a typical experiment.

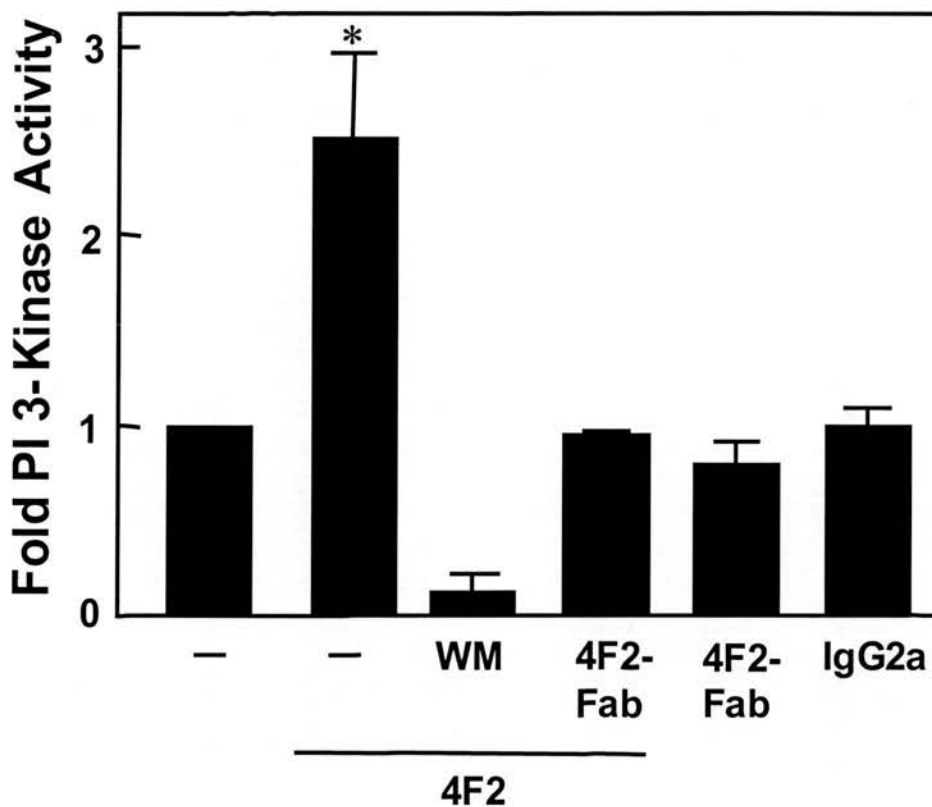


Figure 5.6

The effect of 4F2-Fab and wortmannin on 4F2-induced PI 3-kinase activity.

H69 SCLC cells which had been quiesced overnight and thoroughly washed in serum-free medium were treated with 4F2 (20 $\mu\text{g/ml}$) or 4F2-Fab (20 $\mu\text{g/ml}$) having been pre-treated with wortmannin (100 nM), 4F2-Fab (20 $\mu\text{g/ml}$) or diluent as indicated. PI 3-kinase activity was assayed as described in 'Materials and Methods'. An isotype matched IgG2a control antibody (20 $\mu\text{g/ml}$) is shown for comparison. All data are the mean of three independent experiments performed in triplicate \pm SEM. * $p < 0.05$ compared with control cells.

5.2.4 Cross-linking CD98 activates PKB.

PKB has been identified as a key downstream effector of PI 3-kinase (Hemmings, 1997). We therefore examined the effect of 4F2 on PKB activity using an *in vitro* kinase assay as described in 'Materials and Methods'. Figure 5.7 (upper panel) shows that cross-linking CD98 with 4F2 antibody stimulated PKB activity in a time-dependent fashion. An increase in PKB activity was first evident at 2 minutes (1.3 fold), maximal by 10 minutes (2.5 fold) and had returned to baseline by 20 minutes. This magnitude of response, and time course, was very similar to that seen for PI 3-kinase activation.

These results were confirmed by 'Western' blotting using an anti-phospho-PKB antibody that recognises phosphorylation of PKB at serine 473. Figure 5.7 (lower panel) shows that 4F2 caused an increase in phosphorylation of PKB at 5 and 10 minutes compared with that in untreated control cells or 4F2-Fab treated cells. Therefore, in addition to activating PI 3-kinase, cross-linking CD98 also activates its principal downstream effector, PKB.

5.2.5 Functional effects of cross-linking CD98 with 4F2

PI 3-kinase and PKB have both been shown to be involved in regulating cell growth and survival (Khwaja et al., 1997). Therefore, the functional consequences of cross-linking CD98 with 4F2 in SCLC cells were examined. The ability of cells to grow in soft agarose is a feature of anchorage-independence and is pathognomonic of the transformed phenotype, correlating with tumourigenicity and invasiveness of the tumour (Carney et al., 1980). Cross-linking CD98 with 4F2 markedly enhanced the ability of SCLC cell lines H69, H345 and H510 to form colonies in semi-solid agarose. SCLC basal colony growth was stimulated 200-300% by the addition of 20 µg/ml 4F2 (Figure 5.8). This effect could be blocked by co-incubation with the PI 3-kinase inhibitor, LY294002 (10µM). Furthermore, addition of maximal clonal-stimulating concentrations of neuropeptide growth factors galanin (50nM) and bombesin (10nM) in H510 and H345 cells respectively, caused a supra-maximal stimulation of clonal growth (Figure 5.9).

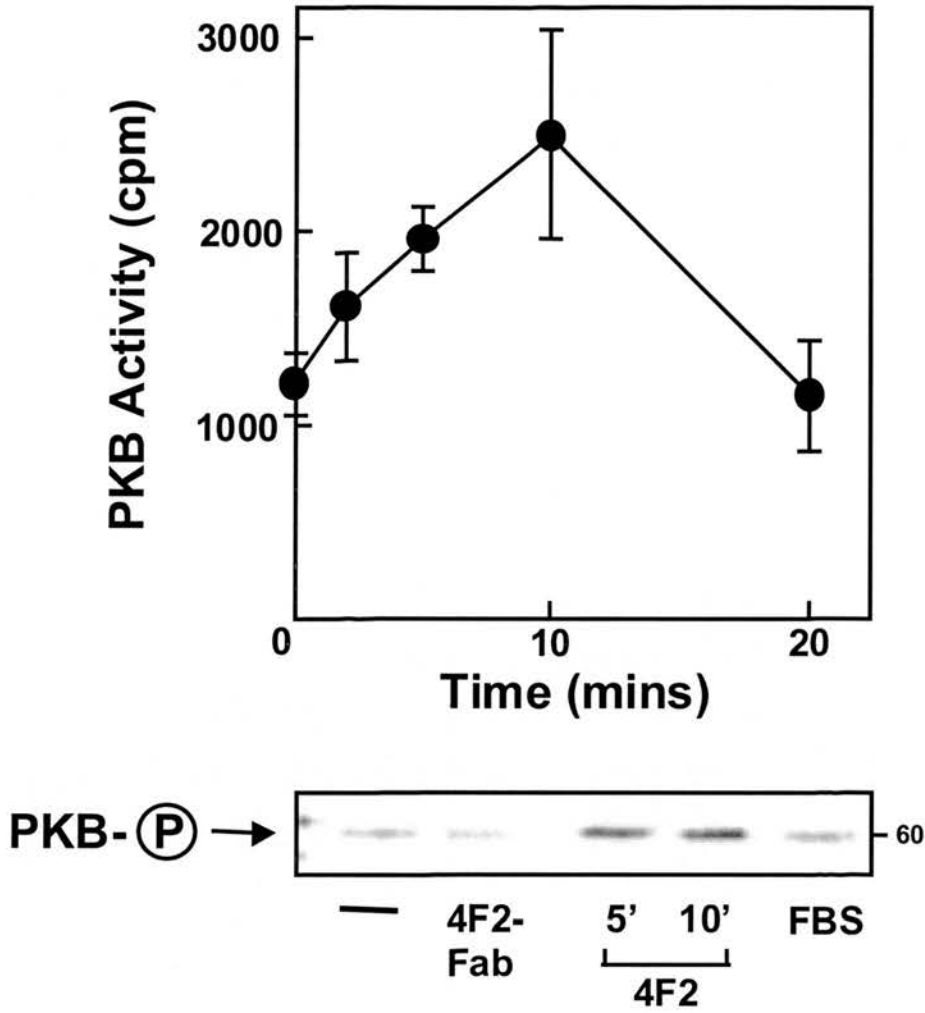


Figure 5.7

Cross-linking CD98 activates PKB. *Upper Panel:* Time course of PKB activation by 4F2 (20 $\mu\text{g}/\text{ml}$). PKB was immunoprecipitated from cell lysates using an anti-PKB PH-domain antibody. PKB activity was assayed as described in 'Materials and Methods'. The figure shows data from a representative experiment. *Lower Panel:* Western blot of phospho-PKB. H69 SCLC cells were incubated in the presence or absence of 4F2-Fab (20 $\mu\text{g}/\text{ml}$ 10 minutes), 4F2 (20 $\mu\text{g}/\text{ml}$ for 5 and 10 minutes) or 10% FBS (5 minutes). 'Western' blots of H69 SCLC cells were probed with an antibody, which recognises phosphorylation of PKB at serine 473 at 60kDa.

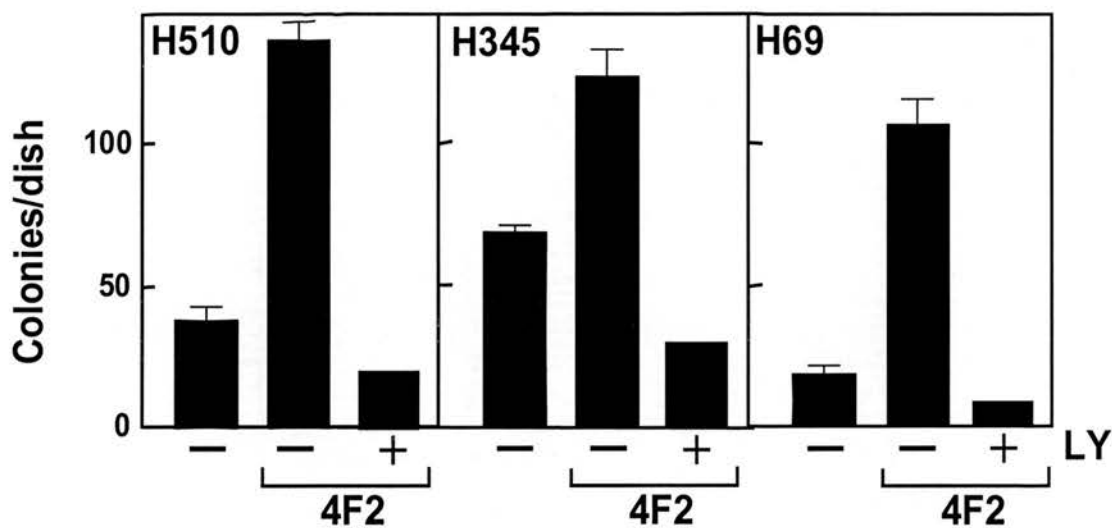


Figure 5.8

Cross-linking CD98 stimulates SCLC clonal growth. H69, H345 and H510 SCLC cells were plated in SITA medium containing 0.3% agarose in the presence or absence of 4F2 (20 $\mu\text{g/ml}$) or LY294002 (10 μM) over a base of 0.5% agarose in culture medium as described in 'Materials and Methods'. After 21 days colonies of $>120 \mu\text{m}$ (16 cells) were counted. Results are expressed as mean \pm SEM colonies/dish of three independent experiments performed in triplicate.

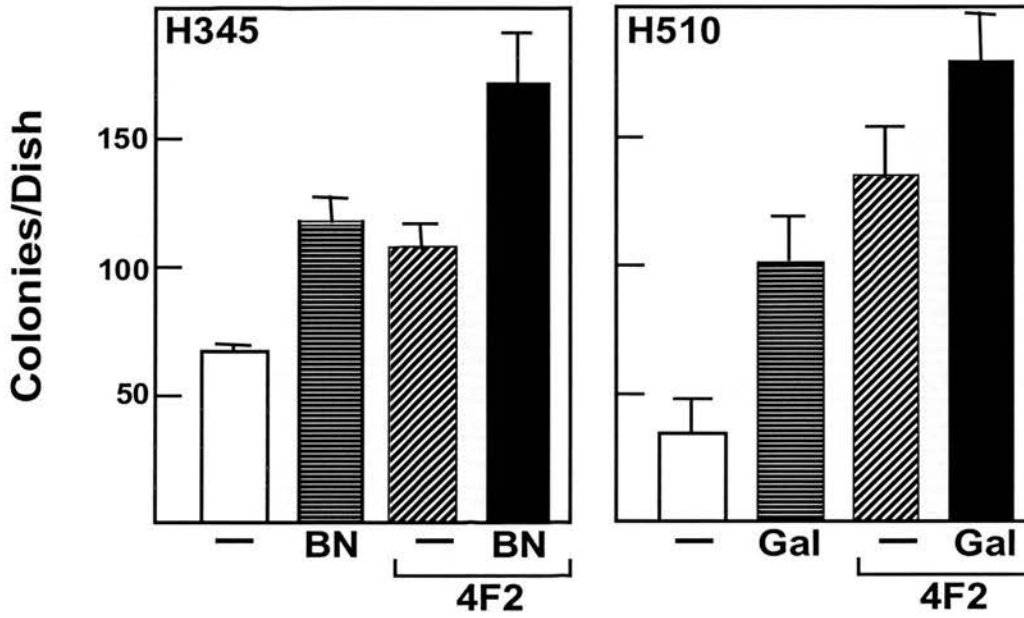


Figure 5.9

The effect of cross-linking CD98 on neuropeptide-stimulated clonal growth in SCLC cells. H345 and H510 SCLC cells were plated in SITA medium containing 0.3% agarose, in the presence or absence of bombesin 10 nM (BN), galanin 50 nM (Gal) and 4F2 20 μ g/ml, over a base of 0.5% agarose in culture medium as described in 'Materials and Methods'. After 21 days colonies of >120 μ m (16 cells) were counted. Results are expressed as mean \pm SEM colonies/dish of three independent experiments performed in triplicate. (Experiments performed by Dr T Sethi).

5.3 DISCUSSION

Until now little has been known about the signalling pathways activated by CD98. Early studies suggested that tyrosine kinase activation may be an early signal transduction pathway activated by CD98 (Warren et al., 1996). This study shows for the first time that cross-linking CD98 with the mAb 4F2 stimulates PI 3-kinase and PKB activity. Furthermore, 4F2 SCLC colony growth in soft agarose. 4F2-mediated colony growth could be blocked by LY294002 showing that it is a PI 3-kinase-dependent pathway. Taken together these data show that PI 3-kinase-dependent signalling is playing a central role in CD98 mediated SCLC cell growth.

The finding that cross-linking CD98 stimulates anchorage-independent growth suggests that it promotes 'integrin-like' intracellular signalling. However, as discussed previously, full oncogenic transformation is believed to require both serum and anchorage-independent growth (Schwartz, 1997). SCLC cell growth is promoted by multiple autocrine and paracrine growth loops involving calcium mobilising neuropeptides (Sethi and Rozengurt, 1991) which activate MAP kinase through G-protein coupled receptors (Seufferlein and Rozengurt, 1996a). Recent work suggests that for full activation of the MAPK pathway by growth factors an integrin-mediated co-signal is required (Renshaw et al., 1997). The finding that CD98 augments neuropeptide-mediated SCLC colony growth supports this hypothesis although it is conceivable that 4F2 and neuropeptides are working through unrelated pathways but having an additive effect on colony cell growth. However, we propose that the CD98-mediated stimulation of PI 3-kinase activity in SCLC cells by 4F2 is mimicking integrin-dependent signal transduction and thereby facilitates neuropeptide-mediated cell growth.

What are the possible mechanisms by which CD98 could generate 'integrin-like' signals? One possibility is that CD98 may physically associate with integrins, providing a mechanism whereby cross-linking of CD98 modifies integrin activity as a consequence of their close proximity. Alternatively, CD98 cross-linking may generate signalling intermediates, which subsequently activate integrin-signalling

pathways. Such a mechanism would not be without precedent. The transmembrane-4 superfamily (TM4SF or tetraspans) associate with a subset of integrins. For example CD9 associates with $\alpha 2\beta 1$ (Jones et al., 1996) and $\alpha 5\beta 1$ (Rubinstein et al., 1994) and CD151 associates with $\alpha 3\beta 1$ (Hemler et al., 1996; Yanez-Mo et al., 1998). An emerging model is that TM4SF proteins recruit signalling enzymes such as PI 4-kinase and PKC into complexes with integrins (Berdichevski et al., 1997; Hemler, 1998). PI 4-kinase mediated PtdIns(4)P and PtdIns(4,5)P₂ are both substrates for PI 3-kinase and regulators of the actin cytoskeleton.

Recent work suggests that CD98 associates either directly or indirectly with $\beta 1$ integrin. First to demonstrate this were Fenczik et al., (1997) who showed that 4F2 antibody promoted $\beta 1$ integrin-mediated adhesion of a SCLC cell line to fibronectin and laminin. The same group has subsequently reported that CD98 associates with integrin $\beta 1$ cytoplasmic domains with a unique integrin class and splice variant specificity. CD98 interacts with the ubiquitous $\beta 1_A$ integrin but not the muscle specific splice variant $\beta 1_D$ or leukocyte specific $\beta 7$ cytoplasmic domains (Zent et al., 2000). Furthermore, Warren et al., (2000) have shown that CD98 co-stimulation of T-lymphocyte activation could be blocked by antibody to $\beta 1$ integrin indicating a functional association between CD98 and $\beta 1$ integrin. However, further work is required to determine which of the mechanisms outlined above mediates CD98/ $\beta 1$ integrin signalling.

There is now increasing evidence that CD98 has oncogenic potential. A recent study by Hara et al., (1999) showed that human CD98 transfected NIH3T3 cells were capable of anchorage-independent growth and that CD98 transfected clones led to tumour development in athymic mice. The data presented here showing that cross-linking CD98 promotes anchorage-independent growth supports this idea. It has previously been shown that constitutively active PI 3-kinase can transform chick embryo fibroblasts (Chang et al., 1997) and that a mutant p85 can transform fibroblasts *in vitro* (Jimenez et al., 1998). Furthermore, recent studies suggest that integrin-mediated PI 3-kinase activation is important for cell migration and can promote carcinoma invasion (Renshaw et al., 1997; Keely et al., 1997). Constitutive

activation of PI 3-kinase as a result of overexpression of CD98 may explain the transformation seen in NIH3T3 cells overexpressing CD98. PI 3-kinase activation may also account for the implication that CD98 is involved in the regulation of cellular activation and progression through the G1 phase of the cell cycle (Suomalainen, 1986; Parmacek et al., 1989). PI 3-kinase regulates progression from G1 to S phase of the cell cycle via its downstream effector p70^{S6k} (Takuwa et al., 1999).

CD98 is transpiring to be a multifunctional molecule: fusion regulator, amino acid transporter, Na⁺/Ca²⁺ exchanger, co-mitogen and now an integrin regulator and potentially an oncogenic protein. The data presented here support the hypothesis that CD98 is able to transmit 'integrin-like' signals and, given what is known about its ability to modulate integrin affinity, it seems likely that it does indeed play a major role in regulating adhesion.

CHAPTER 6

SUMMARY, CONCLUDING COMMENTS AND FUTURE DIRECTIONS

6.1 DISCUSSION

Over the last 20 years medical and surgical intervention has resulted in little change in the five-year survival for lung cancer, with approximately 90% of patients dying within one year of diagnosis. The major breakthrough for management of SCLC occurred in 1969 with the finding that this tumour is particularly sensitive to chemotherapy and radiotherapy. However, despite high initial response rates with combination chemotherapy the majority of patients relapse, so that the five-year survival rate is only 3-8%. Despite multiple trials examining new chemotherapy and radiotherapy regimens current therapies seem to have reached something of a plateau. More and more it is being realised that in order to make further progress towards the development of novel therapies for lung cancer a better understanding of the cellular and molecular processes that govern their growth is required.

If lung cancers did not metastasise far more would be potentially curable by surgery and if SCLC did not relapse and become insensitive to chemotherapy cure rates would be much higher. Thus, the early dissemination of metastases and the development of resistance to chemotherapy are two fundamental processes responsible for the highly malignant phenotype of SCLC. In this thesis the molecular mechanisms that underlie anchorage-dependence, chemoresistance and apoptosis of SCLC have been examined.

PI 3-kinase is now recognised to play a role in multiple cellular processes including several central to the process of tumourigenesis. We have shown that SCLC cells exhibit constitutively active PI 3-kinase and that, as a result, PKB has elevated basal activity. Furthermore, PtdIns(3,4,5)P₃ levels are also elevated in SCLC lines compared with control cells. It is proposed that constitutive activation of PI 3-kinase

may mimic integrin-dependent signal transduction thereby promoting cellular proliferation, anchorage-dependence and tumorigenicity in SCLC. Over the last year a number of other groups have also reported high PI 3-kinase activity in a variety of tumour types. As discussed in Chapter 3, high PI 3-kinase activity can probably occur in several ways. Certain oncogenes such as *ras* and *src* can activate PI 3-kinase, although neither has been described in SCLC. Recently, a novel oncogene, *PIK3CA*, located in 3q26.3 has been described in ovarian and more recently, cervical carcinomas (Shayesteh et al., 1999; Ma et al., 2000). Amplification of 3q26.3 in primary cervical carcinomas and cervical cancer cell lines results in increased copy number of *PIK3CA* and elevated levels of its gene product, p110 α . This is associated with high PI 3-kinase activity. At present, the cause of constitutive PI 3-kinase activity in SCLC cells is not known. The next step might be to use single strand conformational polymorphism to see if there are any mutations present in the coding sequences of the p85 and p110 subunits of PI 3-kinase in our SCLC lines. If any mutations are identified sequencing would be performed to determine the nature of these mutations.

The central role of PI 3-kinase in tumorigenesis makes it a potential target for drug development. Recently two groups have reported the use of either inositol phosphates or phosphatidylinositol analogues to inhibit the growth of various cancer cell lines including the same SCLC cell lines that we utilised. Razzini et al., (2000) showed that inositol 1,3,4,5,6 pentakisphosphate and inositol 1,4,5,6 tetrakisphosphate inhibited IGF-1 induced [³H]-thymidine incorporation in a human breast cancer cell line and also blocked the ability of H69 SCLC cells to grow in liquid medium and form colonies in semi-solid agarose medium. Hu et al., (2000a) obtained similar results using phosphatidylinositol analogues. While these results are very interesting it remains to be seen whether they can be replicated in animal models. In another approach the PI 3-kinase inhibitor LY294002 was used to treat athymic mice that had been inoculated intraperitoneally with an ovarian cancer cell line (Hu et al., 2000b). In the LY294002 treated group mean tumour burden and ascites production was markedly reduced compared with controls. This is the first description of an animal model to test the efficacy of LY294002 *in vivo*. Although

LY294002 is a 'broad-spectrum' PI 3-kinase inhibitor there were apparently no systemic side effects in the control group. An animal model for SCLC has been described previously and it would be most interesting to replicate this type of experiment in such a model (Fridman et al., 1990).

In vivo, tumour cells exist in a state of dynamic interplay between anchorage-dependence and independence. The ability of a cell to regulate adhesion and de-adhesion is central to the processes of migration and metastasis. The mechanisms regulating these processes are poorly understood at this time. In this thesis it has been shown that cell-matrix adhesion appears to be critical to the ability of SCLC cells to survive the effects of chemotherapy. Although we have begun to dissect the basic protective mechanism, many questions remain unanswered. For instance, what are the identities of the various tyrosine phosphorylated proteins that mediate ECM protection? Preliminary data suggests that one is PI 3-kinase. This is supported by recent data that links the PI 3-kinase/PKB pathway to cell survival and protection from apoptosis (Khwaja et al., 1997). However, it is likely that a number of other factor(s) are also involved. Whilst constitutive PI 3-kinase activity in SCLC cells seems to be sufficient to allow anchorage-independent growth, it does not appear to be sufficient to prevent chemotherapy-induced apoptosis because non-adhered cells are chemosensitive. Integrin engagement also appears to be required. At least twenty different proteins including Rho, Ras, FAK, MAPKs and PI 4-kinase as well as PI 3-kinase can be recruited to the ECM ligand/integrin binding site. It is quite possible that two or more pathways may contribute to the ECM-mediated protective effect. Recently, specific inhibitors for a number of tyrosine kinases have been described (Levitzki and Gazit, 1995). If we can define the exact pathways mediated by ECM it may be possible to selectively target key signalling molecules and block the 'protective pathway' thereby augmenting the effects of conventional chemotherapeutic drugs. Further work is also required to dissect the mechanism by which the ECM-mediated tyrosine phosphorylation pathway impinges upon the chemotherapy-induced pro-apoptotic pathway. Preliminary data suggests that ECM-induced tyrosine phosphorylation may prevent cell cycle arrest by modulating cyclin-dependent kinase activity and thus maintaining cell cycling. However, certain

cyclins such as cyclin B/cdk-1 are tyrosine phosphorylation dependent and it might be possible, using selective tyrosine kinase inhibitors, to arrest the cell cycle and thereby induce apoptosis.

In Chapter 5 the role of CD98 in SCLC was examined. CD98 has recently been implicated as a regulator of integrin affinity and may be central to the control of anchorage-dependence. The finding that cross-linking CD98 stimulates anchorage-independent growth suggests that it can mimic integrin-like intracellular signalling. Integrins are unique in their ability to mediate bi-directional signalling. The ability of CD98 to mimic integrin-like signalling is an example of 'outside-in' signalling. As discussed in Chapter 5, the exact mechanism by which cross-linking CD98 activates PI 3-kinase is not known and work is currently in progress to try and elucidate this. Hara et al., (1999) have also shown that CD98 transfected fibroblasts are capable of anchorage-independent growth. However, at present little is known about how CD98 regulates integrin affinity. It has now been shown that cross-linking CD98 can enhance both integrin-dependent adhesion of SCLC cells to ECM proteins (Fenczik et al., 1997) and also promote anchorage-independent growth. Much work remains to be done in order to dissect out the mechanism by which CD98 regulates integrin affinity and avidity by 'inside-out' signalling. As we learn more about the mechanisms regulating integrin adhesion it may become possible using selective antibodies to artificially switch integrins 'on' or 'off' in order to promote or prevent adhesion and thus manipulate the ability of tumour cells to metastasise.

In conclusion one can hypothesise that SCLC cells, *in vivo*, exist in a state of dynamic flux between anchorage-dependence and independence. The regulation of integrin adhesion is governed by complex signalling pathways and feedback loops involving, among others, PI 3-kinase and CD98. At any one time a proportion of cells are anchorage-independent and therefore can potentially migrate and metastasise while others are adhered to ECM proteins and are protected from the effects of chemotherapy. Furthermore, within this specialised microenvironment there exist a variety of mitogenic growth factors, cytokines and angiogenic factors as well as serine proteases and MMPs. This would appear to be a good model to

explain the aggressive nature of SCLC accounting for this tumour's propensity to metastasise early and relapse following chemotherapy.

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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 neurotrophic 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. SCLC⁴ 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.

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⁴The abbreviations used are: SCLC, small cell lung cancer; PI, phosphatidylinositol; PI(4)P, phosphatidylinositol 4-phosphate; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P₃, phosphatidylinositol 3,4,5-triphosphate; SH, Src homology; FAK, focal adhesion kinase; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-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; SITA, RPMI 1640 medium with 25 mM HEPES supplemented with 30 nM selenium, 5 μg/ml insulin, 10 μg/ml transferrin; 0.25% (w/v) BSA; 5 μg/ml L-glutamine; 50 μg/ml penicillin, and 50 μg/ml streptomycin; ECL, enhanced chemiluminescence.

The ability of cancer cells to grow in the absence of cell adhesion to extracellular matrix has been shown to correlate closely with 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^{s6k} 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, San Francisco, 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 and cultured for another 2 days before use. Alter-

natively, 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 4-(2-aminoethyl)benzenesulfonyl fluoride, 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^{s6k} Activity Assay. p70^{s6k} 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 *p*-nitrophenyl phosphate, 1 mM benzamide, 0.1 mM phenylmethylsulfonyl fluoride and 1% (v/v) NP40. Lysates were clarified at 13,000 × g for 10 min and equal quantities of protein equilibrated supernatant incubated with 2 µg polyclonal anti-p70^{s6k} antibody. After 90 min, the lysates were further incubated with 25 µl of Pansorbin. The immunoprecipitates were washed twice in lysis buffer and twice in the same buffer without detergent. This was followed by two washes in a buffer containing 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 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, 0.27 M sucrose, 50 mM sodium fluoride, 10 mM sodium β-glycerophosphate, 5 mM 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 Crosstide, 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 (1×10^5) 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, Luton, 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 1×10^4 viable cells mixed with SITA containing 0.3% (w/v) agarose and

agonist/antagonist at the concentrations indicated (see Fig. 8) 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 (1×10^5) 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).

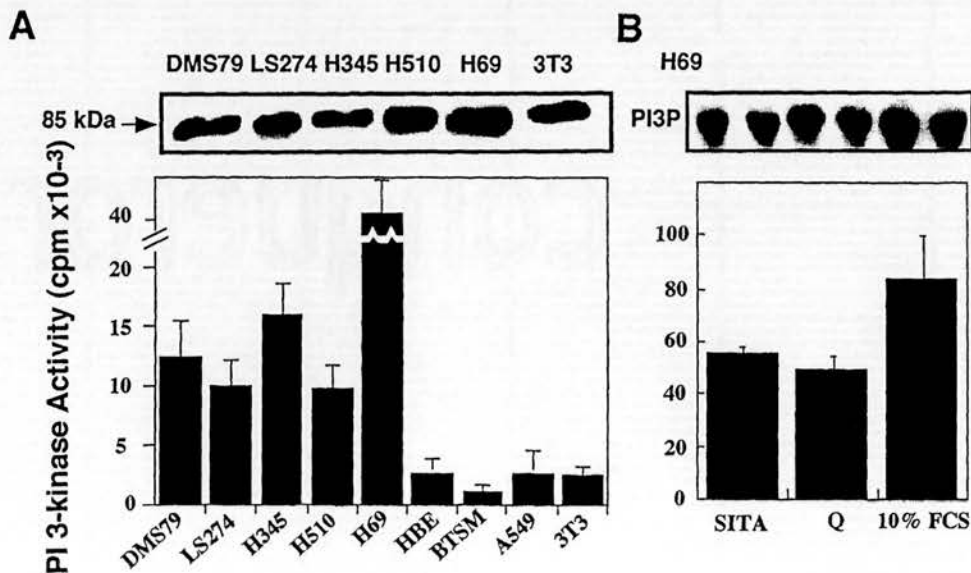
Flow Cytometric Assessment of Cell Cycle Phase. Cells (1×10^5) were cultured in SITA medium in the presence or absence of LY294002 (10 µM). After 24 h, cells were washed twice in PBS and fixed in 70% ice-cold ethanol. Cellular DNA content was determined by staining with 50 µg/ml of propidium iodide and 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^{6k} 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 Crosstide 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.



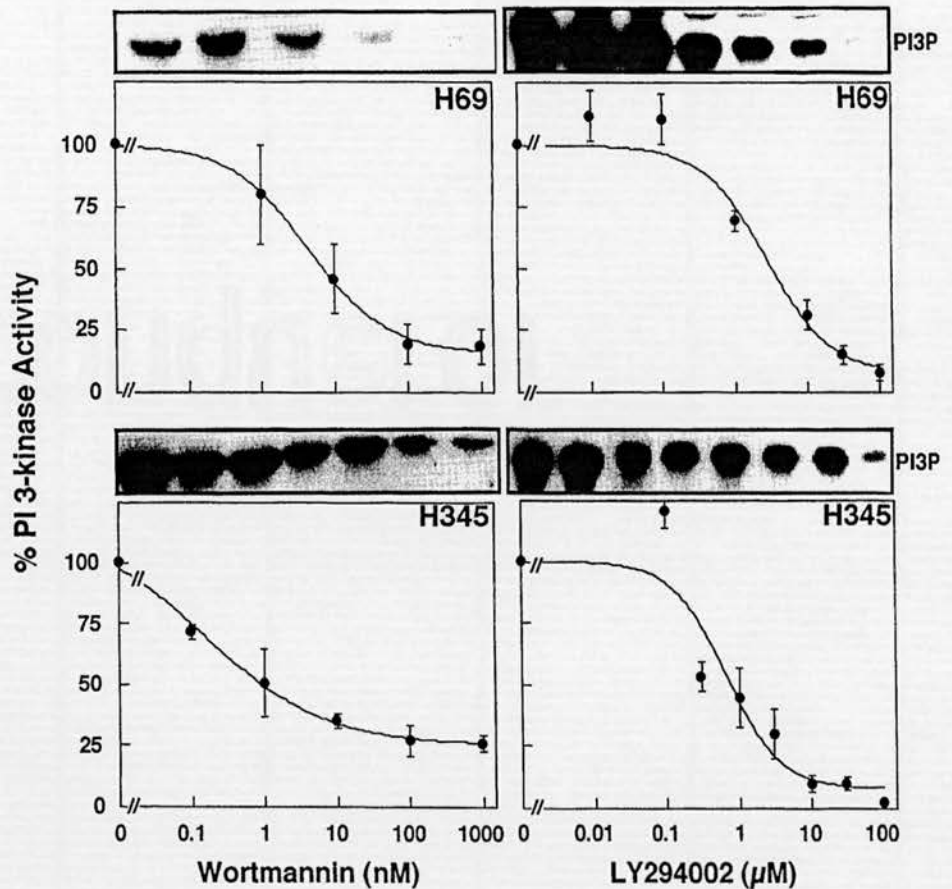


Fig. 2. Effect of PI 3-kinase inhibition on PI 3-kinase activity in SCLC cells. Concentration response of wortmannin (left) and LY294002 (right) on PI 3-kinase activity in H69 (upper) and H345 (lower) SCLC cells. Values shown are the means of two to three independent experiments performed in duplicate; 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^{s6k} 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^{s6k} activation (15). Fig. 3A shows a Western blot analysis of p70^{s6k} expression in SCLC cell lines H69, H345, and H510. We therefore investigated the possibility that the constitutive phosphorylation of p70^{s6k} described previously in SCLC cells (19) causes high basal p70^{s6k} activity that is driven by constitutive PI 3-kinase activity. Immunoprecipitation of p70^{s6k} 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^{s6k} 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^{s6k} 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.

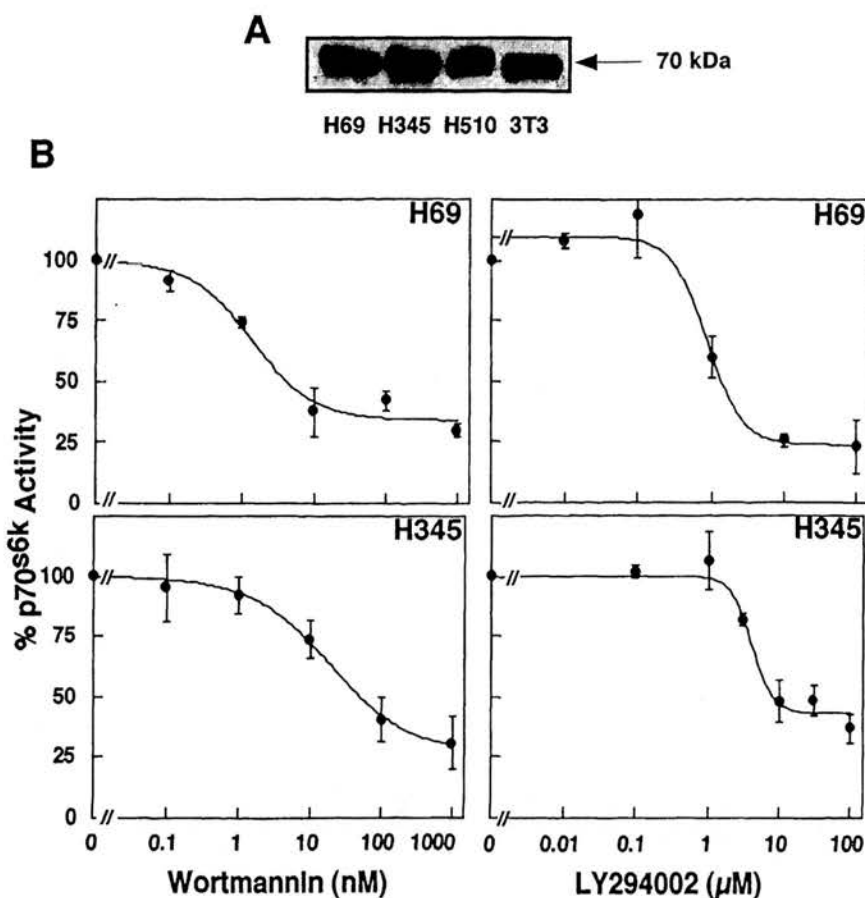


Fig. 3. *A*, expression of the p70 isoform of ribosomal s6 kinase in SCLC cell lines H69, H345, H510 and Swiss 3T3. Cell lysates were Western blotted and probed using a rabbit polyclonal anti-p70^{s6k} antibody. Immunoreactive bands were visualized by ECL. *B*, p70^{s6k} activity in SCLC. Well-washed 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 \pm SE; $n = 3-4$). Similar results were seen in H510 cells with wortmannin and LY294002 (IC_{50} , 4.1 nM and 1.0 μM , respectively). These results are similar to the IC_{50} s 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.

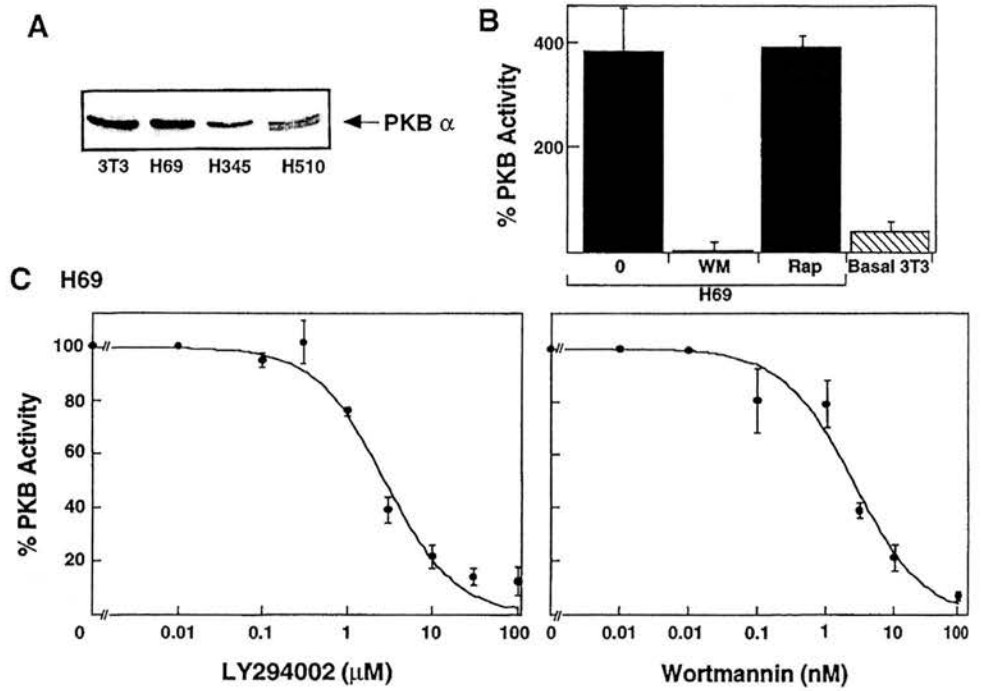
Constitutive PKB Activity in SCLC Cells Is PI 3-Kinase-dependent. In integrin and growth factor signal transduction, PKB has been identified as a key downstream effector of PI 3-kinase (21). Western blot analysis of SCLC cell lines H510, H69, and H345 revealed expression of the α isoform of PKB (Fig. 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 PKB α 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 IC_{50} for LY294002 and wortmannin was 2.7 ± 0.1 μM and 2.0 ± 0.6 nM, respectively (mean \pm 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 (IC_{50} s, 10.0 ± 0.8 , 2.9 ± 0.2 , and 13.3 ± 0.8 μM for H69, H345, and H510 cells, respectively; mean \pm 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,

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 Crosstide 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.

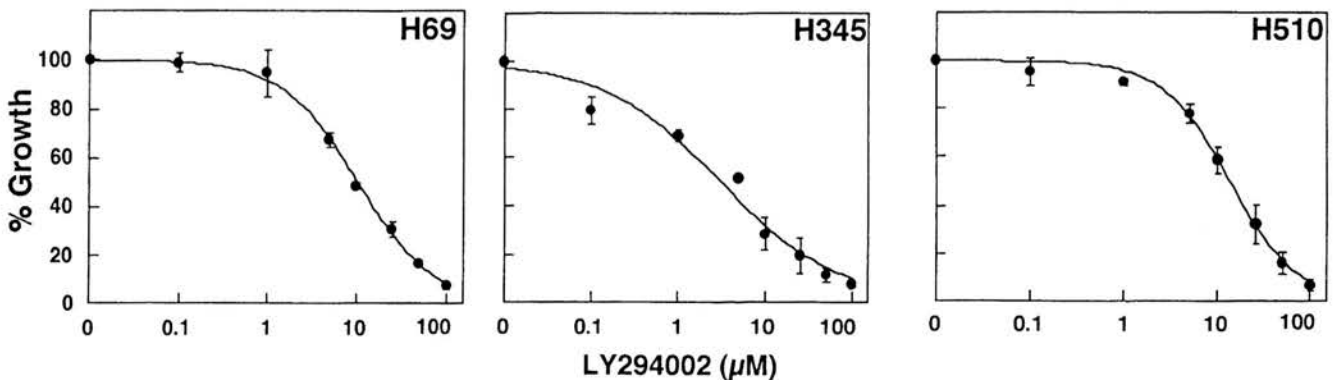


Fig. 5. Concentration-dependent effect of LY294002 on H69, H345, or H510 SCLC growth. SCLC cells (1×10^5) were washed and incubated in fresh SITA medium in the presence of increasing concentrations of LY294002 as shown. Cell number was determined on day 9, and results are expressed as a percentage of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

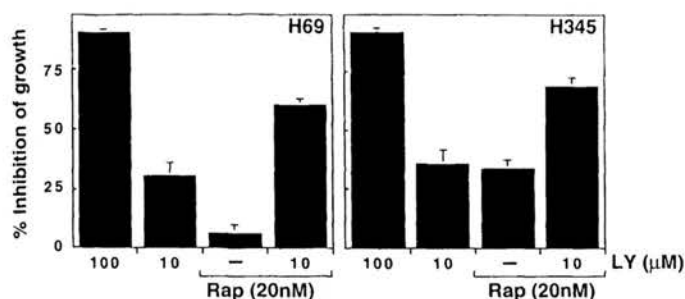


Fig. 6. Effect of LY294002 and rapamycin on H69 (left) and H345 (right) SCLC cell growth. Cells (1×10^5) were washed and incubated in fresh SITA medium in the presence of LY294002 (LY) and rapamycin (Rap) alone or in combination, at the concentrations indicated. On day 9, cell number was measured. Results 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 combi-

nation of a stimulation of apoptosis and a delay in the cell cycle in G₁ 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). Furthermore, 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 (IC₅₀, 3 nM) is noncompetitive with respect to ATP, binding irreversibly to the p110 catalytic subunit of PI 3-kinase (10), whereas LY294002 (IC₅₀, 1.4 μM) behaves as a competitive inhibitor for the ATP binding site of PI 3-kinase (11).

PKB and $p70^{\text{S6k}}$ have been identified as downstream effectors of PI 3-kinase (15, 21). Our results may underlie the constitutive basal

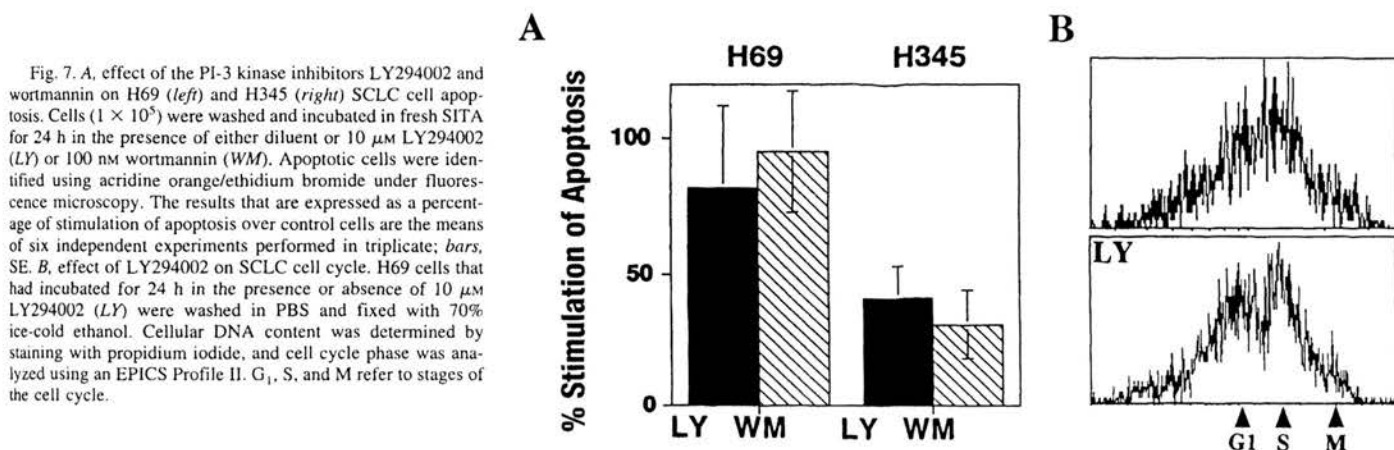


Fig. 7. A, effect of the PI-3 kinase inhibitors LY294002 and wortmannin on H69 (left) and H345 (right) SCLC cell apoptosis. Cells (1×10^5) were washed and incubated in fresh SITA for 24 h in the presence of either diluent or $10 \mu\text{M}$ LY294002 (LY) or 100 nM wortmannin (WM). Apoptotic cells were 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. G₁, S, and M refer to stages of the cell cycle.

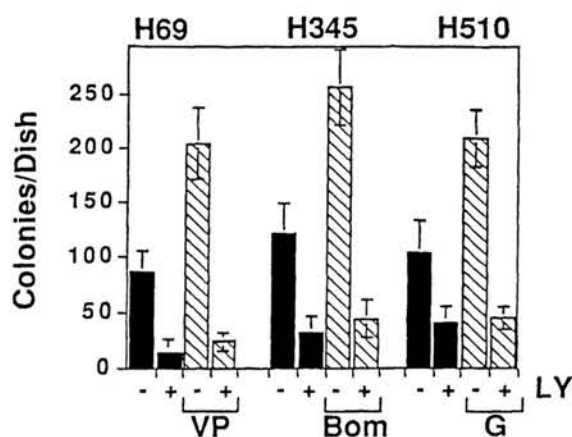


Fig. 8. Effect of LY294002 on SCLC clonal growth in semisolid medium. H69, H345, and H510 SCLC cells, 3–5 days after passage, were washed, and 1×10^4 viable cells/ml were plated in SITA medium containing 0.3% agarose on top of a base of 0.5% agarose in culture medium as described in "Materials and Methods." 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 μ M LY294002 (LY). After 21 days, colonies of $>120 \mu$ M (16 cells) were counted. Results are expressed as colonies/dish, means of triplicates of two independent experiments; bars, SD.

phosphorylation of $p70^{S6K}$ noted previously in SCLC cell lines H510, H69, and H345 (19). This constitutive basal phosphorylation is reflected in the high basal activity of $p70^{S6K}$. Wortmannin and LY294002 inhibit $p70^{S6K}$ activity by 70–80% with IC_{50} s similar to those seen for both PI 3-kinase and $p70^{S6K}$ inhibition. Thus, the constitutive phosphorylation and kinase activity of $p70^{S6K}$ is, in part, driven by constitutive PI 3-kinase activity. However, even when high concentrations of wortmannin (100 nM) and LY294002 (100 μ M) were used, residual $p70^{S6K}$ activity was noted. This may be due to activation of $p70^{S6K}$ by other pathways, e.g., the PKC pathway as described in other cell systems (17). This may also explain why H345 cell $p70^{S6K}$ 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 $p70^{S6K}$ 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 $p70^{S6K}$ 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 $p70^{S6K}$ 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 $p70^{S6K}$ implied that PKB may mediate mitogenic signaling through $p70^{S6K}$ activation (47). Recently, PDK1, the enzyme that phosphorylates Thr308 of PKB, has been shown to directly phosphorylate and activate $p70^{S6K}$, resulting in a PI 3-kinase-dependent mechanism of activation that may circumvent PKB in the proliferative pathway (48). Differential activation of PKB and $p70^{S6K}$ can be achieved under distinct cellular calcium levels; this may be important for integrating signals from multiple signaling inputs (49). Our results support these latter findings. PI 3-kinase regulation of SCLC cell growth is mediated both by $p70^{S6K}$ -dependent and -independent pathways. It appears that $p70^{S6K}$ 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 G_1 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 ($38 \pm 1.3\%$ versus $29 \pm 2.7\%$ 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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Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: A mechanism for small cell lung cancer growth and drug resistance *in vivo*

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Resistance to chemotherapy is a principal problem in the treatment of small cell lung cancer (SCLC). We show here that SCLC is surrounded by an extensive stroma of extracellular matrix (ECM) at both primary and metastatic sites. Adhesion of SCLC cells to ECM enhances tumorigenicity and confers resistance to chemotherapeutic agents as a result of $\beta 1$ integrin-stimulated tyrosine kinase activation suppressing chemotherapy-induced apoptosis. SCLC may create a specialized microenvironment, and the survival of cells bound to ECM could explain the partial responses and local recurrence of SCLC often seen clinically after chemotherapy. Strategies based on blocking $\beta 1$ integrin-mediated survival signals may represent a new therapeutic approach to improve the response to chemotherapy in SCLC.

Lung cancer is the most common fatal malignancy in the developed world. Small cell lung cancer (SCLC), which constitutes 25% of all lung neoplasms, is characterized by early and widespread metastases. Etoposide is the most effective drug for use against SCLC and is commonly used in combination with doxorubicin and cyclophosphamide or platinum-based drugs¹. However, despite initial sensitivity to chemotherapy, this tumor almost invariably relapses and becomes resistant to further treatment; thus, the patient 2-year survival rate remains less than 5% (ref. 2). New therapeutic strategies are urgently needed, and these will most likely result from a better understanding of the cell biology of SCLC.

The integrin family of receptors represents the main extracellular matrix (ECM) receptors. The growth and survival of untransformed epithelial and endothelial cells requires adhesion to ECM proteins. When displaced from ECM, these cells undergo apoptosis. This phenomenon is called 'anchorage dependence'. After being activated by an ECM ligand, integrins stimulate intracellular signals, which can prevent entry into the cell death program^{3,4}. Integrins regulate many intracellular signaling pathways, such as tyrosine phosphorylation and inositol lipid metabolism⁵. This can lead to negative regulation of interleukin-1 β converting enzyme expression⁶, an essential effector of apoptosis. A characteristic feature of cancer cells is their ability to grow without the requirement of adhesion to ECM. This correlates closely with tumorigenicity in animal models⁷. Cytoplasmic oncogenes may provide a constitutively activated signal, usually initiated by ligand-bound integrins, thereby overcoming anchorage dependence. Anchorage-independent

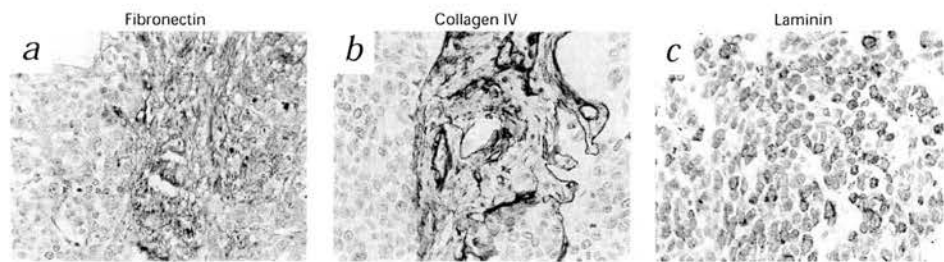
growth is seen *in vitro* in SCLC cells, which grow as non-adherent, free-floating aggregates.

Some forms of chemotherapy exert their cytotoxic effects mainly by inducing apoptosis^{8,9}. Tumor mass is a balance between cell proliferation and cell death (necrosis and apoptosis), and factors affecting this balance have a profound effect on tumor growth. Regulation of apoptosis in tumor cells remains poorly understood. However, the level of protein tyrosine kinase (PTK) activity may determine whether SCLC cells survive and proliferate or die by apoptosis¹⁰. SCLC cell growth *in vitro* may be sustained by multiple autocrine and paracrine loops involving calcium-mobilizing neuropeptides¹¹. This discovery led to the development of agents to block these growth loops for use in clinical trials in patients with SCLC, for example, neuropeptide receptor antagonists such as [D-Arg⁶, D-Trp^{7,9}, N¹⁰-Phe⁸]-substance P (6-11)(ref. 12) and monoclonal antibodies against the growth factor gastrin-releasing peptide¹³. The aim of our study here was to determine if the local environment of SCLC cells *in vivo* could provide a survival signal, or block a death signal, thereby accounting for the protection of SCLC cells from chemotherapy-induced apoptosis.

SCLC cells are surrounded by ECM proteins

We determined the ECM composition of unselected SCLC specimens from pathology files by immunohistochemical staining for fibronectin (Fn), laminin (Ln), collagen IV and tenascin. Normal areas of the lung showed Ln and collagen IV localized to the basement membranes of alveoli, septae, blood vessels and bronchial glands, whereas Fn staining showed diffuse weak pos-

Fig. 1 Immunohistochemical staining of ECM proteins around SCLC cells *in vivo*. Fibronectin (**a**) and collagen IV (**b**) immunoreactivity in SCLC lung. **c**, Intra-/pericellular immunoreactivity for laminin. Brown staining shows strong immunoreactivity. Original magnifications, $\times 40$.



itivity throughout the pulmonary interstitium. No tenascin immunoreactivity was seen in normal lung. In contrast, extensive (> 50% of section area) Fn, collagen IV and tenascin staining were seen in 56%, 87% and 61% of tumors, respectively, with focal areas of immunoreactivity seen in another 35%, 9% and 39% of sections, respectively (Table 1). Fn, collagen IV (Fig. 1) and tenascin (not shown) were expressed mainly in areas of reactive host connective tissue, which were present as extensive areas of scarring or as stromal bands delineating packets of invasive tumor cells. Intracellular staining for Fn and Ln was seen in 26% and 16% of tumors, respectively (Fig. 1 and Table 1). In SCLC sections, the basement membranes of the alveoli were considerably thickened compared with adjacent uninvolved areas, with increased expression of Fn, Ln and collagen IV. In three cases in which matched metastatic lymph nodes were available for examination, Fn, collagen IV and tenascin immunoreactivity was as extensive there as in the primary tumors (data not shown). Thus, *in vivo*, SCLC cells exist in an ECM-rich environment.

SCLC cells adhere to ECM proteins through $\beta 1$ integrins

We next determined the physiological relevance of these *in vivo* results using the classical SCLC cell lines H69, H345 and H510. Expression of individual integrin subunits on SCLC cells was homogeneous with an almost unimodal distribution. The main beta integrin expressed was $\beta 1$. The alpha integrins were those known to be involved in adhesion to collagen and Ln; ($\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 1$) and Fn; ($\alpha 3\beta 1$ and $\alpha v\beta 1$) (refs. 14–16; Table 2). These results were in agreement with those described in other SCLC cell lines¹⁷. In keeping with this, H69 cells adhered to Fn, Ln and collagen IV in a concentration-dependent manner (data not shown). Adhesion induced a profound change in SCLC cell morphology, with reorganization of the actin cytoskeleton. The cells adopted a flattened conformation and formed actin-rich filopodia (Fig. 2a), giving the appearance of differentiation to a neuron-like morphology. However, adhesion to ECM proteins had no effect on the expression of neuroendocrine markers chromogranin A, dopa-decarboxylase and neuron-specific enolase

(results not shown). Adhesion was stimulated by 1 mM Mn^{2+} (similar results were seen with 1 mM Mg^{2+}) and was inhibited by the function-blocking $\beta 1$ antibodies P5D2 and 4B4 (Fig. 2b; similar results were obtained in H510 and H345 cells). These results demonstrate that SCLC cells adhere to Fn, Ln and collagen IV in a mostly $\beta 1$ integrin-dependent manner.

ECM protects SCLC cells from chemotherapy-induced apoptosis

The addition of the chemotherapeutic agents doxorubicin, CIS-4-sulfoethylthio-cyclophosphamide (cyclophosphamide) and etoposide induced a concentration-dependent increase in SCLC cell apoptosis, as judged by morphology, electron microscopy, acridine orange/propidium iodide staining and immunoassay of cytoplasmic histone-associated DNA fragments. Adhesion of SCLC cells to Ln, Fn or collagen IV substantially protected H69, H345 and H510 cells against the apoptosis induced by these chemotherapeutic agents, reducing both the percentage and number of cells undergoing apoptosis (Fig. 2c). For example, in H345 cells, in response to 50 μM etoposide, Fn reduced apoptosis from 75% to 14%, which corresponded to a reduction from 1.05×10^5 to 0.33×10^5 apoptotic cells (similar results were obtained with cis-platinum). Nonspecific adhesion of H69 cells to poly-L-lysine did not protect cells from etoposide-induced apoptosis. Furthermore, the addition of the $\beta 1$ function-blocking antibody P5D2 abolished the ECM-mediated protection from etoposide-induced apoptosis (Fig. 2d). The addition of conditioned medium (from SCLC cells grown in the presence of collagen IV or Fn) did not protect SCLC cells adhered to poly-L-lysine from chemotherapy-induced apoptosis (data not shown). Thus, ECM-mediated protection from apoptosis was not mediated by the release of soluble autocrine growth factors. Therefore, ECM proteins acting through $\beta 1$ integrins protect SCLC cells from chemotherapy-induced apoptosis.

In liquid culture, the presence of Fn stimulated a 150–200% increase in SCLC cell number. Furthermore, Fn also reduced the cytotoxicity of cyclophosphamide and cis-platinum in H345 cells (50% inhibitory concentration (IC_{50}): 1.0–200 μM and 92–450 $\mu g/ml$, respectively; ($n = 3$; Fig. 3a). Similarly, the presence of Ln, Fn or collagen IV substantially reduced the sensitivity of all SCLC cell lines to the cytotoxic agents doxorubicin, cis-platinum, etoposide and cyclophosphamide (results not shown). Adhesion to Fn, Ln and collagen IV had no effect on cell–cell aggregation up to 24 hours, but at later time points, a small increase in cell aggregation was seen, which was abolished by function-blocking $\beta 1$ antibodies. Treating SCLC cells with either $\beta 1$ function-blocking or -stimulating antibodies in the ab-

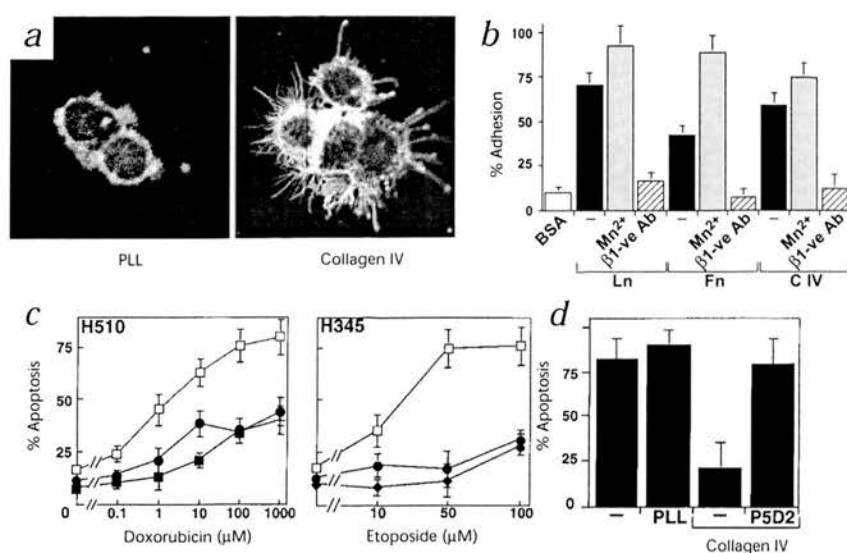
Table 1 Expression of ECM proteins in primary lung SCLC resection specimens

ECM	Number of cases	Stroma			Tumor cells			
		Extensive number	Focal number (%)	Negative number (%)	Positive number (%)	Negative number (%)		
Fibronectin	23	13 (56)	8 (35)	2 (9)	6 (26)	17 (74)		
Laminin	19	0 (0)	0 (0)	19 (100)	3 (16)	16 (84)		
Tenascin	23	14 (61)	9 (39)	0 (0)	0 (0)	23 (100)		
Collagen IV	23	20 (87)	2 (9)	1 (4)	0 (0)	23 (100)		

After immunohistochemistry, ECM protein staining within areas of carcinoma stroma was recorded on a semi-quantitative scale: no staining, focal staining or extensive staining (> 50% of section). Sections were also assessed for intracellular ECM protein expression. Data represent numbers of cases, with percentages in parentheses.

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Fig. 2 β 1-integrin-mediated adhesion to ECM proteins protects SCLC cells from chemotherapy-induced apoptosis. **a**, Adhesion of H69 SCLC cells to ECM. Confocal fluorescence microscopy of cells stained with rhodamine phalloidin after 4 h of attachment to poly-L-lysine (left) or collagen IV (right). **b**, Effect of Mn^{2+} and the β 1 function-blocking antibodies (β 1-ve Ab) P5D2 or 4B4 on SCLC cell adhesion to laminin (Ln) fibronectin (Fn) and collagen IV (C IV). -, negative control. Data represent the mean \pm s.e.m. of three to six independent experiments done in quadruplicate on duplicate plates. **c**, Effect of ECM proteins on chemotherapy-induced apoptosis in SCLC cells. SCLC cells were seeded in the presence of pre-coated fibronectin (\bullet), laminin (\blacksquare) or collagen IV (\blacklozenge) or in the absence of ECM (\square), then increasing concentrations (horizontal axes) of chemotherapeutic agents were added. Apoptosis was determined by acridine orange or Giemsa staining. Each point represents the mean \pm s.e.m. of two to four independent experiments. **d**, The effect of etoposide on H69 SCLC cell apoptosis in the presence of no coating matrix (-), poly-L-lysine (PLL) or collagen IV in the presence or absence



of the function-blocking antibody P5D2. Each bar represents the mean \pm s.e.m. of three independent experiments.

sense of ECM had no effect on SCLC cell growth, cell-cell aggregation or chemotherapy-induced apoptosis (data not shown). Tumor and transformed cells, including SCLC, are able to form colonies in agarose medium. Indeed, there is a positive correlation between the cloning efficiency of cells and the histological tumor involvement of SCLC cells¹⁸. The presence of Fn or Ln in the clonogenic assay caused a 200–300% stimulation of H69 cell colony formation (Fig. 3b). Further addition of neuropeptide growth factors bradykinin (10 nM) and galanin (50 nM) caused an additive effect. Moreover, Fn also reduced the cytotoxicity of etoposide in the clonogenic assay in H69 cells (IC_{50} : 3.2–9.3 μ g/ml; $n = 2$; Fig. 3c). These data indicate that *in vivo* β 1 integrin-mediated adhesion of SCLC cells to ECM proteins around the tumor is associated with enhanced tumorigenicity and increased resistance to standard chemotherapeutic agents.

PTK activation prevents chemotherapy-induced caspase activation

PTK activity in SCLC cells may be essential in determining whether they survive and proliferate or die by apoptosis¹⁰. Adhesion of H69 SCLC cells to Fn stimulates PTK activity in both basal cells (not treated with etoposide) and etoposide-treated cells. This effect was completely blocked by pretreatment with tyrphostin-25 (a selective PTK inhibitor) or a function-blocking β 1 antibody (Fig. 4a). The presence of Fn reduced the cytotoxicity of H69 SCLC cells to etoposide, shifting the concentration-response curve to the right (IC_{50} : 2.2–25 μ g/ml). This effect was blocked by the addition of function-blocking β 1 antibodies (Fig. 4b). Addition of tyrphostin-25 (25 μ M) also blocked the Fn-mediated reduction in cytotoxicity shifting the concentration-response to etoposide back to the left. As a consequence, in the presence of tyrphostin-25, the IC_{50} for etoposide was the same (5 μ g/ml) in the presence or absence of ECM (Fig. 4b). Similar results were obtained with Ln and collagen IV (results not shown). The addition of 25 μ M tyrphostin-25 to SCLC cells for 24 hours had no effect on SCLC cell adhesion to ECM (data not shown). These data show that β 1 integrin-dependent resistance to chemotherapy is mediated by a PTK-dependent mechanism.

Several of the chemotherapeutic agents used here, including

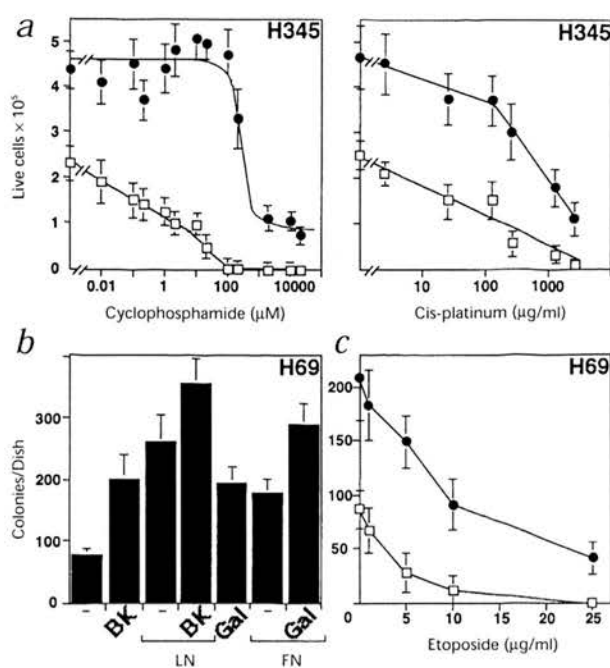
etoposide, function specifically by inhibiting the re-annealing action of topoisomerase II, resulting in DNA damage and apoptosis¹⁹. Caspase-3, one of the essential mediators of apoptosis, is activated by proteolytic cleavage of pro-caspase-3 (ref. 20). After being activated, caspase-3 cleaves the chaperone inhibitor of caspase activated deoxyribonuclease, releasing the DNAase activity, which causes DNA fragmentation in nuclei²¹. In H69 SCLC cells, the etoposide (25 μ g/ml)-induced pro-apoptotic effect and pro-caspase-3 cleavage was completely blocked by co-incubation with either the tyrosine phosphatase inhibitor sodium vanadate (200 μ M) or the broad-spectrum caspase inhibitor z-Val-Ala-DL-Asp-fluoromethylketone (z-VAD; 100 μ M) without affecting the level of pro-caspase-3 expression (Fig. 4c). In addition to blocking pro-caspase-3 cleavage, Fn reduced both the percent (43% to 11%) and number (4.8×10^4 to 1.45×10^4) of cells undergoing apoptosis in response to 25 μ g/ml etoposide. Furthermore, the Fn-mediated protection of H69 SCLC cells from etoposide-

Table 2 Integrin expression in SCLC cell lines

Cell line	H69	H345	H510
Integrin subunit			
β 1	30.8 \pm 3.5	17.1 \pm 1.4	42.3 \pm 3.9
β 2	5.0 \pm 0.3	1.4 \pm 0.2	4.2 \pm 0.4
β 3	6.8 \pm 0.5	6.8 \pm 0.6	4.5 \pm 0.7
β 4	6.7 \pm 0.9	4.6 \pm 0.5	6.0 \pm 0.1
β 5	5.6 \pm 0.8	3.8 \pm 0.2	6.8 \pm 0.6
β 6	5.6 \pm 0.6	5.9 \pm 0.4	2.5 \pm 0.1
α 1	4.3 \pm 0.2	6.1 \pm 0.5	4.9 \pm 0.8
α 2	2.3 \pm 0.3	7.1 \pm 0.6	17.4 \pm 2.2
α 3	36.4 \pm 2.5	38 \pm 2.2	30 \pm 1.8
α 4	5.7 \pm 0.4	6.5 \pm 0.3	3.2 \pm 0.
α 5	3.0 \pm 0.2	2.4 \pm 0.4	4.4 \pm 0.4
α 6	32.4 \pm 2.8	22.5 \pm 2.5	17.1 \pm 2.9
α v	20.6 \pm 3.1	14.7 \pm 2.8	26.3 \pm 1.2

Integrin expression was determined by flow cytometric analysis using an indirect immunofluorescent technique with monoclonal antibodies against integrin subunit. Each value represents the mean fluorescence intensity, arbitrary units (AU) \pm s.e.m., of three to four independent experiments. Background control antibody mean fluorescence values were 2.4 \pm 0.2 AU.

Fig. 3 ECM proteins stimulate SCLC cell growth and tumorigenicity. **a**, The chemosensitivity of H345 SCLC cells to increasing concentrations (horizontal axes) of chemotherapeutic agents was assessed in the presence (●) or absence (□) of fibronectin. Live cells were assessed by trypan blue exclusion. Data represent mean \pm s.e.m. of three independent experiments done in quadruplicate in duplicate plates. **b**, The effect of ECM proteins laminin (Ln) and fibronectin (Fn) on H69 SCLC cell colony formation in the presence of bradykinin (BK) or galanin (Gal). The clonogenic assay was done in triplicate in two independent experiments. Data represent mean \pm s.d. **c**, Inhibition of H69 cell colony formation by increasing concentrations (horizontal axis) of etoposide, in the presence (●) or absence (□) of fibronectin. Data represent mean (of two independent experiments done in triplicate) \pm s.d.



induced apoptosis and pro-caspase-3 cleavage was abrogated by either the addition of a function-blocking β_1 antibody or the PTK inhibitor tyrphostin-25 (25 μ M). This tyrphostin effect was blocked by 100 μ M z-VAD without affecting the level of pro-caspase-3 expression. The function-blocking β_1 antibody had no effect on etoposide-induced apoptosis in the absence of Fn. Apoptosis induced by DNA damage can be suppressed by Bcl-2 (ref. 22) through a mechanism that prevents caspase-3 activation²³. Adhesion to Fn or treatment with etoposide has no effect on the level of Bcl-2 expression in SCLC cells (data not shown). Adhesion to Fn or treatment with tyrphostin-25 did not substantially alter either the expression or activity of topoisomerase II in basal or etoposide-treated cells (Fig. 4d). Furthermore, treatment with z-VAD did not affect etoposide-inhibition of topoisomerase II (results not shown). Thus adhesion to Fn through β_1 integrins protects SCLC cells from etoposide-induced caspase-3 activation and apoptosis by activating PTK signaling downstream of DNA damage. In addition, z-VAD prevents apoptosis at a point downstream of DNA damage, preventing activation and cleavage of pro-caspase-3 by upstream caspases.

Discussion

This study shows that SCLC, *in vivo*, is surrounded by an extensive stroma of ECM at both primary and secondary metastatic sites. This stroma contains, in part, Fn, Ln, collagen IV and tenascin. The extent of ECM deposition around SCLC cells *in vivo* has not generally been appreciated. The origin of ECM proteins in SCLC stroma is unclear, but may be the consequence of complex autocrine and paracrine interactions involving fibroblasts and other surrounding cells. SCLC cells secrete many growth factors, including insulin-like growth factor and neuropeptides such as gastrin-releasing peptide, which are mitogenic both for SCLC cells and fibroblasts^{24,26}. In addition, a basal 'feeder' layer of human fibroblasts can stimulate the clonal growth of SCLC cells. Furthermore, SCLC cell lines also secrete many inflammatory cytokines and mediators involved in tissue repair (T.S., S.C.D. & R.S., unpublished results). These include TGF- β and IL-8 (an essential angiogenic factor for non-small cell lung cancer growth in nude mice²⁷). In cultured fibroblasts, TGF- β modulates the splicing of the primary Fn transcript, leading to the production of other ECM proteins, including Ln and tenascin^{28,29}. SCLC cell lines secrete Fn, as shown by western blot analysis (T.S. & R.C.R., unpublished results), and the detection of intracellular and pericellular staining for Fn, collagen IV, Ln and tenascin in pathological samples raises the possibility that, at least in some cases, SCLC cells may secrete these ECM proteins. ECM may also be a substrate for neovascularization³⁰ further facilitating metastasis. Therefore, *in vivo*, SCLC may create a specialized environment as a consequence of autocrine and

paracrine effects that, using an analogy to inflammation, likens SCLC to 'a wounding reaction' with the laying down and remodeling of ECM, growth factor release and neovascularization.

The exact function of tenascin is unclear. It is known to be an oncodevelopmental protein expressed in fetal tissue³¹ and in adult tissues that are actively remodeling, where it is often co-expressed with matrix metalloproteases³², supporting the idea that the local environment around SCLC is like an ongoing inflammatory reaction. Tenascin may facilitate growth factor-dependent proliferation and protect cells from apoptosis, possibly in conjunction with other ECM proteins³³.

Doxorubicin, cyclophosphamide, etoposide and cis-platinum caused a concentration-dependent stimulation of apoptosis in SCLC cells. Overexpression of the multidrug resistance gene MDR1 is not common in SCLC, indicating that, unlike in other tumors, this is not an important mechanism for drug resistance in these tumors³⁴. The binding of SCLC cells to laminin reduces the cytotoxicity of certain chemotherapeutic agents³⁵. We have shown here that this is a general property of many ECM proteins found in increased amounts around SCLC *in vivo*. This occurs as a result of β_1 integrin-dependent adhesion to Ln, Fn and collagen IV. Despite their anchorage independence, the binding of SCLC cells to ECM proteins confers protection from chemotherapy-induced apoptosis. Furthermore, Ln and collagen IV are the main glycoproteins of basement membranes, and induce migration, probably by haptotaxis³⁶. This seems to be important in metastasis in many tumor cell lines. Thus, at primary and secondary metastatic sites, there seems to be a permissive environment for SCLC cell proliferation, protection from apoptosis and resistance to chemotherapy.

ECM suppression of cytotoxicity is not due to differentiation to a chemoresistant phenotype, a lower growth rate or ECM-stimulated autocrine secretion. Function-blocking β_1 and function-stimulating β_1 antibodies did not substantially affect SCLC cell-cell adhesion. The small increase in cell aggregation seen after prolonged contact with Fn is therefore most likely due to clonal expansion. Changes in cell-cell adhesion do not seem to

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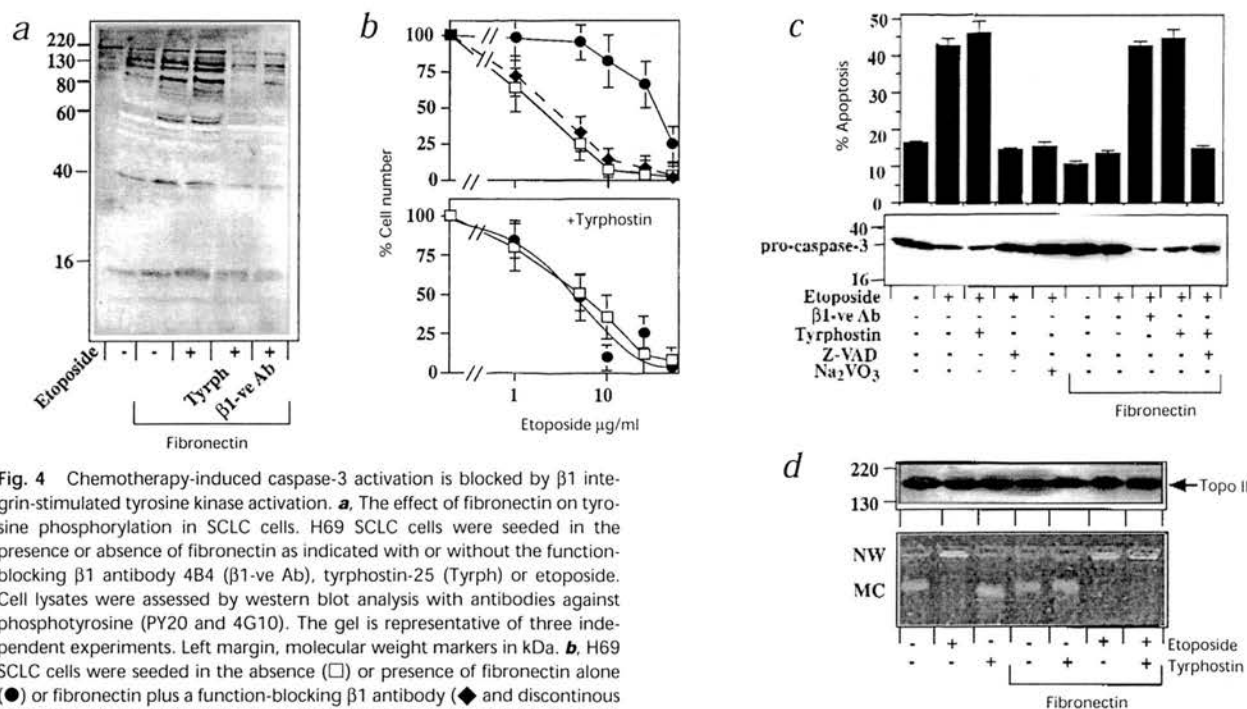


Fig. 4 Chemotherapy-induced caspase-3 activation is blocked by $\beta 1$ integrin-stimulated tyrosine kinase activation. **a**, The effect of fibronectin on tyrosine phosphorylation in SCLC cells. H69 SCLC cells were seeded in the presence or absence of fibronectin as indicated with or without the function-blocking $\beta 1$ antibody 4B4 ($\beta 1$ -ve Ab), tyrphostin-25 (Tyrph) or etoposide. Cell lysates were assessed by western blot analysis with antibodies against phosphotyrosine (PY20 and 4G10). The gel is representative of three independent experiments. Left margin, molecular weight markers in kDa. **b**, H69 SCLC cells were seeded in the absence (\square) or presence of fibronectin alone (\bullet) or fibronectin plus a function-blocking $\beta 1$ antibody (\blacklozenge and discontinuous line). Etoposide (concentrations, horizontal axis) and diluent (upper) or tyrphostin-25 were added, then cells were aspirated and counted. Cell number in the absence of etoposide for each condition was considered 100%. Data represent the mean \pm s.e.m. of three independent experiments done in quadruplicate in duplicate plates. **c**, Effect of fibronectin on etoposide-induced apoptosis and caspase-3 activation. H69 cells were seeded, and etoposide, $\beta 1$ blocking antibody ($\beta 1$ -ve Ab), tyrphostin, z-VAD or sodium orthovanadate (Na_2VO_3) were added to each well. Upper, apoptosis, determined by acridine orange staining; data represent mean (of three independent experiments done in triplicate) \pm s.e.m. Lower, western blot analysis with antibody against

pro-caspase 3; blot is representative of a parallel sample. Left margin, molecular weight markers in kDa. **d**, Effect of fibronectin on topoisomerase II α (Topo II) expression in the presence and absence of etoposide and tyrphostin. Upper, western blot analysis with antibody against human topoisomerase II α . Left margin, molecular weight markers in kDa. Lower, decatenation assay of topoisomerase II α activity; NW, high-molecular-weight interlocking kDNA network; MC, decatenated individual DNA mini circles. Gels shown are representative of two independent experiments done in duplicate.

be important in ECM-mediated resistance to chemotherapy. Our results indicate that the resistance to chemotherapy induced by $\beta 1$ -mediated adhesion to ECM is due to an increase in the level of PTK activity. However, increased intracellular tyrosine phosphorylation had no effect on etoposide-induced topoisomerase II inhibition. Thus, despite chemotherapy-induced DNA damage, ECM-mediated PTK activation blocks caspase activation, which prevents apoptosis. It is not known how integrin-stimulated PTK activation suppresses the early phase of apoptosis in SCLC cells. R-Ras and insulin-like growth factor-1, which activate phosphatidylinositol-3 kinase (PI3K), cooperatively inhibit caspase-3 activation, preventing apoptosis of BaF3 cells³⁷. Activation of PI3K by integrins protects epithelial cells from detachment-induced apoptosis³⁸. Integrin-stimulated PI3K activation may impinge on the nuclear response to DNA-damaging agents.

We made a retrospective analysis of available clinical case notes to determine the translational research implications of these findings. Sixteen patients 62.5 ± 2.3 years of age who had received some standard form of chemotherapy were identified; eight had no or focal Fn matrix and eight had extensive Fn matrix. They had a survival time of 11.5 ± 1.7 months (mean \pm s.e.m.). There was no substantial age or sex difference between the two groups. Patients with extensive matrix around their tumors had a significantly shorter survival time from diagnosis

(8.4 ± 1.7 months) than did patients with focal or no matrix (15 ± 2.6 months; mean \pm s.e.m.; $P < 0.05$, paired Student's *t*-test).

Our *in vitro* and *in vivo* data provide strong preliminary evidence that adhesion to ECM proteins is essential in SCLC cell resistance to chemotherapy. Cancer cells bound to ECM may escape chemotherapy-induced cell death and then, with subsequent genetic damage, drug-resistant clones are selected. This is an excellent model to explain not only SCLC behavior *in vivo* but also why partial responses and local recurrence of SCLC are often seen after chemotherapy. With the development of increasingly specific tyrphostins, identification of the PTKs mediating $\beta 1$ -integrin-dependent survival signals may provide new therapeutic strategies to improve the response of SCLC to chemotherapy.

Methods

Cell Culture. H69, H510 and H345 cell lines from ATCC (Rockville, Maryland) were grown in RPMI 1640 medium (Sigma) supplemented with 10% (volume/volume) heat-inactivated fetal calf serum and 5 mg/ml L-glutamine in an atmosphere of 5% CO_2 . For experiments, cells were grown in SITA (RPMI 1640 supplemented with 30 nM selenium, 5 $\mu\text{g}/\text{ml}$ insulin, 10 $\mu\text{g}/\text{ml}$ transferrin and 0.25% (weight/volume) BSA). Cell viability was routinely greater than 90%, as judged by trypan blue exclusion.

Tissue immunohistochemistry. Formalin-fixed, paraffin-embedded SCLC resection specimens from University of Edinburgh Department of Pathology files (1989–1994) were de-waxed in xylene and rehydrated through graded alcohols to water. Antigen unmasking was done by either pre-incubating

sections in 0.1% trypsin, pH 7.6, at 37 °C for 15 min (for Fn, Ln and tenascin) or boiling them in a microwave oven for 15 min in 0.1 M citrate, pH 6.0 (for collagen IV). Primary antibodies against Fn (1:100 dilution; FN-3E2), Ln (1:50 dilution; LAM-89), tenascin (1:400 dilution; BC-24) and collagen IV (1:100 dilution; Col-94) were all from Sigma. Sections were processed using a Techmate 500 (Dako, Carpinteria, California). Antibody detection was done using a multilink streptavidin-biotin complex method, and visualization was achieved using a di-amino benzidine chromagen method following the manufacturer's instructions (Dako, Carpinteria, California). Slides were counterstained using Harris' hematoxylin. Negative control samples were incubated in primary antibody diluent only. All slides were reviewed independently by two people. Staining was graded on a semi-quantitative scale as no staining, focal staining or extensive (> 50% of section area) staining.

Flow cytometric analysis of integrin expression. Cells (1×10^5) were washed in PBS containing 0.2% (weight/volume) BSA and 0.1% sodium azide (EPICS buffer), plated onto 96-well flexible assay plates and incubated at 4 °C for 30 min with integrin subunit antibodies (1:500 dilution) followed by a 30-minute incubation with species-appropriate FITC-conjugated secondary antibodies (1:40 dilution; Dako, Carpinteria, California). Cells were resuspended in EPICS buffer and analyzed by flow cytometry using an EPICS profile 2 (Coulter, Hialeah, Florida). All photomultiplier settings were standardized throughout the study. Mean fluorescence intensity arbitrary units on a log scale were recorded for each sample. The antibodies against integrin were all from Serotec unless otherwise stated: α v (13C2; M. Horton, Edinburgh, UK), α 2 (AK7), α 3 (11G5), α 4 (HP2/1), α 5 (SAM-1) and α 6 (4F-10 (IgG2b)). β 6 (E7P6G10P0) and β 5 (Pif6) were from D. Salter (Edinburgh, UK); β 4 (3E1) was from Life Technologies; β 3 (PM6/13) and β 2 (MHM23) were from Dako (Carpinteria, California); and β 1 (mAb13) was from Becton Dickinson (San Jose, California).

Adhesion assay. First, 96-well plates (CoStar, Cambridge, Massachusetts) were coated with 100 μ l of 20 μ g/ml fibronectin, 10 μ g/ml laminin (Life Technologies) or 10 μ g/ml collagen IV (Sigma) overnight at 4 °C and blocked with 1 mg/ml BSA (1 h at 25 °C). Then, SCLC cells (5×10^4) that had been washed twice in RPMI were added to the wells in the presence or absence of the function-blocking β 1 antibodies P5D2 (10 μ g/ml; P. Hughes, Scripps Research Institute) or 4B4 (10 μ g/ml; Coulter, Hialeah, Florida) or 1 mM Mn^{2+} . After 45 min of incubation at 37 °C, non-adherent cells were removed by gentle washing and adherent cells were fixed with 3% formaldehyde and stained with 1% methylene blue. After plates were washed with distilled water, 100 μ l of 0.1 M HCl was added to each well and the absorbance of the resulting solution was measured at 630 nm on a plate reader (Dynatech Technologies, Chantilly, Virginia). Each condition was assayed in quadruplicate in duplicate plates.

Chemosensitivity assay. Cells (0.5×10^5 – 1.0×10^6) cultured in SITA medium were seeded into 96-well microtiter plates in the presence or absence (where indicated) of 100 μ l pre-coated fibronectin (20 μ g/ml), laminin (10 μ g/ml) or collagen IV (10 μ g/ml). The cytotoxic agents platinum (cis-platinum (II) diammine dichloride), etoposide, doxorubicin (Sigma) and cyclophosphamide (CIS-4-sulfoethylthio-cyclophosphamide) (Asta Pharmaceuticals, Frankfurt, Germany) were added to each well 1 h after seeding. Where indicated, function-blocking β 1 antibodies (P5D2 or 4B4, both at 10 μ g/ml) were added at the time of cell seeding, and etoposide and/or 25 μ M tyrphostin-25 (Calbiochem, La Jolla, California) were added with the cytotoxic agent (1 h after cell seeding). Each condition was assayed in quadruplicate in duplicate plates. After 72 h, cells were either detached from the plates and aspirated and cytospun onto glass slides. Apoptotic cell populations were then assessed (described below). Live cells were determined by trypan blue exclusion, using a hemocytometer. The total cell number was determined using a Coulter counter (Coulter, Hialeah, Florida).

Assessment of SCLC cell apoptosis. To assess apoptosis, 1 μ l of an ethidium bromide (100 μ g/ml)/acridine orange (100 μ g/ml) (1/1 volume/volume) mixture was added to 200 μ l SCLC cells, and the percentage of cells undergoing apoptosis was determined using fluorescent microscopy as described³⁹. Apoptosis was also assessed using a Cell Detection ELISA^{PLUS} kit

following the manufacturer's instructions (Boehringer), or by staining cytospin preparations using May-Grünwald-Giemsa stain.

Clonogenic assay. Cells (1×10^4) were suspended in a 0.3% (weight/volume) agarose/SITA mix containing neuropeptides (10 nM bradykinin or 50 nM galanin), ECM proteins (10 μ g/ml laminin or 20 μ g/ml fibronectin) and 0–25 μ g/ml etoposide. The cells were layered over a solid base of 0.5% (weight/volume) agarose in SITA containing the same additives, in 35-mm plastic dishes. Cultures were incubated in a humidified atmosphere of 5% CO₂/95% air at 37 °C for 21 d, and were then stained with nitroblue tetrazolium. Colonies greater than 120 μ m in diameter (16 cells) were counted using light microscopy.

Confocal microscopy. SCLC cells (1×10^5) in SITA medium were allowed to attach for 45 min at 37 °C to glass cover slips pre-coated with 25 μ g/ml poly-L-lysine or 10 μ g/ml collagen IV. Unattached cells were gently removed by washing with SITA medium and attached cells were allowed to spread for 4 h. After fixation with 3% paraformaldehyde in PBS for 20 min, residual paraformaldehyde was 'quenched' using 50 mM NH₄Cl/PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 4 min and were blocked with 0.2% fish skin gelatin/PBS before being stained with rhodamine phalloidin (1:400 dilution) for 20 min at room temperature. Immunofluorescence was measured using a BioRad MRC 600 laser confocal microscope with a Zeiss 63 \times objective.

Immunoblotting. To determine the effect of fibronectin on tyrosine phosphorylation, 1×10^6 H69 SCLC cells cultured in SITA medium were seeded in 6-well plates in the absence or presence of 20 μ g/ml fibronectin with or without function-blocking β 1 antibody (10 μ g/ml 4B4). Etoposide (25 μ g/ml) or tyrphostin-25 (25 μ M) were added to each well 1 h after cell seeding. After 72 h, cells were aspirated and pelleted, and lysates were assessed by western blot analysis (see below) with anti-phosphotyrosine antibodies. A 50:50 mixture of PY20 (Santa Cruz Biotechnology, Santa Cruz, California) and 4G10 (Upstate Biotechnology, Lake Placid, New York) was used.

To determine the effect of fibronectin on etoposide-induced apoptosis and caspase-3 activation, H69 SCLC cells (1×10^6) cultured in SITA medium were seeded in 6-well plates. Etoposide (25 μ g/ml), function-blocking β 1 antibody (10 mg/ml), tyrphostin-25 (25 μ M), z-Val-Ala-DL-Asp-fluoromethylketone (100 μ M) or sodium orthovanadate (200 μ M) were added to each well 1 h after cell seeding. After 72 h of culture, apoptosis was determined by acridine orange staining (described above) and the level of pro-caspase 3 was determined by western blot analysis (see below) using an anti pro-caspase 3 antibody (CPP-32; Transduction Laboratories, Lexington, Kentucky).

For western blot analysis, cell pellets were lysed at 4 °C using standard RIPA buffer and 20 μ g of protein were separated by 10% SDS-PAGE. Proteins were transferred to Hybond C nitrocellulose membranes (Amersham), and immunodetection was done using the enhanced chemiluminescence system (ECL; Amersham). The antibody against human topoisomerase II α was from TopoGEN (Columbus, Ohio). Species-appropriate HRP-conjugated secondary antibodies were from Dako (Carpinteria, California).

Decatenation assay. Cells (5×10^6) were cultured in SITA medium in 6-well plates in the presence or absence of 1 ml pre-coated fibronectin (20 μ g/ml). Cells were allowed to 'settle' before the addition of 25 μ g/ml etoposide or 25 μ M tyrphostin-25. Each condition was assayed in duplicate. After 72 h, nuclear extracts were prepared as described⁴⁰. Topoisomerase II activity was measured by the ATP-dependent decatenation of kDNA, in which an interlocking kDNA network is decatenated to individual DNA minicircles and small catenanes. Reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 100 mM KCl, 0.5 mM EDTA, 30 μ g/ml BSA, 1 mM ATP, 0.5 mM dithiothreitol and 100 μ g/ml kDNA) was mixed with drug and enzymes (in that order) in a total volume of 45 μ l, and the reaction mixtures were incubated for 30 min at 37 °C. The reaction was then stopped and reaction products were analyzed by agarose gel electrophoresis in 1% gels using 89 mM Tris-borate (pH 8.3) and 2 mM EDTA buffer.

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