INTEGRIN AFFINITY MODULATION

AND

LUNG CANCER

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University of Edinburgh Medical School

Declaration

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous candidature for a higher degree. All work presented in this thesis was initiated and undertaken by myself, unless otherwise acknowledged in the text. All sources of information in the text have been acknowledged by reference.

Robert Christians Buttery
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Abstract

Integrin Affinity Modulation in Lung Cancer

Lung cancer accounts for the most deaths due to cancer in the United Kingdom and yet has historically been one of the most ignored of neoplastic diseases. The work presented in this thesis explores the fundamental processes that govern cell behaviour in the context of lung cancer and contributes to a deeper understanding of this behaviour at a molecular level. The work covers three main areas, centred upon the molecular regulation of integrins, proteins that are the key communicators between a cell and its local environment and which provide powerful signals governing cellular behaviour, including motility, cell survival and proliferation.

Recent work has shown that the transmembrane protein CD98 is able to influence the affinity with which β1 integrins bind to extracellular ligands. The first part of this thesis presents confocal microscopy and co-immunoprecipitation experiments that confirm the physical juxtaposition of the two proteins within the cell membrane, suggesting a direct link between the two, rather than an extensive signalling cascade. It also demonstrated that cross-linking CD98 stimulates both phosphoinositide 3-kinase intracellular signalling and increased β1 integrin-dependent cellular adhesion. Because of the role of CD98 in integrin affinity modulation, the immunohistochemical expression of CD98 and its ligand, galectin-3, was studied in a variety of human lung diseases including lung cancers. The major finding of this work was a striking distinction between high expression of galectin-3 in non-small cell lung cancer and low expression in small cell lung cancer. This may have

significant implications for the differing clinical behaviours of these two groups of cancers. The final section of this thesis returns to describe experiments aimed at defining the molecular regulators of integrin affinity more clearly. A genetic screen of a cDNA library was undertaken to identify candidate genes coding for proteins able to rescue integrins from the low affinity state induced by the small signalling protein H-Ras. This identified a candidate cDNA 480, recognised to be part of a novel gene *Nessie*, coding for a large protein with multiple transmembrane domains. Both 480 and *Nessie* appear to have the ability to rescue integrin affinity from H-Ras suppression.

This thesis thus moves from the basic molecular science of integrin function to the cellular behaviour of lung cancer cells, both *in vitro* and *in vivo*, and back again. The understanding of cellular behaviour is central not just to lung cancer, but to all cancers and it is only through furthering this understanding that significant advances will be made in treating these diseases.

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List of Abbreviations

AI	Activation Index
ATP	Adenosine 5' triphosphate
AU	Arbitrary Units
Bcl-2	B cell leukaemia oncogene 2
bp	Base pair
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CAAX	Cysteine-Aliphatic amino acid-Aliphatic amino acidSerine/Methionine
$CaCl_2$	Calcium chloride
CD	Cluster of differentiation
cDNA	Complimentary DNA
CHO	Chinese hamster ovary
CMV	Cytomegalovirus
DAG	Diacylglycerol
DMEM	Dulbeccos Modified Eagles Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiotreitol
E. Coli	*Eschericia Coli*
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis (β-aminoethyl ether) N,N,N'N'-tetracetic acid
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorter
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
G12V	$Glycine^{12} \rightarrow Valine^{12}$
G38V	$Glycine^{38} \rightarrow Valine^{38}$
G418	Geneticin
GAP	GTPase activating protein
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
Grb2	Growth-factor-receptor-bound protein 2
GST	Glutathione-*S*-transferase
GTP	Guanine triphosphate
HCl	Hydrochloric acid
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) hemisodium salt
HRP	Horseradish peroxidase
IC50	Inhibitory concentration 50%

ICAM	Immunoglobulin-like cell adhesion molecule
IP_3	Inosito 1,4,5-trisphosphate
IPTG	Isopropylthio-β-D-galactoside
JNK	c-Jun N-terminal kinase
kb	Kilobase
kDa	Kilodalton
LB media	Luria-Bertani media
LN	Laminin
M	Molar
MAPK	Mitogen activated protein kinase
MEK	MAPK/ERK kinase
$MgCl_2$	Magnesium chloride
MKP	MAPK phosphatase
Mn^{2+}	Manganese
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NaF	Sodium fluoride
NSCLC	Non-small cell lung cancer
OD	Optical density
$p70^{s6k}$	Ribosomal protein S6 kinase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	PD098059
PDGF	Platelet derived growth factor
pERK	phosporylated ERK
PI 3-kinase	Phosphoinositide 3-OH kinase
PKC	Protein kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PS	Phosphatidylserine
PtdIns/PI	Phosphatidylinositol
RalGDS	Ral guanine nucleotide dissociation stimulator
RalGEF	Ral guanine exchange factor
RGD	Arginine-glycine-aspartic acid
Rgl	RalGDS-like factor
Rlf	RalGDS-like
R-PE	R-phycoerythrin
RTK	Receptor tyrosine kinase
SCLC	Small cell lung cancer
SDS	Sodium dodecylsulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	Standard error of mean
SH	Src homology
Shc	SH2 domain-containing α2-collagen related protein
SOS	Son of Sevenless
Src	Rous sarcoma virus
T35S	$Threonine^{35} \rightarrow Serine^{35}$

Thr	Threonine
TM	Transmembrane
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothial cell growth factor

Chapter 1

Introduction

Section A: Lung Cancer

1.1 Epidemiology of lung cancer

Headlines from the press over recent years emphasize the increasing social vilification of smoking in Europe and the United States. Together with multibillion-dollar litigation suits against tobacco companies, this might suggest that the tide has turned against lung cancer, one of the most devastating effects of smoking. Sadly this is not the case. Lung cancer remains the most lethal cancer in the world. It has been estimated that more people died in 2002 in the U.S.A. from lung cancer than from prostate, breast, colon and cervical cancer put together (Jemal *et al.*, 2002). In the United Kingdom alone, lung cancer is estimated to have caused 33,600 deaths in 2002, an average of 92 people every day, or one person every fifteen minutes (Cancer Research UK, 2004). In the year 2000, approximately 1.2 million people globally were diagnosed with lung cancer, with estimated deaths of 1.1 million as a direct consequence of lung cancer (Mathers CD, 2001). In particular, the continued high levels of smoking amongst younger generations and a burgeoning population of smokers in developing countries such as China (Liu *et al.*, 1998;Zhang and Cai, 2003), targeted by the tobacco companies, mean that lung cancer will remain an enormous healthcare burden for the foreseeable future. In 1998 it was estimated that approximately one third of the 0.3 billion (i.e. 100 million) Chinese

males then aged 0-29 years would die of tobacco-induced disease; approximately 15% of these would be from lung cancer (Liu *et al.*, 1998).

In populations with low tobacco smoking, the age-adjusted incidence of lung cancer is low, at around 1-3/100,000 (Doll and Peto, 1976). The highest incidences are found in industrialised societies: in 1999, Hungary and Scotland had the highest age-related incidences of lung cancer in the world, amongst both males (120.7/100,000 and 92.7/100,000, respectively) and females (39.6/100,000 and 62.9/100,000, respectively) (IARC, 2003). The incidence of lung cancer rises with age as a result of trends in tobacco smoking over the past 50 years. More recently there has been a decline in the number of male smokers, at least in the U.K., but the number of female smokers has continued to rise. A worrying rise in the incidence of lung cancer in women (Osann, 1998) has been blamed partly upon this, but there is also emerging evidence that women are at greater risk of developing lung cancer for fundamental biological reasons (Osann *et al.*, 1993).

1.2 Aetiology of lung cancer

1.2.1 Tobacco smoke

The clear, causative link between tobacco smoking and lung cancer was established in seminal work by Doll (Doll and Hill, 1954). By studying the smoking habits of a group of over 40,000 British doctors over more than a decade, Doll found clear evidence of a

link between smoking and deaths from lung cancer (Doll and Hill, 1964;Azzarone *et al.*, 1986). Pipe and cigar smokers have a reduced risk compared to cigarette smokers, considered to be due to decreased inhalation of the tobacco smoke. The risk of lung cancer increases with the number of cigarettes smoked and the duration of the smoking habit. Conversely, there is also good evidence that smoking cessation immediately begins to reduce this risk, although it takes many years for it to approach the risk of lifelong non-smokers. More recently, Peto has suggested that smoking cessation rapidly reduces the risk of developing lung cancer, such that cessation by age 50 or age 30 reduces the cumulative risk of lung cancer by age 75 to 6% and 2% respectively (Peto *et al.*, 2000).

There are numerous known carcinogens in tobacco smoke, both in the particulate and the vapour phase, as shown in **Table 1a**. Historically, post-mortem studies showed a strong association between the number of cigarettes smoked during life and the presence of metaplasia and dysplasia found at autopsy (Auerbach *et al.*, 1979). The natural history of such dysplastic lesions is unclear, however, since not all appear to go on to develop into carcinomas.

Table 1a: Carcinogens in particulate and vapour phases of tobacco smoke

Particulate Phase		Vapour Phase
Neutral fraction	Benzpyrene Dibenzanthracene Benzofluoranthenes	Nickel carbonyl Hydrazine Vinyl chloride Nitrogen oxides Nitrosodiethylamine
Basic fraction	Nitrosamines	
Acidic fraction	Tumour-promoting agents	
Residual fraction	Nickel Cadmium Polonium-210	

1.2.2 Other risk factors

Other agents than smoking contribute to the risk of developing lung cancer. These occupational and environmental hazards include asbestos, chromium, nickel, arsenic, chloromethyl ethers, vinyl chloride, uranium, radon and polyaromatic hydrocarbons. They probably account for 5-15% of total lung cancer deaths. During the 1950s it was estimated that air pollution in the U.K., primarily in the form of polycyclic hydrocarbons from domestic coal fires, contributed to approximately 10% of lung cancer in men.

Asbestos exposure is the most pre-eminent of these compounds. Although most notorious for its causative link with mesothelioma, exposure to high levels of asbestos, especially amphibole asbestos (crocidolite and amosite), is associated with increased risk of carcinoma. Tobacco smoking is an important co-factor in this risk and its effect is multiplicative: asbestos exposure alone increases the risk of lung cancer by five times compared with unexposed individuals, but individuals who smoke and have high

asbestos exposure have a 53-times greater incidence of lung cancer than those who have neither risk factor (Hammond *et al.*, 1979).

Fibrotic lung disease appears to increase the risk of lung cancer. Idiopathic pulmonary fibrosis confers a 14-fold increased incidence of carcinoma (Hubbard *et al.*, 2000), similar to that seen in asbestosis. In contrast to the normal population, carcinomas in both conditions tend to arise more in the lower lobes, where the fibrotic process is most pronounced.

Recent studies have implicated viral oncogenes in lung carcinogenesis. For example, in a small study of just 34 cases, papilloma virus (type 18) was identified in a small proportion (two cases) of squamous cell carcinomas (Bohlmeyer *et al.*, 1998) and Epstein-Barr virus in bronchopulmonary lymphoepitheliomas, but only rarely in more common histological subtypes (Higashiyama *et al.*, 1995).

Finally, not all smokers develop lung cancer. Indeed, the majority never develop the disease, which affects only about 10% of smokers. This suggests that genetic predisposition and other factors play important roles in carcinogenesis. However, although epidemiological studies have shown an excess of lung cancer in first degree relatives (Ooi *et al.*, 1986;Lynch *et al.*, 1986), in such studies it is difficult to control for the pervasive confounding factors of smoking and passive smoking within families. By contrast, a study of almost 6,000 pairs of male twins failed to confirm any genetic

predisposition (Braun *et al.*, 1994). Nevertheless, the case for genetic susceptibility to cancer remains persuasive. In particular, enzymes responsible for carcinogen activation, degradation and subsequent DNA repair are being studied, since mutations affecting enzyme activity in these key processes might significantly influence the risk of developing lung cancer.

1.3 Classification of lung cancer

The World Health Organisation has classified lung cancer into four main subgroups, which together comprise 95% of all lung cancers (WHO, 1999): squamous cell carcinoma, adenocarcinoma, large cell carcinoma (collectively described as "non-small cell carcinomas" (NSCLC)) and small cell carcinoma (SCLC). A number of uncommon types are also identified, that include mucoepidermoid and adenoid cystic carcinomas, and carcinoid tumours. Most tumours arise within the airspaces or bronchi from epithelial cells, of which there are four main types – basal, mucous, neuroendocrine and ciliated. Apart from the ciliated cells, which are terminally differentiated, all are capable of division and thus considered to have malignant potential. Amongst lung carcinomas, SCLC and squamous cell carcinomas show the highest rate of growth when measured by volume doubling time (Geddes, 1979;Hasegawa *et al.*, 2000).

1.3.1 Squamous cell carcinoma

These carcinomas arise endobronchially and centrally within the lung and are considered the least malignant phenotype. They show either keratinisation or intercellular tight junctions, or both. They tend to metastasise late, causing symptoms by local mass effect, such as atalectasis or segmental/lobar collapse. Historically, squamous cell carcinoma has been the commonest histological subtype.

1.3.2 Adenocarcinoma

The characteristic of adenocarcinomas is the development of glandular or papillary structures, with mucin production. They metastasise earlier than squamous cell tumours, possibly by aerogenous spread and aspiration into other areas of the lung, forming disseminated satellite lesions. Bronchioloalveolar cell carcinomas are a subtype of adenocarcinoma, in which the tumour growth is along the alveolar walls in a "lepidic" pattern, with preservation of the alveolar framework.

1.3.3 Large cell carcinoma

Large cell carcinomas represent a group of poorly differentiated tumours that display neither squamous differentiation, nor glandular differentiation with mucosynthesis. Rather, the tumour cells appear as monotonous sheets, with plentiful cytoplasm and prominent nucleoli.

1.3.4 Small cell carcinoma

Small cell lung carcinoma represents the most aggressive form of lung carcinoma and is the most strongly associated with tobacco smoking. These are the fastest growing carcinomas and metastasise early, such that over 70% of patients have metastatic disease at presentation (Elias, 1997). The classical "oat-cell" morphology describes cells of a high nuclear/cytoplasmic ratio, in which the nuclei are pyknotic or have a finely granular chromatin pattern and indistinct nucleolus.

There is a distinct, but uncommon subgroup of mixed tumours, in which SCLC cells are found combined with either squamous cell carcinoma or adenocarcinoma. It is difficult to truly assess their incidence, since the presence of SCLC tends to preclude surgical excision and detailed analysis. In a surgical study, Hage *et al.* reported an proportion of 26% mixed tumours in their very small total of 100 patients undergoing resection for SCLC over 17 years (Hage *et al.*, 1998). There is an overt surgical bias in this study and other authors report a lower incidence of mixed tumours, up to 10-14% of SCLC cases (Fushimi *et al.*, 1996). Patients with such mixed tumours fare worse than those with a "pure" NSCLC morphology.

1.3.5 Changing prevalence of histological subtypes

Over recent decades there have been changes in the prevalence of the histological subtypes of lung cancer. There has always been significant variation in reports, depending upon the nature of the population studied and the origin of the tissue for

diagnosis: surgical resection specimens give a more reliable diagnosis than bronchoscopic sampling, but will result in a falsely low incidence of SCLC, since it is rarely considered possible to resect this subtype. Nevertheless, there has been an increase in the proportion of adenocarcinomas (Vincent *et al.*, 1977). Data from the U.S. National Cancer Institute shows that for 1973-77 there were twice as many squamous cell carcinomas than adenocarcinomas in men, whilst adenocarcinomas were marginally more frequent in women. By 1990, adenocarcinomas had increased to levels equivalent to squamous cell carcinomas in men and in women were now 1.5-2 times more frequent. Analyses of birth cohorts suggests that these two related epidemics – of squamous cell carcinomas and more recently of adenocarcinomas – are related to patterns of smoking amongst populations, with both an increase in women smoking and a move towards lower yield, filter-tipped cigarettes (Skuladottir *et al.*, 2000). The effect of filter-tipped cigarettes is that smokers have to take more frequent, deeper puffs to fulfil their nicotine kick. This in turn means that tobacco smoke reaches more distal parts of the bronchoalveolar tree, where adenocarcinomas are more likely to arise than squamous cell carcinomas (Wynder and Hoffmann, 1994).

1.4 Development of lung cancer

1.4.1 Phenotypic changes

The bronchial epithelium exhibits a marked potential for metaplasia. In airway damage, one of the earliest events is basal cell hyperplasia, then replacement of normal ciliated

epithelial cells by stratified squamous epithelium. These cells may undergo further differentiation into mucous cells and ciliated cells, but may differentiate into neuroendocrine cells or metaplastic squamous cells in the presence of carcinogens in hamster models of carcinogensesis (Reznik-Schuller, 1977). Current evidence therefore points towards the bronchial epithelial cell as a common origin for all lung cancers, with the final histological subtype a mirror of the molecular changes that have induced carcinogenesis. This is supported by work showing that neuroendocrine features can be observed in up to 25% of adenocarcinomas (Mabry *et al.*, 1988). Additionally, SCLC can be induced to mutate into a large cell carcinoma phenotype (Mabry *et al.*, 1991) and different histological subtypes can be induced from the same bronchial epithelial cell line (BEAS-2B) following transformation with c-*myc* and c-*raf*-1, producing tumours with phenotypic relations to adenocarcinomas, large cell, squamous cell or small cell carcinomas (Pfeifer *et al.*, 1989;Pfeifer *et al.*, 1991).

Therefore the sequence of hyperplasia → metaplasia → dysplasia → carcinoma-in situ → invasive carcinoma is more clearly understood. Indeed, it has been demonstrated clinically by periodic sputum examination in uranium workers at high risk of evolving bronchial carcinoma. In these subjects squamous metaplasia, then increasing atypia, were followed by frank carcinoma of either SCLC or squamous cell type, the period of dyplastic change being prolonged for an average of 16 years (Saccomanno *et al.*, 1974).

1.4.2 Molecular changes

The molecular changes that accompany these phenotypic changes are the result of multiple, accumulated alterations to the cellular machinery. It is estimated that established lung cancers have acquired 10-20 genetic abnormalities in dominant oncogenes or tumour-suppressor genes (Kerr, 2001). Growth factors and their receptors are not usually affected by mutation, but their upregulation and stimulation of autocrine and paracrine growth loops, gives them a supporting role, which has clearly been established in both SCLC and NSCLC (Sethi and Rozengurt, 1991;Sethi *et al.*, 1992).

The major cellular oncogenes implicated in lung cancer include those of the *ras* and *myc* families. Overexpression of c-*myc* is common in SCLC and NSCLC. c-*myc* encodes a nuclear phosphoprotein transcription factor, which promotes programmed cell death, or apoptosis, in normal cells. However, amplification or over-expression of c-*myc* in transformed cells with impaired apoptotic signalling pathways, whilst not being essential for the transformation process, promotes cellular proliferation, (Gazzeri *et al.*, 1991). Overexpression of *myc* protein is accounted for by either amplification of *myc* genes (in 20% of SCLC and 10% of NSCLC) or by mRNA hyperexpression (45% of lung carcinomas) as a result of other, upstream events such as growth factor activation. In addition, there is evidence to suggest that *myc* mRNA transcripts are abnormally stabilized in some lung cancers *in vitro* (Bernasconi *et al.*, 2000).

The *ras* gene encodes a membrane-associated protein involved in signal transduction from tyrosine kinase growth factor receptors. K-*ras* is a mutation found in 30-50% of NSCLC, causing dysregulation of Ras signal transduction. It can be found in early stage adenocarcinoma and in pre-invasive, atypical, hyperplastic cells and is therefore considered an important early mutation (Westra *et al.*, 1996;Cooper *et al.*, 1997). It can also be induced *in vitro* by nitrosamines and benzo-*a*-pyrene, found in cigarette smoke (Osann, 1998). The presence of K-ras mutation appears to be associated with more poorly differentiated tumours and a poorer outcome after surgery (Slebos *et al.*, 1990;Rodenhuis and Slebos, 1992).

Other oncogenes found to be aberrantly expressed in lung cancer include cyclin D1, normally involved in cell cycle control and, specifically, the transition from G1 to S-phase; bcl2, responsible for protection against apoptosis; c-erb-B2/neu and epidermal growth factor receptor (EGF-R), both resulting in cellular proliferation. All such dominant oncogenes require only one allele to be affected – and therefore one genetic event – for their carcinogenic potential to be unleashed (Kerr, 2001).

Inactivation of tumour-suppressor genes, which normally promote apoptosis or anti-proliferative functions, requires a double "hit" – one allele mutation followed by loss of the normal allele. This resulting loss of heterozygosity is frequently described in lung cancer and may occur during progression from primary tumour to metastasis (Shiseki *et al.*, 1994;Okamoto *et al.*, 1995). Recurrent allelic deletions at specific chromosomal

sites have helped identify tumour-suppressor genes lost in lung cancer. The fragile histadine triad (FHIT) gene has been isolated from a 3p locus; abnormal mRNA transcripts were detected in 80% of SCLC and 40% of NSCLC, while 76% of all tumours exhibited loss of FHIT alleles (Sozzi *et al.*, 1996). The *p53* gene is the commonest genetic alteration in human cancer, normally encoding a transcription factor for G1 arrest or apoptosis of damaged cells. It is altered in 70-80% of SCLC and 50% of NSCLC and is associated with loss of heterozygosity at 17p13 (D'Amico *et al.*, 1992;Mitsudomi *et al.*, 2000). Amongst NSCLC, a recent meta-analysis has suggested that p53 alterations are of adverse prognostic significance for patients with adenocarcinoma rather than squamous cell carcinoma, even though it is less frequent in adenocarcinoma (34% v. 52%, respectively)(Mitsudomi *et al.*, 2000). Similarly, the retinoblastoma gene *Rb* is encoded at a frequent site of allelic deletion, 13q14, and loss of expression occurs in 90% of SCLC and 10-15% of NSCLC. Loss of Rb expression results in loss of control of the G1 arrest phase of the cell cycle. Although Rb may be normally expressed in most NSCLC, its phosphorylation is frequently deregulated by either overexpression of cyclin D1 or loss of the negative regulator of Rb phosphorylation, $p16^{INK4}$ (Kratzke *et al.*, 1996;Brambilla *et al.*, 1999).

The end result of these molecular events is that cells accumulate progressively more genetic damage. Although initially some may be reversible, with increasing damage cells are more likely to escape the natural protective mechanisms of cell cycle arrest and

programmed cell death, such that cellular proliferation and a cancerous cellular phenotype result.

1.5 Lung cancer – the clinical problem

At a clinical level, lung cancer usually presents with features related to local effects in the lung, such as cough, haemoptyis, chest wall pain or breathlessness. Apical lung tumours can produce shoulder or arm pain, with brachial plexus involvement or Horner's syndrome, whilst mediastinal involvement may result in hoarseness due to recurrent laryngeal nerve palsy, superior vena caval obstruction or phrenic nerve damage. Disseminated disease may present with features related to metastatic sites: jaundice and abdominal pain due to hepatic involvement; headache, confusion or seizures from brain metastases; bone pain or fractures from skeletal metastases.

In addition, tumours may present with paraneoplastic features due to polypeptide hormone production. Such hormones are detectable at elevated levels more in SCLC (70%) than NSCLC (15-20%), but only around 5% produce clinically relevant effects. In squamous cell carcinomas this characteristically includes hypercalcaemia due to a parathormone-like peptide, but paraneoplastic syndromes are the feature of SCLC *par excellence*. These most frequently include the syndrome of inappropriate anti-diuretic hormone, Cushing's syndrome (due to adrenocorticotropic hormone) and ectopic production of calcitonin and parathyroid hormone (Spiro, 2003). In addition, production

of anomalous antibodies directed against tumour antigens can result in a variety of neurological disorders, including cerebellar syndromes, peripheral neuropathy and the Lambert-Eaton myasthenic syndrome (De Aizpurua *et al.*, 1988).

1.5.1 Non-small cell lung cancer

Management of NSCLC is dependent upon classical tumour (T) node (N) metastasis (M) staging, initially devised in 1946 and upgraded in 1997 (Mountain, 1997). This remains the best prognostic indicator for outcome in NSCLC and emphasises the importance of tumour size, lymph node involvement, proximity to the main carina, involvement of the mediastinum or involvement of the pleura or chest wall (Mountain, 2000). The most effective treatment for NSCLC remains successful surgery and the staging process is directed at identifying those patients suitable for surgery. However, up to 60% of patients are deemed inoperable due either to extensive disease or to comorbidity.

The TNM classes have been grouped into three stage categories deemed operable (IA and B, IIA and B, IIIA), with different survival rates. Stages IIIB and IV are deemed inoperable. With progression through the stages the prognosis rapidly becomes increasingly bleak (**Table 1b**). Thus, predicted 5-year survival for a patient with clinically staged IA disease (T1N0M0) is approximately 60%, whereas for IB (T2N0M0) it drops to nearer 40%. Ipsilateral lymph node involvement, as in Stage IIA disease (T1N1M0), further reduces this to 34% and by the time Stage IIIA disease is reached (T3N1M0) 5-year survival is only 13% (Mountain, 1997). Unsurprisingly, these

figures are improved if the disease is staged pathologically, post-operatively, but in this lies one of the difficulties of lung cancer management – how to accurately stage the burden of disease pre-operatively, so as to avoid unsuccessful surgery and to target adjuvant therapy more effectively to those patients who will benefit most.

Table 1b: Survival in NSCLC according to clinical (c) or pathological (p) staging.

c/p Stage	Months after treatment (cumulative % survival)				
	12	**24**	**36**	**48**	**60**
cIA	91	79	71	67	61
cIB	72	54	46	41	38
cIIA	79	49	38	34	34
cIIB	59	41	33	26	24
cIIIA	50	25	18	14	13
cIIIB	34	13	7	6	5
cIV	19	6	2	2	1
pIA	94	86	80	73	67
pIB	87	76	67	62	57
pIIA	89	70	66	61	55
pIIB	73	56	46	42	39
pIIIA	64	40	32	26	23

Taken from Mountain *et al.*(Mountain, 1997). cStage, clinical Stage; pStage, pathological Stage.

Radiotherapy

The role of radiotherapy in NSCLC depends upon the context in which it is considered. In 1998 a meta-analysis (PORT, 1998) revealed no survival advantage to radiotherapy following complete resection of T1-2N0-1 tumours or T3N0 tumours. Indeed, a more recent update of this meta-analysis suggests that post-operative radiotherapy is detrimental to outcome, reducing overall survival from 55% to 48% at two years

(Cochrane, 2003). For patients found at resection to have pathological N2 disease, pre-operatively clinically staged as N0-1, management is less clear. These patients had pathologically Stage III (N2) disease and there was no clear adverse effect (Cochrane, 2003). Adjuvant radiotherapy is often given in cases of incomplete resection, although there is no evidence that this reduces the rate of recurrence. Additionally, radiotherapy may be used for a tumour that is potentially resectable, but where an operation is not possible, due either to patient comorbidity or disinclination. Such radical radiotherapy, aiming to cure, involves higher doses of radiation (at least 60 Gray) and there is continuing debate over the best way in which to deliver this dose. Recently, continuous hyperfractionated accelerated radiotherapy (CHART), delivered over two weeks, has been shown to offer a significant survival advantage over conventional fractionation delivered over 6 weeks (Saunders, 2001).

Chemotherapy

NSCLC has been considered to be relatively chemoresistant and historically chemotherapy has played little part in its management. However, this philosophy has been challenged more recently in the context of both inoperable, disseminated (and therefore not readily amenable to radiotherapy) disease and in operable N0-1 disease as adjuvant or neo-adjuvant therapy. In 1995, a major meta-analysis of 52 randomised trials of chemotherapy showed a limited survival advantage with platinum-based treatment of up to 5% at five years in potentially curable disease, or up to 10% at one year in palliative care. This analysis covered 9387 patients in all clinical settings of

NSCLC, from patients treated surgically to those receiving supportive care for incurable disease (Non-small Cell Lung Cancer Collaborative Group, 1995). Since then a global effort has been made to clarify the role of chemotherapy in NSCLC in a number of large-scale trials.

Palliative care

The largest proportion of lung cancer patients falls into this group, being unsuitable for surgery at presentation. Radiotherapy has a clear role in the palliation of symptoms such as thoracic pain, intractable cough, haemoptysis, dysphagia or impending lobar collapse, but no effect on life expectancy (MRC, 1992). The role of chemotherapy remains unclear in this group of patients. In the eight trials pooled in the meta-analysis comparing supportive care alone with supportive care plus chemotherapy, although median survival was increased, long-term survival was less clearly influenced (Non-small Cell Lung Cancer Collaborative Group, 1995). In addition, the risks of chemotherapy increase with poorer performance status, although quality of life overall appears to be no worse after chemotherapy and symptomatic response rates of 60% may be seen (Gregory *et al.*, 2001). It remains important, therefore, that further studies are carried out in this area of care.

1.5.2 Small cell lung cancer

Primary chemotherapy

By contrast with NSCLC, small cell lung cancer is almost invariably inoperable at presentation, yet has a clear response to initial chemotherapy in 50-90% of patients. Rarely, a patient may be confidently assigned to a Stage I or II disease (T1-2N0M0) and go on to surgery, but for the majority of SCLC patients the TNM system is unhelpful and the disease is classified following the Veterans Administration Lung Cancer Group as either limited disease (LD) – confined to one hemithorax, including ipsi- or contralateral hilar nodes and ipsilateral supraclavicular lymph nodes – or extensive (ED), extending beyond this. Patients with ED have a worse prognosis than those with LD, but this is in the context of an untreated median survival for SCLC of about three months. Other poor prognostic factors include older age and male sex, poor performance status, hyponatraemia, and elevated lactate dehydrogenase and alkaline phosphatase (Thatcher *et al.*, 1995).

Even with chemotherapy, median survival only improves to a median of 8 months in average to poor prognosis patients (MRC, 1989). In better prognostic groups, with carefully staged trials, median survival of up to 23 months has been documented with chemotherapy and consolidation radiotherapy given as twice-daily accelerated fractions (Turrisi, III *et al.*, 1999), but there is no definitive chemotherapy regime. Regimes used include: cysplatin plus etoposide; cyclophosphamide, doxorubicin and vincristine; cyclophosphamide, doxorubicin and etoposide; and ifosfamide, carboplatin and

etoposide, with or without vincristine. Primary chemotherapy is usually limited to six cycles for better prognosis patients. In intermediate and poorer prognostic groups this may well be limited to three or four cycles, achieving a partial response rate of 50-70%.

Radiotherapy

Patients with limited disease and a complete radiological response to primary chemotherapy achieve a modest benefit from radiotherapy with 45-55 Gy (Pignon *et al.*, 1992;Warde and Payne, 1992). This group of responders has also been shown to have a reduced incidence of brain metastases (27% v. 49%) and improved survival with prophylactic cranial irradiation compared to controls (Auperin *et al.*, 1999). Radiotherapy is also of benefit in treatment of localised, painful bony metastases.

Relapsed disease

Relapse is almost inevitable in SCLC, being about 50% in the first year and 40% in the second. Conventionally, second-line therapy is with a different regime from primary chemotherapy. However, relapsed SCLC exhibits marked chemoresistance and overall response rates are only in the order of 10-30%. The outlook hereafter is bleak, median survival being only two or three months.

1.6 Chemoresistance

The development of multidrug resistance is not unique to lung cancer, but is seen in a wide range of malignancies, often following impressive initial responses to therapy, as in haematological malignancies, ovarian, germ cell or breast cancer. Many cytotoxic chemotherapeutic agents induce cell death via the final common pathway of apoptosis (Hannun, 1997). This includes etoposide, adriamycin and *cis*-platinum, all of which may be used in treatment of lung cancer. Cells that survive the chemotherapeutic insult are able to pass on at least some of their resistance "factors", as demonstrated in murine models of leukaemia (Koyama *et al.*, 1982;Palyi *et al.*, 1989). Thus, there is an inherited component to multidrug resistance – chemotherapy breeds chemoresistance. In addition, recent evidence has started to suggest that interactions between the cancer cell and its local environment play a significant role in multidrug resistance.

1.6.1 The genetics of chemoresistance

Cancer cells may acquire the ability to reduce intracellular levels of chemotherapeutic agents by exporting the drug. It is now recognised that several ATP-driven efflux pumps can occur, contributing to drug resistance. These include P-glycoprotein (PgP/MDR-1), multidrug resistance-associated proteins (MRP2 – 7), breast cancer resistance protein and lung cancer resistance protein (Biedler and Riehm, 1970;Kartner *et al.*, 1983). The genes for PgP and MRP have been cloned and it is clear that these pumps have a broad specificity, from endogenous proteins such as leukotrienes, to drugs of different classes (Gerlach *et al.*, 1986;Cole *et al.*, 1992). In both SCLC and ovarian tumours, even very

low levels of mRNA for PgP have been associated with resistance to chemotherapy and PgP expression has been shown to be increased in tumours after treatment with chemotherapy (Holzmayer *et al.*, 1992). However, other studies in SCLC have suggested that PgP expression is not necessarily a principal cause of multidrug resistance – PgP mRNA was elevated in only four out of 23 SCLC cell lines and three out of six primary tumours and never to very high levels; in addition, there was no correlation between expression and response to therapy (Narasaki *et al.*, 1996). MRP shows elevated mRNA expression in a significant proportion (35%) of primary NSCLC, with greatest expression in well-differentiated squamous cell carcinoma (Nooter *et al.*, 1996). The role of other drug transporter pumps is not yet clear. Breast cancer resistance protein appears also to have a broad substrate ability and is associated with resistance to methotrexate, mitoxantrone, topetecan and adriamycin in a variety of tumours (Doyle *et al.*, 1998;Lage and Dietel, 2000;Volk *et al.*, 2000;Scheffer *et al.*, 2000;Faneyte *et al.*, 2002). The lung cancer resistance protein localises to nuclear pore complexes and may divert drugs from the nuclear target, rather than necessarily out of the cell. This has been found to be elevated in chemoresistant NSCLC cell lines, acute myeloid leukaemia and prostate and ovarian cancers (Scheffer *et al.*, 1995;List *et al.*, 1996;Volm *et al.*, 1997a;Volm *et al.*, 1997b;Arts *et al.*, 1999;Berger *et al.*, 2000;Van Brussel *et al.*, 2001).

Another way of dealing with a harmful drug is to detoxify it. An example of this is the glutathione-*S*-transferase system, in which toxic compounds are conjugated with glutathione, prior to further metabolism and excretion (Salinas and Wong, 1999). It has

been shown that this mechanism can protect against *cis*-platinum- and doxorubicin-induced DNA damage *in vitro*, but although elevated levels of glutathione-*S*-transferase have been demonstrated in a number of cancers, including NSCLC, it is not clear how important it is *in vivo* (Buser *et al.*, 1997;Nakanishi *et al.*, 1999).

Chemotherapeutic agents such as adriamycin and etoposide induce apoptosis by inhibiting the nuclear enzyme DNA topoisomerase II. This enzyme normally regulates DNA structure by cleavage of both strands, causing transient double strand breaks, and subsequent reannealing at the cleavage site (Smith *et al.*, 1994). By inhibition of the second, reannealing part of this cycle, these agents permit the accumulation of DNA strand breaks, leading to apoptosis via the final common pathway in which caspase-3 (formed from pro-caspase-3) cleaves the chaperone inhibitor of caspase-activated deoxyribonuclease, triggering DNA fragmentation. Several mechanisms of topoisomerase II multidrug resistance mechanisms are now known. It is usually due to a reduction in both topoisomerase II level and activity, although still at a level allowing cell survival (Lage *et al.*, 2000;Kobayashi *et al.*, 2001); mutations resulting in decreased drug sensitivity have also been identified (de Jong *et al.*, 1990). Again, it is unclear of the clinical significance of this mechanism of multidrug resistance: although SCLC have much higher levels of topoisomerase II than NSCLC and are much more etoposide-sensitive, paradoxically other studies have shown worse chemotherapeutic responses with higher levels of the enzyme in SCLC (Dingemans *et al.*, 1999).

1.6.2 The role of the tumour microenvironment

An immunohistochemical study of 23 resected SCLC samples revealed high levels of fibronectin, collagen IV and tenascin in all samples (Sethi *et al.*, 1999b). This high expression was localized to the reactive, desmoplastic stroma around areas of tumour infiltration and the basement membrane in these areas was markedly thickened by comparison with normal, uninvolved lung. Additionally, in up to 25% of tumours, fibronectin and laminin staining was visible within the tumour cells, suggesting that SCLC cells may directly synthesize some of the components of their local ECM. Therefore, SCLC *in vivo* is surrounded by a matrix rich in ECM components, which might influence cell behaviour through cell surface receptors such as integrins (*see below*). It was demonstrated that *in vitro* adhesion to laminin, fibronectin or collagen IV strongly protected SCLC cells from chemotherapy-induced apoptosis by comparison with cells non-specifically adhered to plastic. This protection was mediated through β1 integrins, since the effect was abrogated by co-incubation with function-blocking β1 integrin antibodies, but not with isotype-matched control antibodies.

This phenomenon of ECM-mediated chemoresistance appears to have general application in cancers. An earlier report by Fridman *et al.* showed that matrigel and laminin were able to enhance SCLC tumourigenicity and drug resistance in SCLC (Fridman *et al.*, 1990). In xenograft-derived endothelial cells, culture on gelatin, collagen IV, laminin, fibronectin or the integrin ligand peptide Gly-Arg-Gly-Asp-Ser-

Pro (but not the integrin peptide Gly-Arg-Ala-Asp-Ser-Pro) prevented etoposide-induced DNA damage (Hoyt *et al.*, 1996). Tumour-derived endothelial cells also exhibit cytoprotection when plated on surfaces coated with antibodies to α5, β1 or β3 integrin subunits and by clustering of integrins by soluble antibodies. Myeloma cells adhered to fibronectin are protected against chemotherapy-induced apoptosis in a mechanism mediated through α4β1 and α5β1 integrins (Hazlehurst *et al.*, 2000). In mammary epithelial cells, apoptosis could be enhanced by antibodies to β1 integrin or by stromelysin-1, which degrades the ECM (Boudreau *et al.*, 1995).

The intracellular signalling mechanisms mediating β1 integrin-induced drug resistance are beginning to be defined. Tyrphostin-25, a selective inhibitor of protein tyrosine kinase (PTK), blocks adhesion-mediated drug resisitance in SCLC cells. Phosphoinositide 3-kinase (PI 3-kinase) is an enzyme that acts as a direct biochemical link between a phosphoinositide pathway and a number of proteins containing tyrosine kinase activity. PI 3-kinase requires tyrosine phosphorylation for full activation and would thus be a target for PTK activity. Protein kinase B (PKB) is a principal downstream effector of PI 3-kinase, so the PI 3-kinase/PKB pathway may indeed be a key mediator of integrin-induced chemoresistance. Both PI 3-kinase and PKB suppress anoikis and the induction of chemotherapy-induced chemoresistance (Schmidt *et al.*, 2002). The introduction of a constitutively active form of PKB conferred resistance to anoikis in mammary epithelial cells; the same construct inserted to A549 NSCLC cells also resulted in profoundly decreased chemosensitivity to mitoxantrone and cisplatin.

PKB has been shown by other groups to be constitutively activated in NSCLC and to confer resistance to both radiation- and chemotherapy-induced apoptosis (Brognard *et al.*, 2001).

To better understand the mechanisms underlying the relationship between a cell and its local environment, it is necessary to describe the key cellular proteins involved at this interface. These are the integrin family of proteins and the following section will describe their main features and those of the cellular signalling pathways which they govern.

Section B: Integrins

1.7 Integrins

Integrins are transmembrane receptor proteins that play a key role in cellular adhesion to and communication with the extracellular matrix (ECM) (Tamkun *et al.*, 1986). They mediate signal transduction in both directions: extracellular ligand binding transmits signals into the cell, controlling cytoskeletal events and gene transcription, so-called "outside-in signalling"; conversely, intracellular signals influence the ability of integrins to bind these ligands in a process called "inside-out signalling". By controlling cellular adhesion integrins critically influence cell survival and cell motility and dysfunction of integrin function has been shown to be the underlying problem with an array of human diseases, including atherogenesis, cancer and clotting disorders.

All members of the integrin family comprise an alpha (α) and beta (β) type I transmembrane subunit, non-covalently linked in the cell membrane. To date, a total of 18α and 8β subunits have been described in vertebrates. *In vivo*, the pairing of α and β subunits is limited, such that only 24 different pairings have been identified (Plow *et al.*, 2000;Danen and Yamada, 2001). Early work assigned a confusing array of names to these proteins, such as lymphocyte function associated antigen-1 (LFA-1, now αLβ2), macrophage-1 receptor (Mac-1, now αMβ2) or very late after activation (VLA) antigens, but more recently a simplified nomenclature has been adopted, described by the α and β

subunits involved (Hynes, 1987;Ruoslahti and Pierschbacher, 1987;Albelda and Buck, 1990;Hemler, 1990;Diamond and Springer, 1994;Schwartz *et al.*, 1995).

There is considerable evidence of conservation of integrins through evolution. Families of homologous proteins are described in *Caenorhabditis elegans* and *Drosophila melanogaster* that are responsible for binding to ECM components, specifically laminin and an Arg-Gly-Asp (RGD) motif, which is found in fibronectin, vitronectin and other adhesion proteins (Felding-Habermann and Cheresh, 1993). Here they are responsible for epidermal adhesion, muscle attachment, neurite outgrowth and tissue morphogenesis (Zusman *et al.*, 1990;Brown, 1994;Stark *et al.*, 1997;Brown *et al.*, 2000).

1.8 Integrin complexity

The integrins are now classified into subgroups, dependent upon their β subunit interactions. Although integrins are expressed on almost all mammalian cells, the integrin expression profile of cells varies markedly both between cells and within the same cells under different conditions. For example, αIIbβ3 integrins are expressed on platelets, where they play a critical role in platelet aggregation. In addition, integrin expression changes markedly under different cellular conditions, such as seen in granulocyte maturation (Lund-Johansen and Terstappen, 1993), or during cellular differentiation (Jiang and Grabel, 1995). Complexity and flexibility of the integrin system is further increased by the presence of alternative splicing of some of the integrin

subunits. Alternative splicing of intracellular domains has been described for β1, β3, β4, α3, α6 and α7 subunits (Altruda *et al.*, 1990;Suzuki and Naitoh, 1990;Cooper *et al.*, 1991;Hogervorst *et al.*, 1991;Tamura *et al.*, 1991;Languino and Ruoslahti, 1992;Collo *et al.*, 1993;Belkin *et al.*, 1996), of which perhaps the most extensively described are the β1 variants, β1A-D (Armulik, 2002). By tissue restriction and by modifying the signalling role of the integrin, these vary the overall function of the integrin. For example, β1C appears to inhibit cell cycle progression, whilst β1B may inhibit adhesion during development and β1D, restricted to myocytes, plays a key role in adhesion and inhibition of cell cycle progression (Balzac *et al.*, 1993;Meredith, Jr. *et al.*, 1995;Belkin and Retta, 1998). Extracellular domain slice variants have also been described, for αIIb and α7 (Bray and Shuman, 1990;Ziober *et al.*, 1993).

1.9 Integrins: structure and ligands

Integrins all share a similar protein structure, based upon the interaction of α and β subunits. Each subunit has a large (> 700 residue) NH_2-terminal extracellular domain, a single transmembrane region and a short cytoplasmic region of 13 – 70 residues (except β4, where there is an extensive cytoplasmic tail of > 1000 amino acids)(Hynes, 1992). Electron microscopy shows a globular head portion ligand binding domain, comprising the NH_2-terminal portions of the α and β subunits. This is connected to the membrane by two stalks, the C-terminal portions of the extracellular domains (Hantgan *et al.*,

1999). Mutational analysis has shown that the cytoplasmic domains play a key role in bi-directional signalling and loss of the β tail results in integrins that fail to localise to focal adhesions, and show reduced ligand-binding and signalling ability (O'Toole *et al.*, 1994).

1.9.1 Extracellular ligands

The ligand binding potential of integrins is shown in **Table 1c**. It is complicated by the fact that one integrin may exhibit specificity for multiple ligands and, conversely, one ligand may be bound by multiple integrins, either through the same recognition epitope or different epitopes on the same ECM protein. The key ECM proteins interacting with integrins are laminin, fibronectin, collagen and vitronectin; other important ligands include the soluble protein fibrinogen, which plays a key role in coagulation, and the cellular transmembrane proteins ICAM-1/2 and VCAM-1, which interact with integrins to mediate cell-cell adhesion.

A key component of many of the macromolecules that act as integrin ligands is the peptide sequence Arg-Gly-Asp (RGD), which has been identified as an essential epitope for integrin binding, originally identified as the adhesive sequence in fibronectin (Pierschbacher and Ruoslahti, 1984). Soluble RGD peptides can be used experimentally to block fibronectin-integrin binding, but some monoclonal antibodies to RGD do not inhibit platelet aggregation and recombinant fibrinogen lacking the RGD sequence can still mediate aggregation (Cheresh *et al.*, 1989). This suggests that other epitopes exist,

Table 1c: Integrin nomenclature and binding potential

Integrin	Alternate names	Extracellular ligands
α1β1	VLA-1, CD49a / CD29	Laminin, collagen
α2β1	VLA-2, CD49b / CD29	Laminin, collagen
α3β1	VLA-3, CD49c / CD29	Laminin, collagen, fibronectin
α4β1	VLA-4, CD49d / CD29	Fibronectin, VCAM-1
α5β1	VLA-5, CD49e / CD29	Fibronectin, thrombospondin, Insulin-like growth factor binding protein-1, L1-CAM
α6β1	VLA-6, CD49f / CD29	Laminin, disintegrin
α7β1		Laminin
α8β1		Fibronectin, tenascin-C, vitronectin, osteopontin
α9β1		Tenascin-C
αvβ1		Fibronectin, vitronectin, fibrinogen, L1-CAM
αvβ3		Fibronectin, vitronectin, thrombospondin, osteopontin, bone sialoprotein-1, fibrinogen, von Willebrand factor, L1-CAM
αvβ5	CD51 / CD61	Vitronectin
αvβ6	αvβx, αvβs	Fibronectin
αvβ8		Vitronectin
α6β4		Laminin
α4β7	αvβp, PLAM-1	Fibronectin, VCAM-1, MAdCAM-1
$\alpha_{IEL}\beta7$	αEβ7	E-Cadherin
αIIbβ3	gpIIb / IIIa	Fibronectin, fibrinogen, von Willebrand factor, L1-CAM
αLβ2	LFA-1, CD11a / CD18	ICAM-1, ICAM-2
αMβ2	Mac-1, CD11b / CD18	C3bi (complement), fibrinogen, Factor X, ICAM-1
αXβ2	P150 / 95	C3bi, fibrinogen
αdβ2	CD11c / CD18	

which confer ligand/integrin specificity, as have been identified for fibronectin and fibrinogen (Plow *et al.*, 2000).

The importance of these integrin-mediated cell-ECM and cell-cell interactions is underlined by targeted knockout studies on integrins or their ligands (Hynes, 1996;Beauvais-Jouneau and Thiery, 1997). Many integrin knockouts are severe or lethal - β1 knockout mice die during embryogenesis soon after implantation (Fassler and Meyer, 1995;Stephens *et al.*, 1995), whilst β2 integrin knockouts suffer from impaired leucocyte function and recurrent bacterial infections (Prince *et al.*, 2001).

1.9.2 Proteins associated with the integrin cytoplasmic domain

Integrins were named for their perceived importance in linking the cell surface with the cytoskeleton and an ever-increasing array of proteins has been associated with integrin cytoplasmic domains, reflecting their diverse roles in cell signalling. These associated proteins have been regularly reviewed and are summarized in **Table 1d** (Liu *et al.*, 2000;Berditchevski, 2001). Some key features will be discussed here. The proteins listed include actin-binding proteins, adaptor proteins, enzymes, a transcriptional co-activator and proteins whose function remains unclear.

Integrin β cytoplasmic domain binding proteins

Actin-binding proteins provide a fundamental link role between integrins and the cytoskeleton and are critical in cell spreading, migration and matrix assembly. The first

Table 1d: Integrin cytoplasmic domain binding proteins

Binding partner	Integrin tail
Actin-binding proteins	
Talin	β1A, β1D, β2, β3
Filamin	β1A, β2, β3, β7
α-actinin	β1A, β2
F-actin	α2
Myosin	β3
Skelemin	β1, β3
Signalling proteins	
ILK	β1, β3
FAK	β1, β2, β3
Cytohesin-1	β2
Cytohesin-3	β2
Other proteins	
Paxillin	β1, β3, α4
Grb2	β3
Shc	β3
β3-endonexin	β3
TAP-20	β5
CIB	αIIb
Calreticulin	α
Caveolin-1	α
Rack1	β1, β2, β5
WAIT-1	β7
JAB1	β2
Melusin	β1A, β1B, β1D
MIBP	β1A, β1D
ICAP-1	β1A
CD98	β1A, β3
DRAL/FHL2	α3A, α3B, α7A, β

to be isolated was talin (Horwitz *et al.*, 1986), which binds to both the β tail (Pfaff *et al.*, 1998) and also (in platelet αIIbβ3 integrins) the αIIb tail (Knezevic *et al.*, 1996). Integrin mutants unable to bind talin are unable to localize to focal adhesions and fail to accumulate F-actin following integrin clustering, strongly suggesting that talin connects integrins to the cytoskeleton (Lewis and Schwartz, 1995). Other actin-binding proteins include α-actinin and filamin and disruption of integrin binding to either of these proteins disrupts focal adhesion formation. Filamin also acts as an important adaptor protein for a number of signalling proteins that can regulate cytoskeletal dynamics, such as RalA: in filamin-deficient melanoma cells RalA is incapable of generating filopodia (Ohta *et al.*, 1999).

Since integrins possess no intrinsic enzymatic activity, their ability to conduct bi-directional signalling requires binding of signalling proteins, or adaptor proteins that can recruit such proteins. The best characterised of these are focal adhesion kinase and integrin linked kinase, which will be discussed in more detail later. Another class of signalling molecule is the cytohesin family, of which cytohesin-1 and –3 have been identified as β2-tail-binding proteins (Kolanus *et al.*, 1996;Korthauer *et al.*, 2000). Cytohesins have guanine nucleotide-exchange factor activity for the ADP ribosylation factor (ARF) family of small GTPases (Ogasawara *et al.*, 2000), which appear to be important for integrin-mediated effects on cell spreading and adhesion (Geiger *et al.*, 2000). Adaptor proteins such as paxillin, Grb2 and Shc have also been shown to bind to integrin β tails *in vitro* (Law *et al.*, 1996), although paxillin also binds strongly to the α4

integrin cytoplasmic domain and clearly modulates integrin-mediated signalling (Liu *et al.*, 1999a).

CD98 and β3-endonexin are both proteins that have been found to modulate integrin ligand-binding affinity via their interactions with β integrin tails (Kashiwagi *et al.*, 1997;Zent *et al.*, 2000), as will be discussed later. The novel protein TAP20 is 55% homologous with β3-endonexin and binds and regulates β5 integrins, although this would appear to be a suppression effect, rather than integrin activation as for β3-endonexin (Tang *et al.*, 1999).

Integrin α cytoplasmic domain binding proteins

Whereas there is much sequence similarity between the β cytoplasmic domains, the α cytoplasmic domains are dissimilar, except for a membrane proximal "hinge" region. They are thus potentially critical for differentially regulating integrin-mediated responses to extracellular stimuli. For example, the two β2 integrins expressed on lymphocytes, αMβ2 (Mac-1) and αLβ2 (LFA-1) respond to the same chemokines differently, an effect conferred by the cytoplasmic domains of αM and αL, which may be due to their different abilities to bind to cellular components or to regulate β subunit signalling (Weber *et al.*, 1999).

Of the α cytoplasmic domain-binding proteins, calreticulin is noteworthy for its ability to bind the conserved KXGFFKR membrane proximal region of a number of α integrins, both by co-immunoprecipitation and co-localization studies (Coppolino *et al.*, 1995), where it has been suggested that it may be able to modulate both integrin-mediated cell adhesion and integrin-mediated signalling (Coppolino *et al.*, 1997).

1.9.3 Integrins and tetraspanins

Tetraspanins are a family of over 26 proteins with four transmembrane domains, implicated in a wide range of cellular behaviour, including motility and metastasis (Boucheix and Rubinstein, 2001), proliferation and differentiation (Boismenu *et al.*, 1996). They were first associated with integrins in platelets, where the CD9-αIIbβ3 complex was described in association with integrin activation (Slupsky *et al.*, 1997), but a large number of such integrin-tetraspanin complexes is now known (Berditchevski, 2001). The biochemical function of tetraspanins remains undefined. There is little to suggest that tetraspanins are involved in cell-ECM attachments, such as focal adhesions, but they do appear to be important in intercellular adhesion and in the initial transient cell-substratum contacts that occur in lamellipodia during cell migration (Berditchevski *et al.*, 1997). A number of tetraspanins are physically associated with phosphatidylinositol 4-kinase (Yauch and Hemler, 2000) or protein kinase Cα or βII (Zhang *et al.*, 2001). Tetraspanins may therefore be considered to act predominantly as scaffold proteins or molecular facilitators, controlling the spatial organisation of membrane complexes of integrins and signalling molecules (Maecker *et al.*, 1997).

1.10 Integrin ligand binding sites

Figure 1.1 shows a schematic representation of integrin structure (Takagi and Springer, 2002), which appears to adopt a folded conformation in which the ligand-binding headpiece is folded back onto the tailpiece of the molecule (Xiong *et al.*, 2001). Springer predicted that the N-terminal domain of all α subunits contains a seven-fold repeat region, each repeat being ~60 residues (Springer, 1997). These contain phenyl-analyl-prolyl (FG) and glycyl-analyl-prolyl (GAP) consensus sequences (FG-GAP repeats) (Corbi *et al.*, 1987). Within these FG-GAP repeats are putative Ca^{2+} binding domains, in repeat 4 in some integrins and in repeats 5-7 in all integrins (Tuckwell *et al.*, 1992). The predicted β-propeller structure for the α subunit contains seven β sheets arranged pseudosymmetrically around an axis, each sheet containing four antiparallel β-strands. The overall shape is cylindrical, with a pocket in the upper surface. That this upper surface is critical for ligand binding is evidenced by mutational studies in a variety of α subunits: mutations of residues Y187, W188 and G190 in the α4 subunit abrogated binding to VCAM-1 and fibronectin (Irie *et al.*, 1995); the same residues are involved in ligand binding in α5 (Irie *et al.*, 1995), αIIb (Kamata *et al.*, 1996), and the same region is involved in α4 binding to laminin. In all cases, the residues involved are present on top of the β-propeller. Conversely, the monoclonal antibody CBRM1/20 is predicted to bind to the bottom of the β-propeller of αMβ2 (Mac-1), recognising residues 94 positions apart in the primary structure. Although it requires Ca^{2+} to bind, thereby supporting the likely importance of the putative Ca^{2+} binding domains, CBRM1/20 does

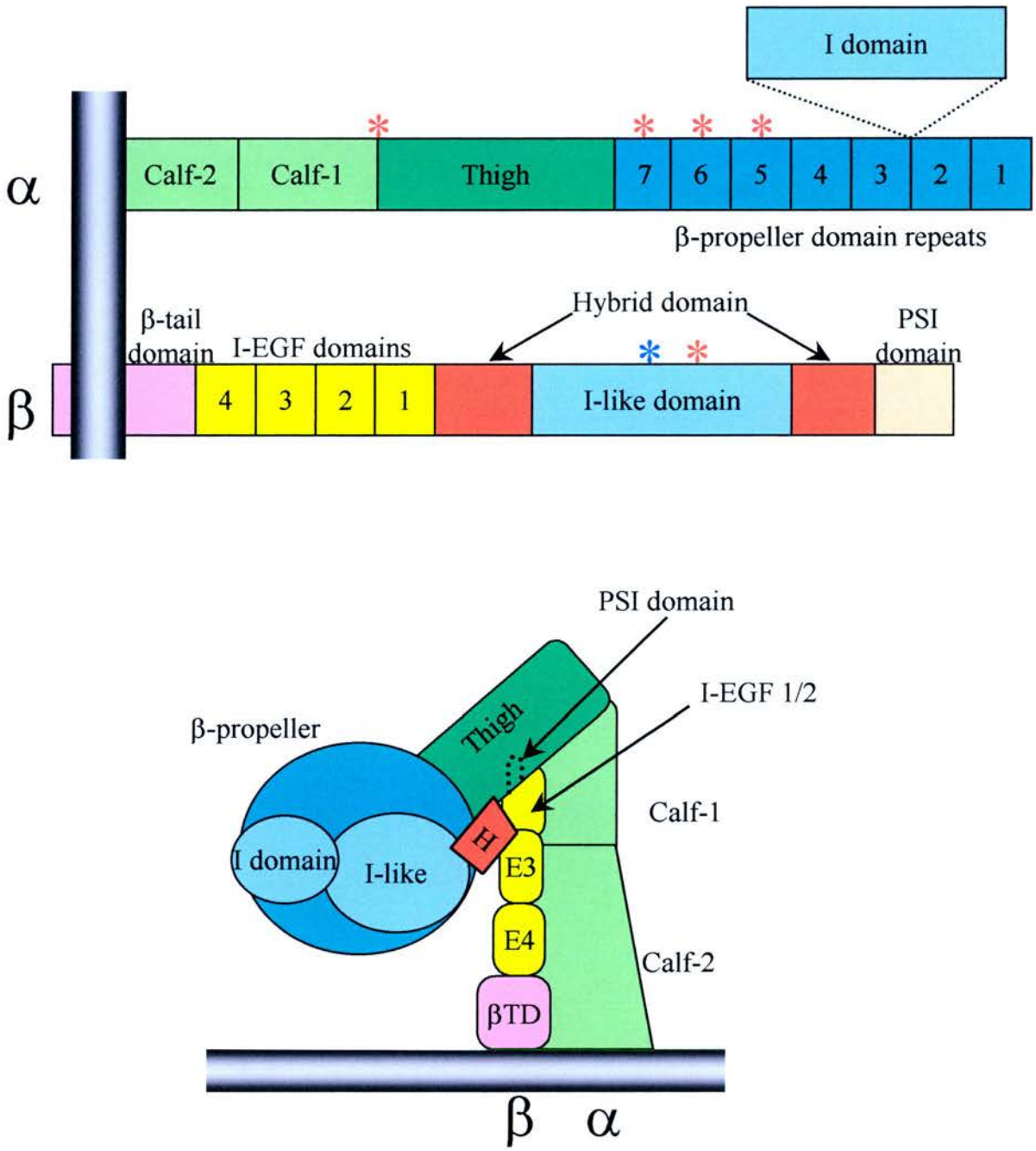


Figure 1.1: Structure of integrins

Upper panel: Organisation of domains within the primary structure of integrins. In some integrins there is an extra I domain within the α subunit, as indicated by dotted lines. Asterisks denote Ca^{2+} (red) and Mg^{2+} binding sites (blue) respectively.

Lower panel: Representative structure of αvβ3, with the I domain added. Domains are colour-coded as for the primary structure shown above.

Figures adapted from Takagi J. and Springer T.A. (2002).

not itself inhibit ligand binding. These findings support the β-propeller model, which would elegantly explain the proximity of these residues (Oxvig and Springer, 1998). More recently, the β-propeller model has been confirmed in the crystal structure of αvβ3 (Xiong *et al.*, 2001).

In at least eight integrins there is also a ~200 residue I domain in FG-GAP repeat 3, whose structure contains a fold similar to that found in nucleotide-binding proteins, small G proteins and heterotrimeric G protein α subunits (Huang and Springer, 1995;Lee *et al.*, 1995a;Lee *et al.*, 1995b). A hydrophilic linker segment is present on the C terminal side of the I domain and suggests a hinge between the I domain and the β-propeller domain. Within the I domain is a metal ion-dependent adhesion site (MIDAS), where two serines, a threonine and two water molecules coordinate Mg^{2+} binding, and a sixth coordination position is available to bind an acidic residue on the ligand. Residues surrounding the MIDAS motif confer ligand specificity to LFA-1, suggesting that in I domain-containing integrins, this is the ligand binding site (Huang and Springer, 1995).

Within the β subunit, a MIDAS motif has also been identified, predicted to fold in an I domain-like manner, and thus likely to represent a ligand binding site on the β subunit (Lee *et al.*, 1995b). Like the β-propeller, this has now been demonstrated in the crystal structure of αvβ3 (Xiong *et al.*, 2001).

The role of divalent cations in ligand binding has been controversial. The presence of the MIDAS motif and putative Ca^{2+} binding domains strongly suggests their central importance. Early studies suggested that the divalent cation binding sites of the αIIb chain were directly involved in ligand binding (D'Souza *et al.*, 1991;Gulino *et al.*, 1992;D'Souza *et al.*, 1994) and early studies on αIIbβ3 showed that removal of Ca^{2+} could induce subunit dissociation and inhibit ligand binding. However, in the absence of Ca^{2+} but the presence of Mg^{2+} ligand binding appears to be stimulated in other integrins such as αvβ3 (Dransfield *et al.*, 1992;Hu *et al.*, 1995;Hu *et al.*, 1996). The demonstration by Oxvig *et al.* that a Ca^{2+}-dependent mAb bound to the bottom of the β-propeller structure, away from the ligand binding site, suggested that cations might influence binding indirectly (Oxvig and Springer, 1998). This is supported by other work in α4β1 integrins showing that mutations in divalent cation binding sites did not impair binding of the soluble ligand VCAM-1, but did impair cellular resistance to detachment under shear flow. This suggests that these cation binding sites influence adhesion strengthening, but not necessarily initial ligand binding (Pujades *et al.*, 1997).

1.11 Modulation of integrin-ligand binding – integrin activation

The ability of an integrin to interact with a ligand depends not only upon the ligand specificity conferred by the ligand binding site, but also upon the biochemical state of the integrin itself, its state of "activation". In a highly activated state, integrin-ligand binding is greatly increased, whereas in the low activation (or suppressed) state, there is

greatly reduced ligand-integrin interaction. This rapid switching between activated or suppressed states is of fundamental importance to the functional effects of integrin-ligand binding, controlling many biological responses, most classically cellular migration and platelet aggregation. The activation status of integrins is therefore tightly controlled by intracellular signalling events, which collectively comprise "inside-out signalling" (Bazzoni and Hemler, 1998).

Integrin activation may be a result of two separate mechanisms termed "avidity modulation" and "affinity modulation". Avidity modulation results from increased concentrations of integrins within the plasma membrane, resulting in local increases in integrin clustering. Affinity modulation results from a conformational change in the individual integrin, producing a change in ligand binding affinity at the ligand binding site.

1.11.1 Avidity modulation

Avidity modulation has been well described in β2 integrins, especially αLβ2 (LFA-1). Activation of this integrin plays a key role in the response of leucocytes to injury or infection, when they must migrate from the blood to the affected tissues. Although initial adhesion to the vascular endothelial cells is via selectins, stronger adhesion is then mediated by integrins like LFA-1 (Stewart and Hogg, 1996) (Lub *et al.*, 1995). In lymphocytes, α4β7 has been demonstrated to be capable of initiating adhesion and tethering to lamina propria of veinules, in a selectin-independent manner (Berlin *et al.*,

1995). It has been shown that cross-linking of the T-cell receptor (TcR)/CD3 complex on T-cells causes rapid clustering of LFA-1 and enhanced LFA-1-mediated adhesion to ICAM-1 molecules on the endothelial cells (Dustin and Springer, 1989). The importance of this process is clear in the disease leucocyte adhesion deficiency-1, where individuals lack a normal β2 subunit: unable to mount a satisfactory leucocyte response to injury, they suffer from recurrent severe infections (Bunting *et al.*, 2002). Avidity modulation by integrin clustering has also been demonstrated to play a significant role in the later, irreversible phase of platelet aggregation via αIIbβ3 integrin (Fox *et al.*, 1996;Simmons and Albrecht, 1996).

As would be predicted, avidity modulation is highly dependent on the actin cytoskeleton and disruption of this by compounds such as cytochalasin D are able to inhibit both integrin clustering and adhesion (van Kooyk and Figdor, 2000).

1.11.2 Affinity modulation

Affinity modulation has been demonstrated in the β1, β2, β3 and β7 families (Altieri *et al.*, 1988;Faull *et al.*, 1993;Crowe *et al.*, 1994) and has been studied most extensively in platelets, where the αIIbβ3 integrin is pivotal in the process of platelet aggregation (Shattil *et al.*, 1998). Platelets normally circulate as non-nucleated single, non-adherent cells. However, at points of endothelial injury they will adhere to exposed extracellular matrix by an integrin-independent process. This attachment triggers an intracellular signalling cascade, resulting in a rise in intracellular ADP and triggering a

conformational change in the αIIbβ3 integrin, whose normal ligand is fibrinogen (Shattil *et al.*, 1985). In the suppressed state found in quiescent platelets, αIIbβ3 does not bind fibrinogen, but following integrin activation an activated platelet will bind approximately 45,000 fibrinogen molecules per cell, all of which may act as links to more platelets or the extracellular matrix. Initial ligand binding must occur to surface-expressed αIIbβ3, but internal pools of αIIbβ3 are then released from α-storage granules, which further contribute to ligand binding and platelet aggregation (Nurden *et al.*, 1996). Thus activation of the αIIbβ3 integrin is central to the cascade of platelet aggregation and thrombus formation.

Shattil *et al.* were able to identify the high affinity state αIIbβ3 fibrinogen receptor with a highly specific, ligand-mimetic, monoclonal antibody, PAC1 (Shattil *et al.*, 1985). This has facilitated further study of affinity regulation: the presence of the RGD sequence in the heavy chain of the PAC1 antibody allows specific recognition of the high affinity state (Kunicki *et al.*, 1996). PAC1 binding is independent of the degree of integrin clustering, since PAC1 Fab fragments also recognise the high affinity state; however, PAC1 Fab binding does not generate intracellular tyrosine phosporylation (Abrams *et al.*, 1994). Using PAC1 binding as a marker for integrin activation, it has been shown that αIIbβ3 can be activated by multiple platelet agonists such as thrombin, ADP, adrenaline and thromboxane A_2 (Shattil *et al.*, 1998). Activation does not rest on biochemical changes to the ligand binding site, since the chemical structure remains identical between the activated and suppressed states (Yan and Smith, 2000). However,

there is now good evidence that conformational changes occur on activation, witnessed by an increase in resonance energy transfer seen between fluorochromes labelling the αIIb and β3 subunits, suggesting a closer proximity of the labels (Sims *et al.*, 1991). The mechanisms of this conformational change, together with the cellular triggers that govern them, are now being elucidated.

It has become clear that the interaction of the α and β cytoplasmic domains is central to the regulation of activation (O'Toole *et al.*, 1994). In the membrane proximal regions of all α and most β subunits there are conserved amino acid sequences, GFFKR and LLv-iHDR respectively (**Figure 1.2**). Deletion of these regions activates integrins, independently of cell type or physiological signalling pathways, suggesting that this "hinge" region acts as a structural constraint that normally locks integrins into a default state of low affinity (Crowe *et al.*, 1994;O'Toole *et al.*, 1994). This may be due to charge interactions between the α and β subunits whose membrane proximal regions lie closely associated with each other (Lu *et al.*, 2001). For αIIbβ3, a salt bridge is proposed between αIIb Arg^{995} and β3 Asp^{723} (Hughes *et al.*, 1996). Using a lipid vesicle model, Vinogradova *et al.* have shown that the αIIb cytoplasmic domain adopts a closed formation with its COOH-terminal acidic region, but mutations inducing activation of αIIbβ3 open this closed conformation (Vinogradova *et al.*, 2000). At present, studies have provided contradictory evidence of definite interactions between the α and β tails,

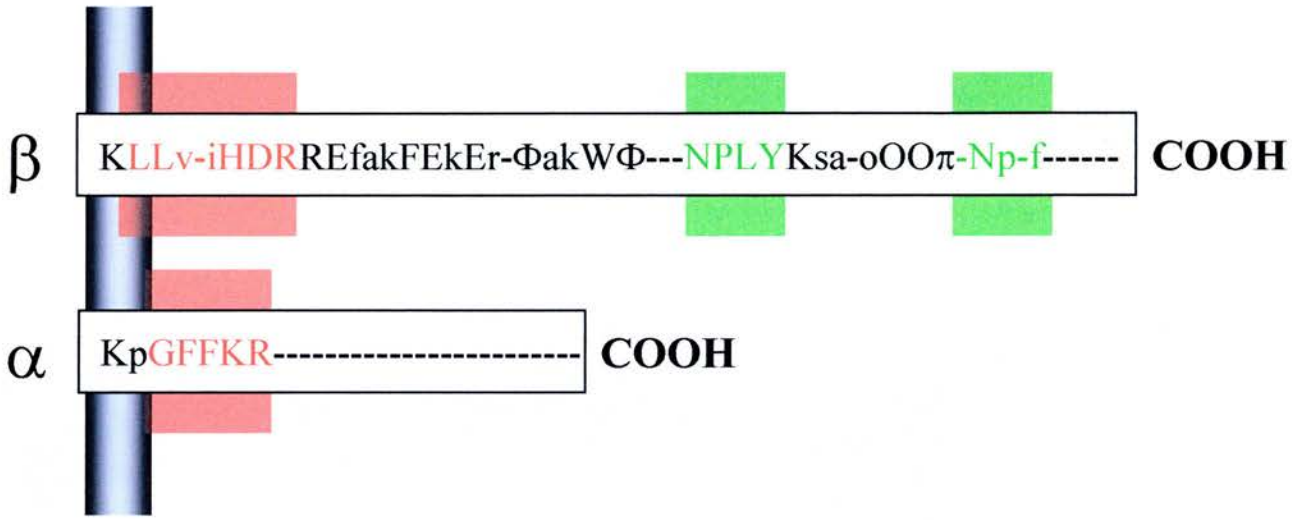


Figure 1.2: Membrane-proximal region and cytoplasmic tail of integrin heterodimers

The cytoplasmic tail region starts with the first charged lysine (K) residue adjacent to the transmembrane domain. The highly conserved membrane-proximal residues (red shading) form a putative "hinge" region, maintaining a low affinity default state. Additional conserved regions in the β integrin tail, such as the NPXY and NPXF motifs (green shading), act as important binding regions for cytoskeletal and regulatory proteins. Highly conserved amino acid residues are shown in capital letters; lower case letters represent residues conserved in three or more subunits; - = non-conserved residue/gap; O = hydroxylated residue; π and Φ represent apolar and polar residues, respectively. (From Hughes *et al.*, 1996.)

which may be due to different methodologies (Ginsberg *et al.*, 2001;Li *et al.*, 2001b;Weljie *et al.*, 2002).

The cytoplasmic interactions are transduced to the extracellular domains via the transmembrane domain. The transmembrane domain begins after an extracellular proline residue. There then follows a stretch of hydrophobic residues, culminating in a positively charged amino acid, arginine or lysine. This is generally followed by a short stretch of hydrophobic residues, so that the K/R is flanked by hydrophobic regions – this is felt to be the beginning of the cytoplasmic domain (Williams *et al.*, 1994). It is likely that the 4-6 residue hydrophobic stretch just COOH-terminal to the conserved K/R is membrane-embedded, at least in some integrin subunits such as α2, α5, β1 and β2 (Armulik *et al.*, 1999). Changes in the hydrophobicity of this region, such as are seen in some activating mutations (O'Toole *et al.*, 1994;Hughes *et al.*, 1996), may alter the length of the transmembrane domain itself in a "piston-like" motion, with resultant conformational changes in the extracellular domain (Williams *et al.*, 1994;Liddington, 2002). In this context, it is noteworthy that there are proteins known to bind to integrin cytoplasmic tails, such as talin and cytohesin-1, and that cytohesin-1 binds to β2 integrin at the conserved hydrophobic region (Nagel *et al.*, 1998). The intracellular signals thought to regulate integrin activation will be described in more detail below.

In the extracellular domain, three major regions have been implicated in ligand binding. In the α subunit these are the β propeller, whose upper surface contains a ligand-binding

site (Xiong *et al.*, 2002) and, in many α subunits, the I domain, with its MIDAS motif (Lee *et al.*, 1995b). Finally, β subunits also contain a MIDAS motif predicted to be part of an I-like domain (Lee *et al.*, 1995b) and demonstrated in the crystal structure of αvβ3 (Xiong *et al.*, 2002). Studying the way collagen binds to α2β1, Emsley *et al.* have described how conformational changes in the α2 I domain are induced by ligand binding. A 10Å shift in the COOH-terminal helix of the I domain allows a strong bond to be formed between the Mg^{2+} of the MIDAS motif and a glutamic acid in collagen (Emsley *et al.*, 2000). Similar conformational changes probably underpin affinity regulation. Loftus *et al.* have proposed a model for integrin activation in platelets in which the I-domain-like region of β3 is incapable of binding ligand in resting platelets, but occludes the ligand binding site of the αIIb subunit. Activation then causes a conformational change in the β3 I-domain-like region, exposing its ligand binding site, and causing unmasking of the αIIb binding site (Loftus and Liddington, 1997) (**Figure 1.3**). Following the publication of the crystal structure of αvβ3 (Xiong *et al.*, 2001;Xiong *et al.*, 2002), Liddington *et al.* have suggested that following activation, initial ligand binding is to the exposed MIDAS domain of the β I-like domain. Occupancy itself activates integrins with structural changes (Du *et al.*, 1993). These conformational changes result in a loosening of contacts between the β I-like domain and the α subunit propeller and a separating of the head pieces of the α and β subunits. This supports other work suggesting that the activated state requires separation of the head pieces and their ligand binding epitopes (Mould *et al.*, 1997;Hantgan *et al.*, 1999).

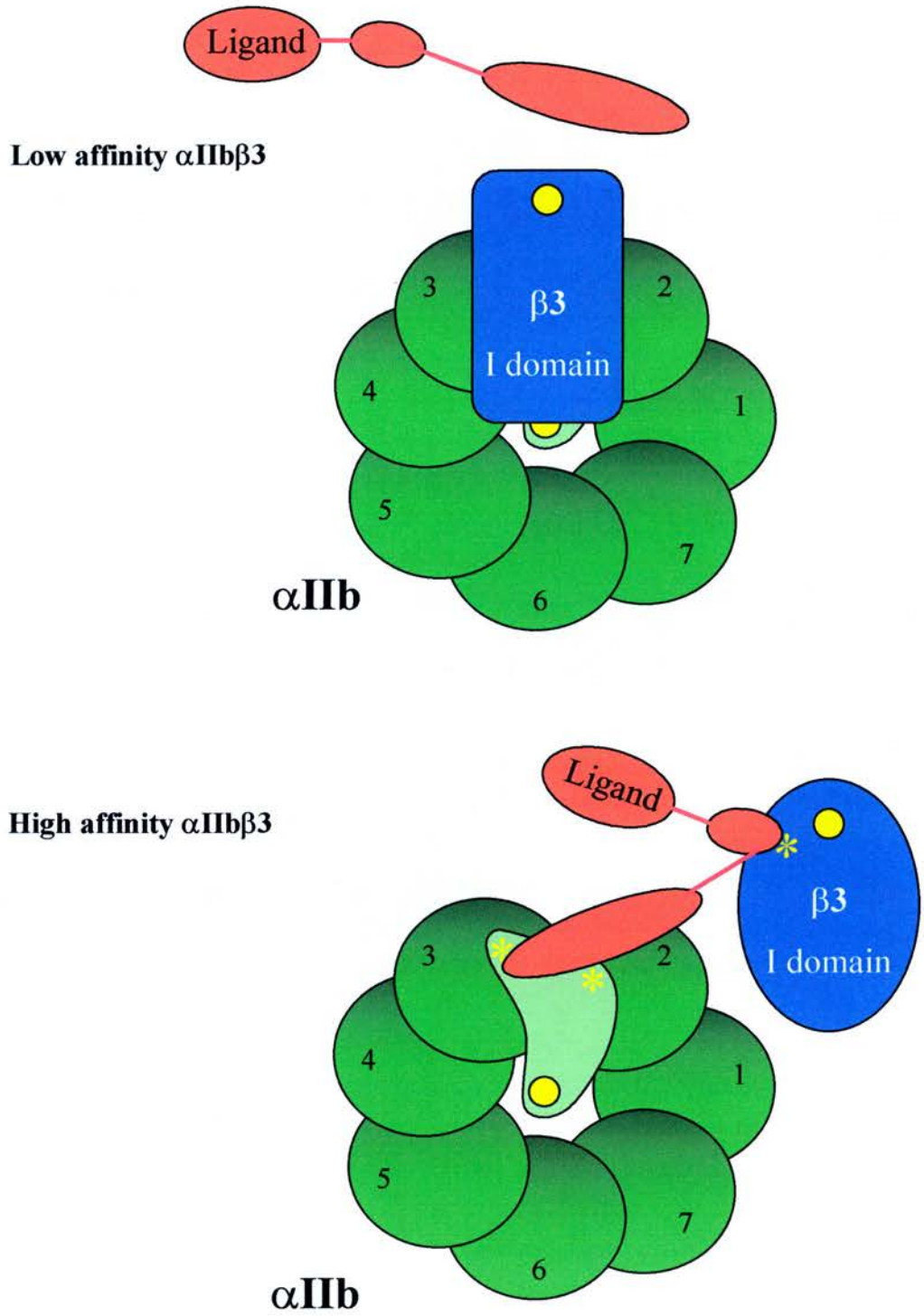


Figure 1.3: Schematic of high affinity binding in integrin αIIbβ3

Schematic diagram demonstrating an overhead view of integrin αIIbβ3 in the low affinity (*above*) and high affinity states (*below*). In the low affinity state the β-propeller of the α subunit contains divalent cations (●) and ligand binding sites (✳)that are partially masked by the overlying β subunit. Platelet activation results in inside-out signals that cause conformational changes, such that the β3 I domain ligand binding site becomes exposed and the orientation of the β and α subunits alters, exposing the binding sites on the α subunit for high affinity ligand binding. (Adapted from Shattil *et al.*, 1998.)

With the two head pieces separated, the ligand binding epitopes become fully exposed and ligand binding affinity becomes maximised.

1.11.3 Inside-out signalling

A limited number of proteins have been demonstrated to influence integrin affinity. In itself, integrin affinity modulation is best measured by binding of soluble ligand, such as fibronectin, or ligand-mimetic antibodies such as PAC1, in order to distinguish it from avidity changes (Hughes and Pfaff, 1998).

G-protein-coupled receptor pathways

In platelets, the affinity of the αIIbβ3 integrin has been shown to be upregulated by ADP, thrombin, collagen and adrenaline (Bennett and Vilaire, 1979), which bind to G-protein-coupled receptors. Several important signalling cascades may result from this. One is the activation of phospholipase C_β by the activated α subunit of G_q. This results in hydrolysis of phosphatidylinositol and the generation of diacylglycerol and IP_3; platelets in mice that are $G\alpha_q$ null fail to aggregate in response to thrombin, ADP or thromboxane A_2 agonists (Offermanns *et al.*, 1997). The link between the generation of IP_3 and diacylglycerol and integrin activation remains unclear. IP_3 stimulates a rise in intracellular Ca^{2+}, although this is not sufficient for activation (Shattil and Brass, 1987), whilst diacylglycerol stimulates activation through protein kinase C (Shattil *et al.*, 1992).

G-protein-coupled receptors also activate a number of nonreceptor protein tyrosine kinases, such as Src, Syk and Pyk2 (Jackson *et al.*, 1996;Raja *et al.*, 1997). Tyrosine phosphorylation or dephosphorylation events are likely to be important in inside-out signalling, since tyrosine kinase inhibitors inhibit fibrinogen binding in platelets, whereas protein tyrosine phosphatase inhibitors enhance platelet activation (Jackson *et al.*, 1996). One key target for tyrosine kinases might be phosphoinositide 3-kinase, which phosphorylates PI(4)P and PI(4,5)P_2 to PI (3,4)P_2 and PI(3,4,5)P_3 respectively. PI 3-kinase can be co-immunoprecipitated with the protein tyrosine kinases Src and Syk in activated platelets (Rittenhouse, 1996). Inhibition of PI 3-kinase reduces platelet aggregation and αIIbβ3 activation in response to agonists (Rittenhouse, 1996;Zhang *et al.*, 1996a), suggesting that PI 3-kinase is an activator of integrins, possibly via isoforms of PKC that are found in platelets (Toker and Cantley, 1997). Other potential links between PI 3-kinase and integrins include proteins that contain pleckstrin-homology (PH) domains and are therefore targets for the PI 3-kinase product PI(3,4,5)P_3, such as cytohesin-1. Cytohesin-1 is known to interact with the β2 cytoplasmic domain of αLβ2 (Kolanus *et al.*, 1996) and PI 3-kinase activation is associated with recruitment of cytohesin-1 to the plasma membrane where it may interact with the β2 cytoplasmic domain (Nagel *et al.*, 1998). However, in CHO cells PI 3-kinase does not appear to be such a critical activator of integrins (Oertli *et al.*, 2000) and it may be that PI 3-kinase is more important for outside-in signalling than for affinity modulation.

Ras proteins

Several members of the Ras superfamily of GTP-binding proteins have been shown to play important roles in integrin affinity regulation. They are also key components of the multitude of downstream signalling cascades for outside-in signalling (*see below*).

The *ras* gene was first identified in viral-induced rat sarcomas, from which the Harvey and Kirsten strains of murine sarcoma retrovirus were isolated (HARVEY, 1964;Kindig and Kirsten, 1967). The viral oncogenes were derived from normal 21kDa rat cellular proteins, Harvey-Ras (H-Ras) and Kirsten-Ras (K-Ras) and the dominant oncogenes of several human tumours were found to be mutant forms of the *ras* gene, homologous to the viral H-*ras* and K-*ras* (Bos, 1989). N-Ras was later identified in a neuroblastoma cell line (Shimizu *et al.*, 1983). The *ras* genes are all highly conserved between species and the promoter sequence suggests they are cellular housekeeping genes, each of importance. The Ras proteins consist of 189 residues, of which 1-86 are identical and 87-164 are highly homologous; the terminal 25 residues represent the "hypervariable domain", where the sequences diverge. The proteins bind guanine nucleotides and have an intrinsic GTP-ase activity: they cycle between an active GTP-bound state and an inactive GDP-bound state (Bourne *et al.*, 1990). Since the vast majority of Ras within the cell is in the inactive GDP-bound state (Scheele *et al.*, 1995), activation results in replacement of GDP by GTP, by means of guanine exchange factors (GEFs) such as SOS (Quilliam *et al.*, 1995). GTP is then hydrolysed back to GDP by the intrinsic GTPase activity under the control of GTPase Activating Proteins (GAPs) that stimulate

this hydrolysis. Whilst in the active GTP-bound state Ras triggers downstream events in a variety of signalling pathways.

Using the chimæric integrin αIIbα6aβ3β1 in CHO cells, Hughes *et al.* identified H-Ras and its effector kinase Raf-1 as key players in a pathway that suppressed integrin activation without influencing integrin phosphorylation, mRNA translation or protein synthesis (Hughes *et al.*, 1997). This suppression also involved activation of the ERK/MAP kinase pathway, although subsequent evidence has thrown doubt over the necessity for ERK/MAPK activity for affinity modulation (Hughes *et al.*, 2002). In other cell lines, ectopic expression of a constitutively active H-Ras induced activation of the α4β1 and α5β1 integrins, independent of MAPK activation, but dependent upon phospholipase C activation (Shibayama *et al.*, 1999;Liu *et al.*, 1999b). Further, in marrow-derived mast cells, H-Ras again activated α5β1, but in these cells activation was dependent upon PI 3-kinase, being inhibited by wortmannin (Kinashi *et al.*, 2000). It seems likely, therefore, that H-Ras affinity modulation of integrins is dependent upon the target integrin, the cell type and the upstream activators involved.

By contrast, the closely related Ras subfamily protein R-Ras, which has more than 50% homology to H-Ras but also has a 26 amino acid N-terminal extension, has so far been shown only to activate integrins. Zhang *et al.* showed that expression of activated R-Ras converted cells that normally grew in suspension into highly adherent cells and upregulated integrin-ligand binding (Zhang *et al.*, 1996b). Since then, the mechanism by

which R-Ras activates integrins has been sought. In CHO cells, Sethi *et al.* showed that R-Ras did not directly activate integrins, but antagonized the effects of H-Ras suppression in these cells (Sethi *et al.*, 1999a). This antagonism did not appear to be mediated through simple competition for the same downstream Raf pathway effectors, although R-Ras and H-Ras can both bind Raf1, Ral-GDS and PI 3-kinase (Urano *et al.*, 1996;Marte *et al.*, 1997;Rodriguez-Viciana *et al.*, 1997). Mutational studies suggest that despite the homology of their effector loop binding domains, R-Ras and H-Ras couple to and activate their common effectors in different ways (Oertli *et al.*, 2000). In addition, R-Ras can stimulate PI 3-kinase activity, supporting other data that implicates PI 3-kinase in integrin affinity activation (*see above*) (Marte *et al.*, 1997;Marte and Downward, 1997).

The small GTP-binding protein Rho may also play a role in integrin activation. Inhibition of Rho by C3 exoenzyme blocks α4β1-dependent adhesion of lymphocytes and αLβ2-dependent adhesion of neutrophils to VCAM-1 and fibrinogen respectively (Laudanna *et al.*, 1996); however, in platelets, where C3 exoenzyme blocks platelet aggregation in response to thrombin, no evidence has been found that this is through αIIbβ3 integrin affinity modulation and it has been suggested that Rho effects may therefore be mediated through cytoskeletal regulation or avidity modulation, rather than affinity modulation (Morii *et al.*, 1992;Leng *et al.*, 1998).

Rap-1 is another member of the Ras family of small GTPases and recently this was reported to activate LFA-1 integrin in a nonadherent, lymphoid cell line (Katagiri *et al.*, 2000). This activation involved changes in conformation, affinity and adhesion to ICAM-1, but was not dependent upon PI 3-kinase activation.

CD98 and galectin-3

CD98 is a disulphide-linked 125 kDa heterodimeric membrane glycoprotein that is a ubiquitously expressed cell surface antigen, highly conserved between species. Early studies of peripheral blood T and B-lymphocytes implicated CD98 in the regulation of cellular activation, but did not define a specific function (Haynes *et al.*, 1981). It is highly expressed in the fœtus, but expression thereafter diminishes. In the adult, however, CD98 appears to be upregulated in proliferating cell populations and particularly in tumours (Warren *et al.*, 1996;Diaz, Jr. and Fox, 1998).

CD98 has also been identified as a unique and highly specific regulator of integrin affinity, using a cloning strategy based upon changes in integrin affinity to identify potential modulators of integrin activation (Fenczik *et al.*, 1997b). β1 integrin-mediated adhesion of the SCLC cell line H345 to fibronectin and laminin can be markedly upregulated by cross-linking CD98 (Fenczik *et al.*, 1997b). Other studies also implicate CD98 in the regulation of integrin-mediated cell adhesion. In breast cancer cell lines, α3β1-mediated adhesion to thrombospondin-1 was upregulated by cross-linking CD98 (Chandrasekaran *et al.*, 1999b). In the promonocytic U937 cell line, attempts have been

made to tease apart the interaction between CD98 and β1 integrins in cell adhesion: ligation of CD98 induced adhesion that was at least partly mediated through β1 integrins, since some β1 integrin antibodies (but not all) were able to inhibit the effect (Cho *et al.*, 2001). In lymphocytes, CD98 ligation induced αLβ2/ICAM-1 mediated cell adhesion via PI 3-kinase (Suga *et al.*, 2001). There is therefore accumulating evidence for an important role for CD98 in regulating integrin-mediated adhesion.

Putative ligands for CD98 include galectin-3, a member of a family of carbohydrate-binding proteins. Using lysates from a murine macrophage cell line, Dong and Hughes isolated a number of glycoproteins that bound to galectin-3, of which CD98 was one (Dong and Hughes, 1997). Galectin-3 was initially described as the Mac-2 antigen, when it was shown to be upregulated on murine peritoneal macrophages, specifically in response to stimuli such as thioglycollate (Springer, 1981;Ho and Springer, 1982). Galectin-3 is synthesized without a signal sequence and lacks a transmembrane sequence, yet it is able to be secreted from the cytoplasm via pathway independent of the endoplasmic reticulum and may be found at the cell surface (Sato *et al.*, 1993). Galectin-3 exists as a monomer and it has been shown to self-associate and form polymers through interactions of either its N-terminal domain (Birdsall *et al.*, 2001) or its C-terminal domain (Yang *et al.*, 1998), giving rise to a potential to cross-link CD98 at the cell surface. Like CD98, galectin-3 is upregulated in T-lymphocytes in response to mitogenic stimuli, such as Il-2, Il-4 or Il-7 (Joo *et al.*, 2001). There is no clear evidence directly linking galectin-3 to integrin affinity modulation, although early work noted that

at relatively high concentrations galectin-3 inhibited adhesion and spreading of baby hamster kidney cells to laminin, suggesting a possible influence on integrin function (Sato and Hughes, 1992).

PEA-15

PEA-15 is a small death effector domain-containing protein that was isolated using a cDNA expression cloning screen designed to isolate proteins able to reverse H-Ras-mediated integrin suppression (Ramos *et al.*, 1998). Interestingly it did this through a pathway blocked by a dominant negative mutant of R-Ras, suggesting that R-Ras acts downstream of PEA-15. Subsequently, PEA-15 has been shown to activate the MAPK kinase and ERK pathway in a Ras-dependent manner, bypassing the usual anchorage dependence of ERK activation (Ramos *et al.*, 2000). This effect was independent of its effect on integrin activation, but suggests that PEA-15 is able to protect against apoptosis and to enhance Ras-ERK signalling, and may thus play an important role in oncogenesis.

β3-endonexin

β3-endonexin has been identified as a regulator of αIIbβ3 integrin, the key platelet integrin. It was initially identified in a yeast two-hybrid system as a novel protein that bound β3 integrin cytoplasmic tails in a manner that was integrin subunit specific, since it did not bind to αIIb, β1 or β2 integrins (Shattil *et al.*, 1995). CHO cells transfected with β3-endonexin demonstrated an increase in PAC1-αIIbβ3 binding and increased

fibrinogen-dependent aggregation (Kashiwagi *et al.*, 1997). Again using a yeast two-hybrid system, it has been shown that β3-endonexin binds to the β3 integrin cytoplasmic domain through motifs located in the membrane-proximal and membrane-distal regions of the β3 integrin tail (Eigenthaler *et al.*, 1997). Experiments with chimæric integrin cytoplasmic tails suggest that these motifs are sufficient to confer specificity of binding with β3-endonexin.

1.12 Cellular roles for integrins

An expanding number of roles are now understood to be dependent on integrins and it is apparent that they are critical to key cellular processes, including cellular survival.

1.12.1 Adhesion

Cellular adhesion was the role in which integrins were initially identified in the 1980s and ranges from adhesion of cell layers in *Drosophila* wing (Brown, 1994) to egg fertilization, by interaction between α6β1 on the oocyte and the sperm disintegrin receptors (Almeida *et al.*, 1995). Integrin-mediated adhesion occurs at an intercellular level, but most prominently it occurs between cells and extracellular proteins – such as fibrinogen in the case of thrombus formation – or components of the extracellular matrix.

Cell-extracellular matrix interactions are critical for the survival and function of normal epithelial cells. Not only does adhesion generate important cell survival signalling, but it

permits a structural integrity between the ECM and the cell cytoskeleton which is critical for biological roles such as cellular migration and cell growth. Adhesion occurs via integrins at specialised sites called focal adhesions (FAs) or focal contacts (FCs). The larger FAs are located at the ends of actin stress fibres; FCs are located at the tips of extending filopodia and lamellipodia (Petit and Thiery, 2000). Both sites comprise a number of molecules linking the cytoplasmic tails of ligand-bound integrin to the underlying actin cytoskeleton. This protein complex is essential for integrin-mediated signalling, both "inside-out" and "outside-in". It is also critical for the generation of physical tension within the cell, without which cells are unable to migrate. Lauffenburger and Horwitz (1996) argue that cell motility is a balance between contractile forces pulling the cell forward over a surface and adhesive forces between the cell and substratum. If the adhesive forces are too strong the cell becomes unable to move, but if there is too little adhesion no tensile force can be generated to transmit to the cytoskeleton and allow movement (Lauffenburger and Horwitz, 1996). These models predict a bell-shaped curve for the rate of migration against the strength of cell adhesion. Some credence is lent to this proposal, since it has been demonstrated that high affinity integrin activity can impair cell migration at a given ligand concentration (Palecek *et al.*, 1997).

Formation of FAs follows ligand-induced integrin clustering and requires Rho activity (Ridley and Hall, 1992;Hotchin and Hall, 1995). Actin-binding proteins such as talin, filamin and α-actinin, together with focal adhesion kinase (FAK) form a complex with

integrin cytoplasmic tails (Miyamoto *et al.*, 1995a;Miyamoto *et al.*, 1995b;Schaller *et al.*, 1992;Hanks *et al.*, 1992). This induces FAK activation and autophosphorylation, producing an SH2 binding domain capable of recruiting Src kinase (Schaller *et al.*, 1992;Schaller *et al.*, 1999). Src kinase activity results in tyrosine phosphorylation of FAK, inducing the formation of SH2 and SH3 binding sites for a variety of signalling proteins, including p130cas/crk (Vuori *et al.*, 1996) and Grb2/SOS (Schlaepfer *et al.*, 1994;Wary *et al.*, 1996), through which integrins influence fundamental cellular behaviours such as cell cycle progression in response to mitogens (Giancotti and Ruoslahti, 1999).

1.12.2 Migration and invasion

There is extensive evidence that integrins play a key role in cell migration. As discussed above, they provide a key link to the actin cytoskeleton and the involvement of different integrins in migration has been demonstrated in a number of cell types. Leucocyte adhesion to epithelial cells via αLβ2 (LFA-1) has already been discussed; there is also strong evidence that other, non-β2 integrins, such as α4β1 (VLA-4), α5β1 (VLA-5) and αvβ3, play a key role in the subsequent migration of leucocytes across the endothelium, through extracellular matrix and on to sites of inflammation (Sixt *et al.*, 2001;Burns *et al.*, 2001). In asthma α4β1 also seems to be important for an eosinophilic migratory response (Bocchino *et al.*, 2000). Monocyte migration has been shown to be dependent on a combination of Mac-1, αLβ2 and α4β1, since blockade of these three effectively inhibits monocyte accumulation at inflammatory sites (Issekutz, 1995).

Integrins play a key role in embryonic development, in a manner that is likely to be exquisitely timed and regulated. Administration of RGD peptides or monoclonal antibodies directed against αvβ1 integrins disrupts migration of avian cranial neural crest cells (Bronner-Fraser, 1985;Darribere *et al.*, 1990), whereas truncal neural crest cell migration appears to be dependent more on αvβ3 and αvβ5 (Delannet *et al.*, 1994). The β1 integrins are also implicated in migration of retinal ganglion cells (Cann *et al.*, 1996), oligodendrocytes (Milner *et al.*, 1996) and glioma cells (Friedlander *et al.*, 1996). These events represent *controlled* invasion, but integrins are equally implicated in invasion in disease states, such as neoplasia (*see below*).

1.12.3 Cellular proliferation and differentiation

There is considerable evidence that integrins are important in cellular proliferation. Optimal growth factor signalling in many cell lines is only seen in adherent cells (Khwaja *et al.*, 1997;Short *et al.*, 1998), whilst Bohmer *et al.* have reported that in human fibroblasts adhesion is required throughout most of the G1 phase when mitogen stimulation occurs (Bohmer *et al.*, 1996). Extracellular matrix adhesion increases the number of PDGF receptors by blocking their ubiquitin-dependent degradation (Baron and Schwartz, 2000). Some integrins physically associate with growth factor receptors, such as the PDGF, insulin and VEGF receptors with αvβ3, or β1 integrins with the EGF receptor (Giancotti and Ruoslahti, 1999). T cell proliferation has been shown to result from cross-linking of α4β1, α5β1 and α6β1 (Davis *et al.*, 1990;Shimizu *et al.*, 1990);

for thymocytes, α3β1 and α6β1 are costimulatory for anti-CD3-induced proliferation (Chang *et al.*, 1995). These proliferative responses are specific to the integrin heterodimer and are not universal: for example, the β1C and β1D cytoplasmic domain splice-variants appear to be inhibitory to proliferation (Meredith, Jr. *et al.*, 1995;Belkin and Retta, 1998;Meredith, Jr. *et al.*, 1999).

Integrin-mediated effects on the cell cycle are mediated through the plethora of outside-in signalling pathways that are regulated by integrin-ligand binding. Although complex and not yet fully understood, some signalling cascades linking integrins to the cell cycle machinery have been established. These include stimulation of focal adhesion kinase (FAK), $p130^{cas}$ and paxillin, phosphoinositide 5-kinase and the Na^+/H^+ antiporter (Schwartz *et al.*, 1995). Integrin and growth factor receptor signalling results in upregulation of cyclin-D1 and downregulation of Cdk-2 inhibitors (Fang *et al.*, 1996;Roovers and Assoian, 2000). Of these pathways, FAK signalling is most clearly understood and has been shown to be required for cell cycle progression through G1 phase, in part by activating Jun kinase (Oktay *et al.*, 1999). Integrin-mediated FAK activation may also potentiate growth factor signalling by impinging on other pathways such as the ERK/MAP kinase cascade (Renshaw *et al.*, 1999). It seems likely that sustained ERK signalling as a result of integrin and growth factor stimulation is necessary for upregulation of cyclin D1 expression (Roovers and Assoian, 2000). It has also been shown that PI 3-kinase is necessary for expression and stability of cyclin D1

and integrins are known to regulate PI 3-kinase activity (Gille and Downward, 1999;Takuwa *et al.*, 1999).

Whilst most integrins appear to promote cell proliferation, there are areas where this has been found not to be the case and where integrin- or matrix-specific effects are seen. For example, elevated α6β1 levels in myoblasts inhibit ERK and promote withdrawal from the cell cycle and differentiation (Sastry *et al.*, 1999). In fibroblasts and endothelial cells, α2β1 stimulation does not promote proliferation either (Wary *et al.*, 1996), although this may be a cell-specific effect, since it has been shown to promote mammary epithelial tube formation (Zutter *et al.*, 1999).

Cellular adhesion has been shown to be important in tissue morphogenesis, with changes in cellular phenotype associated with changing integrin expression patterns. Disruption of the cell-ECM interactions results in disrupted tissue development and has been shown in a variety of contexts, including submandibular gland (Kadoya *et al.*, 1995), kidneys (Wada *et al.*, 1996), mammary epithelium (Streuli *et al.*, 1991) and myoblast differentiation (Sastry *et al.*, 1996).

1.12.4 Anoikis and programmed cell death

Programmed cell death, or apoptosis, has been described since the early 1980s. The key features are a massive destruction of DNA by intranucleosomal cleavage, with condensation of chromatin and nuclear fragmentation (Hengartner, 2000). *In vivo*,

apoptotic cells are rapidly recognised and phagocytosed, thereby minimising the release of harmful intracellular proteases and bystander damage. A variety of signals, including intracellular stress and receptor-mediated signals, feed into the evolutionarily conserved caspase family of cysteine-proteases; these converge on the "final common pathway" involving activation of caspase-3 and its downstream effectors. The subject has been extensively reviewed and an overview will be given here (Kaufmann and Hengartner, 2001;Assuncao and Linden, 2004).

The three major pathways leading to caspase-dependent apoptosis are illustrated in **Figure 1.4**. (i) The death receptor pathway involves cell surface receptors, such as Fas/CD95, whose ligation promotes the binding of adaptor molecules and then binding and cleavage of procaspase-8 (Budihardjo *et al.*, 1999). Activated caspase-8 then directly cleaves and activates procaspase-3, or it may cleave the Bcl-2 family protein Bid, to stimulate the mitochondrial pathway to apoptosis. (ii) In the mitochondrial pathway, Bcl-2 family cytoplasmic proteins such as Bax and Bak translocate to and insert into the mitochondrial membrane, promoting release of cytochrome c (Burlacu, 2003). This promotes the assembly of Apaf-1 and procaspase-9 into the apotposome, which is then capable of cleaving and activating procaspase-3. Mitochondrial cytochrome c release may occur as a result of DNA damage. (iii) A third pathway involves regulation of anti-apoptotic proteins such as Inhibitor of Apoptosis Protein (IAP), which normally bind to and inhibit active caspases-3, -7 and –9 (Salvesen and Duckett, 2002). Such IAP family proteins may be sequestered away from caspases

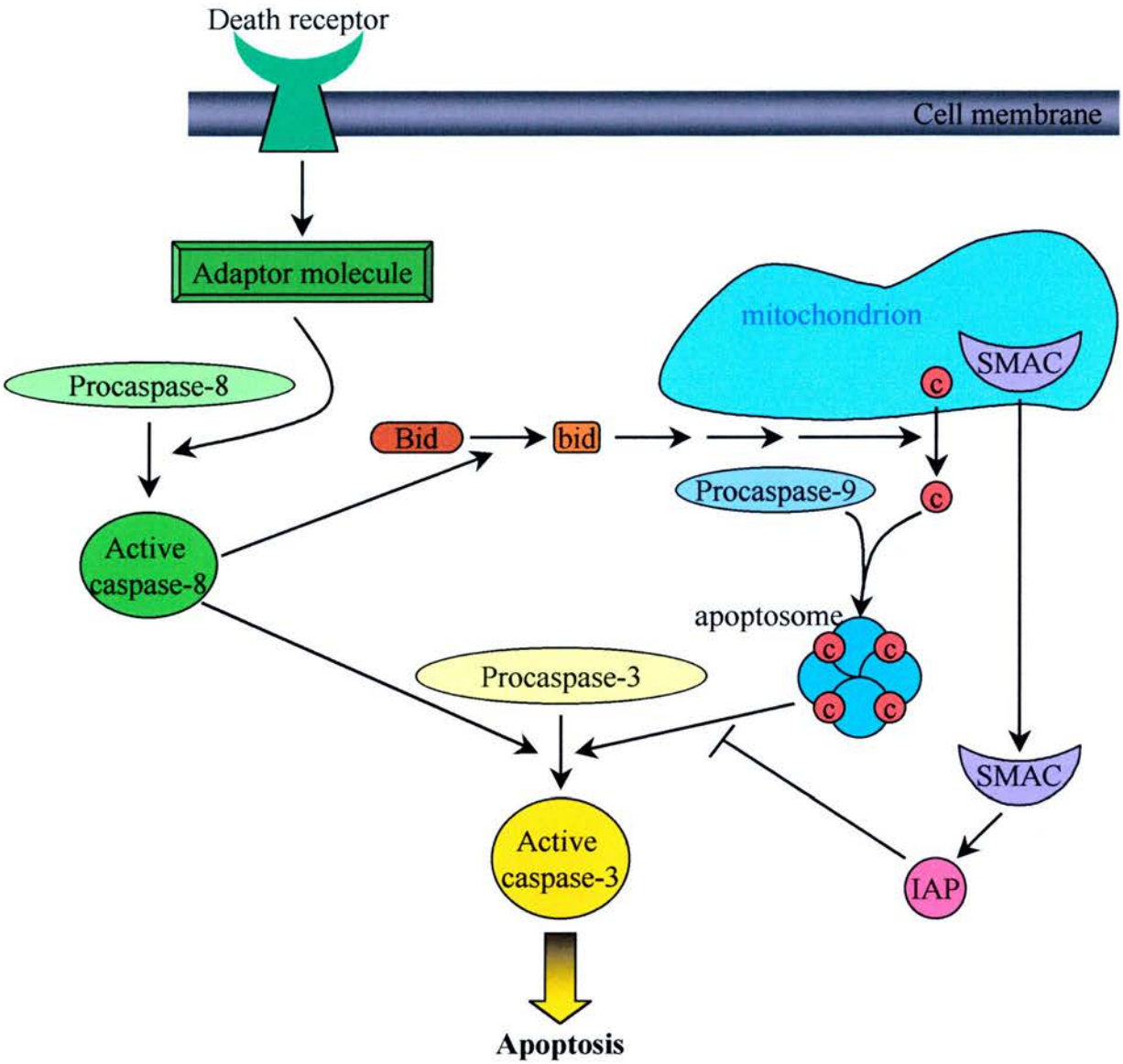


Figure 1.4: Pathways regulating caspases in apoptosis

Figure summarising the three major pathways regulating leading to caspase-3 activation. The death receptor pathway is initiated by activation of a number of cell surface death receptors, such as Fas/CD95, tumour necrosis factor-α receptor 1, and death receptors 4 and 5. Activated caspase-8 provides a link with mitochondrial pathways, by cleaving Bid. The truncated C-terminal fragment of Bid (bid) interacts with other Bcl-2 family proteins to promote the release of cytochrome c (c) into the cytoplasm; here it forms a tetrameric complex with procaspase-9, the apoptosome. Both the apoptosome and caspase-8 activate caspase-3, triggering apoptosis. A second mitochondrial pathway arises by the release of SMAC, which sequesters inhibitor of apoptosis proteins (IAPs), thus promoting caspase-3 cleavage. Adapted from Kaufman SH (2001) and Abraham MC (2004).

through activity of SMAC/Diablo ("Second Mitochondrial Activator of Caspases"), which itself is released from the mitochondria.

Caspase-independent pathways to programmed cell death also exist, since caspase inhibition does not always block programmed cell death with apoptotic morphology (Mateo *et al.*, 1999;Assefa *et al.*, 2000). Proteins that mediate these alternative cell death pathways include apoptosis-inducing factor (Joza *et al.*, 2001), endonuclease G (Li *et al.*, 2001a) and leucocyte elastase inhibitor-DNase II (Torriglia *et al.*, 1999).

It has been recognised that cells require constant stimulation form their environment to suppress their constitutive apoptotic pathway, since almost all cells (in a mouse model) can be induced to undergo apoptosis (Weil *et al.*, 1996). This environmental stimulation may take the form of soluble factors, cell-cell contact or cell-ECM interactions, where it has been termed "anoikis". ECM-dependent growth, or anchorage-dependence, has important developmental functions, such as allowing the generation of hollowed-out canals, like the vertebrate semicircular canals (Coucouvanis and Martin, 1995;Fekete *et al.*, 1997), and in preventing the survival of cells in an inappropriate environment. The signals regulating anchorage dependence are mediated through integrins at the focal adhesions and are intimately related to those that regulate cell proliferation, described above. Activated FAK supports anchorage-independent cell survival, since transfection of constitutively activated FAK rescues epithelial cell lines from anoikis (Frisch *et al.*, 1996), as do protein tyrosine phosphatase inhibitors such as vanadate (Meredith, Jr. *et*

al., 1993). PI 3-kinase activation appears to be an important component of the cell survival signal in many cell types (Khwaja *et al.*, 1997) and other groups have suggested that in some cell lines an increase in Bcl-2 expression upon attachment to fibronectin is critical (Zhang *et al.*, 1995;Frisch *et al.*, 1996). In mammary epithelial cells β1-mediated adhesion to ECM suppresses activity of caspase-1 (or interleukin-1-converting enzyme, ICE), providing a direct link to inhibition of the apoptosis pathway (Boudreau *et al.*, 1995).

More recently, it has been proposed that, whilst integrin-mediated cell signalling may indeed promote survival, an alternative would be that unoccupied integrins transmit signals that promote anoikis – signals that must be actively switched off to promote survival (Schwartz, 2001). It has already been observed that unoccupied α5β1 in non-adherent cells has a dominant effect inhibiting growth and cell survival (Giancotti and Ruoslahti, 1990;Varner *et al.*, 1995); in addition, by promoting α5β1 transcription, the CDK inhibitor INK4a sensitises cells to anoikis when the ligand is unavailable (Plath *et al.*, 2000).

1.13 Outside-in signalling

The complexities of outside-in integrin-mediated cell signalling and the integration of the roles described above are incompletely understood. It is unclear why there should be so much apparent redundancy in a system that finds at least eight integrins able to bind

to fibronectin, or five to bind laminin; and it is unclear how different integrins, with no intrinsic enzymatic activity themselves, activate specific cellular signalling pathways resulting in diverse cellular responses. Signalling is centred upon the focal adhesion complexes to which an array of signalling proteins is recruited. The major pathways are outlined below.

Focal adhesion kinase

It has long been recognised that focal adhesion kinase (FAK) localises with integrins at FAs (Schaller *et al.*, 1992). Elegant studies using monovalent ligand or non-inhibitory antibodies have highlighted the early role of FAK in integrin signalling (Miyamoto *et al.*, 1995b). Integrin occupation by monovalent ligand was able to induce integrin redistribution in the plasma membrane, but no major signalling events; occupation by non-inhibitory mAbs aggregated integrins and led to accumulation of FAK and tensin, but not maximal signalling. Only the combined stimuli of integrin ligand occupancy, plus integrin aggregation were sufficient to promote complete signalling complexes, able to synergise with growth factor signalling (Miyamoto *et al.*, 1996).

Engagement of integrins induces auto-phosphorylation of FAK on tyrosine-397 and activation of FAK tyrosine kinase activity (Huang *et al.*, 1993). A cascade of signalling events is then unleashed, by tyrosine and serine phosphorylation of a number of proteins such as paxillin and tenascin, by FAK or its mediators (Richardson and Parsons, 1995). A number of important linking proteins are now implicated in events downstream from

FAK phosphorylation. Fibronectin binding in 3T3 fibroblasts induces FAK to complex with the tyrosine kinase Src, the adaptor protein Grb2 and the nucleotide exchange factor SoS (Schaller and Parsons, 1994;Schlaepfer *et al.*, 1994). This results in activation of the ERK pathway and provides a link between integrin signalling and the Ras/ERK pathway. In v-Src-transformed NIH3T3 cells, the association of v-Src, Grb2 and Sos with FAK is independent of cell adhesion to fibronectin and may be important in the acquisition of anchorage-independent growth.

More recently, FAK has been shown to associate with p130Cas, a highly phosphorylated protein isolated in vSrc- and vCrk-transformed cells (Sakai *et al.*, 1994). Together with human enhancer of filamentation-1 (HEF1) and embryonal Fyn substrate (Efs), p130Cas makes up the Cas (Crk-associated substrate) family of proteins, which have multiple interaction domains and appear to act as docking proteins for SH2-domain-containing proteins. Cyclical alteration between phosphorylated and dephosphorylated states would permit the Cas proteins to modulate the stable assembly of such signalling complexes (O'Neill *et al.*, 2000).

Ras/ERK pathways

Ras (H-Ras, K-Ras and N-Ras) plays a pivotal role in control of cell growth and differentiation. It is the downstream effector for receptor tyrosine kinases such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) and is classically involved in promoting mitogenic signalling via activation of Raf, MEK

(MAPK/ERK kinase) and ERK (extracellular signal-regulated kinase, or mitogen-activated protein kinase/MAPK) (Avruch *et al.*, 1994). As described above, it is also directly linked to FAK signalling, by the Grb2 adaptor protein and subsequent recruitment of its GEF SoS. Maximal ERK pathway stimulation seems also to depend upon phosphorylation of the Shc adaptor protein at the FAK-Src complex, as well as involvement of the PI 3-kinase pathway, since mutation of the FAK Tyr^{397} autophosphorylation site disrupts binding of both the Shc SH2-domain and the p85 SH2-domain, and in each case reduces ERK signalling (Schlaepfer and Hunter, 1998).

An increasing number of downstream Ras effectors have been identified, which merely includes the classical pathway of Ras-Raf-MEK-ERK. Downstream events in the pathway include translocation of ERK to the nucleus, where it phosphorylates nuclear transcription factors such as Elk1, c-Myc, TCF and Jun. Other Ras effector pathways include PI 3-kinase and Ral-guanosine exchange factors (Ral-GEFs), such as Ral guanine nucleotide dissociation stimulator (RalGDS), Ral-GDS-like 1/2 (Rgl1/2) and RalGDS-like factor (Rlf). The major pathways are summarised in **Figure 1.5**. Both Ras and its downstream effector Raf are capable of oncogenic transformation in some cells, but activated Ras G12V effector mutants with differential binding to the major downstream effectors have demonstrated that the transforming potential of the various Ras pathways varies and that they may be synergistic (White *et al.*, 1995).

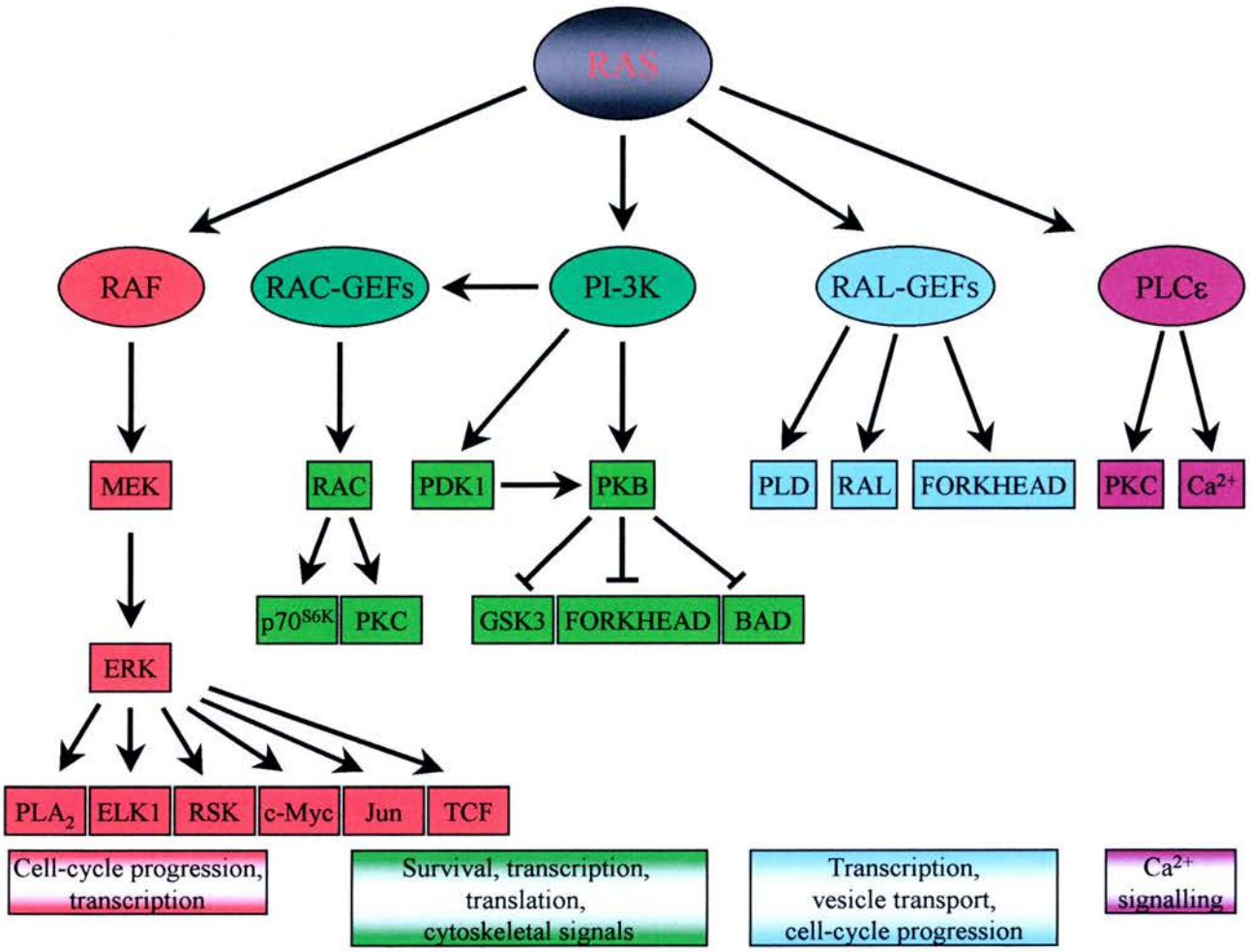


Figure 1.5: Ras signalling pathways.

Activated, GTP-bound Ras interacts with a number of effector proteins, as illustrated. The classical Ras-Raf-MEK-ERK pathway results in activation of a number of transcription factors, such as ELK1, RSK and Jun, involved in transcription and cell-cycle progression. PI 3-kinase links Ras directly with cell survival signalling via PKB and inhibition of the pro-apoptotic factor BAD, but also with cytoskeletal signalling events via Rac. Activation of the RAL-GEF proteins such as RAL-GDS controls Ral activity and transcription factors like Forkhead. Phospholipase Cε activation catalyses PI 4,5-bisphophate into diacylglycerol and inositol triphosphate, activating PKC and mobilising intracellular calcium.

Key: PLCε, phospholipase Cε; $p70^{S6K}$, p70 ribosomal S6 kinase; PDK1, phosphatidylinositol trisphosphate-dependent kinase 1; PLA_2, phospholipase A_2; PLD; phospholipase D; RSK, p90 ribosomal S6 kinase, GSK3, glycogen synthase kinase 3; TCF, ternary complex factor; ELK-1, ETS(E26 transformation specific)-like gene 1;PKC, protein kinase C; PKB, protein kinase B.

PI 3-kinase

Through its p85α regulatory subunit, PI 3-kinase has been shown to associate with activated FAK and to be activated by this association (Chen *et al.*, 1996). PI 3-kinase also interacts reciprocally with Ras, as described above (Rodriguez-Viciana *et al.*, 1994). PI 3-kinase activity appears to be essential for actin-cytoskeletal rearrangements and cell survival in Ras-induced transformation (Kauffmann-Zeh *et al.*, 1997;Rodriguez-Viciana *et al.*, 1997;Matsuguchi and Kraft, 1998). These effects are mediated by the phosphorylated lipid products of PI 3-kinase, which can regulate many protein targets. Of these, protein kinase B (PKB, also known as AKT) phosphorylates glycogen synthase kinase 3 (Cross *et al.*, 1995) and indirectly activates p70 S6 kinase ($p70^{S6K}$) (Burgering and Coffer, 1995); it also protects against apoptosis in a number of cell systems including protecting against anoikis in epithelial cells (Khwaja *et al.*, 1997). This appears to be through regulation of the apoptosis machinery at multiple points, with inhibition of pro-apoptotic factors BAD, caspase-9 and NF-κB (Datta *et al.*, 1999). Given this protective role in cell survival it is unsurprising to find that PKB is constitutively activated in a number of human tumours, including lung cancers, both *in vivo* (Bellacosa *et al.*, 1995) and *in vitro* (Moore *et al.*, 1998).

Rho pathways

Rho has been implicated in downstream signalling for both cell cycle regulation and FA assembly. Treatment of Swiss 3T3 cells with lysophosphatidic acid induces Rho-mediated FA assembly in an ECM-dependent manner; in this setting it was concluded

that Rho was necessary for FA formation and subsequent integrin signalling (Hotchin and Hall, 1995). In the same cell type, Rho activity is also required for integrin-mediated activation of ERK (Renshaw *et al.*, 1996). In suspended cells, activation of endogenous Rho can also lead to ERK activation, partially mimicking the effects of adhesion (Renshaw *et al.*, 1996), which is intriguing since Rho is the target for nucleotide exchange of the oncogenes *dbl* and *lbc* (Toksoz and Williams, 1994). It has been demonstrated that *dbl* and *lbc* can induce anchorage-independent but serum-dependent growth in fibroblasts (Schwartz *et al.*, 1996), which may therefore be a result of constitutive Rho-mediated signalling. The downstream effectors of the Rho pathway have not been confirmed, but are likely to include a series of Rho-activated serine/threonine kinases (Ishizaki *et al.*, 1996).

Integrin-linked kinase

Integrin-linked kinase (ILK) was identified by a yeast two-hybrid screen as a protein able to interact with the cytoplasmic domain not only of β1 integrin (Hannigan *et al.*, 1996), but can also β2 and β3 subunits. ILK appears to have an important role in anchoring actin filaments of the cytoskeleton to cell matrix contact sites, which may modulate the physical strength of the connection to the ECM as well as cell signalling at these sites (Wu and Dedhar, 2001). Overexpression of ILK in epithelial cells results in activation of PKB and glycogen synthase kinase 3 (Persad *et al.*, 2001a;Persad *et al.*, 2001b), with resultant activation of the transcription factor AP-1, thus providing a direct link influencing gene expression (Dedhar, 2000). One of the consequences of

constitutive activation, or overexpression, of ILK in mammalian cells is suppression of anoikis, through suppression of the apoptosis protease caspase-3 (Persad *et al.*, 2000).

1.14 Integrins and disease

The importance of integrins to the organism as a whole is emphasised by the effects of complete loss of some integrin subunits, as seen in knockout studies. For example, β1 knockout mice, with the loss of 10 heterodimers, die *in utero* (Fassler and Meyer, 1995) and knockouts of β1-associated α subunits show similar embryonic lethality, with the exception of α1. In addition, a number of rare diseases have now been pinpointed to specific aberrations in integrin function.

Glanzmann's thrombasthenia is an autosomal recessive disorder characterized by excessive gum bleeding or menorrhagia. The fault lies in the platelet integrin αIIbβ3, with loss of functional integrin at the platelet surface and consequent loss of platelet aggregation (Hogg and Bates, 2000). Mutations are recognised in either subunit (Newman *et al.*, 1991). Conversely, understanding of integrin-mediated platelet aggregation has led to the development of specific αIIbβ3 inhibitors (GpIIb-IIIa inhibitors), which are used clinically to induce a Glanzmann's thrombasthenia-like state and have been shown to reduce the risk of clot formation in acute coronary syndromes and following coronary artery stenting.

As has been noted above, leucocyte adhesion deficiency-1 is a disease characterized by recurrent soft tissue infections. The molecular basis for this is a loss of β2 integrin expression, with loss of an array of β2 heterodimers, including LFA-1 and Mac-1. Without these key cell-ECM protein links, neutrophils are unable to extravasate and migrate to sites of injury (Anderson and Springer, 1987).

Given their role in cellular migration and adhesion, it is unsurprising that integrins are important in the function of the cells of the immune system. This cellular recruitment may be beneficial, such as neutrophils responding to infection; or it may prove harmful, for example, where eosinophils are recruited to an allergenic stimulus, in a process mediated through α4β1 and ICAM-1 on bronchial epithelium and β2 integrins on the eosinophils (Hillis and MacLeod, 1996). Integrins are now also implicated in atherosclerosis, since the development of atherosclerotic plaques, with a local influx of T lymphocytes and monocytes, is a β2-dependent process (Mine *et al.*, 2002).

1.14.1 Integrins and neoplasia

Just as they are key regulators of cellular adhesion and migration, and of cellular proliferation and survival, so the corollary is that integrin dysfunction has been implicated in neoplasia, where cells escape the normal bounds that control these functions. Two cardinal features of neoplasia are the ability to proliferate free of normal mitogenic stimuli, witnessed *in vitro* as serum-independent growth, and the ability to escape anoikis, anchorage-independent growth. The development of anchorage

independence has been associated with integrin dysregulation in many tumours (Varner and Cheresh, 1996). In mammary tumours, higher grade tumours exhibit increasingly aberrant integrin expression in both *in vitro* models and *in vivo* (Koukoulis *et al.*, 1991;Howlett *et al.*, 1995). Such effects are cell- and integrin-specific: *in vitro* work shows that blocking β1 integrins can cause reversion to a non-transformed phenotype in a model of breast cancer development, whilst blocking anti-β4 or anti-α6 antibodies promote neoplasia (Weaver *et al.*, 1997). In CHO cells overexpression of α5β1 inhibited tumour growth (Giancotti and Ruoslahti, 1990) and increased αv expression or β3 expression has been associated with the development of malignant melanoma, both *in vivo* and *in vitro* (Albelda *et al.*, 1990;Mitjans *et al.*, 1995). It is unclear exactly how these changes in integrin profile result in the development of neoplasia and invasive growth, but it is likely to be a many-facetted problem. Signalling proteins known to be regulated in part through integrins, in particular PI 3-kinase, have been shown to be constitutively elevated in tumour cells, including small cell lung cancer (Moore *et al.*, 1998) – and would be expected to promote cellular proliferation. In addition, there is some suggestion that tumour invasiveness is enhanced by matrix metalloproteinases and that integrins can induce expression of these proteins (Seftor *et al.*, 1992;Varner and Cheresh, 1996). More recent work in colon cancer cell lines has shown that higher cell density induces αvβ6 expression in a PKC-dependent manner, which in turn induces gelatinase B secretion and degredation of the local extracellular matrix (Niu *et al.*, 2001); in this model, therefore, cancer cell growth is driven in part by the induced expression of αvβ6. This integrin has been implicated in tumour invasion in head and

neck squamous cell carcinomas as well, by upregulating matrix metalloproteinases-9 and -2 (Thomas *et al.*, 2001).

1.15 Aims of this thesis

Cancer cells therefore show evidence of abnormal integrin regulation, with profound effects on cellular behaviour. Constitutive activation of integrin-mediated pathways is likely to be fundamental in the acquisition of serum-independent and anchorage-independent growth, the hallmarks of neoplasia. Furthermore, integrin-mediated adhesion is important in the development of chemoresistance in cancer cell lines (Sethi *et al.*, 1999b), probably mediated through the PI 3-kinase pathway (Brognard *et al.*, 2001;Schmidt *et al.*, 2002), and in cellular migration and the development of metastasis. These two behavioural features of lung cancer – the development of chemoresistance and of metastasis – are most directly responsible for its high mortality.

The primary aim of this thesis, therefore, was to determine how integrins were regulated, in order to try and modulate these two fundamental processes. It has been shown that CD98 stimulates adhesion in SCLC and that small GTP-binding proteins of the Ras family regulate integrin function and therefore I addressed the following questions:

1. How does CD98 regulate integrin function and what are the functional consequences?

2. In what way is galectin-3, a natural ligand of CD98, related to lung cancer phenotype?
3. How does R-Ras mediate reversal of integrin suppression?
4. Can a genetic screen be used to identify novel proteins involved in integrin regulation?

Chapter 2

Materials and Methods

2.1 Materials

The following reagents were purchased from Life Technologies (Paisley, UK): Dulbecco's Modified Eagles Medium (DMEM); Hanks' buffered salt solution (HBSS); penicillin; streptomycin; dialysed foetal bovine serum (FBS); BCA protein reagent; L-glutamine; 30% albumin (w/v); non-essential amino acids; 30%(w/v) acrylamide/bis solution; LipofectAMINE^{TM} Plus reagent. The following were purchased from Calbiochem-Novabiochem Corporation (Nottingham, UK): wortmannin, LY294002. The following were purchased from Amersham Life Science Ltd. (Cheshire, UK): Hybond C nitrocellulose membrane, enhanced chemiluminescence (ECL) reagent, [γ^{32}P]ATP. Unless otherwise stated, all chemicals were purchased from Sigma Chemical Company (Dorset, UK).

Primary antibodies

PAC1 antibody (IgG_{2a}) was a generous gift from Dr. S. Shattil (Scripps Research Institute, La Jolla, USA). Anti-galectin-3 antibody (B2C10) was a generous gift from Dr. F.T. Liu (University of California, Sacramento, USA). TS2/16 (IgG_1) and 4F2 (IgG_{2a}) were cultured in-house (see Section 2.2).

The following primary antibodies were purchased as indicated:

Antibody	Target	Isotype	Source
SC-7095	CD98	Goat polyclonal	Santa Cruz Biotechnology
RDI CD98 CabG	CD98	Goat polyclonal	Research Diagnostics Inc.
C-14	ERK2	Rabbit polyclonal	Santa Cruz Biotechnology

Antibody	Target	Isotype	Source
PI 3-kinase p85, N-SH	p85 α subunit, PI 3-kinase	IgG_1	Upstate Biotechnology
4B4	β1 integrin	IgG_1	Coulter Immunology
18	β1 integrin	IgG_1	Transduction Laboratories
K20/K20-FITC	β1 integrin	IgG_{2a}	DAKO
CD71 (L01.1)	CD71	IgG_{2a}	Becton Dickinson
A-17	FAK	Rabbit polyclonal	Santa Cruz Biotechnology
P1B5	α3 integrin	IgG_1	Chemicon International, Inc.

Santa Cruz Biotechnology (Santa Cruz, California, USA); Upstate Biotechnology (Lake Placid, USA); Coulter Immunology (Hialeah, USA); Transduction Laboratories (Lexington, USA); Becton Dickinson (Erembodegem, Belgium); Chemicon International, Inc. (Temecula, California, USA).

Secondary antibodies

All anti-species specific horseradish peroxidase-conjugated secondary antibodies were purchased from DAKO (Bucks, UK). For immunofluorescence studies, all anti-species specific and anti-FITC specific ALEXA^{TM}-conjugated secondary fluorochromes were purchased from Molecular Probes (Leiden, The Netherlands).

DNA constructs were obtained as follows: Tac-α5 (permission from Dr. S.E. LaFlamme, Centre for Cell Biology and Cancer Research, NY, USA); pDCR-H-Ras G12V (permission from Dr. M.H. Wigler, Cold Spring Harbor Laboratory, NY, USA) and pSG5-R-Ras G38V (permission from Prof. J. Downward, ICRF, London, UK) were provided by Dr. M.H. Ginsberg (Scripps Research Institute, La Jolla, USA) and have been described previously (LaFlamme *et al.*, 1992;White *et al.*, 1995;Wennstrom and Downward, 1999;Oertli *et al.*, 2000).

The human promonocytic leukaemia cell line U937 H4 was obtained from the UK Human Genome Mapping Project Resource Centre (Cambridge, UK).

Tissue sections for immunohistochemical studies were obtained from archival samples from University of Edinburgh, Department of Pathology files.

2.2 Production of 4F2 monoclonal antibody

The hybridoma cell lines for the monoclonal antibodies 4F2 (C13) and TS2/16 (Hb-243) were purchased from the American Type Tissue Culture Collection and cultured in-house in DMEM containing 15%FBS, 50IU/ml penicillin, 50IU/ml streptomycin and 5μg/ml L-glutamine and OPI media supplement. Secreted antibody was purified using protein-A affinity chromatography (Amersham, Pharmacia, Uppsala, Sweden).

2.3 Cell culture

Chinese hamster ovary (CHO) cell lines

The $\alpha\beta$-py CHO cell line was a generous gift from Dr. Mark Ginsberg (Scripps Institute, La Jolla, California, USA). These cells stably express a chimaeric integrin comprising αIIbα6aβ3β1, of which the extracellular domain is represented by αIIbβ3 (Hughes *et al.*, 1997). Expression of the chimeric integrin is maintained through G418 selection and its expression is driven by the polyoma T antigen. The $\alpha\beta$-py cell line was cultured in DMEM, supplemented with 10%(v/v) FBS (heat inactivated at 57°C for 1 hour), 1%(v/v) non-essential amino acids, G418 antibiotic (400μg/ml) (Promega), 50IU/ml penicillin and 50IU/ml streptomycin. Cells were passaged on reaching confluence, every three to five days. Beyond a passage number of 12, fresh stocks were utilized.

Small cell lung cancer (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). These cell lines have been well characterized (Carney *et al.*, 1985a;Carney *et al.*, 1985b).

All SCLC cell lines were cultured in Rosewell Park Memorial Institute Medium 1640 (RPMI 1640) containing 25mM HEPES supplemented with 10% (v/v) heat-inactivated FBS, 50IU/ml penicillin and 50IU/ml streptomycin. Cells were grown in 25-50mls medium in $75cm^2$ flasks. Cells were passaged every four to five days and passage numbers of greater than forty were discarded. Prior to experimentation, cells were washed in HBSS and cultured in quiescent medium, comprising RPMI 1640 containing 25mM HEPES, 0.25% (w/v) BSA, 50IU/ml penicillin and 50IU/ml streptomycin.

Macrophages

Human peripheral leukocytes were isolated using discontinuous density centrifugation through Percoll gradients (Szefler *et al.*, 1987;Dransfield *et al.*, 1994). Whole blood was obtained by venepuncture using 19 gauge needles (Sarstedt UK), from healthy volunteers on no current drug therapies. Volunteers were screened to ensure that they did not have elevated eosinophil levels, by flow cytometry and light microscopic assessment of cytocentrifuge preparations stained with haematoxylin/eosin according to previous protocols (Ward *et al.*, 1999). Purity was assessed by flow cytometry using 20 microlitre samples of isolated cell suspensions, to assess the composition of each preparation with respect to individual leukocyte populations. Flow cytometric scatter profiles were used to gauge percentages of monocytes and lymphocytes, before proceeding to further monocyte purification. This was performed by using in vitro adhesion to tissue culture plastic. For adhesion-mediated purification, cells were allowed to adhere to tissue culture plastic (Falcon or BioWhittaker, UK), at a concentration of $4x10^6$ per ml for one hour in warm Iscove's medium without serum at 37°C/5%CO_2. Non-adherent cells were then removed by circumferential washing of cell culture wells

three times with cation replete PBS, using sterile plastic pastettes. Further cell culture occurred in Iscove's supplemented with 10% autologous serum.

2.4 Transformation of *E. coli*

TOP10/P3 competent stocks were purchased from Invitrogen (Groningen, The Netherlands). Frozen aliquots of cells were thawed on ice and 25mM β-mercaptoethanol (final concentration) was added for 10 minutes prior to addition of DNA. DNA (1-10ng) was gently mixed with the cells and allowed to rest for 30 minutes at 4°C. Cells were heat-shocked at 42°C in a thermomixer (Eppendorf, Cambridge, UK) for one minute and returned to 4°C for a further two minutes. Transformed cells were allowed to grow by addition of antibiotic-free SOC media for 60 minutes at 37°C (225rpm in a thermomixer), prior to spreading on appropriate antibiotic selection plates and incubating overnight at 37°C. Individual colonies were picked into LB broth containing appropriate antibiotic selection and grown overnight for DNA purification and restriction enzyme digest verification.

2.5 DNA purification

Small scale (1–10ml) DNA purifications were performed using Wizard SV Miniprep kits (Promega) according to the manufacturers' instructions. Diagnostic restriction enzyme digests were performed with appropriate restriction enzymes (Promega) and resolved on 1% agarose (Seakem, Rockland, Maine, USA) gels containing 0.3μg/ml ethidium bromide to enable UV visualization.

Large scale (100–500ml) DNA purifications were performed using the Qiagen Endotoxin Free Maxiprep kit (Qiagen, Crawley, UK) according to the manufacturers' instructions. For low copy plasmids, Qiagen Midiprep kits were used, with their low copy number plasmid protocol. Purified DNA was checked with diagnostic restriction

enzyme digests and quantified using a Pharmacia Biotech Ultrospec 2000UV spectrophotometer (Amersham Pharmacia Biotech, Amersham, UK)

2.6 Cellular transfection

Transient transfections of αβ-py and CHO-K1 cells were performed with LipofectAMINE™ Plus reagent as described by Chen *et al.* (Chen *et al.*, 1994). Cells were seeded at a density to achieve 50% confluency overnight in 100mm or 60mm dishes (Corning, High Wycombe, UK). Purified DNA was placed in 5ml BD Falcon tubes (Becton Dickinson, Oxford, UK) in a volume ranging from 1–40μl. The DNA was pre-complexed with 110μl of the Plus reagent mixture (comprising Plus reagent 0.25%(v/v) diluted in DMEM containing 1%(v/v) non-essential amino acids) and gently mixed. After a 15 minute incubation at room temperature, 110μl of LipofectAMINE™ mixture (comprising LipofectAMINE™ 0.25%(v/v) diluted in DMEM containing 1%(v/v) non-essential amino acids) was added and incubated for a further 15 minutes at room temperature. The mixture was then made up to a total volume of 4ml with pre-warmed DMEM containing 1%(v/v) non-essential amino acids and placed onto the washed cells. Cells were cultured for five hours at 37°C, before addition of complete media (4ml), containing 10% FCS. After 24 hours, media containing DNA was removed and replaced with fresh complete media. For experiments where protein kinase activities were to be assessed, the transfection media was replaced with quiescent media. Cells were assessed 48 hours after transfection, either for lysis and SDS-PAGE analysis, or for integrin affinity determination.

2.7 Cell lysis

Cells were washed with ice cold PBS and lysed in buffer containing 50mM HEPES pH 7.4, 0.3M NaCl, 1.5mM $MgCL_2$, 1.2mM EDTA, 0.5% Triton X-100, 20mM β-glycerophosphate, 100mM sodium fluoride, 10mM sodium pyrophoshphate, 1mM

sodium vanadate, 0.5mM dithiothreitol. One Complete™ protease inhibitor tablet (Boehringer Mannheim, Lewes, UK) was added per 50ml of lysis buffer. Lysates were clarified by centrifugation at 13,000rpm for 10 minutes at 4°C and analysed for protein concentration. Samples were heated in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at 99°C for 10 minutes (See 2.9 for buffer composition).

2.8 Assay of protein concentration

Protein concentrations of lysates were quantified using a BCA protein assay (Pierce, IL, USA). Samples were diluted 1:5 or 1:10 in dH_2O and 10μl incubated with 200μl of test solution for 30 minutes at 37°C in 96-well plates. Plates were read using an automated plate reader (MRX microplate reader, Dynatech, Chantilly, USA). Samples were assayed in triplicate and graded against a standard curve (0.1–0.5mg/ml) using bovine serum albumin.

2.9 SDS Page and Western blotting

Samples were resolved on SDS polyacrylamide gels using a vertical electrophoresis tank Biorad Mini Protean II system (Biorad, Hemel Hempstead, UK). Samples were electrophoresed at 80–150volts using electrophoresis buffer for 1–2 hours, adjacent to pre-stained molecular weight markers (Life Technologies, Paisley, Glasgow). For optimal protein separation, 8% gels were used for 60–120kDa proteins, 10% gels for 40–70kDa and 12% gels for 10–40kDa proteins.

Proteins were transferred onto Hybond C nitrocellulose membranes in a methanol-based transfer buffer at 100volts for 60–90 minutes, in a Mini Protean II blotting tank (Biorad, UK). Protein loading was confirmed by staining with 1% Ponceau S for 5 minutes to visualize protein bands. Non-specific binding sites were blocked by incubation with

PBS-Tween 20 containing 5% non-fat dried milk powder for 1 hour at room temperature. Membranes were probed with appropriate antibodies diluted in PBS-Tween 20 containing 5% non-fat dried milk powder overnight at 4°C. They were then washed vigorously in PBS-Tween 20 for 1 hour, before exposure to appropriate species-specific horseradish peroxidase-conjugated (HRP) secondary antibodies for 1 hour, diluted in PBS-Tween 20 containing 5% non-fat dried milk powder. Membranes were then washed further in PBS-Tween 20 and immunoreactive bands identified using enchanced chemiluminescence (ECL) according to the manufacturer's instructions. For assessment of phosphorylated proteins, blocking and subsequent incubation steps were carried out using 3% albumin in PBS.

Buffers

1 x SDS-PAGE sample buffer

50mM Tris-HCl, 10%(v/v) glycerol, 2%(v/v) SDS, 0.1%(v/v) bromophenol blue, 10%(v/v) β-mercaptoethanol, pH 6.8.

SDS polyacrylamide gel

Separating Gel: 0.375M Tris base (pH 8.8), 0.1%(v/v) SDS, 8–12%(v/v) acrylamide, 0.1%(v/v) ammonium persulphate, 0.02%(v/v) TEMED.

Stacking Gel: 0.13M Tris base (pH 6.8), 0.15%(v/v) SDS, 4.6%(v/v) acrylamide, 0.11%(v/v) ammonium persulphate, 0.02%(v/v) TEMED.

Gel electrophoresis buffer

50mM Tris base, 250mM glycine, 0.1%(v/v) SDS.

Transfer buffer

210mM glycine, 24.7mM Tris base, 20%(v/v) methanol.

PBS-Tween 20 Wash buffer

PBS containing 0.2%(v/v) Tween 20

2.10 Flow cytometric assessment of antigen expression

Aliquots of $5x10^5$ cells were washed in PBS and resuspended in 50–100μl of PBS, containing either primary antibody or an isotype control antibody. Primary antibody incubation was for 30 minutes at room temperature, all subsequent steps being carried out at 4°C. After primary antibody incubation, cells were washed in PBS and then incubated with appropriate species-specific FITC- or PE-conjugated secondary antibody for a further 30 minutes in the dark. Samples were then washed again in ice-cold PBS and resuspended in 500μl PBS for analysis by flow cytometry on a FACS-Caliber (Becton, Dickinson, Erembodegem, Belgium). A minimum of 5000 cells were analysed per condition.

2.11 Integrin affinity determination by flow cytometry

The integrin affinity status of transfected $\alpha\beta$-py cells was assessed by three-colour flow cytometry, 48 hours after transfection, using the PAC1 antibody to label activated $\alpha_{IIb}\beta_3$. Cells were transfected as described in 2.6 with test 0–10μg DNA, as well as 2μg Tac-α_5 transfection reporter construct. Transfected cells were trypsinised and resuspended in a total volume of 50μl containing 0.4%(v/v) PAC1 ascites in HEPES/NaCl buffer (comprising 20mM HEPES, 140mM NaCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$ and 2mg/ml glucose) for 30 minutes. For each sample, internal controls were performed containg either 5mM EDTA (for maximal integrin suppression) or 100μM $MnCl_2$ (for maximal integrin activation). Cells were washed in cold PBS and all subsequent steps were carried out at 4°C. Samples were incubated in 50μl DMEM containing 4%(v/v) anti-mouse IgM-FITC (Biosource, Nivelles, Belgium) for 30 minutes in the dark. They were washed again and incubated finally in 50μl DMEM containing 2%(v/v) anti-Tac-R-

phycoerythrin (R-PE) (DAKO, Ely, UK). Cells were then washed and resuspended in cold PBS. Immediately prior to analysis on a FACS-Caliber flow cytometer, ToPro3 (Molecular Probes, Leiden, The Netherlands) was added to a final concentration of 1μM per sample, to permit live/dead cell by analysis of FL-4 fluorescence.

Data analysis

Live/dead cell analysis was performed using FL4 fluorescence to gate out dead (FL-4 positive) cells. For the live cell population, PAC1 binding was assessed in the most highly transfected cells. The integrin Activation Index (AI) was calculated as follows, as described by Hughes *et al.* (Hughes *et al.*, 1997):

Activation Index (%) $AI = (F_N - F_I) / (F_A - F_I) \times 100$

F_N: Geometric mean fluorescence intensity (MFI) of PAC1 binding to native integrin

F_I: MFI of PAC1 binding in the presence of 5mM EDTA, i.e. maximally suppressed

F_A: MFI of PAC1 binding in the presence of 100μM $MnCl_2$, i.e. maximally activated

2.12 Co-immunoprecipitation

Cells (30 x 10^6) were washed with Hanks' buffered salt solution, before being lysed using 20mM HEPES, 150mM NaCl, 2mM $MgCl_2$, 0.5mM $CaCl_2$, 1% 3-[(3-cholamido-propyl)dimethylammonio]propanesulfonate, and Complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). All steps were subsequently performed at 4°C. Lysates were clarified by centrifugation at 13,000rpm. To remove non-specific contaminants, lysates were then incubated with 30μl of albumin-agarose (Sigma-Aldrich) for 30 mins. After further centrifugation, the residual lysates were incubated overnight with 2μg of either 4F2, mouse anti-human CD71 as a negative control antibody, mouse anti-β1 integrin (K20), or a mouse IgG_1 negative control antibody (DAKO, Bucks, UK). Incubation with Protein G agarose (50μl) was used to precipitate

the antibodies over 1 hour. Agarose beads were then washed twice in 20mM HEPES, 150mM NaCl, 2mM $MgCl_2$ and 0.5mM $CaCl_2$ before being boiled with SDS-PAGE sample buffer. Samples were run on 10% Sepharose gel and transferred to nitrocellulose membrane (Amersham Biosciences UK, Bucks, UK). After blocking with 5% nonfat milk, Western blots were probed with antibody to β1 integrin (Transduction Laboratories, Lexington, KY) and a secondary anti-mouse horseradish peroxidase conjugate (DAKO, Bucks, UK). Visualization was by enhanced chemiluminescence.

2.13 PI 3-kinase activity assay

Cultures of SCLC cells were washed twice in HBSS, resuspended in quiescent medium and incubated at 37°C for 24 hours. The following day the cells were washed twice in HBSS at 37°C and disaggregated by gentle passage through a 21-gauge needle. They were then counted and aliquotted into 6-well plates (Costar, Cambridge, USA), using 10 x 10^6 cells per point. Cells equilibrated for 1 hour at 37°C before addition of agonist or antagonist. Following addition of the agent, plates were transferred to a water/ice bath (4°C), the cells were aspirated and centrifuged at 3000x*g* for 1 minute. The supernatant was aspirated and the cells lysed by addition of 250μl of ice-cold lysis buffer (50mM HEPES (pH 7.4), 15mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 10mM sodium pyrophosphate, 100mM sodium fluoride, 10%(v/v) glycerol, 1%(v/v) Triton X-100, 0.5mM dithiothreitol, 1mM sodium orthovanadate, 50μM 4-2-aminoethyl-benzenesulfonyl fluoride, 5μg/ml leupeptin, 20μg/ml aprotinin, and 10 μg/ml soybean trypsin inhibitor) to the cells and to any residual cells in the 6-well plate. After a minimum thirty minutes' lysis at 4°C, the plate and eppendorf lysates were amalgamated and then clarified by centrigugation at 13,000x*g* for 10 minutes. The protein concentration of each assay point lysate was then assessed and samples were equilibrated for protein, ~500μg per assay point.

Equilibrated samples were incubated with 1μg of anti-p85-SH3 PI 3-kinase antibody overnight at 4°C. The following day, 30μl of goat anti-mouse IgG agarose beads, washed in lysis buffer, were added for a further 2 hours. The beads were then pelleted and washed twice with lysis buffer, once with a high salt buffer containing 100mM Tris-HCl and 500mM LiCl (pH 7.6) and twice with a solution containing 200mM HEPES, 40mM $MgCl_2$ and 600mM NaCl (pH 7.4). PI 3-kinase assays were performed in a final volume of 200μl containing 50mM HEPES (pH 7.4), 10mM $MgCl_2$, 150mM NaCl, [γ^{32}P]ATP (5μCi, 5000Ci/mmol), 50μM ATP, and 0.5mg/ml sonicated phosphatidylinositol: phosphatidylserine (3:1, w/w). Reactions were carried out for 10 minutes at 37°C and terminated by adding 750μl of chloroform:methanol:0.1M HCl (40:80:1, v/v/v). Phase partition was carried out by adding 250μl chloroform and 250μl 0.1M HCl. After centrifugation, the lower phase was washed twice with synthetic upper phase (methanol:1M HCl:chloroform, 48:47:3, v/v/v), dried in a Speedivac and resupended in 30μl chloroform, prior to separation of the lipids by thin layer chromatography (TLC). 3'-phosphorylated lipids were identified by autoradiography using Kodak "X-OMAT" film. Radioactivity was quantified by liquid scintillation counting using "Flo-Scint IV" (Packard, Biosciences, Groningen, The Netherlands) and a Packard 1900 TR liquid scintillation analyzer.

2.14 Adhesion assay

96 well plates (Costar) were coated with 100μl of 10μg/ml laminin (Life Technologies) overnight at 4°C and blocked with 1mg/ml BSA (1 hour at 25°C). SCLC cells were washed twice in RPMI 1640 and quiesced overnight in quiescent media. 200μl of cells ($2x10^5$/ml) were added to the wells in the presence or absence of 4F2 (20μg/ml), function-blocking β1 antibody 4B4 (1:100) (Coulter Immunology), function-blocking α3 antibody P1B5 (1:100)(Chemicon International), or 2mM EDTA. After 1 hour of incubation at 37°C, non-adherent cells were removed by gentle washing with PBS and adherent cells were fixed with 100% methanol. For control wells, plates were

centrifuged for 2 minutes at 1000rpm and the supernatant was aspirated; all cells were then fixed with 100% methanol. Following fixation, cells were stained with 1% methylene blue. Plates were washed with distilled water, 100μl of 0.1M 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 triplicate. Adhesion was expressed as a percentage of maximum possible adhesion, represented by the control wells in which all cells had been centrifuged and fixed.

2.15 Immunohistochemistry

Tissue

Archival tissue samples of lung tumours were obtained from University of Edinburgh Department of Pathology files (1997-2001) and consisted of human lung biopsy samples taken by bronchoscopy, percutaneous biopsy or thoracic surgery. Control slides were of normal lung taken from non-involved lobes of lung cancer cases. Samples had been fixed in 4% paraformaldehyde and embedded in paraffin wax.

Immunohistochemistry

3–5μm sections were dried on to slides overnight at 37°C. For immunohistochemistry, sections underwent deparaffinization in xylene, followed by antigen unmasking in Vector Unmasking Solution (1:500) (Vector Laboratories, UK) for 3x5 minutes at 99°C. Immunohistochemistry was performed following the DAKO Techmate protocol (DAKO Ltd., UK), using the indirect streptavidin-biotin method. The primary antibodies used were goat polyclonal antibody to CD98 (SC-7095, Santa Cruz, California, USA) at 1:100 dilution and mouse monoclonal antibody to galectin-3 (gift, Dr. F.T. Liu) at 1:400 dilution. Negative control sections were incubated in carrier solution only. Positive control sections were taken from reactive lymph nodes. The immunostained sections were counterstained with haematoxylin.

Scoring

Sections were assessed together by standard light microscopy by two assessors (the author and a pathology registrar) and independently by a third, a consultant pathologist. Where differences were identified, the cases were settled by conference. Immunoreactivity for CD98 and galectin-3 was scored semi-quantitatively in areas of tumour: (a) for percentage of cells staining (absent = 0; < 20% = 1; 20-80% = 2; > 80% = 3); and (b) for strength of staining (absent = 0; weak = 1; moderate = 2; strong = 3). The cellular localization of CD98 and galectin-3, whether nuclear, cytoplasmic or membranous, was noted. The distribution of CD98 and galectin-3 in other cell types (interstitial and alveolar macrophages, lymphocytes, fibroblasts, bronchial epithelium, vascular endothelium and mesothelium) was also documented for all sections, where present.

Statistical analysis

Median scores for (a) percentage of cells staining and (b) strength of staining were compared between groups. Since the number of possible outcomes for each sample was limited (0 – 3), the distribution of data was assumed to be non-Gaussian, so that non-parametric analysis was required: the Kruskal-Wallis test looked for significant variation between group medians, with post hoc between-group comparison by Dunn's Multiple Comparison Test.

2.16 Immunofluorescence

The following primary antibodies were used: 4F2 conjugated with ALEXA^{TM}-Fluor 568 (Molecular Probes, Leiden, The Netherlands) (designated 4F2-AR), K20-FITC (DAKO, Glostrup, Denmark), 4B4 (Beckman Coulter, Fullerton, CA), TS2/16 conjugated with FITC (Sigma-Aldrich)(designated T2/16-FITC)) and CD71-FITC (BD Biosciences). The following secondary and tertiary antibodies were used: to amplify the FITC signal, ALEXA^{TM}-Fluor 488 rabbit anti-fluorescein and ALEXA^{TM}-Fluor 488 goat anti-rabbit

IgG (Molecular Probes); to localize 4B4, ALEXA^{TM}-Fluor 488 goat anti-mouse IgG (Molecular Probes). Mouse IgG_1 and IgG_{2a} antibodies directed against *Aspergillus niger* glucose oxidase (DAKO) were used as negative controls.

To assess the native state of CD98 and β1 integrin, SCLC cells were plated onto glass coverslips and fixed with 3% paraformaldehyde. Formaldehyde groups were quenched by immersing the coverslips in 50mM NH_4Cl for 10 minutes. Nonspecific binding sites were then blocked using 0.2% fish skin gelatin in PBS. Cells were then incubated sequentially with: (i) 4F2-AR and K20-FITC or IgG1 and IgG2a negative control antibodies; (ii) secondary ALEXA^{TM}-Fluor 488 rabbit anti-fluorescein; and (iii) tertiary ALEXA^{TM}-Fluor 488 goat anti-rabbit IgG. To assess the effect of cross-linking CD98 with mAb 4F2, primary antibody incubation with 4F2-AR and K20-FITC was carried out prior to fixation. Secondary and tertiary antibody labeling was carried out as above. To assess the effect of β1 integrin function-stimulating or function-blocking antibodies, cells were incubated with TS2/16-FITC or 4B4, respectively, before fixation and subsequent secondary and tertiary antibody labeling. In these latter experiments incubation with 4F2-AR was performed last of all, after secondary and tertiary labeling of the β1 integrin. As a further negative control, localization of 4F2-AR and β1 integrin was compared with that of the transferrin receptor (CD71), by using an isotype-matched (IgG2a) mouse anti-human CD71 antibody (CD71-FITC). In all experiments cells were gently washed twice with PBS between steps. Finally, cells were mounted from distilled water in Mowiol. Confocal microscopy was performed with a TCS NT confocal microscope system (Leica, Heidelberg, Germany) and image analysis was performed using TCS NT software (Leica).

2.17 Library amplification

The mammalian cDNA expression library H4 was obtained from the Human Genome Mapping Project (Cambridge, UK). This library was extracted from human

promonocytic leukaemia U937 cells, activated with phorbol 12-myristate 13-acetate. The library had been cloned into the pCDM8 vector (Invitrogen, Groningen, The Netherlands), which contains a CMV promoter and the polyoma origin of replication (that facilitates replication in αβ-py cells). Insert sizes were greater than 500bp and cloned non-directionally. Library cDNA was transformed into TOP10/P3 *E. Coli* strains as described and the transformed cells grown overnight at 37°C on 25x25 cm LB-Agar plates (Corning, High Wycombe, UK), containing ampicillin 50μg/ml and tetracycline 10μg/ml for selection. Transformations were repeated six times to obtain sufficient colonies to provide enough DNA to cover the quoted complexity of the library by a factor of 10. Colonies were scraped from the incubation plates into antibiotic-free LB broth and DNA was purified from the culture using the Qiagen Midiprep kit. Purified DNA was quantified by UV spectrophotometry and confirmed to contain vector and library fragments by Xho 1 restriction enzyme digestion. DNA from individual preparations was pooled for use in the genetic screen

2.18 Library screening and sorting

Transfection of αβ-py cells was performed in 100mm tissue culture dishes, according to the protocol described above. In total, 19 plates were transfected with DNA: pCDM8 control (4μg), H-Ras G12V (3μg), and 17 plates with H4 library DNA (4μg). All plates also received 2μg Tac-α_5 reporter construct.

After 48 hours integrin affinity was assessed by flow cytometry as described above, adjusted for volume. Sixteen library-transfected plates were trypsinised and resuspended in a total of 1.35ml HEPES/NaCl buffer. They were divided into 6 cell suspensions of cells (6x225μl) and were incubated with 25μl of PAC1 ascites fluid to give a final concentration of 0.4%(v/v). Cells were washed with cold PBS and incubated at 4°C with 250μl DMEM containing 4%(v/v) anti-mouse IgM FITC. After further washing, cells

were incubated with 250μl DMEM containing 2%(v/v) anti-Tac-PE. Cells were finally washed and resuspended in cold PBS at a concentration of $2x10^6$/ml.

Cell sorting was carried out on a FACSVantage cell sorter (Becton Dickinson) with a 100μm flow nozzle. Prior to cell sorting 7-AAD (Molecular Probes, Leiden, The Netherlands) was added to a final concentration of 20ng/ml as a live/dead marker. Gates were set up using 7-AAD-negative (live) and 7-AAD-positive (dead) populations; in addition, by using cells transfected with pCDM8 and Ras G12V, gates were set for transfected cells (Tac-positive) and cells with high integrin activation (PAC1-positive). Cells were sorted to collect those with high integrin activation status.

Following sorting, cells were subject to HIRT extraction of plasmid DNA (Hirt, 1967). They were transferred to 1.5ml eppendorfs, centrifuged at 1000rpm for 10 minutes and resuspended in 400μl of lysis solution (0.6% SDS(w/v) and 10mM EDTA). After a 20 minute incubation at room temperature, 100μl 5mM NaCl was added and the mixture incubated overnight at 4°C. Lysates were then clarified by centrifugation at 13,000rpm for 10 minutes, then subjected to two cycles of phenol/chloroform purification (lysate:phenol/chloroform, 1:1). DNA within the aqueous phase was subjected to ethanol precipitation with 20μg of glycogen (Boehringer Mannheim, Lewes, UK). DNA resuspended in 100μl dH_2O was precipitated with 3M sodium acetate 10%(v/v) and 300%(v/v) ethanol by centrifugation at 13,000rpm for 20 minutes. The DNA pellet was washed in 70% ethanol and resuspended in a final volume of 8μl dH_20.

DNA was digested overnight with 10 units Dpn1 restriction enzyme at 37°C and then used to transform TOP10/P3 cells as described. Individual colonies were assessed for the presence of the H4 library pCDM8 vector by PCR. Positive colonies were grown individually for transformation into $\alpha\beta$-py cells and re-screened in pools of ten for their effect on integrin affinity status.

2.19 Polymerase chain reaction (PCR)

To determine whether colonies of bacteria carried the pCDM8 vector, initially samples were cultured in 5mls of LB broth under ampicillin (50μg/ml) and tetracycline (10μg/ml) restriction. cDNA were then obtained by Miniprep, as described in Section 2.5, and subjected to PCR. In a 50μl aliquot, the following components were added:

Reagent	Final concentration	Volume for 50μl
Sterile deionised water	-	30
10x PCR buffer	1x	5
2mM dNTP mix	0.2mM of each	5
10mM Forward primer	0.2mM	1
10mM Reverse primer	0.2mM	1
***Taq* DNA polymerase**	1unit/50μl	1
25mM $MgCl_2$	2.5mM	5
Template DNA	Variable	2

Samples were kept on ice until the final addition of *Taq* DNA polymerase, then transferred to a thermomixer for incubation. All PCR regimes went through the same sequence of steps: initial denaturation, denaturation, primer annealing, extension and a final extending phase. Where large numbers of bacterial colonies needed to be screened, a "master mix" of reagents was prepared and aliquotted into a 96-well PCR plate, with a total volume of 25μl per sample. Samples were then taken directly from the bacterial colonies and added to individual wells prior to PCR.

2.20 Statistical analysis

Unless otherwise stated, data was assumed to follow a Gaussian distribution and statistical analysis was performed using one-way analysis of variance (ANOVA) and post hoc analysis by Newman-Keuls Multiple Comparison Test.

Chapter 3

Associations between CD98 and β1 Integrin

3.1 Introduction

CD98 is a disulphide-linked 125 kDa heterodimeric membrane glycoprotein composed of a glycosylated 80 kDa heavy chain and a non-glycosylated 48 kDa light chain. It is a ubiquitously expressed cell surface antigen that is highly conserved between species. Early studies of peripheral blood T and B-lymphocytes implicated CD98 in the regulation of cellular activation, but did not define a specific function for this antigen (Haynes *et al.*, 1981;Gerrard *et al.*, 1984). Expressed at low levels on the surface of quiescent cells, CD98 is quickly induced following cellular activation (Suomalainen, 1986). It is rapidly upregulated early in transition from G0 to G1 phase and remains at elevated levels until the cell cycle is complete. CD98 is strongly expressed on adherent human embryonic and newborn fibroblasts but expression gradually diminishes from 100% to 1% on fibroblasts from normal adults (Azzarone *et al.*, 1984;Azzarone *et al.*, 1986). CD98 expression is reconstituted to high levels on sarcomatous cell lines (Azzarone *et al.*, 1985); moreover, overexpression of CD98 in NIH3T3 cells results in transformation (Hara *et al.*, 1999). These expression patterns suggest that the function of CD98 is coupled to cellular activation, though a definitive role and mechanism of action have not been ascribed.

Six CD98 light chains have been identified, all of which are associated with L-type amino acid transport activity (Mannion *et al.*, 1998) (Kanai *et al.*, 1998) (Torrents *et al.*, 1998) (Estevez *et al.*, 1998) (Mastroberardino *et al.*, 1998) (Pfeiffer *et al.*, 1999) (Pfeiffer *et al.*, 1998;Tsurudome *et al.*, 1999). CD98 heavy chain has been shown to control delivery of the L-amino acid transporter (LAT-2) transporter protein to the membrane in a *Xenopus* oocyte model system (Pineda *et al.*, 1999;Rossier *et al.*, 1999). LAT-2 is predominantly expressed in renal proximal tubules. It has been proposed that the CD98-LAT-2 heterodimer is important in amino acid transport in kidney proximal tubules, whereas CD98-LAT-1 is involved with cellular amino acid uptake, reflected in the more widespread distribution of LAT-1.

There appears to be only a single heavy chain and recent evidence implicates this in an association with integrins. CD98 has been identified as a unique and highly specific regulator of integrin affinity, using a cloning strategy based upon changes in integrin affinity to identify potential modulators of integrin activation (Fenczik *et al.*, 1997a). It has been shown that β1 integrin-mediated adhesion of the SCLC cell line H345 to fibronectin and laminin can be markedly upregulated by cross-linking CD98. In addition, CD98 has been shown to stimulate adhesion of breast cancer cells to laminin via the integrin α3β1. Other studies also implicate CD98 in the regulation of integrin-mediated cell adhesion (Chandrasekaran *et al.*, 1999a). CD98 may also act together with β1 integrins during T-cell co-stimulation (Warren *et al.*, 2000).

Monocytes/macrophages are a key cell type in the control of inflammatory processes and CD98 has a critical role in the functional reprogramming of monocyte behaviour. Anti-CD98 monoclonal antibody promotes monocyte-monocyte interactions that ultimately lead to polykaryon (multinucleated giant cell) formation, a phenotype associated with chronic inflammatory conditions. Compelling evidence also exists for a connection between CD98 and virus-induced cell fusion. Antibodies to the heavy chain of CD98 promote cell fusion induced by Newcastle disease virus and by the gp160 envelope glycoprotein of human immunodeficiency virus (Okamoto *et al.*, 1997;Ohta *et al.*, 1994;Suga *et al.*, 1997). Thus, mounting evidence suggests that CD98 may be important in cancer, inflammation and viral disease through its effects on cellular activation and integrin-mediated adhesion.

Both intracellular signalling via CD98 and the cytoplasmic tail of CD98 appear to be crucial for its action (Tabata *et al.*, 1997). CD98 that lacks either a cytoplasmic domain or the unpaired cysteine in the extracellular domain disrupts virus-induced cell fusion and does not inhibit Tac-β1 suppression of integrin affinity (Fenczik *et al.*, 2001). Tyrosine kinase activation may be an early signal transduction pathway activated by CD98. Tyrosine kinase inhibitors such as genistein and herbimycin inhibit CD98 activity in haematopoietic cells and cell fusion mediated by HIV gp160 (Warren *et al.*, 1996;Tabata *et al.*, 1997). In this context, the phosphoinositide 3-kinase (PI 3-kinase) family of enzymes may also be influenced by CD98, acting as they do as a biochemical link between the phosphoinositide pathway and proteins with tyrosine kinase activity.

Evidence also suggests that CD98 is involved in the regulation of intracellular calcium concentration [Ca^{2+}] through the Na^{+}/Ca^{2+} exchanger: this has been assessed in skeletal muscle, myocytes, parathyroid cells and lymphocytes and its effect seems to be cell type specific (Michalak *et al.*, 1986;Posillico *et al.*, 1987;Freidman *et al.*, 1994).

The understanding of the mechanisms and biological relevance of CD98 function may have far-reaching practical applications. Blocking CD98 activity in tumour cells may be one route to preventing tumour progression and modulating cancer metastasis. Analysis of the mechanisms by which CD98 regulates virus-induced cell fusion may result in the development of novel anti-viral compounds. In addition, if inflammatory cells use CD98 to stimulate activation and adhesion at sites of tissue injury, then agents that inhibit CD98 activity may result in the development of novel anti-inflammatory compounds.

3.2 CD98 is highly expressed by SCLC

To confirm that experimental SCLC cell lines expressed CD98, these cell lines were analysed by flow cytometry and Western blot for CD98 protein expression. For flow cytometry, aliquots of 5 x 10^6 cells were first washed in PBS, then incubated with the monoclonal antibody 4F2, or an isotype-matched control, at a concentration of 20μg/ml, as described in "Materials and Methods". Secondary labelling was performed with a goat anti-mouse $F(ab)_2$-FITC, prior to flow cytometric analysis. The mean fluorescence

exhibited with 4F2 relative to that with isotype-matched control represented CD98 expression levels. The results, shown in **Figure 3.1 (*upper panel*)**, confirmed that the SCLC lines H69, H345 and H510 all expressed high levels of CD98 (mean fluorescence ratio relative to control ± SEM: 49.8 ±20.6; 41.2 ± 4.9; 44.0 ± 24.0 respectively), in contrast to low levels of binding seen with Swiss 3T3 cells (3.0 ± 2.0) or bovine tracheal smooth muscle cells (1.0 ± 0).

In separate experiments, CD98 expression by SCLC was confirmed in Western blot analysis of whole cell lysates. Control cell lines used were Cos-7 cells, which normally express CD98 at moderate levels; in addition, these were transfected with cDNA for human CD98. Lysates were equilibrated for protein and Western blots probed for the presence of CD98 with goat polyclonal antibody directed against CD98 (SC-7095). **Figure 3.1 (*lower panel*)** shows that in H69, H345 and H510 cells a protein band was observed at ~85 kDa, corresponding to the heavy chain of CD98 and also observed in the transfected Cos-7 cells; it was observed at lower levels in native Cos-7 cells. These data support the thesis that CD98 was highly expressed in SCLC experimental lines.

3.3 Co-immunoprecipitation of CD98 with β1 integrins

3.3.1 Initial experiments and modifications

Establishing satisfactory conditions for co-immunoprecipitation of β1 integrin and CD98 was extremely difficult. Initial pilot experiments with H69 and H345 SCLC cells

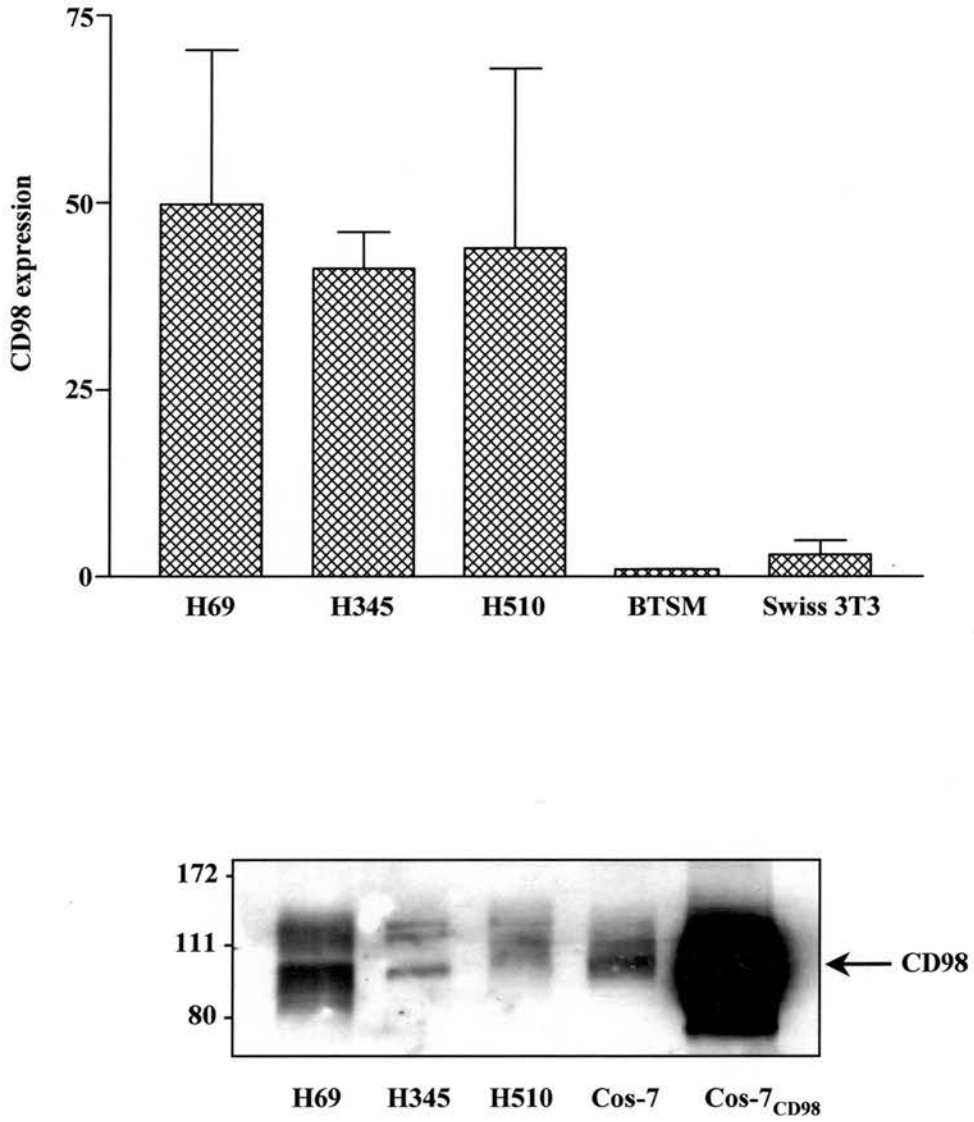


Figure 3.1: SCLC strongly express CD98.

Upper panel: Expression of CD98 in H69, H345 and H510 SCLC cell lines, assessed by flow cytometry. Cell lines were incubated with 4F2 (20μg/ml) or isotype-matched control, followed by secondary anti-mouse FITC. CD98 expression is shown as a ratio of fluorescence compared to controls, for each cell line. Comparisons were made with other cell lines, bovine tracheal smooth muscle (BTSM) and mouse Swiss 3T3.

Lower panel: Western blot of CD98 expression by H69, H345 and H510 SCLC lines. Lysates were equilibrated for protein and probed with goat anti-CD98 (SC-7095). Simian Cos-7 cells transfected with CD98 (Cos-7_{CD98}) were used as positive control and expressed moderate levels of CD98 in their native state.

suggested that although both cell lines expressed β1 integrin, as has previously been shown (Hirasawa *et al.*, 1994;Falcioni *et al.*, 1994), this was in relatively low quantities compared with cells with extensive cytoplasm and greater surface area. Human macrophages derived from donors expressed high amounts of both CD98 and β1 integrin, but even with these, inconsistent results were obtained. It was necessary to establish a control cell line into which cDNA for human CD98 could be transfected. From these cells, CD98 would then be immunoprecipitated and its association with β1 integrin assessed. Amongst easily transfectable cell lines, Cos-7 (simian) and A549 (human) were shown to express β1 integrin strongly, along with other proteins of potential interest, the p85 subunit of PI 3-kinase and FAK (**Figure 3.2, *upper panel***). By contrast, our other commonly used cell line, CHO-K1 cells, did not appear to express β1 integrin strongly (**Figure 3.2, *lower panel***); this may in part be a species problem with the antibody used for probing the Western blot. Cos-7 cells were therefore used for further studies.

Cos-7 cells were cultured in 60mm culture dishes and transfected at a concentration of approximately 5 x 10^5 cells/plate, using the standard LipofectAMINE technique as described in "Materials and Methods". Satisfactory transfection was demonstrated in two ways. Firstly, at harvest, cells were lysed in 100μl MAPK lysis buffer and, following clarification and protein assay, 30μg of cellular protein was run out on Western blot and probed for CD98 expression with the goat polyclonal antibody SC-7095. **Figure 3.3a** shows that untransfected Cos-7 cells express a small amount of

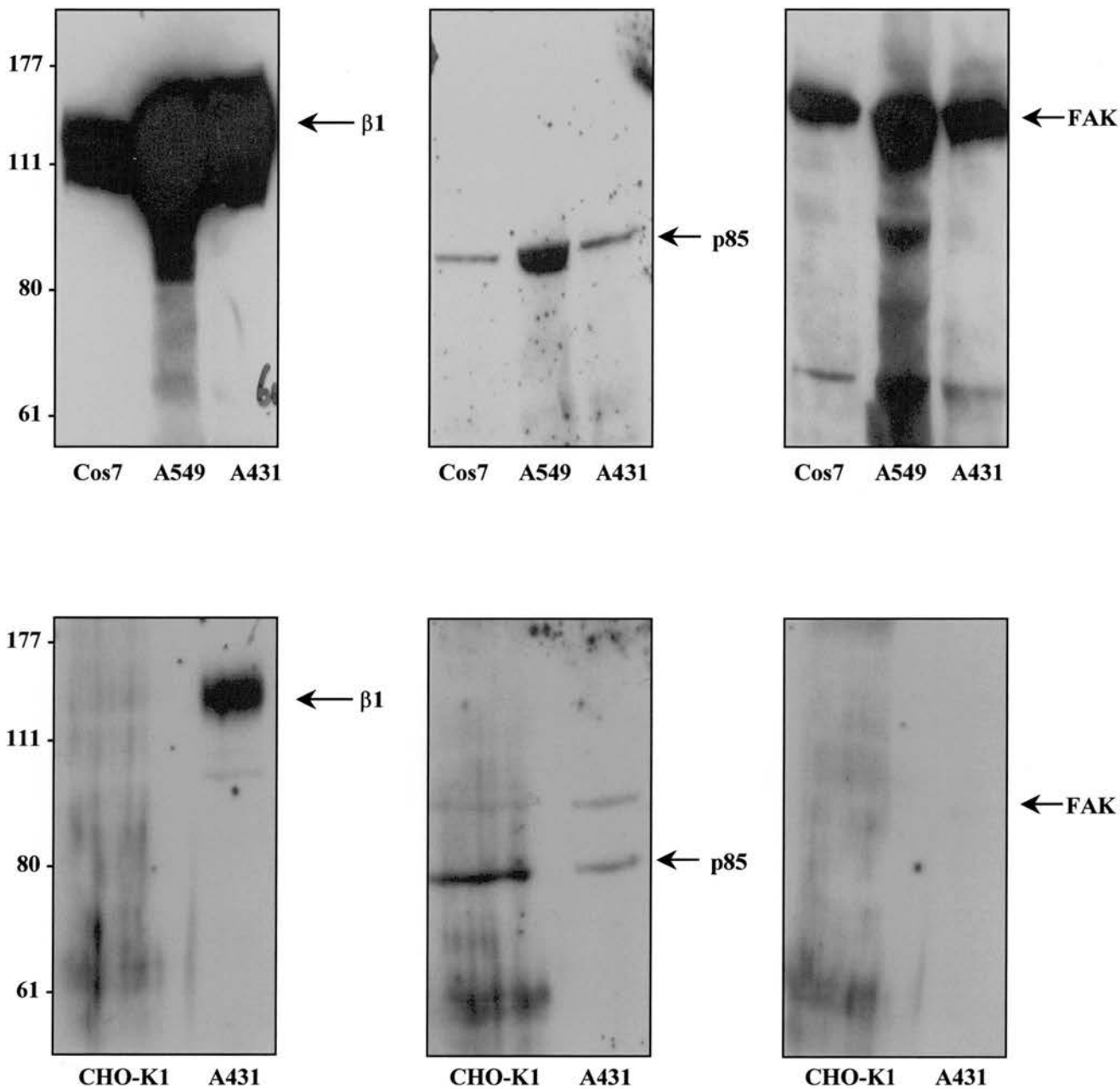


Figure 3.2: Cos-7 cells, but not CHO-K1 cells, strongly express β1 integrin, p85 and FAK. Western blots demonstrating expression of relevant target proteins of potential interest in Cos-7 cells (*upper panel*) and CHO-K1 cells (*lower panel*). Whole cell lysates, equilibrated for protein, were probed with antibodies directed against β1 integrin, p85 and focal adhesion kinase (FAK) and compared with human A549 cells and A431 cells (manufacturer's positive control). Cos-7 and CHO-K1 cells were of interest as easily transfectable cell lines, but CHO-K1 cells clearly did not express target proteins, particularly β1 integrin at high levels (detectable by this antibody).

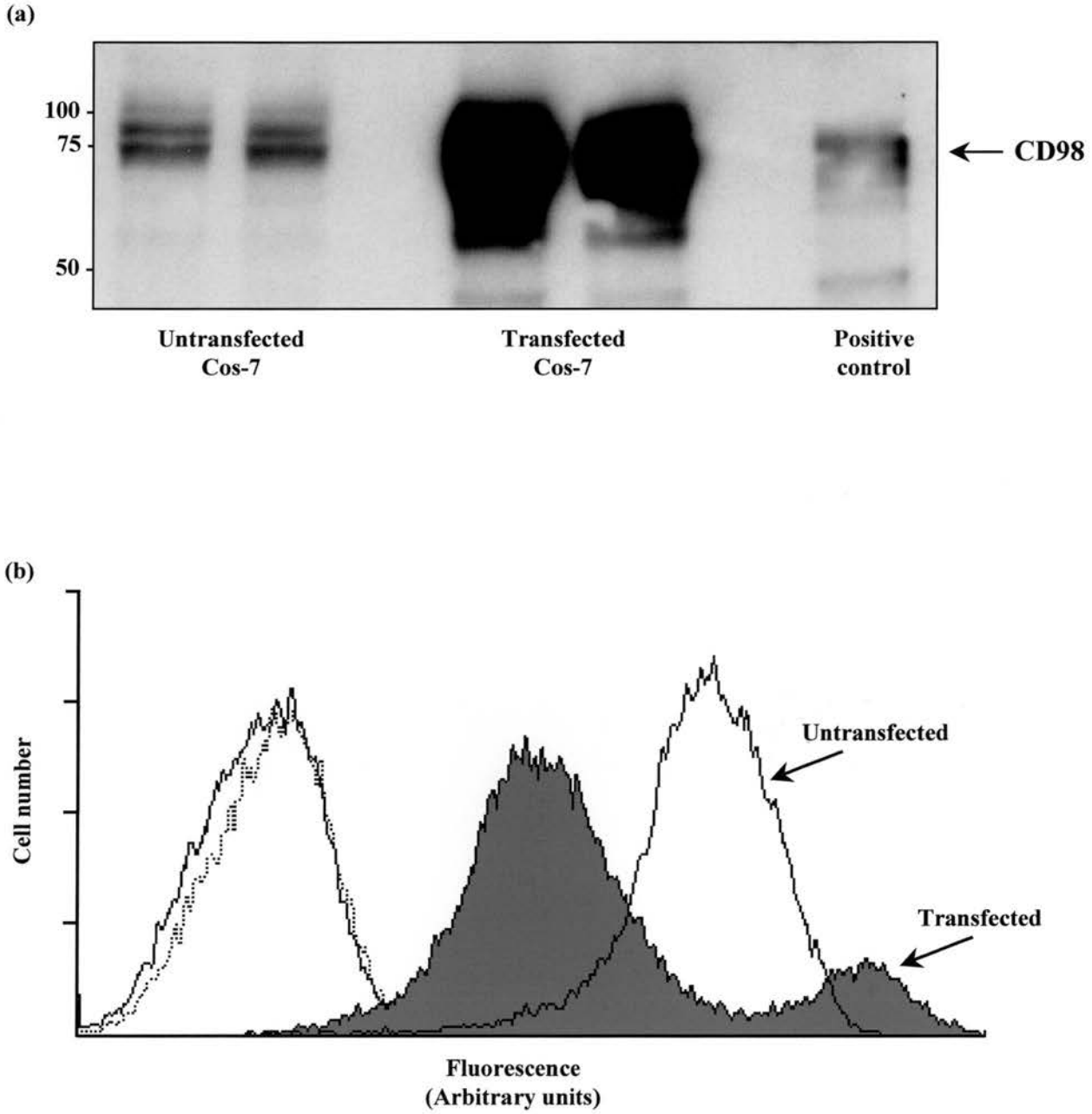


Figure 3.3: Successful transfection of Cos-7 cells with cDNA for CD98.

(a) Lysates from untransfected cells (*lanes 1 and 2*) or cells transfected with CD98 (*lanes 3 and 4*) (30μg protein per lane) were probed with SC-7095 goat anti-CD98 and secondary anti-goat biotin.

(b) Cos-7 cells transfected with CD98 were incubated with carrier (*dotted line; mean fluorescence 6.2 arbitrary units*), IgG_{2a} control antibody (20μg/ml) (*dashed line; 6.8 AU*) or 4F2 (20μg/ml) (*solid line, grey infill; less transfected 109.2 AU, highly transfected 2531.4 AU*), then secondary anti-mouse FITC. Untransfected cells also bound 4F2 (*solid line; 566 AU*).

endogenous CD98 (*lanes 1 and 2*), whilst transfected cells express very high levels (*lanes 3 and 4*). Secondly, cells were trypsinised at harvest and expression of CD98 assessed by flow cytometry using 4F2 monoclonal antibody (20μg/ml), **Figure 3.3b** demonstrates that, although untransfected Cos-7 cells express moderately high levels of CD98, following transfection there is a population of transfected cells that express CD98 to extremely high levels. Thus, by two different methods, and with two different CD98-specific antibodies, it was clearly demonstrated that Cos-7 cells were successfully transfected with, and expressed, CD98.

To assess whether CD98 and β1 integrin co-immunoprecipitated, transfected Cos-7 cells were incubated with 4F2 (20μg/ml) for 10 minutes at 37°C. Cells were washed twice in ice-cold HBSS and lysed with MAPK lysis buffer, as described in "Materials and Methods". Following clarification of the lysates, the 4F2 antibody was immunoprecipitated using Protein G agarose beads. These were washed gently in lysis buffer, before being boiled in reducing sample buffer. Samples were then subject to Western blotting and probed for the presence of β1 integrin, but this was not observed (**Figure 3.4**). This could have been for a number of reasons: (i) CD98 and β1 integrins did not co-immunoprecipitate; (ii) they did co-immunoprecipitate, but the β1 integrins were lost in the assay; (iii) or, the CD98 was never immunoprecipitated in the first place.

Co-immunoprecipitation experiments are highly sensitive to buffer conditions and it was considered probable that the lysis buffer used was too stringent, with the potential for

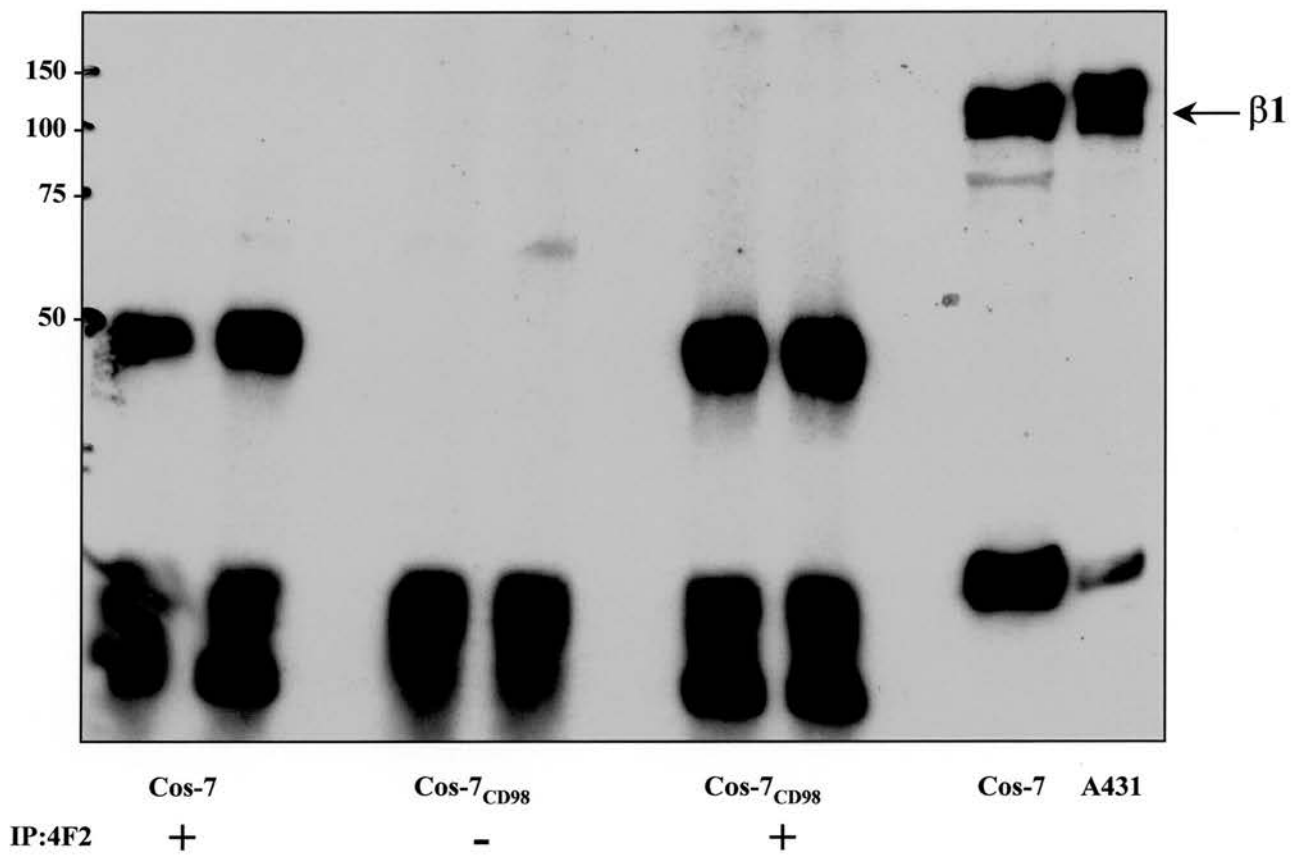


Figure 3.4: β1 integrin was not co-immunoprecipitated with CD98 in transfected Cos-7 cells.
Cos-7 cells transfected with CD98 ($Cos\text{-}7_{CD98}$), or controls (Cos-7) were incubated with 4F2 (20μg/ml), lysed and CD98 immunoprecipitated with protein G agarose beads. Beads were boiled in 1x sample buffer and probed for the presence of β1 integrin with mouse anti-β1 mAb and secondary goat anti-mouse biotin. Cos-7 and A431 whole cell lysates, which express β1 integrin strongly, were used as positive controls.

loss of co-localising proteins during the assay. Therefore, a CHAPS/deoxycholate-based, non-reducing lysis buffer was adopted (see "Materials and Methods"). With this adaptation to the protocol, it was first shown that CD98 was being successfully pulled out by 4F2 and Protein G agarose (**Figure 3.5a**); secondly, β1 integrin was co-immunoprecipitated with CD98, since it was isolated on Western blot in those samples treated with 4F2, but not in those treated with an IgG_{2a} negative control antibody (**Figure 3.5b**). This suggested that, in transfected Cos-7 cells at least, CD98 and β1 integrin co-immunoprecipitated.

3.3.2 CD98 and β1 integrin co-immunoprecipitate in human macrophages

In order to assess whether CD98 and β1 integrin co-immunoprecipitated in untransfected cell types, human macrophages were first studied, since they are known to express both proteins to high levels. Peripheral blood monocytes were kindly donated by colleagues, isolated by discontinuous density centrifugation through Percoll gradients as described in "Materials and Methods" (Szefler *et al.*, 1987;Dransfield *et al.*, 1994) and matured into macrophages by culture for 5-7 days. On day 5-7 of culture, macrophages were washed twice in HBSS, before lysis in CHAPS/deoxycholate buffer. Following protein assay and clarification, immunoprecipitation was performed by overnight incubation at 4°C with 2μg of either 4F2 or IgG_{2a} isotype control antibody, together with Protein G agarose. **Figure 3.6** shows that β1 integrin was co-immunoprecipitated by 4F2 (*lane 2*), but not by the IgG_{2a} isotype control (*lane 1*); in addition, the β1 integrin band was not due to the 4F2 antibody itself, since it was not seen when 4F2 alone was treated with

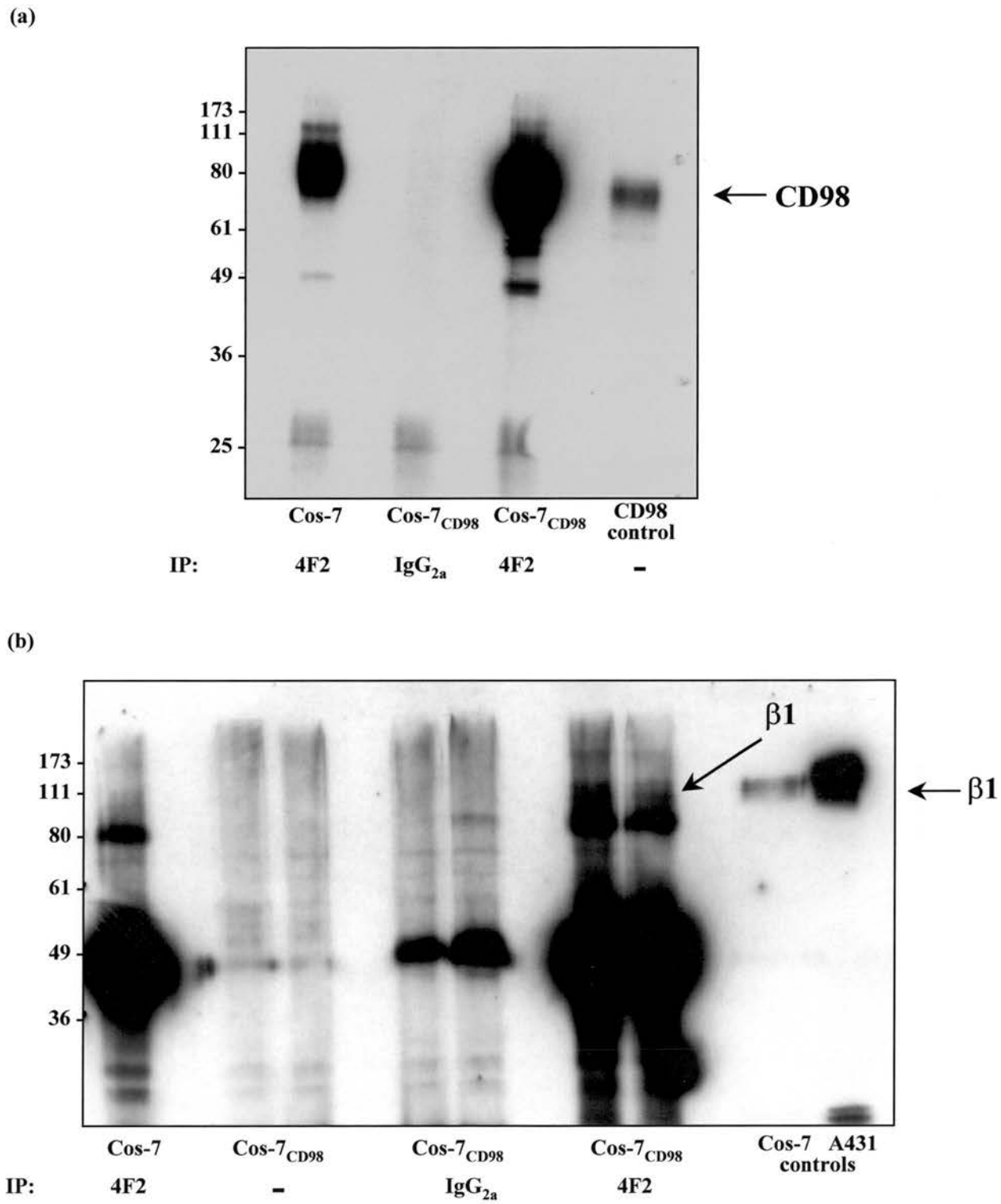


Figure 3.5: CD98 and β1 integrin co-immunoprecipitate in CD98-transfected Cos-7 cells.

(a) Probing with SC-7095 goat anti-CD98, CD98 is immunoprecipitated by 4F2 (20μg/ml) from untransfected cells (Cos-7) and cells transfected with CD98 ($Cos\text{-}7_{CD98}$)(*lanes 1 & 3*), but not from transfected cells with an isotype IgG_{2a} control antibody (*lane 2*).

(b) Probing with mouse anti-β1 mAb, β1 integrin is co-immunoprecipitated from transfected cells ($Cos\text{-}7_{CD98}$), when CD98 is immunoprecipitated by 4F2 (*lanes 6 & 7*), but not by carrier (*lanes 2 & 3*) or control IgG_{2a} control antibody (*lanes 4 & 5*). The co-immunoprecipitation of β1 integrin from untransfected cells (*lane 1*) is more difficult to ascertain. Results representative of three experiments.

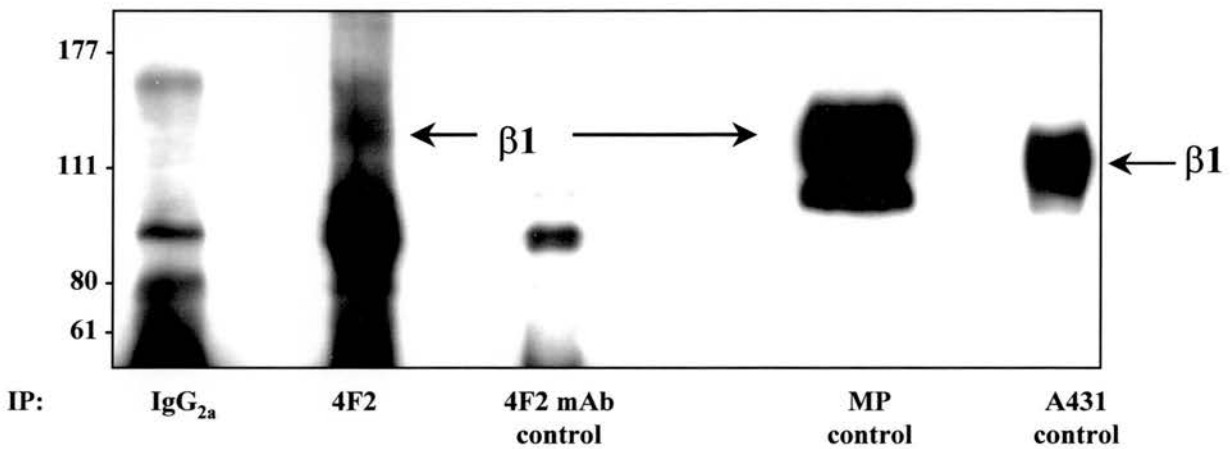


Figure 3.6: CD98 and β1 integrin co-immunoprecipitate in human macrophages.
Probing with mouse anti-β1 mAb, β1 integrin is identified in immunoprecipitates from human macrophages when immunoprecipitated with 4F2 (2μg) (*lane 2*), but not with an isotype IgG_{2a} control antibody (*lane 1*). Whole cell lysates demonstrate that β1 integrin expressed in human macrophages (*lane 4*) runs slightly heavier than in A431 cells (*lane 5*); no equivalent weight protein is identified when "pure" 4F2 mAb (2μg) is run out on the Western blot (*lane 3*). Results representative of three experiments.

sample buffer and run on the Western blot (*lane 3*). Thus, CD98 and β1 integrin were observed to co-immunoprecipitate in human macrophages.

3.3.3 CD98 and β1 integrin co-immunoprecipitate in H69 SCLC

In addition to human macrophages, SCLC cells were assessed for evidence of a physical association between CD98 and β1 integrin. Attempts to confirm this association using the conditions described above for macrophages were fruitless – no definite co-immunoprecipitation was observed. However, another group published evidence suggesting that β1 integrin and CD98 did co-imunoprecipitate in polarised cells (Merlin *et al.*, 2001), using a different CHAPS-based lysis buffer. In addition, because of the low cytoplasm/nucleus ratio of SCLC, large numbers of SCLC were used for these experiments.

Thus, using 30 x 10^6 cells per point, H69 SCLC were lysed with the new lysis buffer. Lysates were clarified and incubated overnight at 4°C with precipitating antibodies, which were then pulled down with Protein G agarose as before. Following boiling in reducing sample buffer, samples were run out on Western blot and probed for the presence of either CD98 or β1 integrin. **Figure 3.7a** shows clearly that CD98 was isolated by precipitation with 4F2 as expected (*lane 3*). **Figure 3.7b** shows that β1 integrin was isolated by both the β1-specific antibody K20 (*lane 3*) and by 4F2 (*lane 1*); however, it was not immunoprecipitated by an IgG_{2a} monoclonal antibody directed against CD71 (*lane 2*). CD71 is the cell surface transmembrane receptor for transferrin,

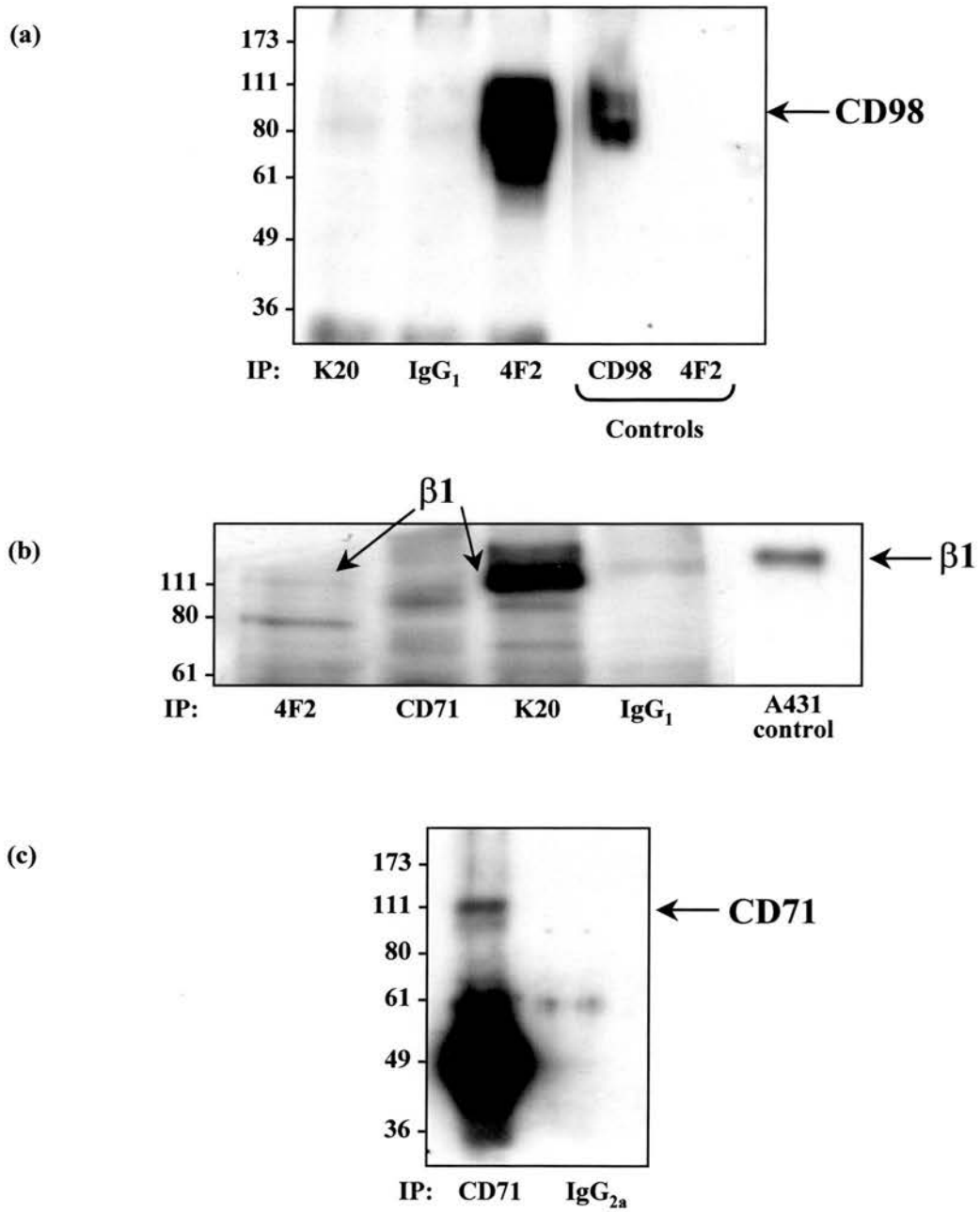


Figure 3.7: CD98 and β1 integrin co-immunoprecipitate in H69 SCLC cells.

(a) Probing with SC-7095 goat anti-CD98, CD98 is immunoprecipitated by 4F2 (2μg) from H69 SCLC, but not by mouse anti-β1 (K20, 2μg, *lane 1*) or an isotype IgG_1 control antibody (*lane 2*).

(b) Probing with mouse anti-β1 mAb, β1 integrin is immunoprecipitated from H69 SCLC cells by mouse anti-β1 (K20, 2μg, *lane 3*) and co-immunoprecipitated by 4F2 (2μg), but not by control IgG_{2a} antibody against CD71 (*lane 2*) or negative IgG_1 control antibody (*lane 4*).

(c) Probing with anti-CD71 antibody, a protein of approximately 110kDa is isolated by immunoprecipitation with anti-CD71 antibody (*lane 1*), but not by control IgG_{2a} antibody (*lane 2*).

Results representative of three experiments.

which is strongly expressed by SCLC; this antibody was thus an excellent isotype control to use for comparison with the IgG_{2a} monoclonal antibody 4F2 directed against CD98, also a transmembrane protein. **Figure 3.7c** shows that when probed for the presence of CD71, a protein band was isolated at approximately the correct molecular weight for CD71 in the lane with antibody against CD71 (*lane 1*), but not in the presence of an IgG_{2a} isotype control (*lane 2*). This suggested that the CD71 antibody was specific and was indeed successfully pulling out the transferrin receptor CD71.

Taken together these results suggested that β1 integrin and CD98 did indeed co-immunoprecipitate in SCLC H69 cells, as they had been shown to do in both human macrophages and transfected Cos-7 cells.

3.4 Confocal microscopy of CD98 and β1 integrin

Although the co-immunoprecipitation experiments above suggested that CD98 and β1 integrin are physically associated, these data are fraught with interpretational difficulties, due to the biochemical constraints that exist in such experiments. Confocal microscopy was therefore used as a complementary technique to validate or refute these results, i.e. whether or not CD98 and β1 integrins co-localise. The proteins of interest were labelled with protein-specific antibodies, which were then localised by the use of fluorophores, attached either to secondary antibodies or directly conjugated to the primary antibodies.

3.4.1 Early experiments

The objective of these studies was to assess CD98 and β1 integrin in intact, native cells, specifically SCLC; as strong expressers of both target proteins, human macrophages were also used. The establishment of reproducible experimental conditions to accurately assess antibody binding and thence co-localisation was fraught with technical and interpretational difficulty. An experienced observer was essential in interpretation and the expert technical assistance of Dr. Linda Sharp was greatly appreciated.

Experiments followed a broadly similar design, as described in "Materials and Methods". After washing in PBS, cells were fixed to a glass slide or coverslip, using 3% paraformaldehyde (PFA). Aldehyde groups were quenched with 50mM NH_4Cl and blocking of non-specific binding was performed using 10% sheep serum/0.2% fish skin gelatin in Ca^{2+}/Mg^{2+}-free PBS. Cells were permeabilised with 0.1% Triton in Ca^{2+}/Mg^{2+}-free PBS. Cells were then incubated with primary antibodies directed against CD98 and β1 integrin, followed by appropriate species-specific secondary antibodies. Because of the necessity to prevent cross-reactivity between the secondary fluorophores and primary antibodies, the primary antibodies needed to be raised in different species. The primary antibodies used initially were a mouse monoclonal directed against β1 integrin (8E3) and a goat polyclonal against CD98 (SC-7095). The secondary fluorophores were donkey anti-goat Alexa Fluor™ 568 (AlexaRed) and goat anti-mouse Alexa Fluor™ 488 (AlexaGreen). A number of problems were encountered.

(a) In the earliest experiments SCLC H69 cells, after washing in PBS, were deposited onto slides by cytospin at 300rpm, prior to fixation with 3% paraformaldehyde (PFA). Aldehyde groups were then quenched with 50mM NH_4Cl and blocking of non-specific binding, primary and secondary antibody incubations were performed as described in "Materials and Methods". However, it was observed that cells examined in this way were both somewhat flattened and accompanied by a large quantity of cellular debris and/or residual serum. This resulted in messy, non-specific staining which was felt to be unrepresentative of the situation in viable, intact cells (**Figure 3.8a**). Cytospin preparations were therefore abandoned.

(b) As an alternative method of adhering cells to slides in preparation for immunofluorescence, slides or coverslips were coated with laminin (10μg/ml) for two hours. Excess laminin was removed by gentle washing with PBS, before cells were plated out and allowed to adhere. Fixation, blocking and antibody incubations were performed as above. However, with both macrophages and SCLC cells, the presence of laminin gave rise to an unacceptably high level of non-specific antibody binding to the laminin (**Figure 3.8b**). Therefore the use of laminin in these experiments was also abandoned.

(c) It was clearly demonstrated that the use of 10% sheep serum as part of the blocking step resulted in increased non-specific binding of the secondary fluorophore donkey anti-goat AlexaRed. As illustrated in **Figure 3.8c**, cells blocked in 0.2% fish

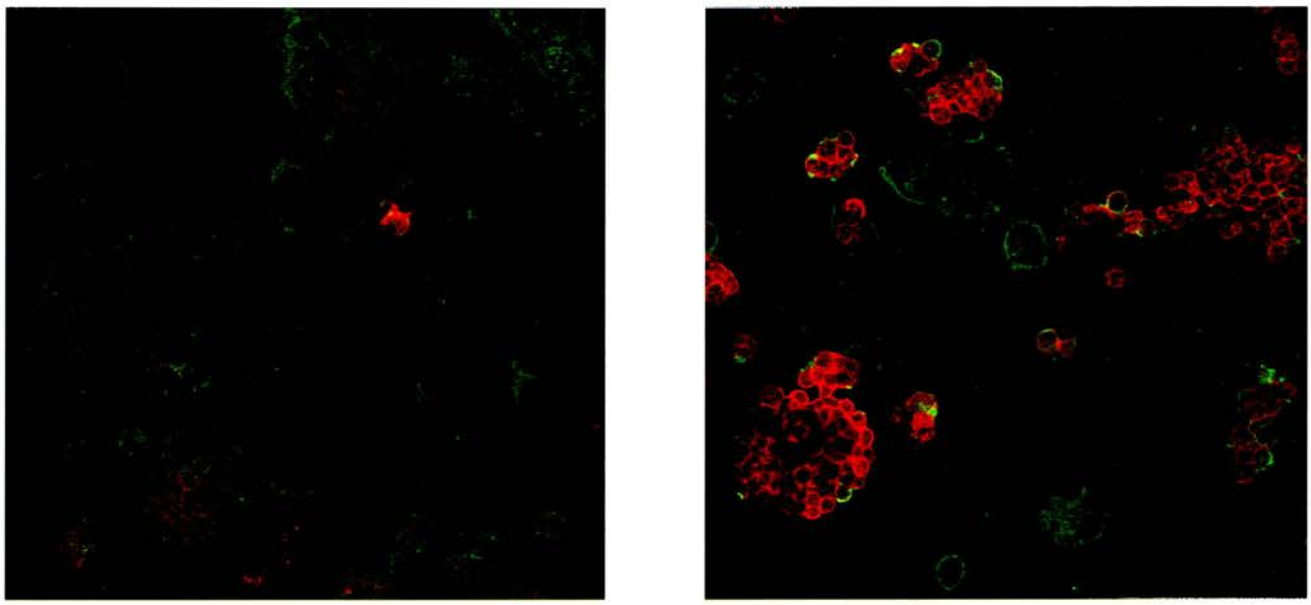
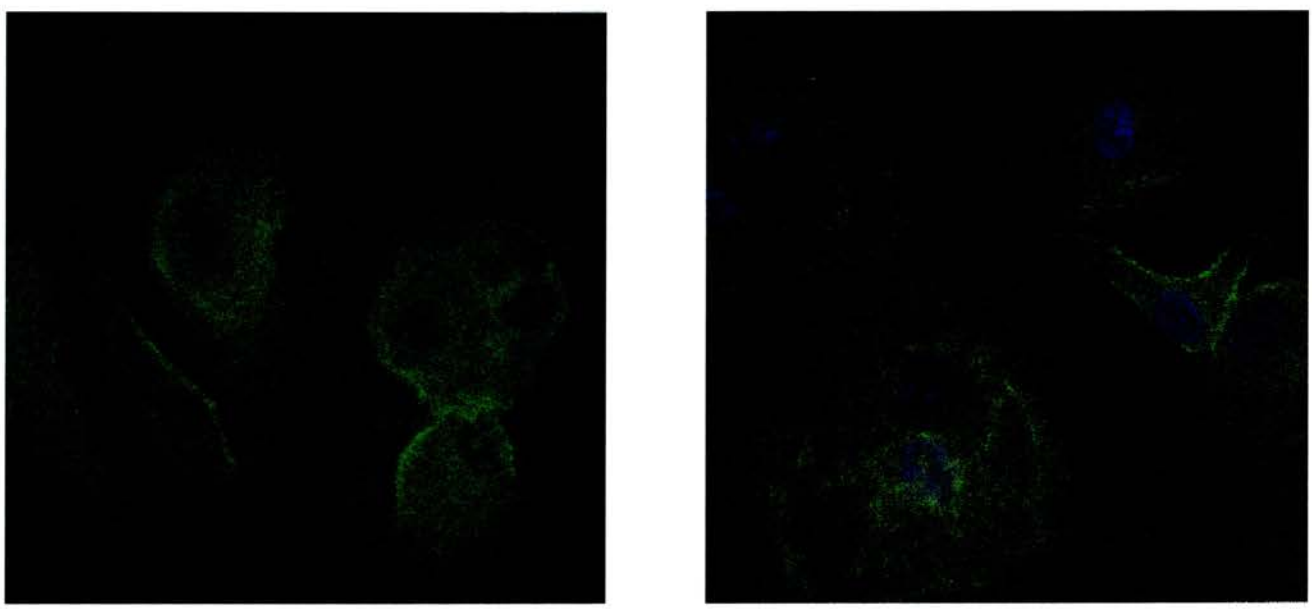
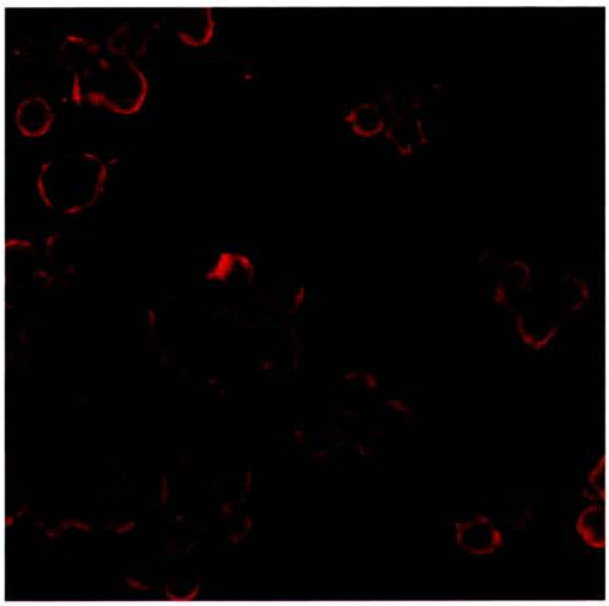


Figure 3.8: (a) Cytospin preparation produces excessive debris (both slides).
Typical appearances of cells, plus profuse cellular debris and serum proteins following cytospin preparation. (The cells were labelled with 4F2 (20μg/ml), followed by goat anti-mouse Alexa Fluor™ 568, then FITC-conjugated mouse anti-β1 antibodies.)

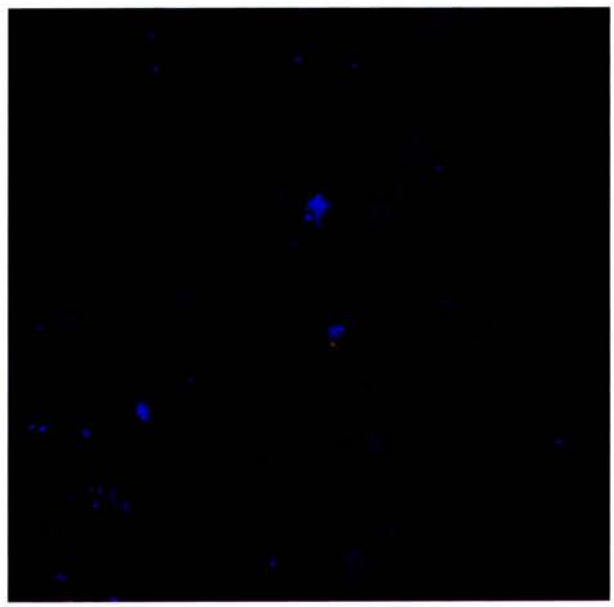


Laminin **Glass**

Figure 3.8: (b) Plating cells on laminin results in more background staining.
Macrophages plated onto laminin (10μg/ml) or straight onto glass were incubated with antibodies against β1 integrin, then labelled with secondary Alexa Fluor 488. Plating cells onto laminin resulted in higher background staining and less specific staining of cells.

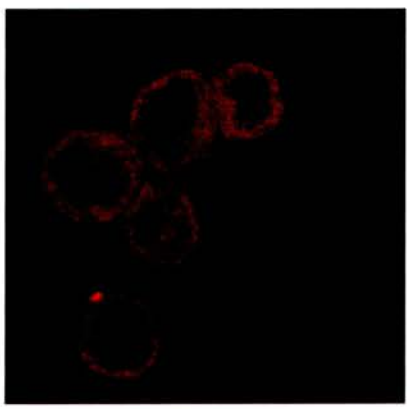


10% sheep serum

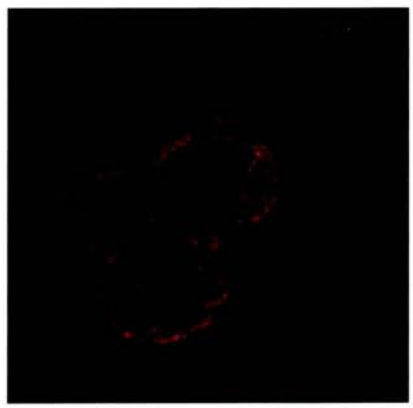


No sheep serum

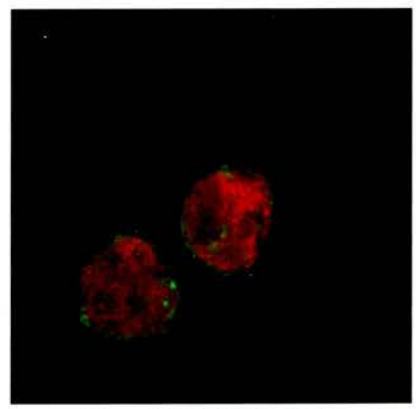
Figure 3.8: (c) Sheep serum block strongly binds secondary antibody fluorophore.
H69 SCLC were blocked in 0.2% fish skin gelatin in PBS, with or without 10% sheep serum. Incubation was with donkey anti-goat Alexa Fluor™ 568. To localise the cells, nuclei were labelled with To-Pro-3 (1μM) (filtered out in the left hand slide).



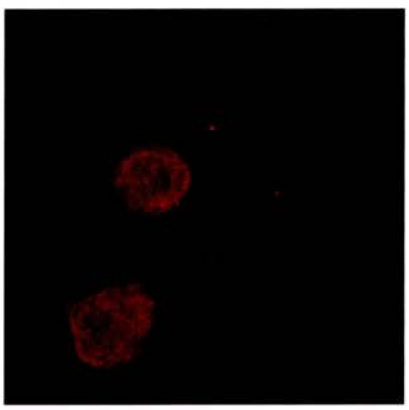
Goat CD98



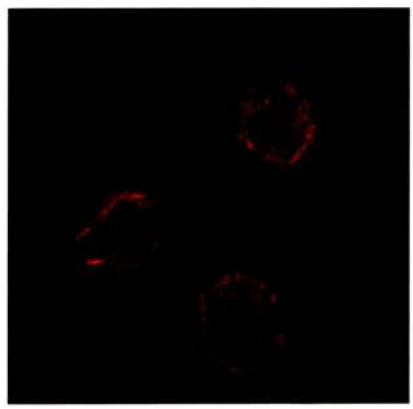
Goat CD98 + peptide block



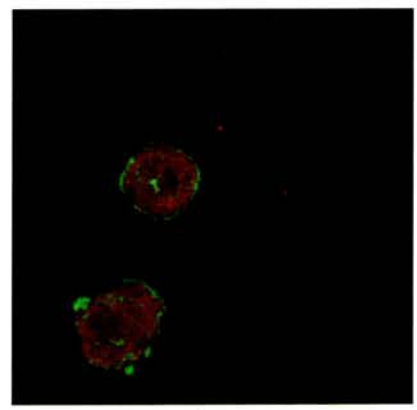
Goat CD98 + 4F2



Goat β1



Goat β1 + peptide block



Goat β1 + 4F2

Figure 3.8: (d) Unconjugated primary goat antibodies lead to poor specificity of staining.
H69 cells incubated with goat polyclonal antibody against CD98 exhibited poor specificity of staining, even if co-incubated with a peptide block (*upper panel, red*). By contrast, the monoclonal antibody 4F2, with secondary Alexa Fluor 488 labelling (*green*), exhibits highly specific staining of CD98. Similar results are demonstrated with goat primary antibody against β1 integrin (*bottom panel, red*).

skin gelatin alone exhibited negligible cellular or background fluorescence following incubation with donkey anti-goat AlexaRed alone, whereas cells blocked with 10% sheep serum/0.2% fish skin gelatin in Ca^{2+}/Mg^{2+}-free PBS exhibited high levels of diffuse fluorescence, rendering meaningful interpretation impossible. The use of sheep serum was, therefore, excluded, with no apparent increase in non-specific antibody binding.

(d) Finally, it was also concluded that indirect immunofluorescence, i.e. the use of unconjugated primary antibodies - by necessity of different species - with appropriate species-specific secondary fluorophores, led to unacceptable levels of cross-reactivity between the secondary fluorophores and primary antibodies (**Figure 3.8d**). It was therefore decided to abandon the use of these unconjugated primary antibodies as far as possible and to turn to pre-conjugated primary antibodies against CD98 and β1 integrin.

3.4.2 Conjugation of 4F2 with Alexa Fluor™ 568

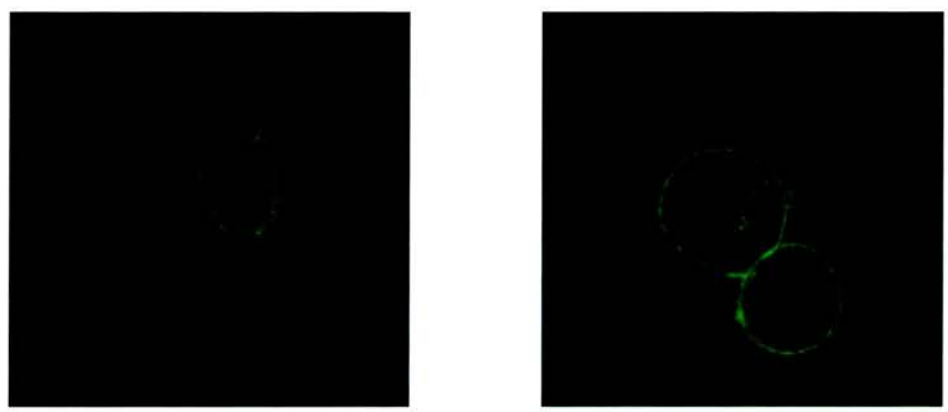
The two key mouse monoclonal antibodies used for further studies were 4F2 and K20. 4F2 was synthesised as described in "Materials and Methods". To conjugate this with the Alexa Fluor™ 568 fluorophore, the Alexa Fluor™ 568 Protein Labelling Kit from Molecular Probes was used. Following the manufacturer's instructions, a conjugate of 4F2 and Alexa Fluor™ 568 was synthesised, designated "4F2-AR", with a labelling ratio of 3.2 moles of dye per mole of protein. 4F2-AR was used in subsequent immunofluorescence experiments at a concentration of 20μg/ml.

3.4.3 Titration of β1 integrin labelling

The antibody K20 (DAKO) is accepted internationally as a specific mouse monoclonal directed against β1 integrin. For use in immunofluorescence, the pre-conjugated product K20-FITC (DAKO) was used. Fluorescein isothyocyanate (FITC) can itself be used for immunofluorescence, but bleaches far more rapidly under laser excitation than the more stable Alexa Fluor™ fluorophores. In addition, conjugation of FITC to K20 was in a low ratio (approximately 1:1), resulting in weak, albeit specific fluorescence when used alone, even at concentrations of 1/10 (10μg/ml). **Figure 3.9** shows that in H69 SCLC this signal could be amplified by use of secondary and tertiary labelling, using rabbit anti-FITC Alexa Fluor™ 488 (1:400) and donkey anti-rabbit Alexa Fluor™ 488 (1:200) respectively, with no significant increase in non-specific labelling.

3.4.4 CD98 and β1 integrin co-localise in human macrophages

To assess whether CD98 and β1 integrin co-localise in macrophages, monocytes were isolated from peripheral blood and cultured as described above. On days 5-7 they were harvested by trypsinisation, then allowed to settle onto coverslips in serum-free Iscove's medium for 4 hours. Cells were washed twice with HBSS and incubated with primary antibodies 4F2-AR (20μg/ml), K20-FITC (1/10) or IgG_1 isotype control antibody in HBSS for 15 minutes at 37°C. The cells were then washed twice in HBSS at 4°C and fixation, quenching of aldehydes and blocking with 0.2% fish skin gelatin in Ca^{2+}/Mg^{2+}-free PBS was performed as above. Incubations with secondary and tertiary antibodies



K20-FITC alone

K20-FITC + secondary anti-FITC + anti-rabbit Alexa Fluor 488™

Figure 3.9: Amplification of β1 integrin signal.

H69 SCLC cells were probed with K20-FITC (1/10), directed against β1 integrin, with or without secondary/tertiary amplification. Amplification was with secondary rabbit anti-FITC Alexa Fluor 488™ (1:400) and tertiary anti-rabbit Alexa Fluor 488™ (1:200).

were performed in 0.2% fish skin gelatin in Ca^{2+}/Mg^{2+}-free PBS. For the isotype control antibody, secondary labelling was with goat anti-mouse Alexa Fluor 488™ (1:200). **Figure 3.10** shows that under these conditions, whilst there was no significant binding of IgG_1 isotype control antibody, there was specific binding of 4F2-AR and K20-FITC. In addition there was clear evidence of overlap of the immunofluorescence signals, indicating that CD98 and β1 integrin co-localise in human macrophages.

3.4.5 CD98 and β1 integrin co-localise in H69 SCLC

Using a similar design, H69 SCLC were first washed in HBSS twice and incubated overnight in quiescent medium. They were then disaggregated by gentle passage through a 21-gauge needle, washed again and incubated in eppendorfs with primary antibodies in HBSS at 37°C. Following primary incubation, cells were washed twice in HBSS at 4°C then resuspended in 20-30μl of HBSS to be placed as a droplet on a coverslip. Fixation, quenching, blocking and secondary/tertiary incubations were performed as before. **Figure 3.11** shows that, as with macrophages, 4F2-AR and K20-FITC bound specifically and that the two signals frequently, but not universally, co-localised. This suggested that CD98 and β1 integrin co-localised in H69 SCLC.

3.4.6 CD98 and β1 integrin co-localisation is independent of activation state

To assess whether this co-localisation was dependent on whether β1 integrin was in the suppressed or activated state, the proteins were interrogated using β1 integrin-inhibiting and -stimulating antibodies respectively. Titration experiments for the β1 integrin

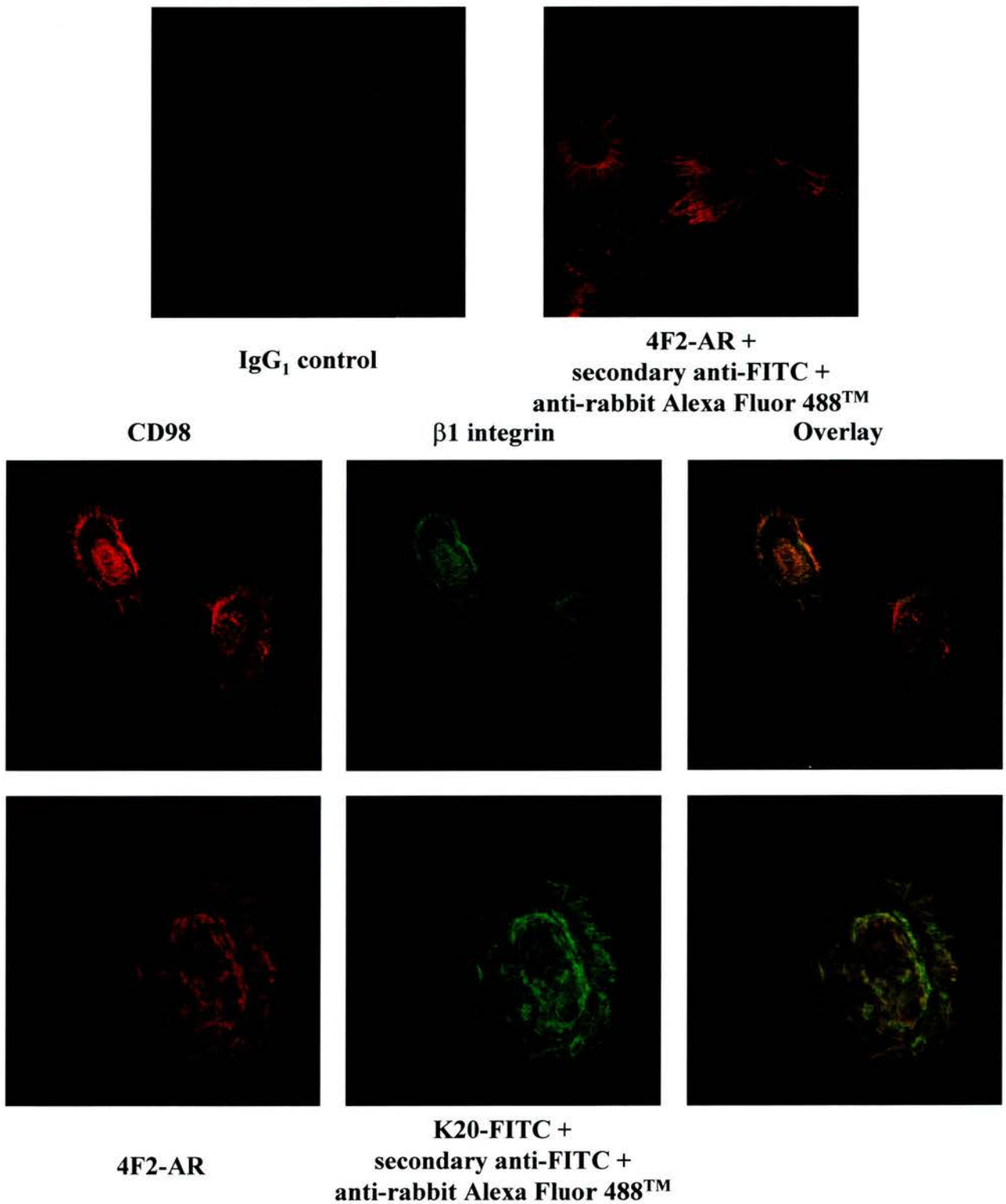


Figure 3.10: CD98 and β1 integrin co-localise in human macrophages.
Macrophages were probed with 4F2-AR or K20-FITC, directed against CD98 and β1 integrin respectively. Amplification of the FITC signal was with secondary rabbit anti-FITC Alexa Fluor 488™ and tertiary anti-rabbit Alexa Fluor 488™. A mouse isotype control IgG_1 antibody did not bind to macrophages and there was no cross-reactivity between 4F2-AR and the secondary/tertiary AlexaFluor 488™ antibodies (*upper panel*). Where CD98 and β1 integrin co-localised, the overlay image produced a gold signal (*lower two panels*). Representative of at least three experiments.

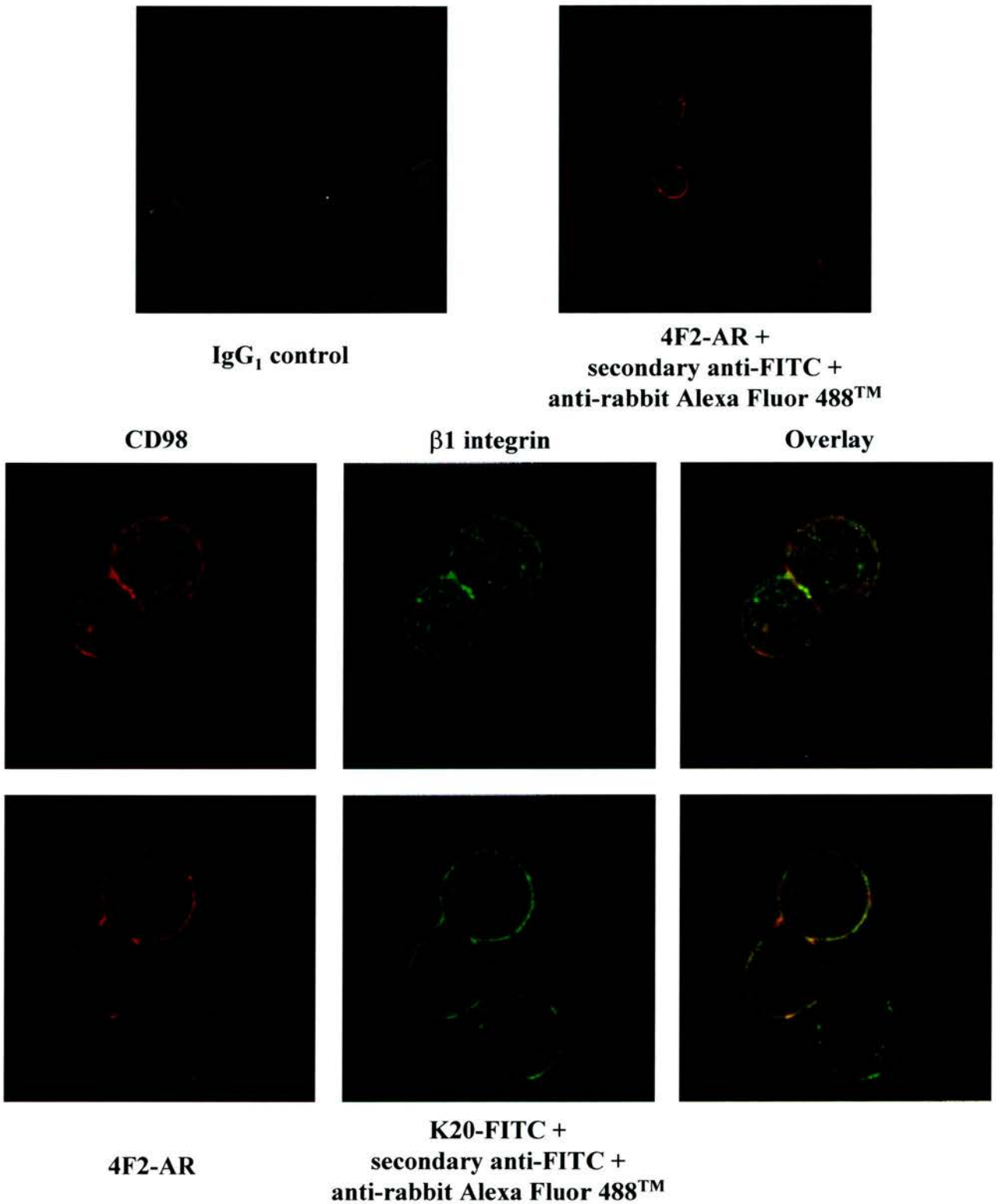


Figure 3.11: CD98 and β1 integrin co-localise in human SCLC H69 cells.

H69 SCLC cells were probed with 4F2-AR or K20-FITC, directed against CD98 and β1 integrin respectively. Amplification of the FITC signal was with secondary rabbit anti-FITC Alexa Fluor 488™ and tertiary anti-rabbit Alexa Fluor 488™. A mouse isotype control IgG_1 antibody did not bind to SCLC and there was no cross-reactivity between 4F2-AR and the secondary/tertiary AlexaFluor 488™ antibodies (*upper panel*). Where CD98 and β1 integrin co-localised, the overlay image produced a gold signal (*lower two panels*). Representative of at least three experiments.

function-blocking antibody 4B4 (Coulter Immunology) suggested that a concentration of 1/30 – 1/100 was optimal for immunofluorescence studies (data not shown). Since this was a mouse antibody like 4F2-AR, care was taken that all incubations with 4B4 and secondary goat anti-mouse AlexaGreen were performed prior to incubation with 4F2-AR (which was therefore the last incubation), to prevent any cross-reactivity. **Figure 3.12a** shows that when SCLC were incubated with the β1 integrin function-blocking antibody 4B4, the β1 integrin and CD98 signals continued to exhibit co-localisation.

The β1 integrin-stimulating antibody TS2/16 was produced in-house by culture of the appropriate hybridoma and subsequent immunoglobulin purification, as described in "Materials and Methods". This was conjugated to the fluorophore FITC and titration experiments confirmed that a concentration of 1/50 (4μg/ml) was optimal for immunofluorescence, using using rabbit anti-FITC Alexa Fluor™ 488 and donkey anti-rabbit Alexa Fluor™ 488. Again, since TS2/16 is a mouse monoclonal antibody, incubation with 4F2-AR was performed as the final incubation to prevent any cross-reactivity. **Figure 3.12b** shows that when SCLC were incubated with the β1 integrin-stimulating antibody TS2/16, the β1 integrin and CD98 signals continued to exhibit co-localisation.

By contrast, when SCLC were incubated with CD71-FITC, a pre-conjugated mouse IgG_{2a} antibody directed against the cell surface transferrin receptor, there was no clear

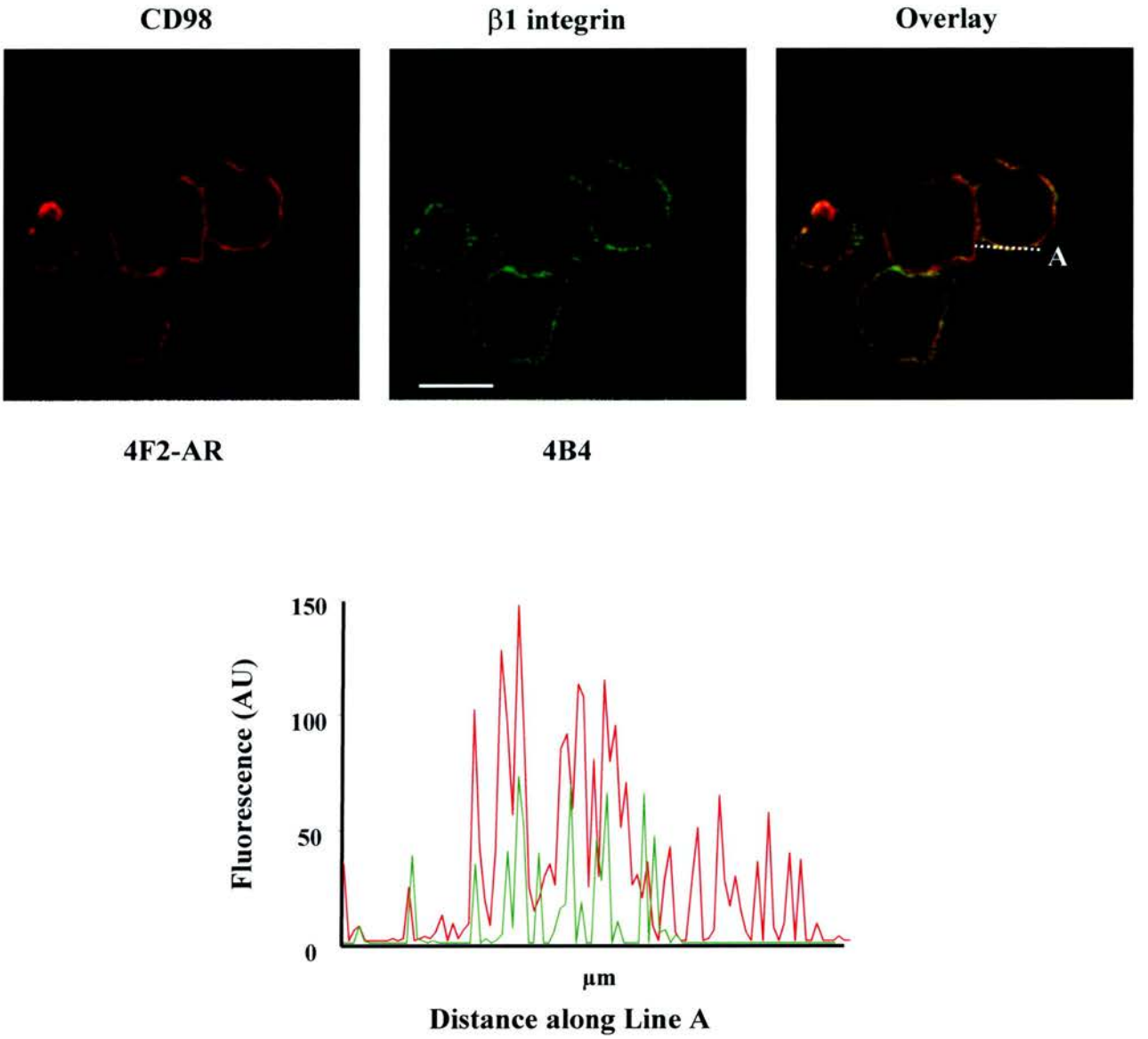


Figure 3.12a: CD98 and β1 integrin are constitutively co-localized.
H69 SCLC cells were probed with the β1 integrin-inhibiting antibody 4B4 (1/30) or IgG_1 and IgG_{2a} isotype controls (not shown), followed by secondary goat anti-mouse Alexa Fluor 488™. CD98 was then isolated with 4F2-AR. The fluorescence of the individual signals along Line A (*Overlay image*) indicates that there is a high degree of co-localization between CD98 and β1 integrin (*graph*). The scale bar represents 10μm. Representative of three experiments.

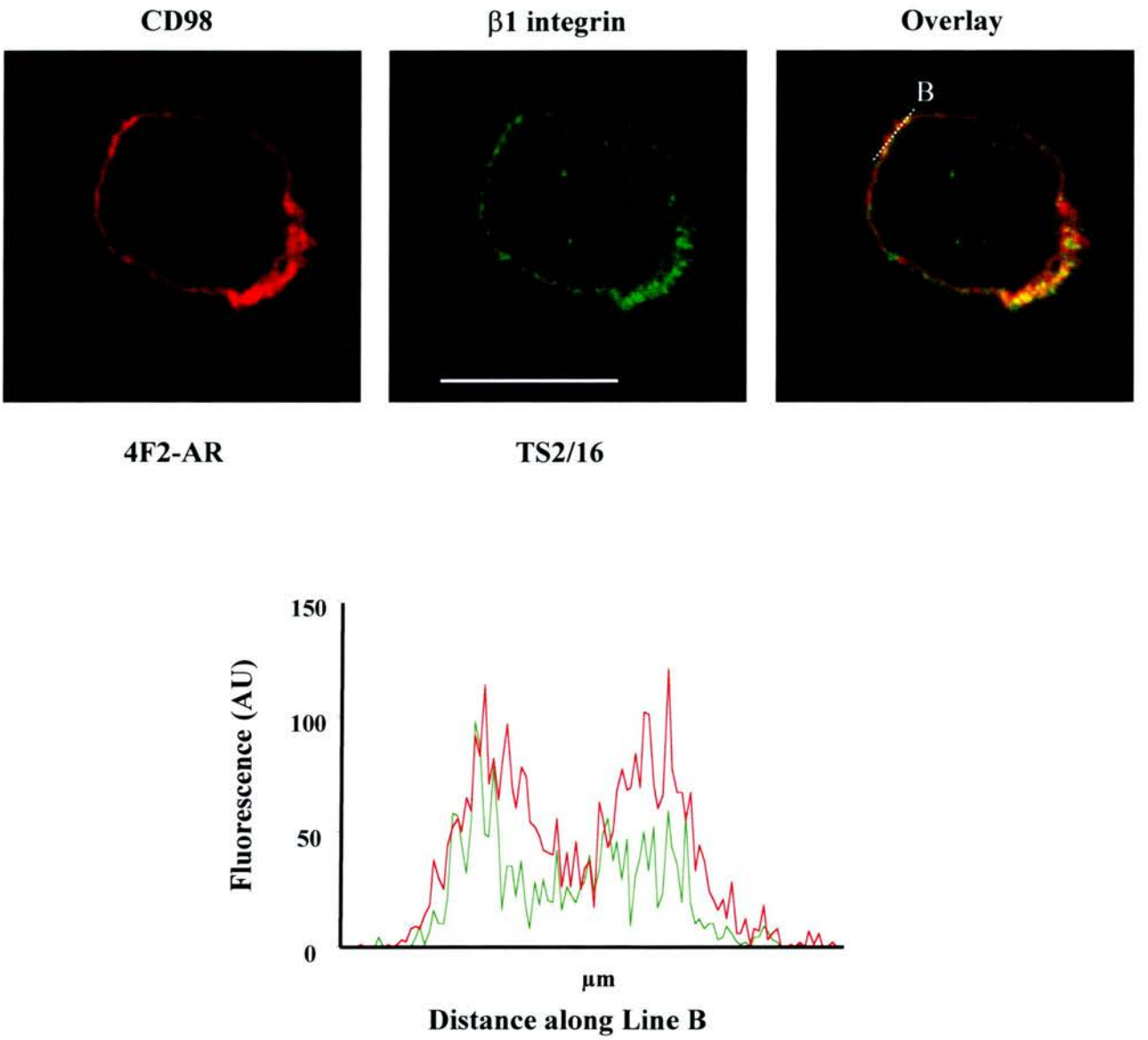


Figure 3.12b: CD98 and β1 integrin are constitutively co-localized.
H69 SCLC cells were probed with the pre-conjugated β1 integrin-stimulating antibody TS2/16-FITC (1/10) or IgG_1 isotype control (not shown), followed by secondary and tertiary amplification. CD98 was then isolated with 4F2-AR. The fluorescence of the individual signals along Line B (*Overlay image*) indicates that there is a high degree of co-localization between CD98 and β1 integrin (*graph*). The scale bar represents 10µm. Representative of three experiments.

evidence of overlap between the fluorescence signals for the transferrin receptor and CD98 (**Figure 3.13**).

Taken together, these experiments suggested that not only were CD98 and β1 integrins co-localised at the cell surface, but that this physical association was independent of the activation status of the β1 integrin. In addition, it was a physical association specific to these two proteins, since it was not seen with another cell surface protein, the transferrin receptor, which is highly expressed by SCLC (EC_{50} of 1.4 μg/ml to H69 SCLC cells).

3.5 Functional effects of cross-linking CD98

3.5.1 Cross-linking CD98 with 4F2 stimulates PI 3-kinase

The cytoplasmic tail of CD98 appears to be critical for its action and there is evidence to suggest that CD98 is involved in the regulation of intracellular signalling (Tabata *et al.*, 1997). The effect of cross-linking CD98 on intracellular signalling was therefore examined. To specifically examine the effect of cross-linking CD98 the monoclonal antibody to the heavy chain of CD98 (4F2) was used. 4F2 did not stimulate mobilization of intracellular [Ca^{2+}] in fura-2 loaded SCLC cells (Dr. R.C. Rintoul, personal communication). Since PI 3-kinase plays a key role in integrin activation, cellular activation and transformation the effect of 4F2 on PI 3-kinase activity in SCLC cells was examined. PI 3-kinase activity from p85α immunoprecipitates was measured as described in "Materials and Methods". The identity of the PI(3)P has previously been

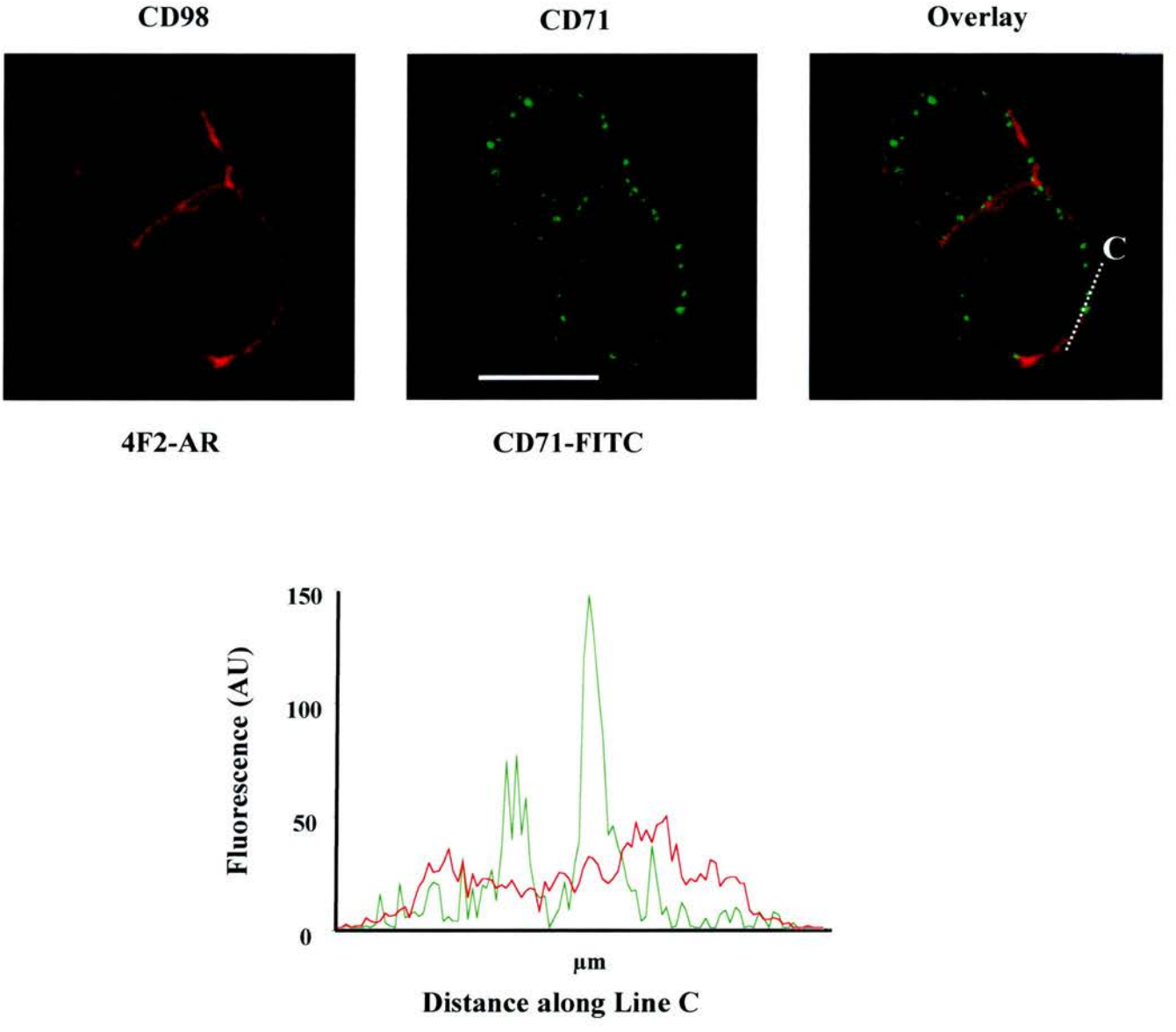


Figure 3.13: CD98 and CD71 are not co-localized.

H69 SCLC cells were probed with the pre-conjugated CD71 antibody CD71-FITC (1/2). CD98 was then isolated with 4F2-AR (20µg/ml). The fluorescence of the individual signals along Line C (*Overlay image*) indicates that there is no significant co-localization between CD98 and CD71 (*graph*). The scale bar represents 10µm. Representative of three experiments.

confirmed by monomethylamine deacylation and HPLC analysis, using a SAX 5 column and $(NH_4)_2HPO_4$ gradient and authentic tritiated standards as markers (Dr. R.C. Rintoul, PhD thesis). **Figure 3.14 (*upper graph*)** shows that following 10 minutes' incubation, 4F2 (20μg/ml) caused a significant increase in PI 3-kinase activity relative to basal levels (mean fold increase ± SEM = 1.60 ± 0.17). PI 3-kinase activation by 4F2 could be blocked by pre-incubation with the PI 3-kinase inhibitor wortmannin (100nM) (0.43 ± 0.14). The IgG_{2a} isotype control antibody against the CD71 transferrin receptor (10μg/ml) did not stimulate PI 3-kinase activity relative to baseline levels, at either 5 minutes (1.04 ± 0.05) or 10 minutes (1.03 ± 0.12). **Figure 3.14 (*lower graph*)** shows further that there is a timecourse of PI 3-kinase activation by 4F2, increasing above baseline at 2 minutes (mean fold increase ± SEM = 1.46 ± 0.23) and 5 minutes (2.49 ± 0.46) and dropping back by 20 minutes (1.73 ± 0.38) (courtesy Dr. R.C. Rintoul). In addition, monovalent Fab'-4F2 fragments (20μg/ml) had no effect on PI 3-kinase activity (0.95 ± 0.01) and were able to block the effect of intact antibody 4F2 (0.83 ± 0.03). It has separately been demonstrated that although the monovalent Fab'-4F2 fragments on their own had no effect on PI 3-kinase activation, cross-linking them by the addition of anti-mouse Fab' antibodies reproduced 4F2-mediated PI 3-kinase activation. These data confirm that cross-linking of CD98 is required for PI 3-kinase activation. Moreover, this is a specific effect of incubation with 4F2, since neither saturating concentrations of negative control IgG_{2a} antibodies, nor an IgG_{2a} antibody (10μg/ml) to the CD71 transferrin receptor, had any effect on PI3-kinase activity.

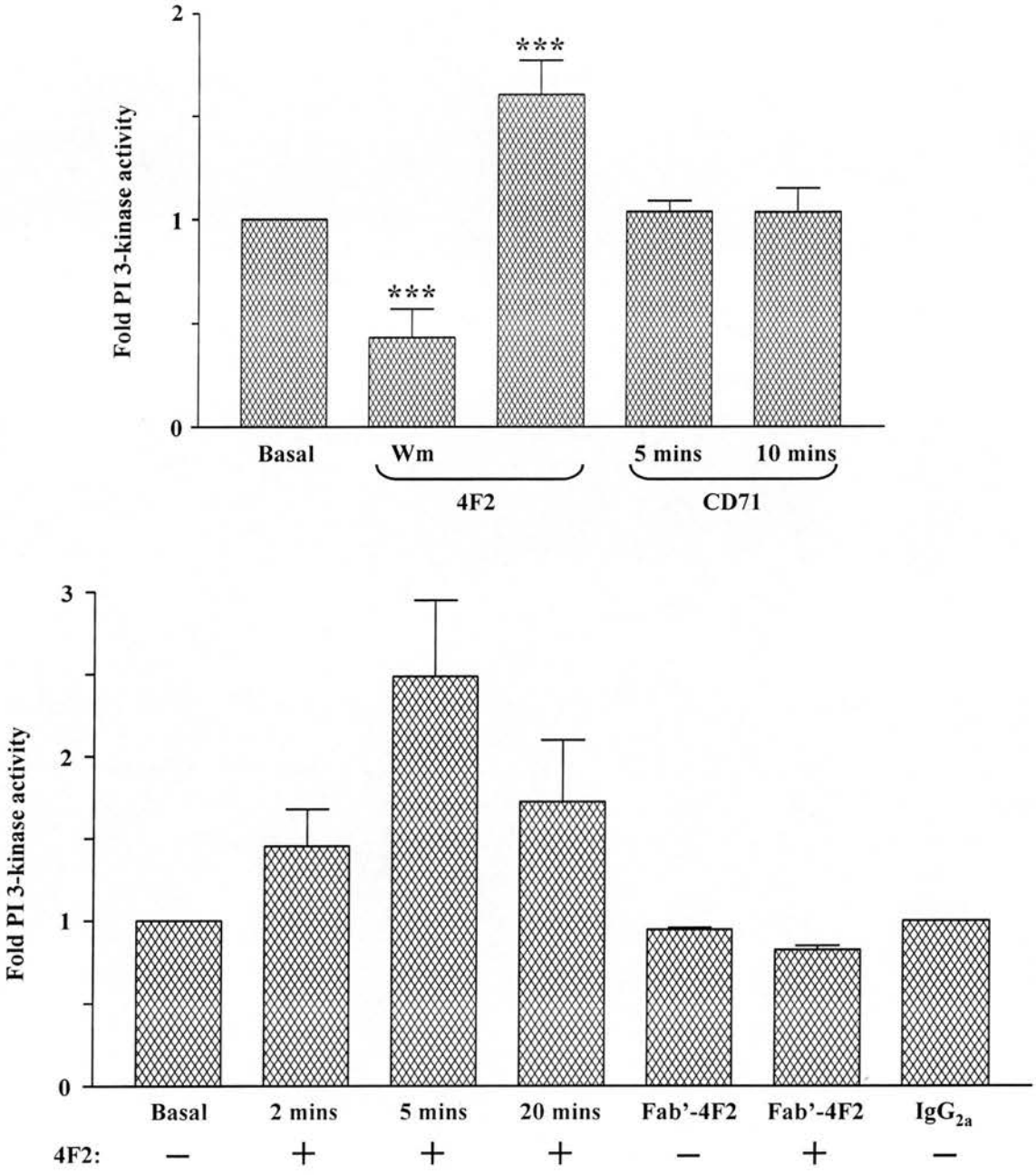


Figure 3.14: Cross-linking CD98 activates PI 3-kinase.

Upper graph: Quiescent H69 SCLC cells were incubated with 4F2 (20μg/ml) for 10 minutes, in the presence or absence of wortmannin (Wm), or with anti-CD71 antibody (10μg/ml) for 5 or 10 minutes. PI 3-kinase activity was then assayed. Results are the mean +/- SEM of three experiments. (*** $p < 0.001$ relative to Basal.)

Lower graph: Quiescent H69 SCLC cells were incubated with 4F2 (20μg/ml) for varying times, or with Fab'-4F2 (20μg/ml) with and without 4F2, or an isotype IgG_{2a} control antibody. Results are the mean +/- SEM of three experiments. (Courtesy Dr. R.C. Rintoul.)

3.5.2 Cross-linking CD98 with 4F2 promotes β1-dependent cell adhesion

To further examine the functional effects of cross-linking CD98, particularly relating to interactions with β1 integrins, the influence of cross-linking CD98 on cell adhesion was assessed. Using a 96-well plate, coated with a layer of laminin (10μg/ml), quiescent H69 SCLC were first disaggregated and counted, then incubated for an hour at a concentration of 1 x 10^5/ml, in the presence or absence of 4F2 (20μg/ml), with EDTA or specific integrin function-blocking antibodies. Non-adherent cells were then gently washed off and adherent cells fixed and stained with 1% methylene blue. The number of cells remaining was thus proportional to the strength of methylene blue staining. Comparing the number of adherent cells with the maximum possible (if all cells were fixed in the well, *100% control*) and the number of cells adherent to untreated plastic alone, **Figure 3.15** demonstrates that although laminin increased adhesion relative to plastic alone, this was not statistically significant. However, incubation with 4F2 resulted in significantly increased cellular adhesion (mean % adhesion ± SEM = 70% ± 13.5), compared to either plastic (28.7% ± 10.1) ($p < 0.01$) or laminin alone (41.8% ± 5.0) ($p < 0.05$). This increased adhesion with 4F2 was strikingly reduced by the presence of 2mM EDTA (22% ± 5.0), the β1 integrin function-blocking mAb 4B4 (1/100) (32.5% ± 9.4) or the α3 integrin function-blocking mAb P1B5 (1/100) (48.3% ± 5.0), none of which exhibited adhesion significantly differing from that achieved on plastic alone. These results suggested that one of the functional effects of cross-linking CD98 was to stimulate α3β1 integrin-dependent cellular adhesion.

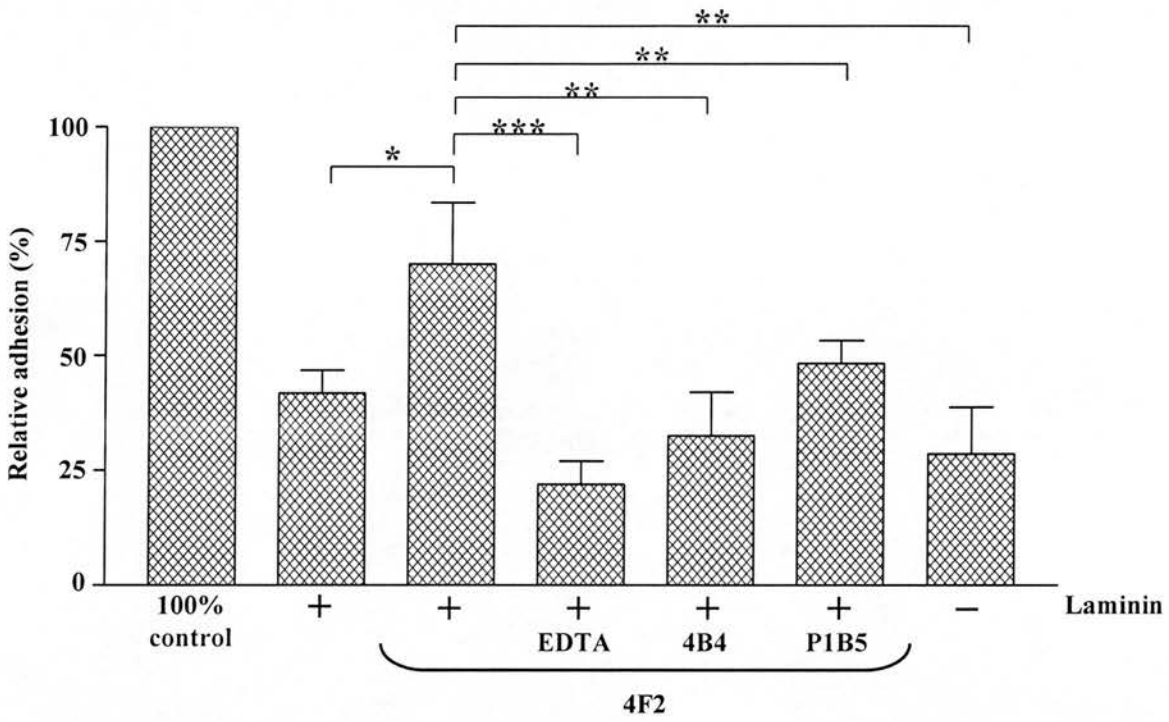


Figure 3.15: Cross-linking CD98 stimulates integrin-mediated adhesion .
Quiescent H69 SCLC cells were allowed to settle for 1 hour in 96-well plates coated with or without laminin, in the presence of 4F2 (20μg/ml). In addition to 4F2, incubations were also performed in the presence of EDTA (2mM), β1 integrin function-blocking antibody 4B4 (1:100), or α3 integrin function-blocking antibody P1B5 (1:100). Adhesion was quantified as described in "Materials and Methods" and expressed as a percentage of maximum possible adhesion (defined as 100%, with all cells centrifuged into the well). Results shown are the mean +/- SEM of three experiments performed in triplicate. (*$p < 0.05$; ** $p < 0.01$; $p < 0.001$).

3.6 Discussion

This chapter presents the results of a number of studies directed at investigating the role of CD98 in modulating β1 integrins. CD98 is a transmembrane protein widely expressed in the foetus and in activated cells and the specificity of this link to β1 integrins was thrown into light when it was demonstrated that CD98 was specifically able to rescue β1 integrins from dominant suppression by β1A integrin tails (Fenczik *et al.*, 1997a). However, the physical and molecular bases of this link are still unclear.

The most immediate question is whether CD98 and β1 integrins are linked by direct association, or by an indirect signalling cascade. Having established that CD98 was highly expressed by SCLC cell lines, this chapter presents substantial evidence that the two proteins are physically associated in the cell membrane. Co-immunoprecipitation experiments demonstrated that β1 integrin could be co-immunoprecipitated with CD98 from a number of cell lines: these included Cos-7 cells transfected with CD98, human macrophages derived from whole blood and the human H69 SCLC cell line. In validation of these experiments, confocal microscopy was used to visualise the two target proteins, in both human macrophages and H69 SCLC cells. In both cell types, CD98 and β1 integrins were co-localised. Importantly, in neither confocal nor co-immunoprecipitation experiments was CD98 seen to be associated with another cell surface protein, the transferrin receptor (CD71). This suggests that the association with

β1 integrin was a specific association and did not occur simply because the two proteins were both expressed in the cell membrane.

Finally, this chapter presents results investigating the functional effects of cross-linking CD98 with the monoclonal antibody 4F2 in SCLC cells. Cross-linking CD98 stimulated intracellular signalling through PI 3-kinase, whose activity was upregulated by 4F2, but not by monovalent Fab'-4F2 or antibodies against CD71. In addition, 4F2 stimulated increased integrin-dependent SCLC cell adhesion to laminin. These findings suggest an integral role for CD98 in events taking place at the cell membrane, with influence over downstream cellular functions.

Reviewing these findings, it was unsurprising to find that CD98 was highly expressed in our SCLC lines. This is entirely in keeping with other work published by other groups (Nikolova *et al.*, 1998;Pedersen *et al.*, 2001;Azzarone *et al.*, 1985), demonstrating that CD98 is upregulated in cancerous cells, both *in vivo* and *in vitro*. Indeed, high CD98 expression was observed in other transformed cell lines, such as CCD19-LU and human bronchial epithelial cells (HBE-16) (*data not shown*), both of which are cell lines transformed with SV40. Earlier work has shown that transfection with CD98 is sufficient to induce cellular transformation (Hara *et al.*, 1999;Shishido *et al.*, 2000). Thus increased CD98 expression correlates with the transformed phenotype. In part this may reflect the fact that CD98 is involved in amino acid transport regulation, since all six of the light chain proteins so far described have been shown to be homologous to

y+L and L-type amino acid transport proteins (Mannion *et al.*, 1998) (Kanai *et al.*, 1998) (Torrents *et al.*, 1998;Estevez *et al.*, 1998;Mastroberardino *et al.*, 1998;Pfeiffer *et al.*, 1998;Pfeiffer *et al.*, 1999;Tsurudome *et al.*, 1999). It makes biological sense that a transformed cell, with higher levels of cell cycle activity, should have increased transporter systems in place to permit this – such systems might be expected to be necessary to sustain transformation. This indeed appears to be the case, since transformation of BALB 3T3 cells by CD98 required its association with the light chain and mutations preventing this association removed its transformational ability (Shishido *et al.*, 2000). Whether increased expression of CD98 *heavy* chain is to be explained in this way, or whether it has influence on the transformation process in other ways is currently unclear.

What has become apparent is that CD98 is intimately linked with β1 integrins. The co-immunoprecipitation experiments presented in this chapter were technically difficult and may be criticised in their interpretation because they were highly dependent upon the conditions under which immunoprecipitation took place. Multiple unsuccessful attempts to co-immunoprecipitate CD98 and β1 integrin preceded successful experiments and these seemed at least in part due to the more stringent effects of the MAPK lysis buffer used initially. Although a CHAPS/deoxycholate lysis buffer was then effective in co-immunoprecipitation from both transfected Cos-7 cells and normal human macrophages, it was unsuccessful for SCLC, for which further modification of the lysis buffer and experimental conditions was required. These adjustments indicate that the molecular

bond between CD98 and β1 integrin is sufficiently weak as to be lost under more stringent lysis conditions, but also that other conditions such as cell numbers are critical in such experiments. Using cell lines such as H69 SCLC, which are small and have a relatively large nucleus and small cytoplasm/cell surface area, even though CD98 and β1 are both highly expressed, increasing the cell numbers was critical in raising the sensitivity of the assay. These co-immunoprecipitation experiments, therefore, were both cell type-dependent and sensitive to assay conditions and hence open to simple criticism – the very conditions that permit co-immunoprecipitation may be producing a false positive result. However, the finding that CD71 did not co-immunoprecipitate with β1 integrins suggests that conditions were not so lax that any protein in the cell membrane would be co-immunoprecipitated.

Under these circumstances, the complementary data provided by confocal microscopy has been supportive. Initial experiments highlighted the difficulties of trying to cope with cross-reactivity between different primary and secondary antibodies and results were strikingly more specific using conjugated primary antibodies. Confocal microscopy then supplied powerful data supporting the co-localisation of CD98 and β1 integrin, including whether it was constitutive or dependent upon integrin activation state, by use of integrin function-stimulating and -blocking antibodies. This had not been assessed before. Limitations include that the cells were assessed only at one point in time, for which they were fixed in paraformaldehyde, so a true assessment of changes in CD98-integrin association was not possible. To do this would require real-time

imaging in live cells during β1 integrin-dependent processes, such as adhesion/deadhesion, together with a more refined imaging analysis, such as fluorescent resonance energy transfer.

Importantly, the findings also agree with other *in vitro* data emerging on the relationship between CD98 and β1 integrin. Zent *et al.* have shown that CD98 binds to the cytoplasmic domain of integrin β1A and β3, but very poorly to the muscle-specific variant β1D or the leucocyte-specific β7 (Zent *et al.*, 2000). It is interesting to note that very mild detergent conditions were required (0.05% Triton X-100), correlating with experience described here. In addition, binding of CD98 in this system correlated with the ability to complement dominant suppression, since it was far less effective at reversing suppression induced by Tac-β1D or Tac-β7. By mutational analysis, it was observed that the last seven residues of the β1A tail were necessary for this interaction and that part of the importance of the CD98-β1 integrin interaction was a participation in the polarisation and regulation of amino acid transporters.

This has been further supported in two whole cell systems. Merlin *et al.* have shown that in polarized intestinal epithelial cells, CD98 is co-localised with β1 integrin (Merlin *et al.*, 2001) on the basolateral cell membrane. This group have suggested that since overexpression of CD98 disrupts the normal β1 integrin surface distribution and actin cytoskeleton re-organisation, CD98 may be intimately involved in β1-related cell

signalling, although this may have been an artefact of overexpression in this system. Using rhabdomyosarcoma and fibrosarcoma cells, Kolesnikova *et al.* have recently shown that CD98 constitutively associates with α2β1, α3β1, α5β1 and α6β1 integrins, but not α4β1, suggesting that the integrin α chain does contribute to the specificity of this interaction (Kolesnikova *et al.*, 2001). This study used reciprocal immunoprecipitation and since EDTA did not influence the association, it supports the present data that it was a constitutive interaction. β1 integrin and CD98 were associated in low density "light membrane" fractions on a sucrose gradient, not in the dense fractions containing most of the β1 integrin – this would be consistent with an interaction in the context of "lipid rafts" in the cell membrane, which may prove important for signalling. Interestingly, this group also reported that the presence of the light chain modified the targeting of CD98 to the lipid fraction.

Earlier studies had suggested that at least some of the functional effects of CD98, such as homotypic cell aggregation, were mediated through tyrosine kinase activation (Warren *et al.*, 1996;Tabata *et al.*, 1997). Since PI 3-kinase is a major downstream target of receptors with intrinsic or associated tyrosine kinase activity, it was therefore unsurprising to find that cross-linking CD98 stimulated PI 3-kinase activity. This work has been extended in our group: it has been shown that cross-linking CD98 activates protein kinase B, the key downstream effector of PI 3-kinase, as well as MAP kinase. This pattern of signalling is in keeping with an integrin-mediated signalling pathway and subsequently CD98 signalling has been shown to be β1 integrin and FAK-dependent

(Rintoul *et al.*, 2002). The signalling function of CD98 heavy chain and the amino acid transport function of the light chain seem to be mutually independent, since cross-linking CD98 with 4F2 had no effect on amino acid uptake and the amino acid transport blocking agent BCH had no effect on CD98 signalling. These features are in keeping with an earlier finding by Fenczik's group that distinct regions of CD98 are responsible for amino acid transport and integrin-related interactions (Fenczik *et al.*, 2001). Using chimæric CD98/CD69 proteins, the extracellular domain of CD98 heavy chain was identified as important for amino acid transport, whereas the cytoplasmic and transmembrane domains were required for its effects on integrin function.

In summary, this chapter has provided evidence of a physical association of CD98 with β1 integrins and of downstream effects of cross-linking CD98, both in cell signalling and cell behaviour. These findings are increasingly supported by a literature that suggests a direct involvement of CD98 in β1 integrin signalling, which is distinct from its role in amino acid transport. This picture of a multifunction molecule gives credence to the finding of CD98 upregulation in transformed cell systems, such as lung cancer.

Chapter 4

CD98 and Galectin-3 Expression in Human Lung Disease

4.1 Introduction

Lung carcinoma is categorised broadly by histology into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC almost invariably present with widespread metastatic disease and, despite initial response to chemotherapy, the tumours relapse and become resistant to further chemotherapy. By contrast, NSCLC are less likely to have metastasised at presentation, but are relatively insensitive to chemotherapy. About 10-14% of cases of SCLC exhibit areas of NSCLC morphology; these mixed tumours have both a poorer response to treatment and poorer survival (Fushimi *et al.*, 1996).

Chapter 3 has presented evidence strongly supporting a role for CD98 as a protein intimately involved in the modulation of integrin function and with a potential for promoting oncogenic transformation. Most of the earlier work has been *in vitro* and there has been relatively little work looking at CD98 expression *in vivo*. Broadly speaking, both lines of investigation have suggested that tumours exhibit increased expression of CD98 compared with non-tumour tissue. Papetti *et al.* described increased CD98 expression in a variety of tumour cell lines as well as paraffin-embedded sctions of prostate cancer; treatment with an antibody to CD98 resulted in growth inhibition

(Papetti and Herman, 2001). In squamous cell laryngeal carcinoma, a histological study observed increased CD98 expression only in those tumours showing metastatic behaviour (Esteban *et al.*, 1990). Transfection of the epidermal growth factor (EGF) receptor negative SCLC cell line, GLC3, with mutant EGF receptor resulted in upregulation of CD98 expression (Pedersen *et al.*, 2001). However, in leukaemia, higher CD98 levels have been associated with improved outcomes, with more prolonged periods of remission in both adults and children (Taskov *et al.*, 1996;Nikolova *et al.*, 1998). In lung disease, including lung cancer, there have been no histological studies examining CD98. Given its potential role in neoplasia, it was decided that an assessment of CD98 expression should be undertaken in a series of routine pathological sections, including a range of lung cancers.

As previously discussed in Chapter 1, Dong and Hughes identified galectin-3 as a ligand for CD98 (Dong and Hughes, 1997). Galectin-3 is a β-galactoside binding protein of ~30kD which is expressed in a variety of cells, including cells of the monocyte/macrophage lineage and epithelial cells (Hughes, 1994;Barondes *et al.*, 1994b). Galectin-3 consists of a carboxyl-terminal carbohydrate binding domain, a repetitive collagen-like sequence and a functionally distinct amino-terminal domain (Barondes *et al.*, 1994a;Gong *et al.*, 1999). It exists as a monomer but can self-associate and form polymers through interactions of either its N-terminal domain (Birdsall *et al.*, 2001) or its C-terminal domain (Yang *et al.*, 1998), giving rise to a potential to cross-link proteins at the cell surface. Histologically, the role of galectin-3 in malignant

transformation is controversial. Upregulation of galectin-3 expression has been observed in thyroid, gastric, pancreatic and head and neck malignancies (Schaffert *et al.*, 1998;Baldus *et al.*, 2000;Cvejic *et al.*, 2000;Honjo *et al.*, 2000), but other studies have found down-regulation of galectin-3 in ovarian and breast cancers (Castronovo *et al.*, 1996;van den Brule *et al.*, 1996a), and conflicting results have been obtained in colonic carcinomas (Lotz *et al.*, 1993;Bresalier *et al.*, 1998). This may reflect cell type specificity and the influence of galectin-3 on tumour cell behaviour. To date there have been no immunohistochemical studies comparing galectin-3 expression in lung cancers, although recently Yoshimura *et al.* have described differential expression between SCLC and NSCLC when measured by reverse transcription-polymerase chain reaction (Yoshimura *et al.*, 2003).

4.2. Assessment of 4F2 for immunohistochemistry

Earlier studies had been performed using the mouse monoclonal antibody 4F2 directed against CD98 in immunohistochemistry studies (Rintoul, PhD thesis, 2002). However, staining on these samples was considered poor, with high background staining, and the samples themselves were old, historical samples from the University of Edinburgh Department of Pathology files. It was deemed necessary to reassess the accuracy of 4F2 for use in immunohistochemical studies.

4.2.1 4F2 is an unreliable antibody for immunohistochemistry

Five recent biopsy samples, embedded in paraffin wax, were confirmed to have SCLC by a consultant pathologist, Dr. Donald Salter. Samples were treated with methanol: acetone (1:1, v/v) at –20°C for 20 mins, then permeabilised with 0.1% Triton X-100 in PBS for 5 mins, before being washed in PBS twice for 5 mins. They were then blocked with 10% normal rabbit serum in PBS for 30 mins at r/t and then incubated with 4F2 (135μg/ml) for 30 mins at r/t. Subsequent washes and secondary staining was performed using the indirect streptavidin-biotin method as described in "Materials and Methods".

Assessment of these sections revealed that 4F2 staining was universally poor (**Figure 4.1**). This included areas of inflammation which, being rich in CD98-rich polymorphonucleocytes and macrophages, would be expected to be strongly positive internal controls. This suggested that the antigen was not being recognised by the antibody. This could be because the epitope had been masked by fixation and not successfully retrieved, or because the antibody was not applicable for this technique. However, no external positive control was utilised. It was concluded that no comment could be made about CD98 expression in these specimens using this technique and that a positive control slide was essential.

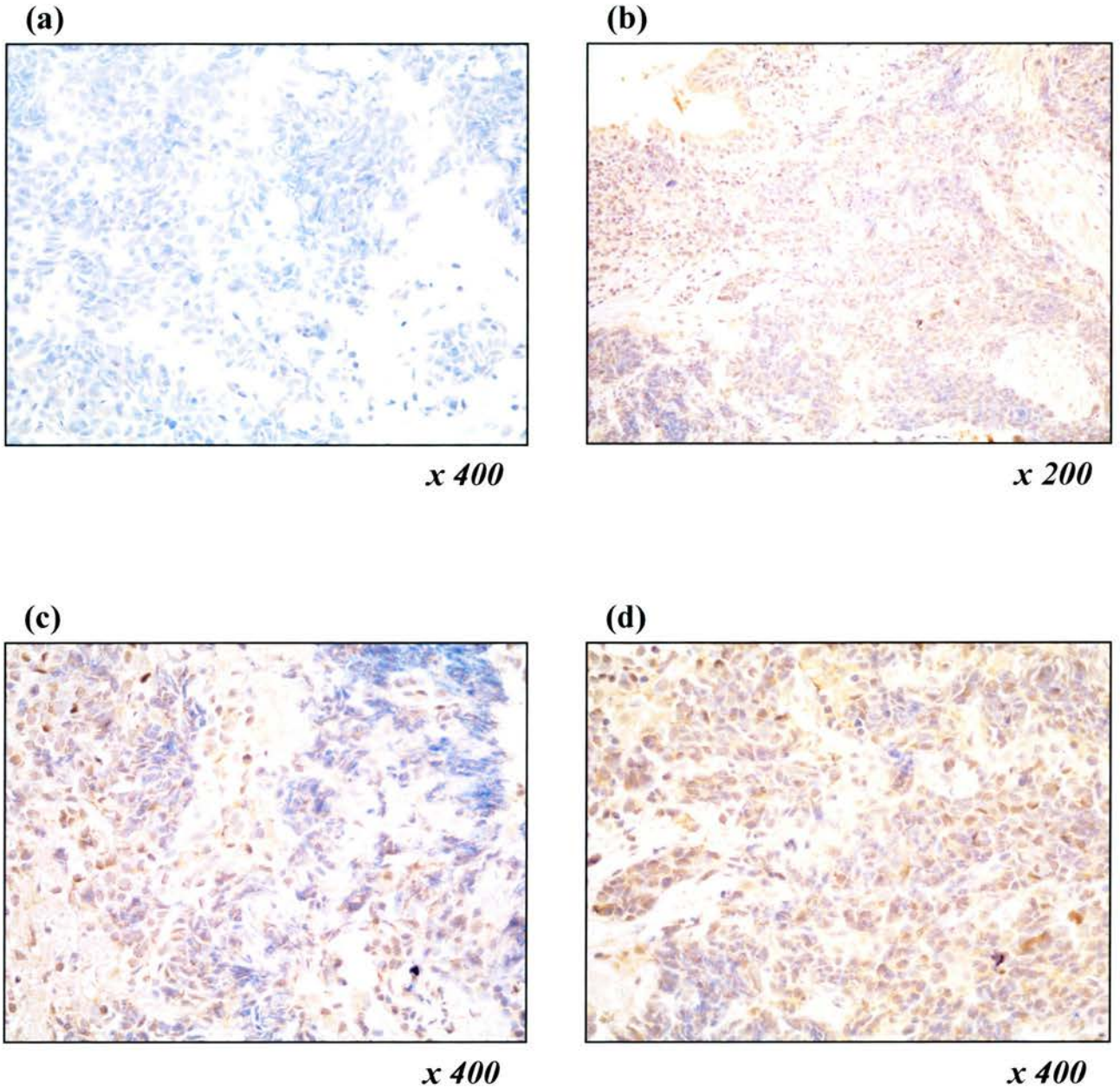


Figure 4.1: 4F2 produces poor results in historical paraffin-embedded specimens.
Historical, paraffin-embedded specimens of SCLC were probed with 4F2 (135μg/ml) or carrier solution, then by secondary rabbit anti-mouse HRP, followed by DAB substrate. Negative control samples showed no binding (*a*), but test samples shown at lower power (*b*) or higher power (*c and d*) exhibited a high degree of non-specific binding of 4F2 to background proteins.

4.2.2 Polyclonal and monoclonal antibodies against CD98 may be used in frozen section samples

As positive controls, two different cell samples were taken: (a) frozen sections of lymph nodes infiltrated by lymphoma; (b) CHO cells transfected with the expression vector for CD98-COOH. Untransfected CHO cells, which express low levels of CD98, were used as negative controls. CHO cells were transfected according to standard protocol with 2μg cDNA for CD98-COOH per 10cm dish. At 48 hours they were harvested, being trypsinized and allowed to re-adhere for 4 hours on glass slides. Cells were then fixed with 3% paraformaldehyde for 20 mins at r/t, and aldehydes quenched with 50mM NH_4Cl for 10 mins. All samples were then subject to the same immunohistochemical staining protocol as in "Materials and Methods", including a peroxidase block (0.5% H_2O_2 in H_2O for 10 mins). Three different antibodies were used directed against CD98: 4F2 mouse monoclonal; SC-7095 goat polyclonal; RDI-CD98 CabG goat polyclonal.

All samples showed positive staining with either of the commercial goat polyclonal antibodies (**Figure 4.2**). This included untransfected CHO cells, although these were markedly less positive than transfected CHO-CD98 cells. Using 4F2 mouse monoclonal as the primary antibody, untransfected CHO cells appeared negative, but CHO-CD98 cells remained strongly positive, as were lymphoma sections. Thus all antibodies appear to work in frozen sections.

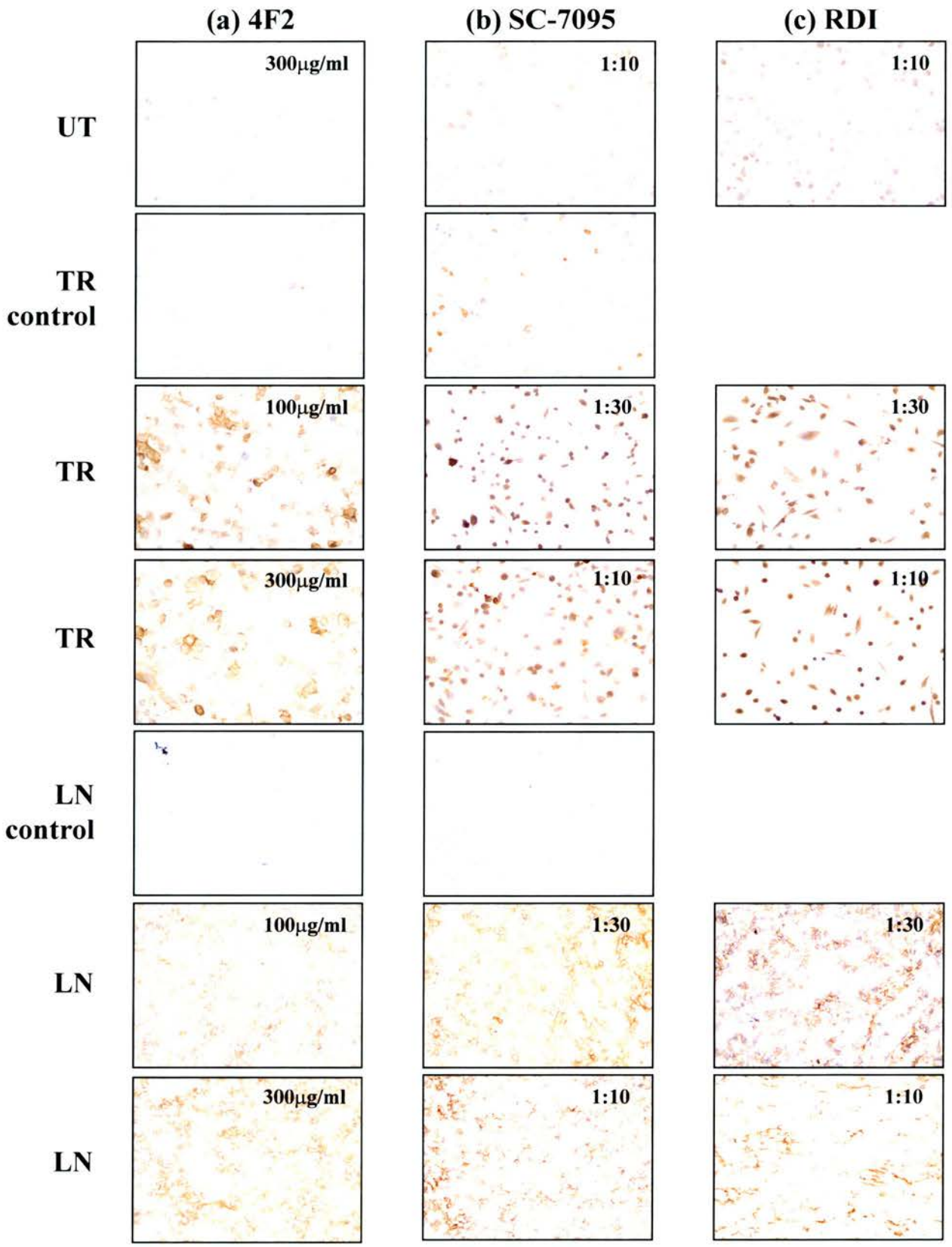


Figure 4.2: Monoclonal and polyclonal antibodies to CD98 in frozen section

CHO cells, either untransfected (UT) or transfected with CD98 (TR), and sections of frozen human lymph node (LN) were exposed to increasing concentrations of (a) monoclonal 4F2 or polyclonal antibodies: (b) SC-7095; or (c) RDI CD98 CabG (RDI). Appropriate secondary antibodies were used and negative controls were exposed to carrier alone during primary incubations.

In order to confirm successful transfection of CD98 into CHO cells, an associated pilot experiment was performed in which transfected CHO-CD98 cells were harvested and assessed for CD98 expression by flow cytometry using 4F2, as described in "Materials and Methods". The use of 4F2 in flow cytometry has previously been well established as a marker of CD98 expression. In transfected CHO-CD98 cells 4F2 bound strongly (mean fluorescence 46.2 AU), in contrast to the fluorescence seen with either an isotype control antibody or carrier solution (3.4 and 3.1 AU respectively); however, untransfected CHO cells exhibited no increase in 4F2 binding (mean fluorescence 2.7 AU) over isotype or carrier controls (2.6 and 2.4 AU respectively) (*data not shown*). This confirmed that CHO cells used as positive controls in immunohistochemical studies were capable of high levels of detectable CD98 expression following transfection, but expressed negligible amounts of CD98 in the native state.

4.2.3 4F2 is ineffective in paraffin-embedded histology sections

No improvement in specificity of staining in frozen sections was observed by pre-adsorbing the primary antibodies in 20% human/20% rabbit serum (*data not shown*). The potential use of 4F2 was therefore reassessed in paraffin-embedded sections of lymph nodes involved by lymphoma, since these would be the type of test sample used. Standard immunohistochemical technique was followed using incremental doses of 4F2. Antigen retrieval was performed either with Vector Unmasking Solution or with 1mM EDTA pH8. **Figure 4.3** demonstrates that, despite containing clearly visible germinal centres, none of these sections revealed any positive CD98 staining.

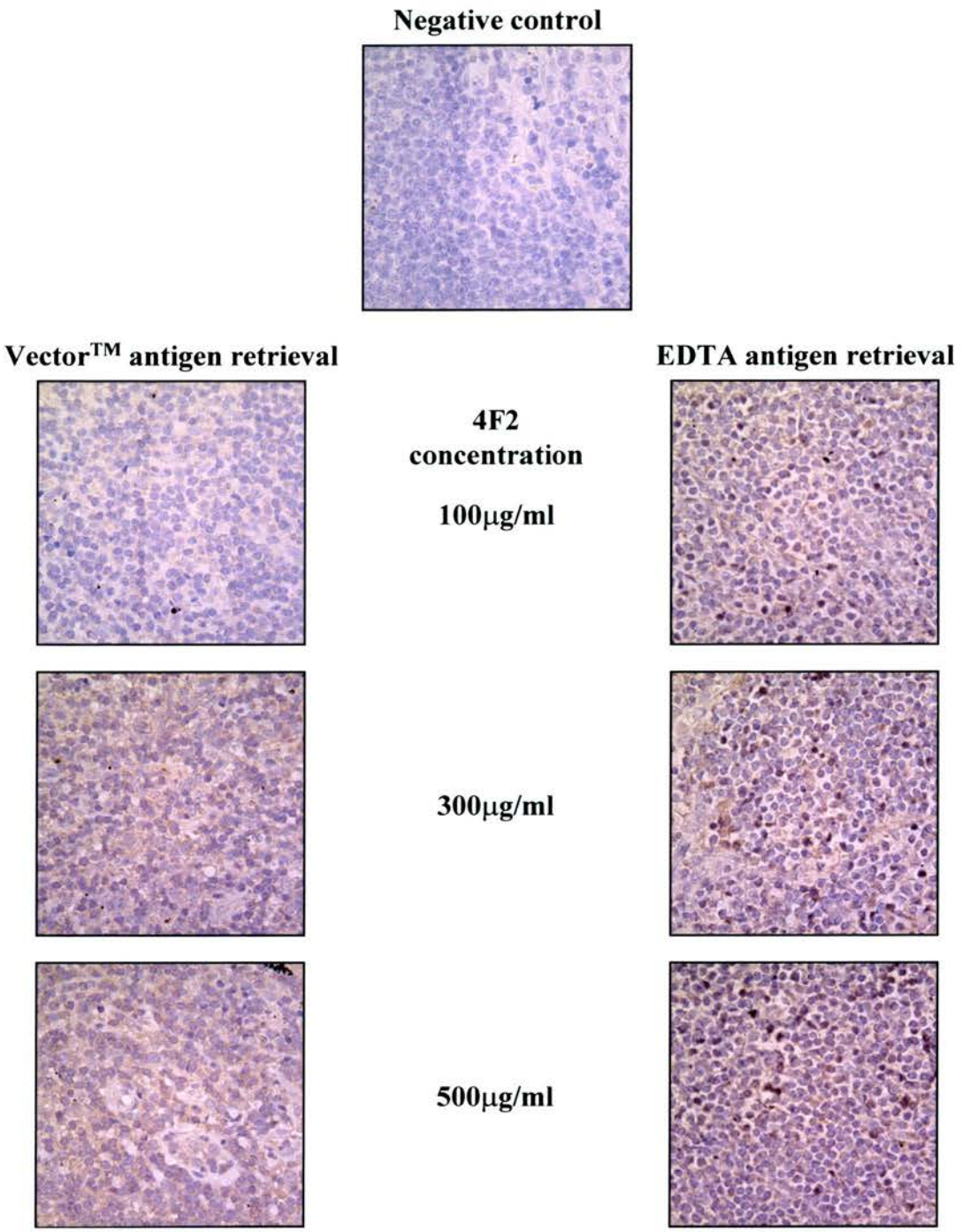


Figure 4.3: 4F2 is not useful in paraffin-embedded histology sections.
Parrafin-embedded sections of human lymph node, known to be involved with lymphoma, were prepared using two different standard methods of antigen retrieval, either the proprietary Vector™ Unmasking Solution or 1mM EDTA pH8. Immunohistochemistry was performed with incremental concentrations of 4F2 (100 – 500μg/ml). No clear antigen specificity was achieved with either antigen retrieval regimen, and the non-specific background signal was high. All sections are shown at x400 magnification.

From this set of experiments it was concluded that 4F2 could be used in frozen sections or paraformaldehyde-fixed cells, or with live cells in flow cytometry, as elsewhere in this thesis. However, the absence of staining in paraffin-embedded positive control tissue sections, despite standard antigen retrieval techniques and high doses, implied that 4F2 was unsuitable as a primary antibody under these conditions. 4F2 was therefore abandoned for further immunohistochemical studies and the commercial polyclonal antibody SC-7095 assessed for further use.

4.2.4 Titration of SC-7095

Paraffin-embedded lymph node control sections were used to assess the sensitivity of the goat polyclonal SC-7095 in detecting CD98 under a standard immunohistochemical protocol with two different standard antigen unmasking conditions. A dose-response study was then performed using the DAKO Techmate system to optimize the antibody concentration to be used, since future studies would use this system to process multiple slides. **Figure 4.4** shows that SC-7095 localized clearly to the germinal centers of lymph nodes, with the clearest results seen using Vector Unmasking Solution; an optimal dilution of 1:100 was identified under these conditions. Negative control samples were probed using carrier solution only in place of primary antibody, but with appropriate biotinylated secondary rabbit anti-goat as for the test samples. These exhibited no positive immunostaining.

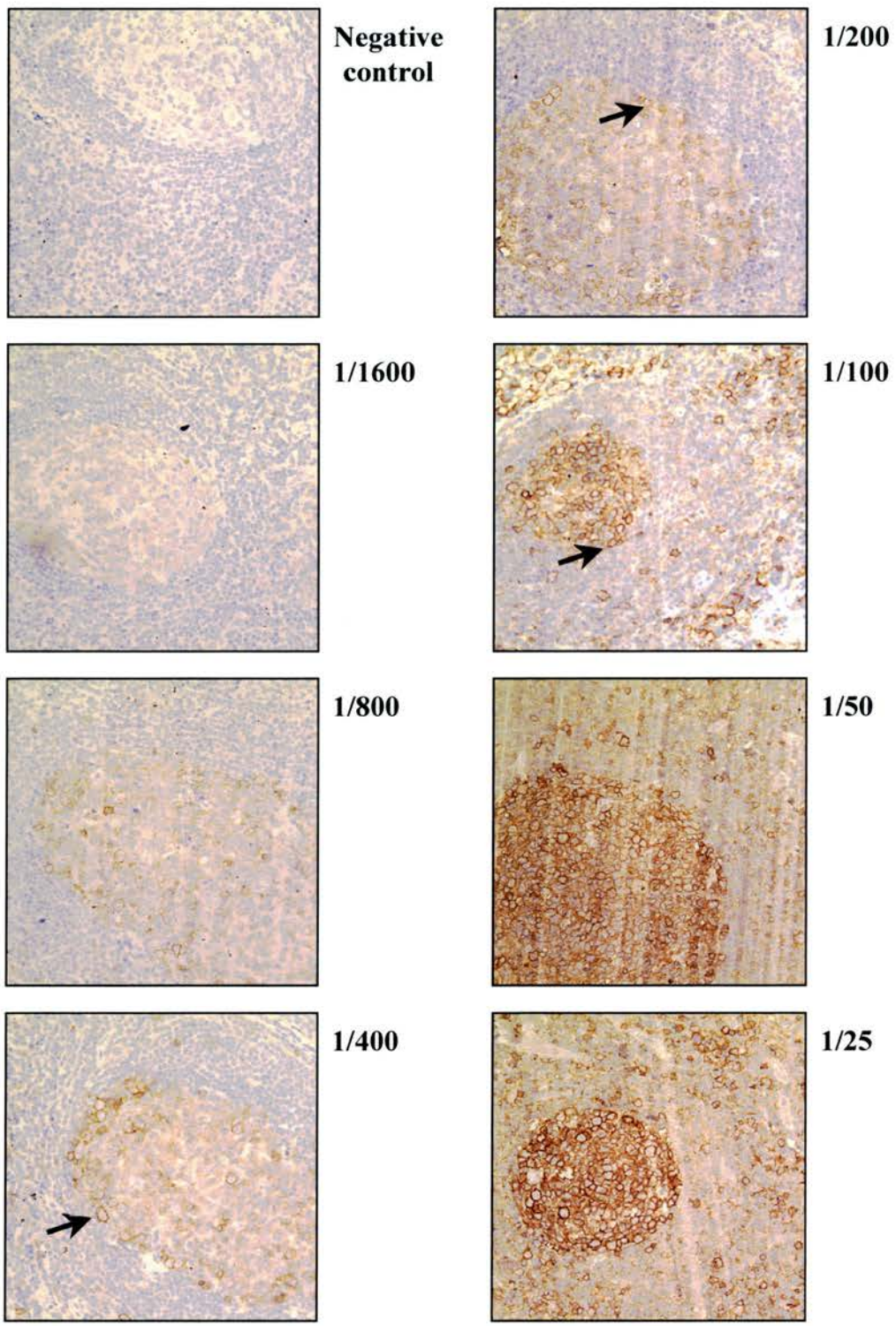


Figure 4.4: Titration of SC-7095 anti-CD98 antibody in paraffin-embedded histology sections. Parrafin-embedded sections of human lymph node, known to be involved with lymphoma, were prepared using Vector™ Unmasking Solution. Immunohistochemistry was performed with incremental concentrations of the goat polyclonal anti-CD98 antibody SC-7095 (1/1600 – 1/25). A clear, specific signal was observed, picking out membranous expression of CD98 by proliferating lymphocytes in lymph node germinal centres (*arrowed*). With increasing antibody concentration, decreasing specificity of staining was observed. All sections are shown at x200 magnification.

4.2.5 Assessment and titration of mAb B2C10 against galectin-3

It was first confirmed that the mouse monoclonal antibody B2C10 (gift, Dr. F.T. Liu), raised against galectin-3, demonstrated immunoreactivity against the appropriate target protein. This antibody has previously been well described and was initially shown to recognize the amino-terminal of galectin-3 (Liu *et al.*, 1996). *E.coli* were transformed with cDNA encoding hamster galectin-3 according to standard protocol as described in "Materials and Methods". *E.coli* were then cultured and selected for carriage of the correct plasmid by antibiotic selection. Individual colonies were then boiled in 1x lysis buffer, run on SDS-PAGE and probed with B2C10 for expression of galectin-3. B2C10 isolated a protein of molecular weight ~30kD from transformed cells (*data not shown*). This would correspond well with the predicted expression of galectin-3 and confirmed that B2C10 was directed against galectin-3.

B2C10 was titrated using the DAKO Techmate system and with a standard protocol. As above, lymph node sections provided appropriate positive control samples. **Figure 4.5** shows that compared with negative controls (diluent only), B2C10 specifically localized to germinal centers, with galectin-3 expression by macrophages. An optimal signal was achieved with a dilution of 1:400, which was therefore used in all subsequent experiments.

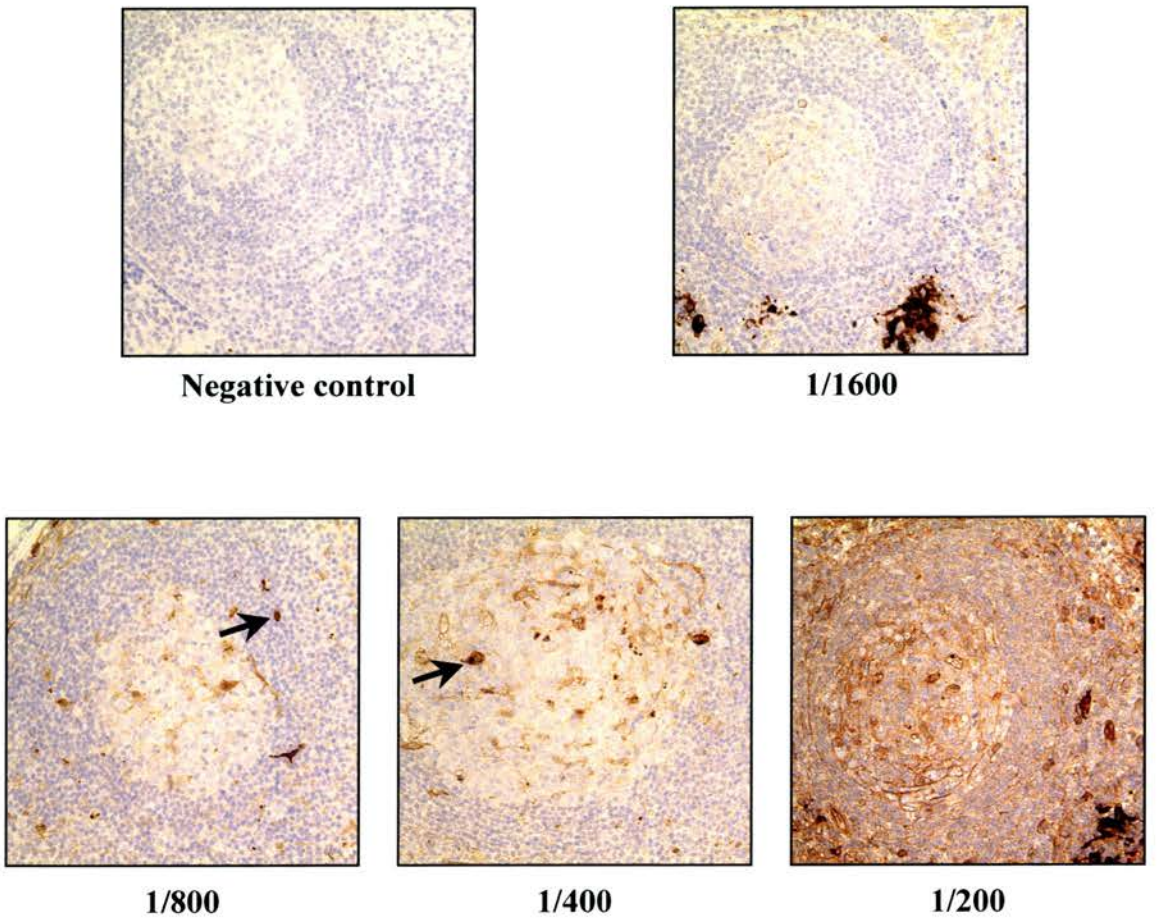


Figure 4.5: Titration of anti-galectin-3 antibody B2C10 in paraffin-embedded histology sections. Titration of B2C10 in lymphoma-involved human lymph node paraffin sections. Immunohistochemistry was performed with incremental concentrations of B2C10 (1/1600 – 1/200). A clear, specific signal was observed, picking out expression of galectin-3 by macrophages (*arrowed*). With high antibody concentration, decreased specificity of staining was observed. All sections are shown at x200 magnification.

4.3 Test sections involving human lung neoplasms

To assess the expression of CD98 and galectin-3 in normal and pathological processes in the lung, a broad cross-section of lung biopsy and lung resection samples was identified from the University of Edinburgh Department of Pathology files. Samples dated from the period 1997–2001 and were pathology specimens identified from routine clinical practice. Control samples of normal lung were taken from non-involved lobes of lung cancer cases. All samples had been fixed in 4% paraformaldehyde and embedded in paraffin wax.

For immunohistochemistry, 3-5μm sections were dried on to slides overnight at 37°C. They then underwent deparaffinization in xylene, followed by antigen unmasking in Vector Unmasking Solution (1:500) for 3x5 minutes at 99°C. Immunohistochemistry was performed following the DAKO Techmate protocol, using the indirect streptavidin-biotin method as described in "Material and Methods". The primary antibodies used were goat polyclonal antibody to CD98 (SC-7095) at 1:100 dilution and mouse monoclonal antibody to galectin-3 (B2C10) at 1:400 dilution. Negative control sections were incubated in carrier solution only. Positive control sections were taken from reactive lymph nodes; in addition, other cell types within the same sections (especially macrophages) acted as internal positive controls. The immunostained sections were counterstained with haematoxylin.

Sections were assessed together by standard light microscopy by the author and Dr. Monaghan, a pathologist with a specialist interest in respiratory disease, and independently by a third (Dr. Salter, consultant respiratory pathologist). Where differences were identified, cases were settled by conference. Immunoreactivity for CD98 and galectin-3 was scored semi-quantitatively in areas of tumour: (a) for percentage of cells staining (absent = 0; < 20% = 1; 20-80% = 2; > 80% = 3) and (b) for strength of staining (absent = 0; weak = 1; moderate = 2; strong = 3). The cellular localization of CD98 and galectin-3, whether nuclear, cytoplasmic or membranous, was noted. The distribution of CD98 and galectin-3 in other cell types (interstitial and alveolar macrophages, lymphocytes, fibroblasts, bronchial epithelium, vascular endothelium and mesothelium) was also documented for all sections, where present.

A total of 115 samples were studied, represented in **Table 4a**. These covered the major subtypes of bronchogenic carcinoma, mesothelioma, carcinoid and a variety of inflammatory conditions. The expression of CD98 and galectin-3 observed in each group was as described below.

Table 4a: Samples studied for CD98 and galectin-3 expression

Lung condition	Sample number
Normal	5
Squamous cell carcinoma	25
well differentiated (8)	
moderately differentiated (9)	
poorly differentiated (8)	
Adenocarcinoma	22
well differentiated (10)	
moderately differentiated (4)	
poorly differentiated (8)	
Small cell carcinoma	19
pre-chemotherapy (18)	
post-chemotherapy (1)	
Carcinoid tumour	10
Mesothelioma	11
epithelioid (9)	
spindle cell (1)	
mixed cell type (1)	
Interstitial pulmonary fibrosis	4
Sarcoidosis	4
Bronchiolitis obliterans organizing pneumonia (BOOP)	5
Extrinsic allergic alveolitis	5
Langerhans cell histiocytosis	5

4.3.1 Normal Lung

All CD98 expression was membranous. It was expressed at moderate levels by both tissue macrophages and alveolar macrophages, and weakly by type II pneumocytes and bronchial epithelium. Otherwise, CD98 expression was absent in normal lung (**Figure 4.6a**), with no expression identified to any degree by type I pneumocytes, endothelium, lymphocytes, mesothelial cells or parenchymal fibroblasts.

(a) CD98

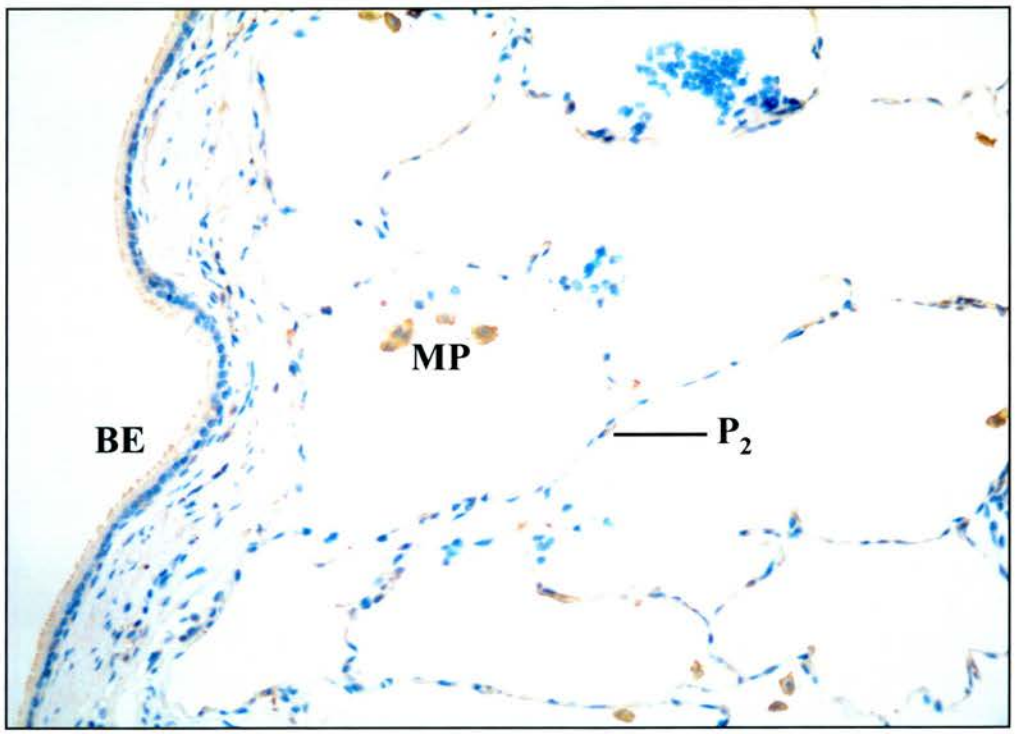


Figure 4.6(a): CD98 expression in normal lung.

Low (x200) and high (x400) power views demonstrating characteristic CD98 expression in normal lung. Expression is identified in bronchial epithelium (BE), macrophages (MP) and type II pneumocytes (P_2).

(b) Galectin-3

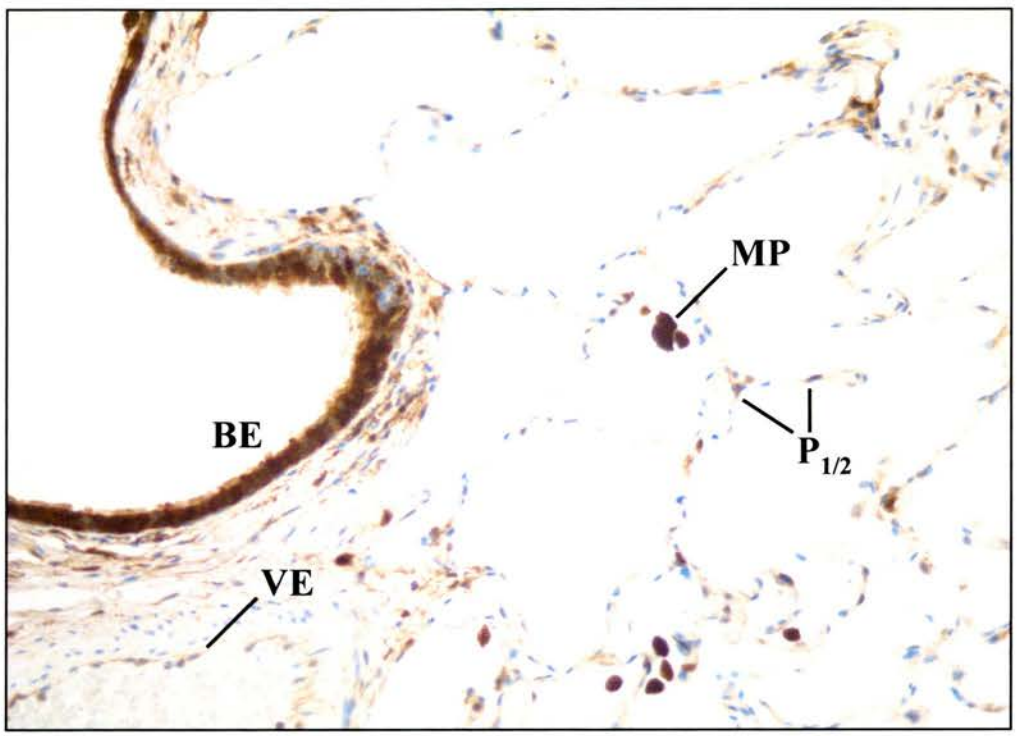


Figure 4.6(b): Galectin-3 expression in normal lung.
Low (x200) and high (x400) power views demonstrating characteristic CD98 expression in normal lung. Expression is identified in bronchial epithelium (BE), macrophages (MP), type I and II pneumocytes ($P_{1/2}$) and vascular endothelium (VE). Mesothelial cells and fibroblasts were also positive (*not demonstrated on this section*).

In normal lung, bronchial epithelium expressed galectin-3 moderately to strongly; tissue and alveolar macrophages expressed it strongly. There was weak/moderate galectin-3 expression by type II pneumocytes and weak expression by type I pneumocytes, mesothelial cells, interstitial fibroblasts and vascular endothelium (**Figure 4.6b**).

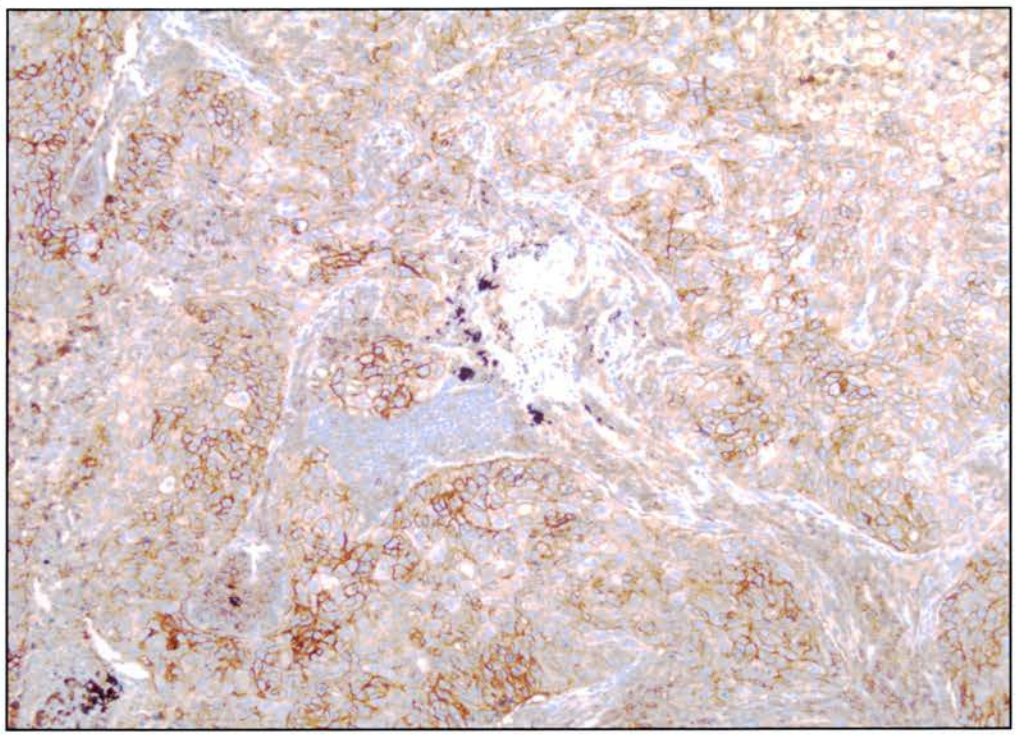
These general observations of CD98 and galectin-3 expression in normal lung held true for non-involved lung in all subsequent sections.

4.3.2 Squamous cell carcinoma

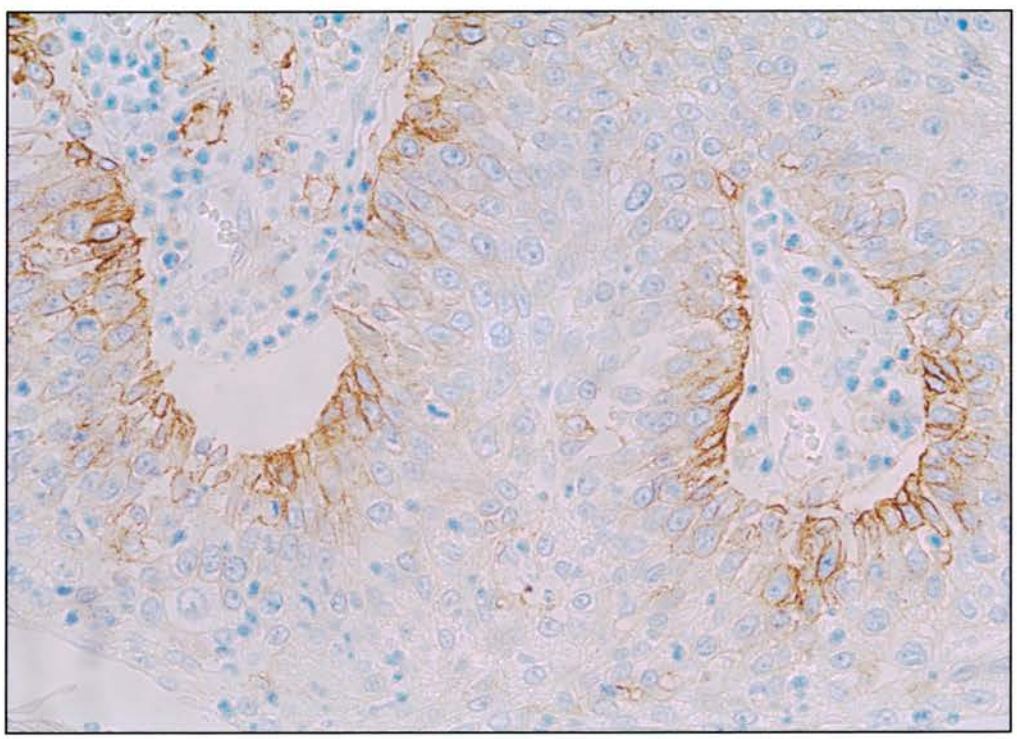
CD98 expression was membranous and was expressed in 25 of the 26 squamous cell carcinoma specimens. Strength of expression was moderate / strong (mean score = 2.42) by the majority of cells (mean score = 2.87). CD98 expression exhibited a degree of "zoning", in that it was most strongly expressed in the basal layer cells of carcinomas, diminishing with distance from the basement membrane (**Figure 4.7a**). In addition, in the most well differentiated squamous cell carcinomas, which exhibited areas of keratinization, CD98 expression was noted to diminish within keratin pearls. Overall, CD98 expression by squamous cell carcinomas was significantly different from that exhibited by both adenocarcinomas and carcinoid tumours ($p<0.001$).

Galectin-3 expression was predominantly cytoplasmic and nuclear, and was seen in the majority of cases (20/25). Overall, strength of expression was moderate (mean score = 1.50). Membranous expression was noted in some regions. There was a suggestion of

(a) CD98



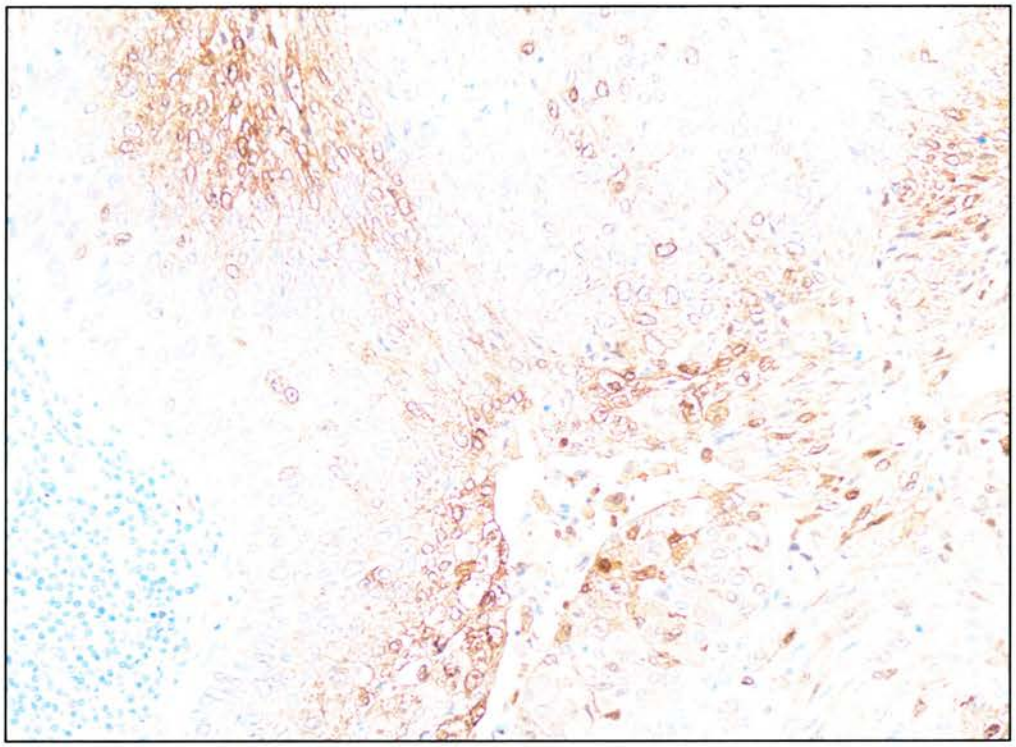
x 100



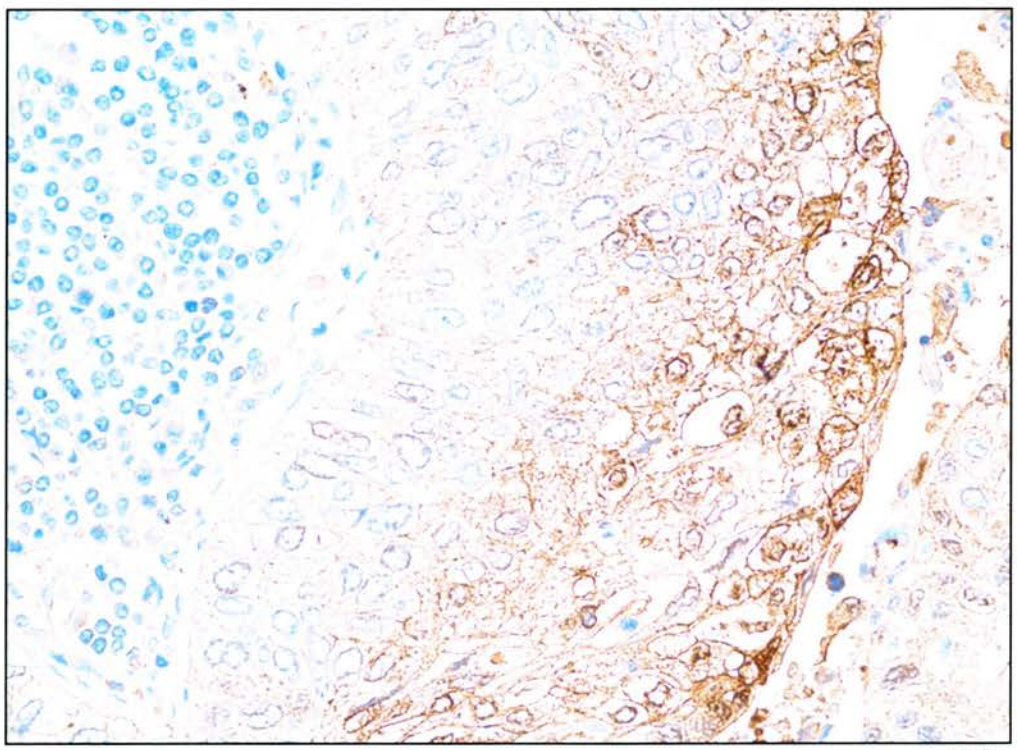
x 400

Figure 4.7(a): CD98 expression in squamous cell carcinoma.
Low (x100) and high (x400) power views demonstrating characteristic CD98 expression in squamous cell carcinoma. Expression was greatest in basal layers, diminishing with distance from the basement membrane (*high power view*).

(b) Galectin-3



x 200



x 400

Figure 4.7(b): Galectin-3 expression in squamous cell carcinoma.
Low (x100) and high (x400) power views demonstrating characteristic galectin-3 expression in squamous cell carcinoma. Expression was greatest towards areas of central necrosis and keratin pearls.

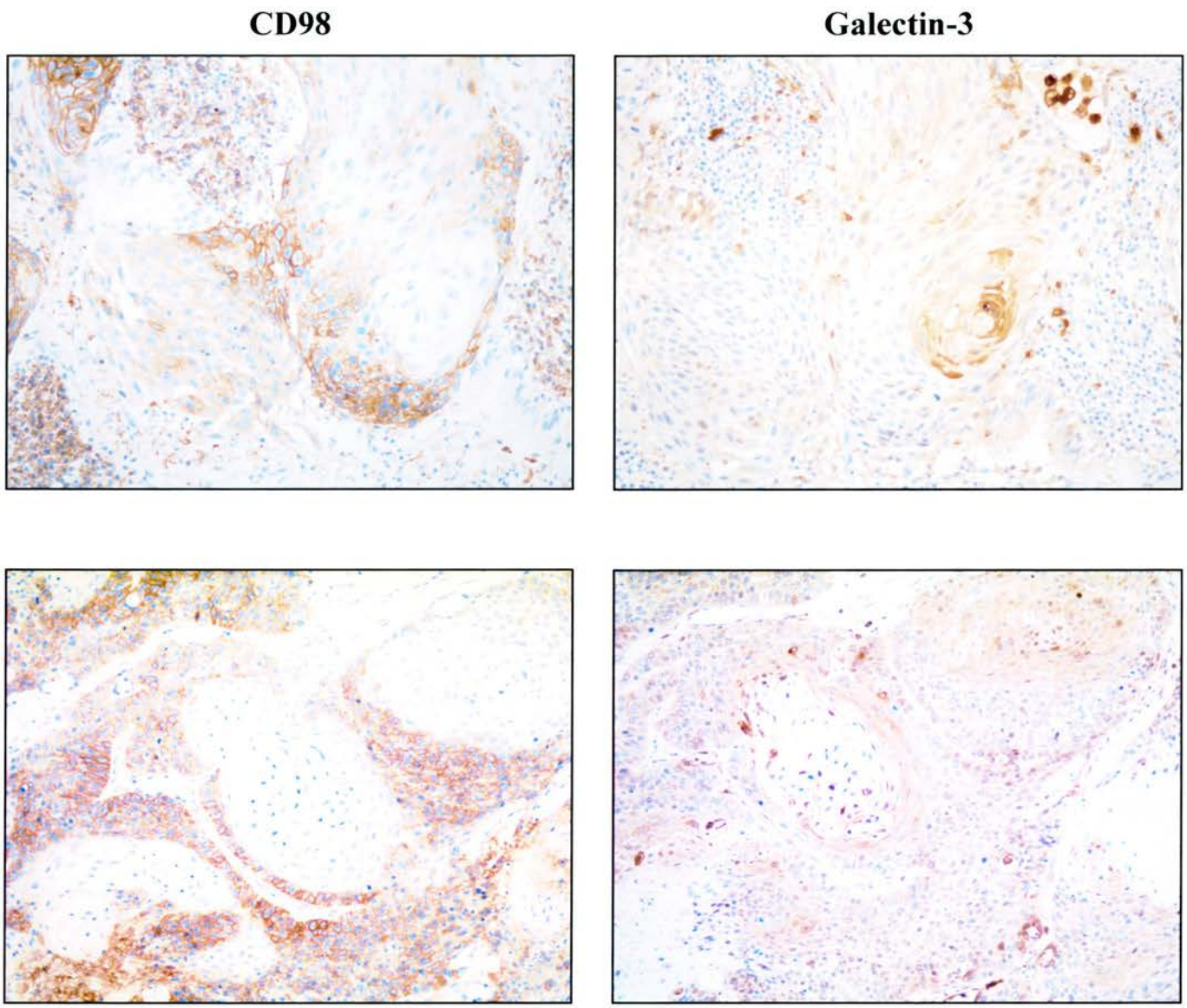


Figure 4.7(c): Complementary expression of CD98 and galectin-3 in squamous cell carcinoma. Intermediate (x200) power views of paired samples of well-differentiated squamous cell carcinoma. CD98 expression tends to diminish within keratin pearls, whereas galectin-3 expression is upregulated in these regions.

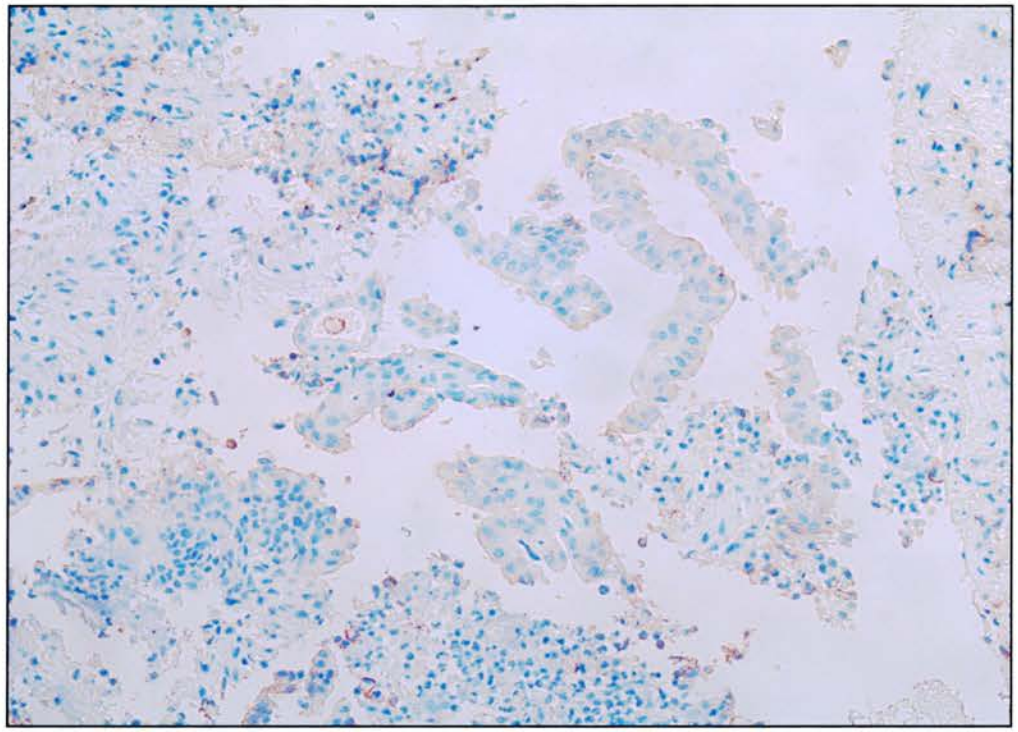
differential expression between well and poorly differentiated carcinomas, with higher expression in well-differentiated carcinomas (mean score = 2.06) and weaker expression in poorly differentiated samples (mean score = 1.43), but this did not achieve significance. Zoning was also evident in the pattern of galectin-3 expression (**Figure 4.7b**), but by contrast with CD98, galectin-3 was increasingly expressed by tumour cells towards areas of central necrosis and within keratin pearls, such that CD98 and galectin-3 expression appeared complementary (**Figure 4.7c**). Overall, galectin-3 expression by squamous cell carcinomas was significantly different from that exhibited by SCLC and carcinoid tumours ($p<0.01$).

Thus, in squamous cell carcinomas, CD98 expression was broadly upregulated, with a tendency to reduced expression in the more differentiated, keratinised areas. By contrast, galectin-3, although highly expressed, tended to be highest in the most well differentiated samples.

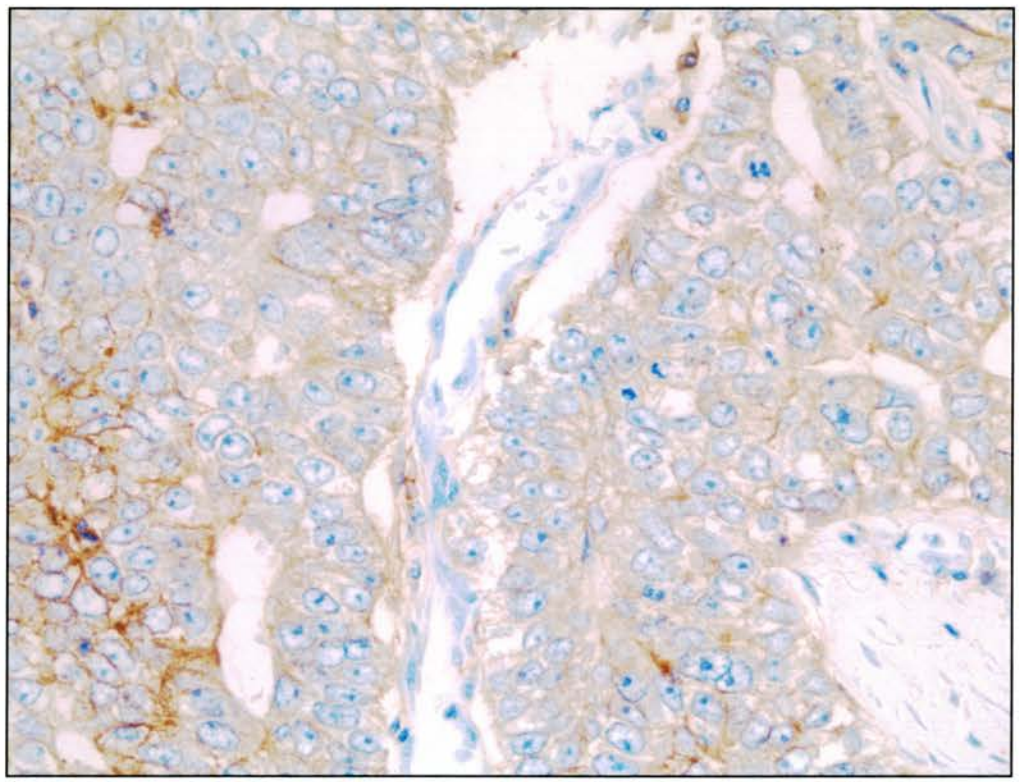
4.3.3 Adenocarcinoma

Of 22 specimens, 20 adenocarcinomas expressed CD98 in a membranous pattern to varying levels (mean strength score = 1.32), and by the majority of tumour cells in each sample (mean score = 2.53) (**Figure 4.8a**). CD98 expression by adenocarcinomas was significantly different from expression by SCLC ($p<0.05$) and significantly different from CD98 expression by squamous cell carcinomas ($p<0.001$).

(a) CD98



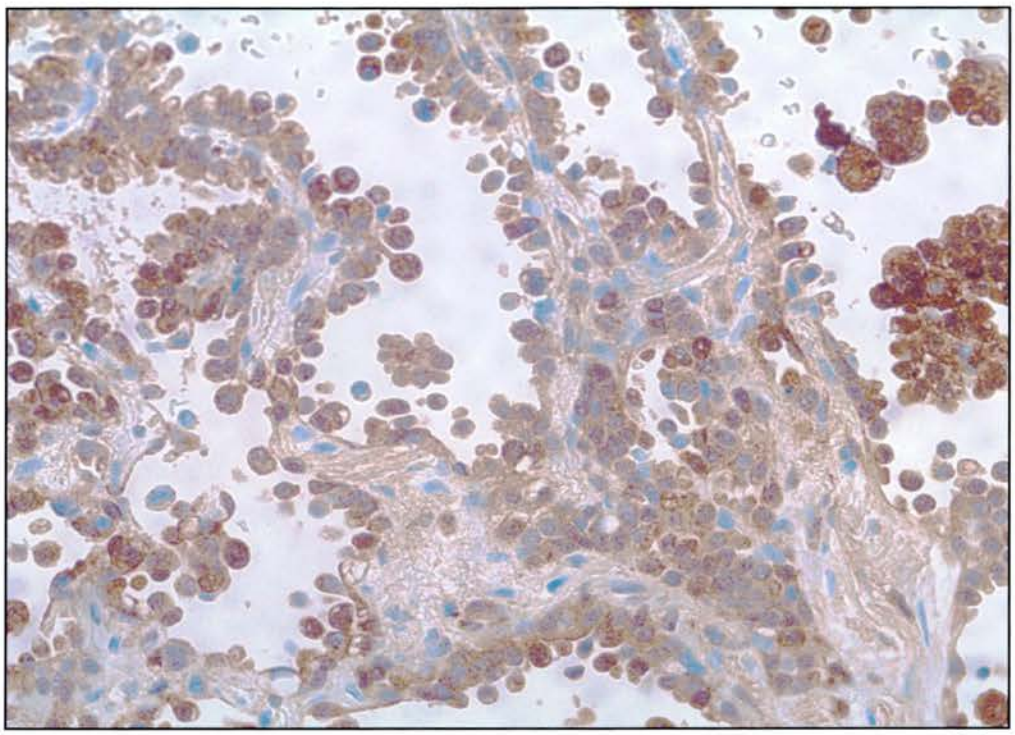
x 200



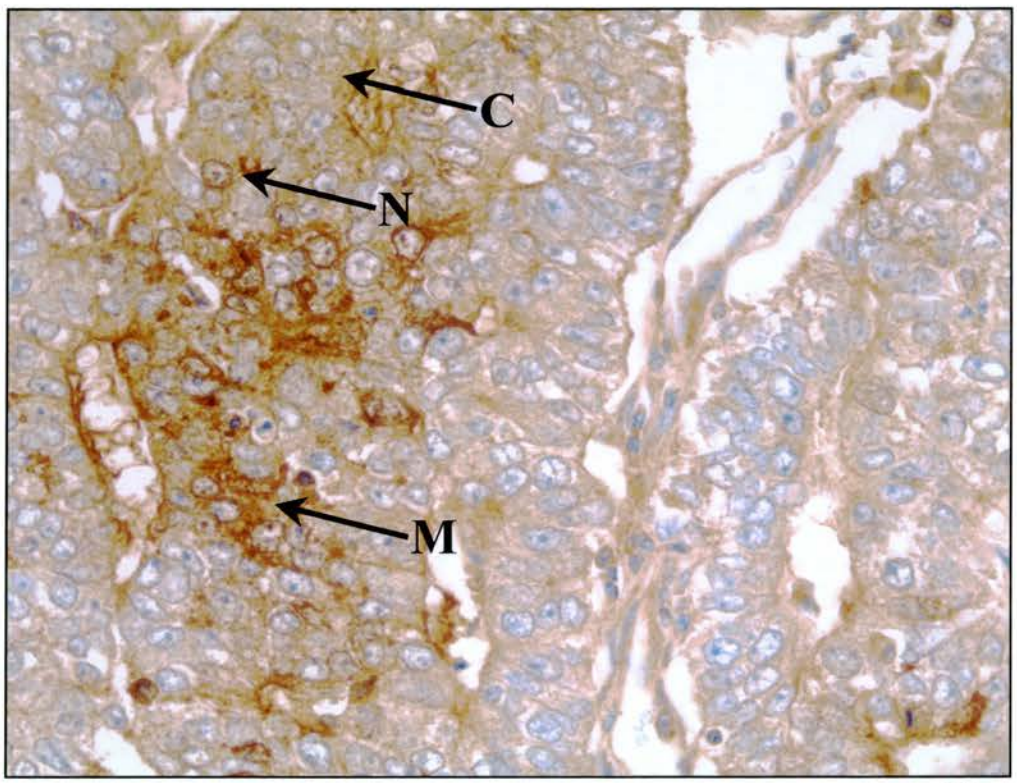
x 400

Figure 4.8(a): CD98 expression in adenocarcinoma.
Intermediate (x200) and high (x400) power views demonstrating characteristic membranous CD98 expression in adenocarcinoma.

(b) Galectin-3



x 400



x 400

Figure 4.8(b): Galectin-3 expression in adenocarcinoma.

High (x400) power views demonstrating characteristic galectin-3 expression in adenocarcinoma. Bronchoalveolar cell carcinomas sometimes exhibited a classical lepidic growth pattern (*upper panel*). Galectin-3 expression was observed in nuclear (N), cytoplasmic (C) and membranous (M) compartments (*lower panel*).

The strength of galectin-3 expression by adenocarcinomas was moderate to high (mean score = 2.36) and in all cases expression was seen in the majority of cancerous cells (mean score = 3). Galectin-3 was again expressed in a predominantly cytoplasmic and nuclear pattern (**Figure 4.8b**). There was no discernible difference between well and poorly differentiated adenocarcinomas. Overall, galectin-3 expression by adenocarcinomas was significantly different from galectin-3 expression by SCLC and carcinoid tumours ($p<0.001$).

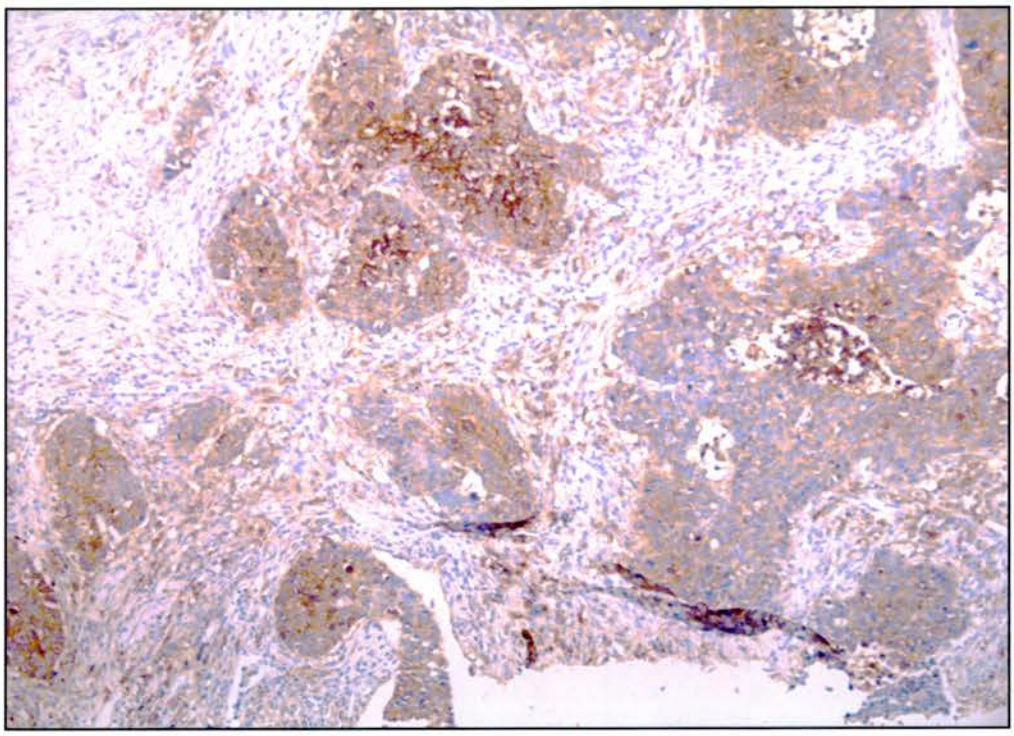
Therefore, in adenocarcinomas, CD98 and galectin-3 are both highly expressed.

4.3.4 Small cell carcinoma

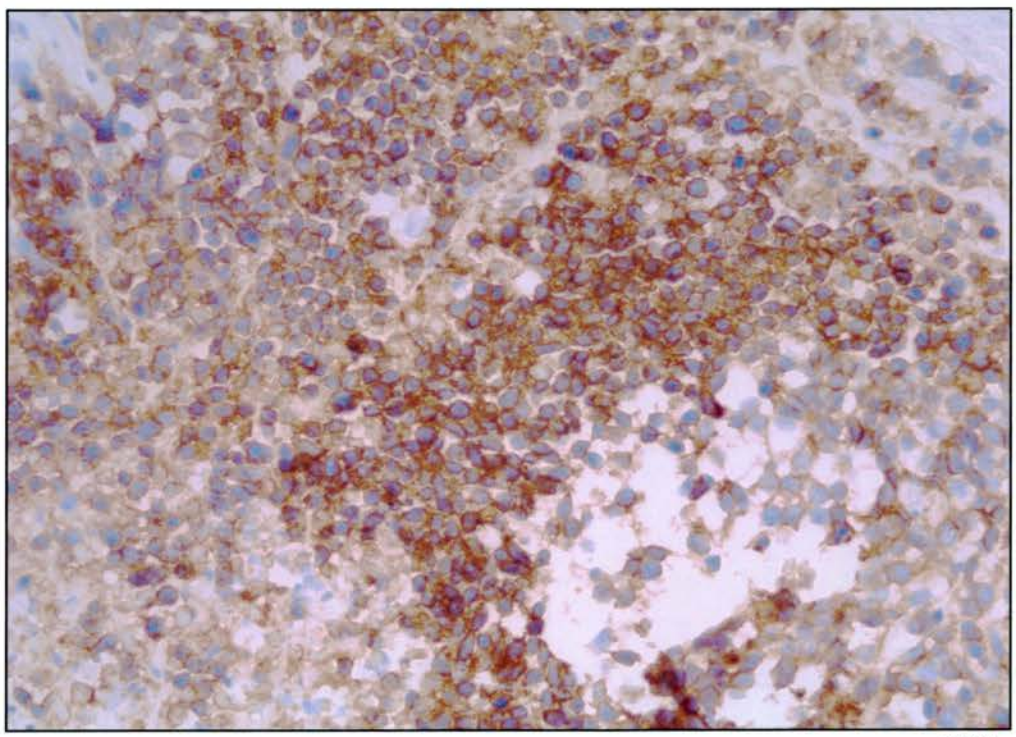
One case of SCLC represented a tumour sample taken post-chemotherapy. This was considered separately from all the other SCLC cases, which were taken pre-chemotherapy. All these small cell carcinomas expressed CD98 in a membranous pattern, the majority to high levels (mean score = 2.23) and by the majority of the carcinoma cells in each sample (mean score = 2.85) (**Figure 4.9a**). This was significantly different from CD98 expression observed in both adenocarcinomas ($p<0.05$) and carcinoid tumours ($p<0.01$).

Galectin-3 expression was negative in the majority (13/18) of SCLC cases (mean strength score = 0.28) (**Figure 4.9b**). Of the five that exhibited any galectin-3 expression, all expressed galectin-3 only extremely weakly. Overall, galectin-3

(a) CD98



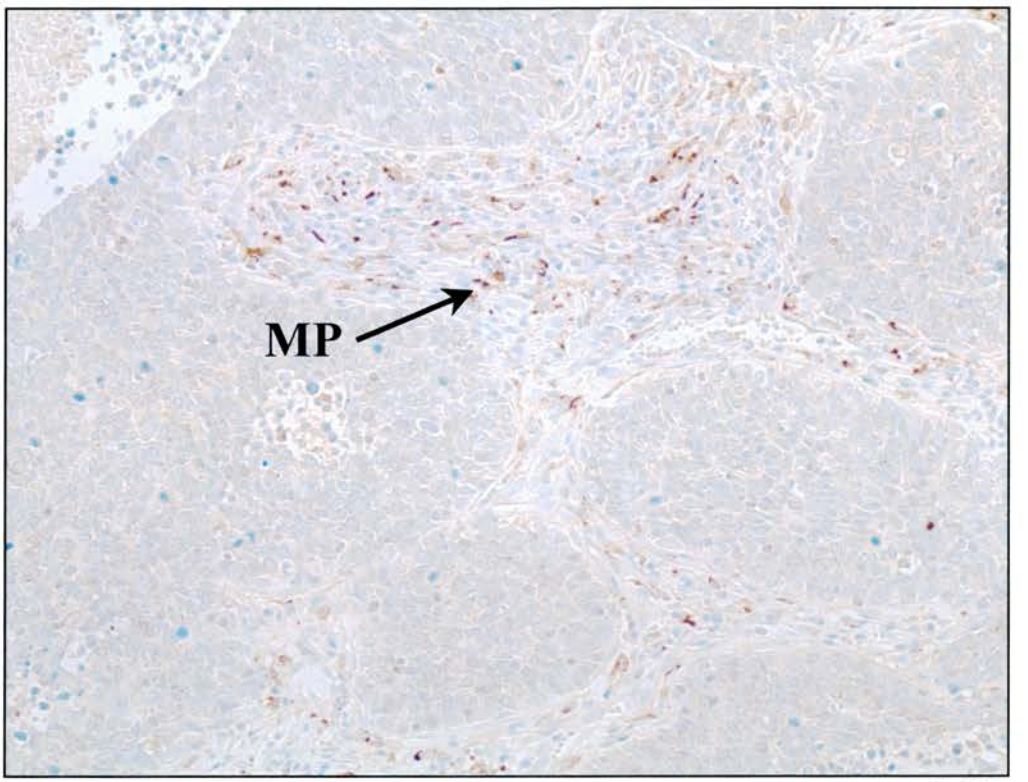
x 100



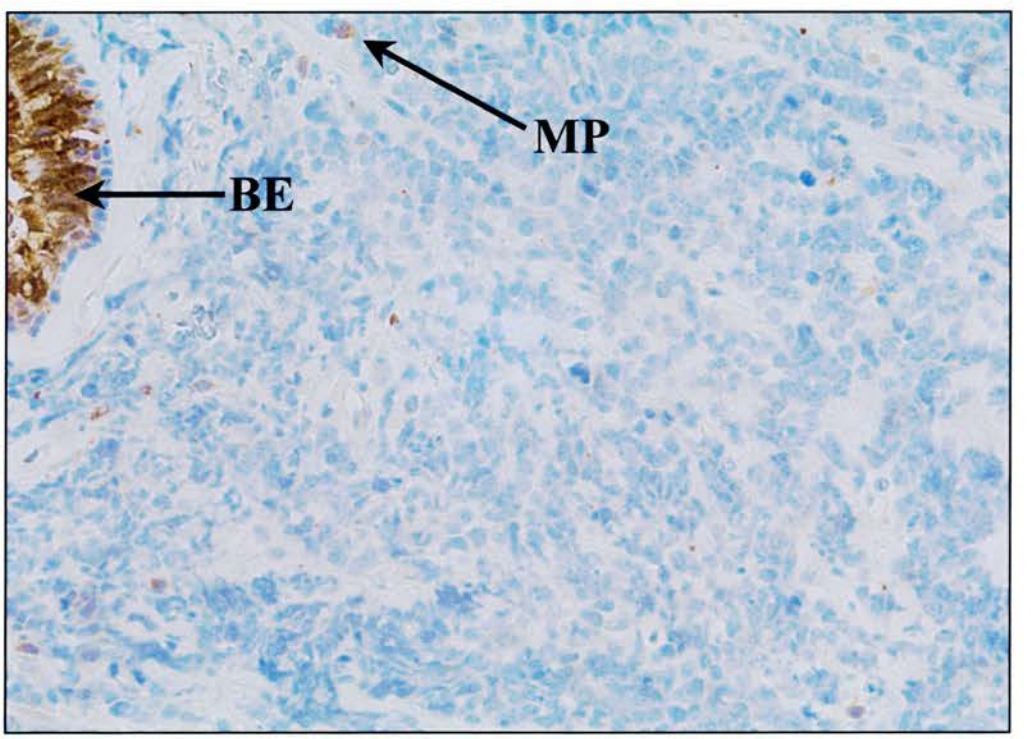
x 400

Figure 4.9(a): CD98 expression in small cell carcinoma.
Low (x100) and high (x400) power views demonstrating characteristic membranous CD98 expression in SCLC.

(b) Galectin-3



x 200



x 400

Figure 4.9(b): Galectin-3 expression in small cell carcinoma.
Intermediate (x200) and high (x400) power views demonstrating characteristic galectin-3 expression in SCLC. Internal controls include macrophages (MP) and bronchial epithelium (BE).

expression by SCLC was significantly different from that observed in squamous cell carcinomas ($p<0.01$) and significantly different from adenocarcinomas ($p<0.001$).

In SCLC, therefore, although CD98 expression is uniformly high, galectin-3 expression is absent or only at very low levels. This is in contrast to the situation seen in NSCLC.

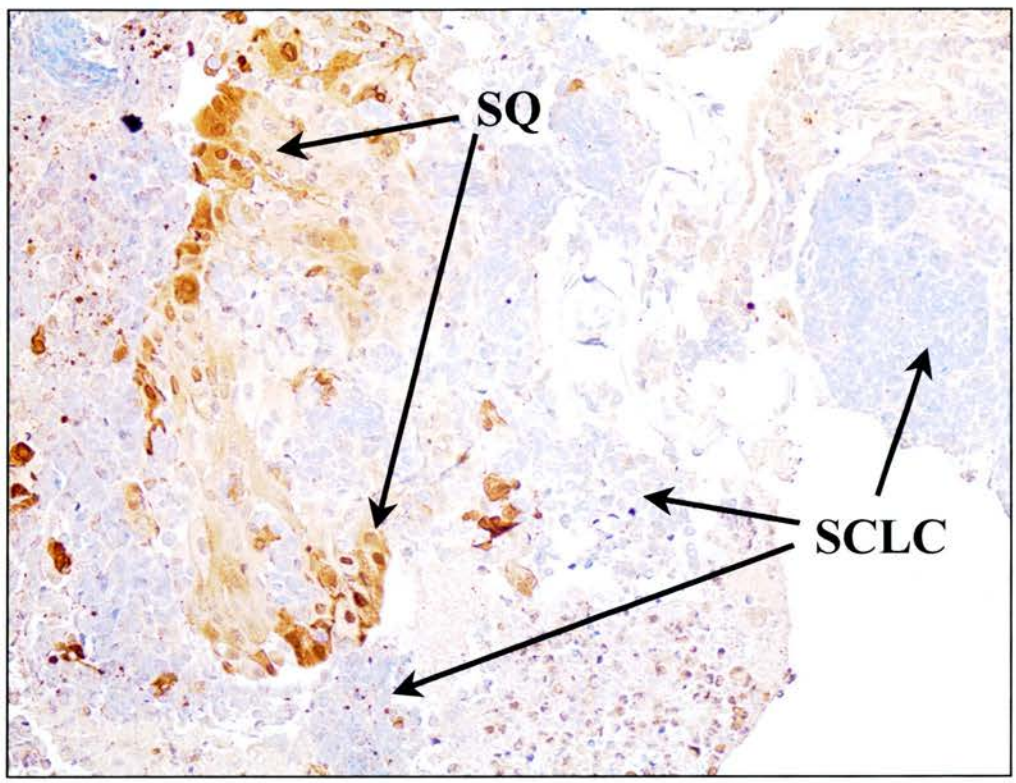
One case of SCLC represented a mixed tumour. Within this, the areas of small cell phenotype were negative for galectin-3, whilst areas of squamoid differentiation exhibited high galectin-3 expression (**Figure 4.10**). This suggests that galectin-3 may be useful in histological assessment of such mixed tumours, by clearly discriminating between SCLC and NSCLC populations within the tumour.

A further case of SCLC was identified that differed from all others in that, for clinical reasons, the biopsy had been obtained after a full course of treatment with chemotherapy. This was the only post-chemotherapy sample examined. Although there was no difference in CD98 expression from other SCLC cases, all malignant cells in this sample exhibited very strong galectin-3 expression, which contrasted markedly with all other SCLC cases (**Figure 4.11**). This was reproducible on repeated immunostaining.

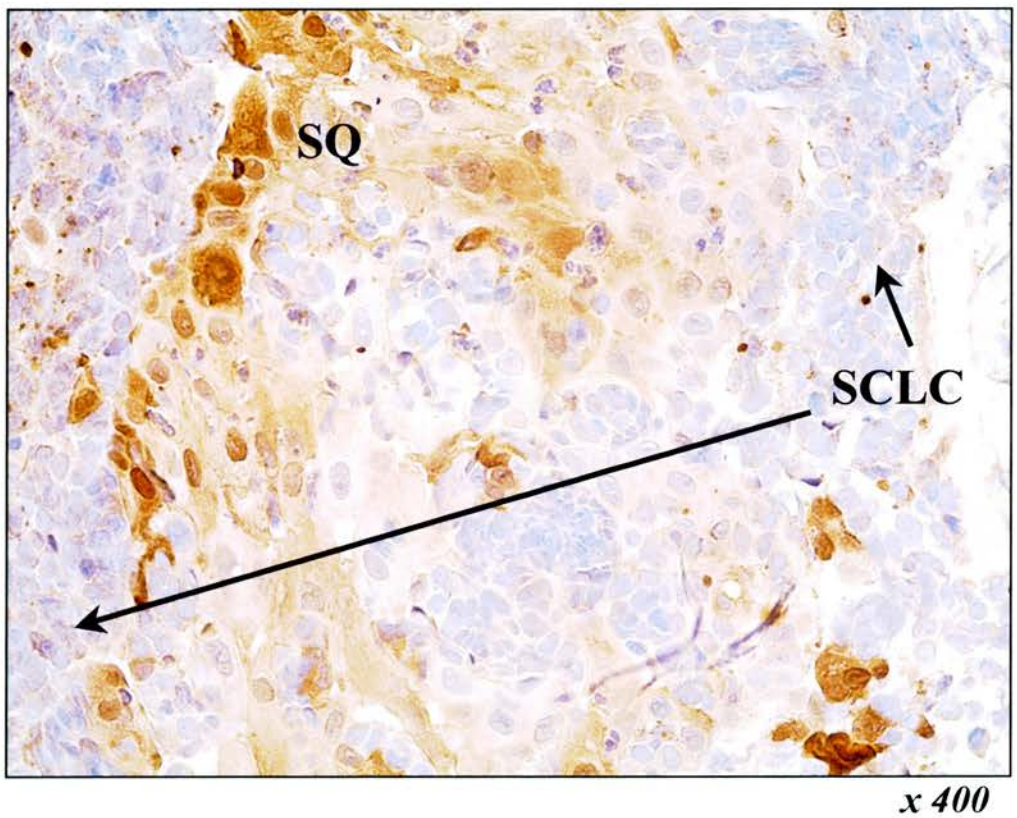
4.3.5 Carcinoid tumour

CD98 expression by carcinoid tumours was focally weak, where present at all (mean score = 0.65) (**Figure 4.12a**). This was significantly different from CD98 expression

Galectin-3



x 200



x 400

Figure 4.10: Galectin-3 expression in a mixed tumour.

Intermediate (x200) and high (x400) power views of a mixed tumour, in which areas of small cell phenotype (SCLC) exhibit low/absent galectin-3 expression, in contrast to areas of squamoid phenotype (SQ), where galectin-3 expression is high.

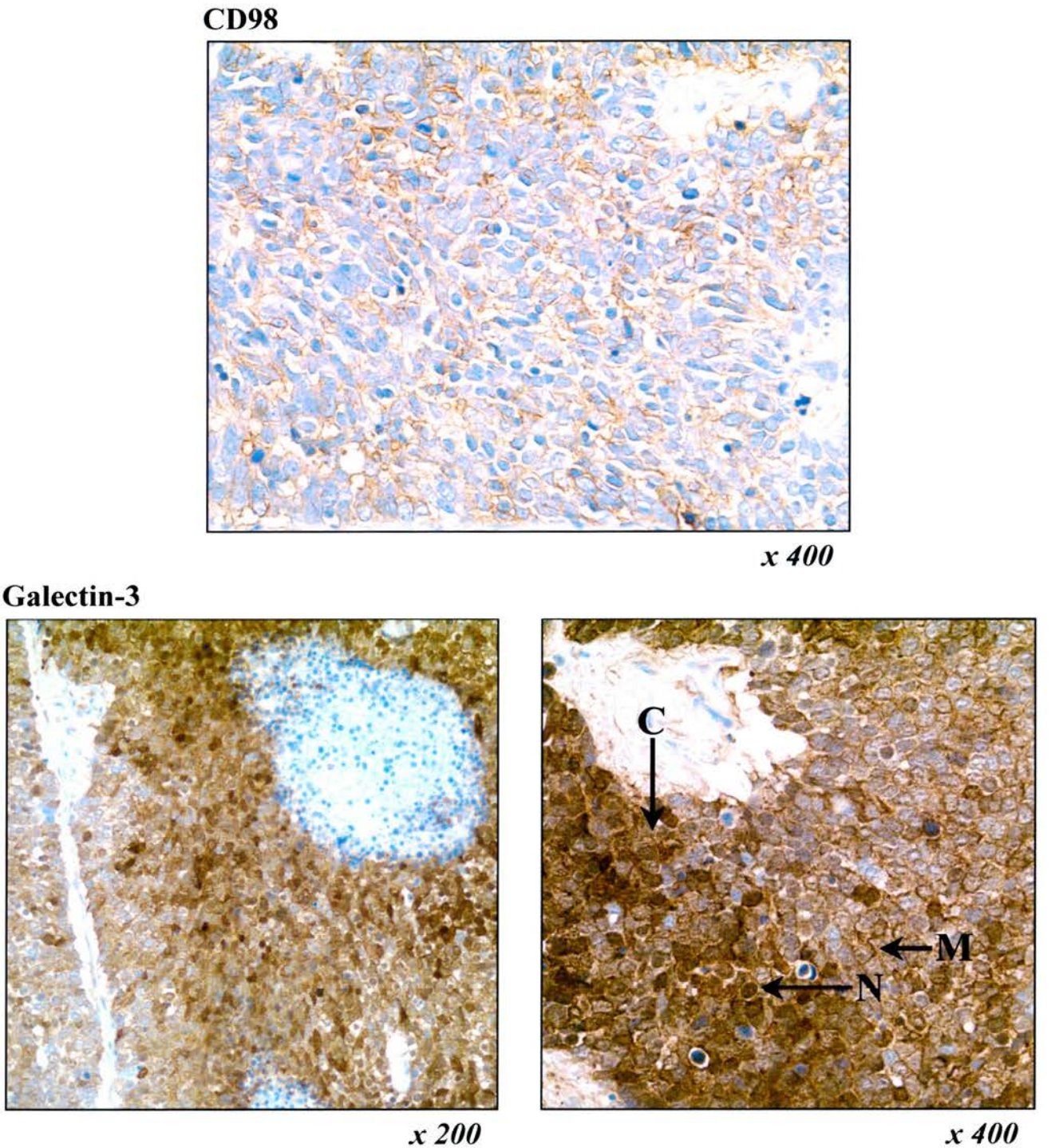
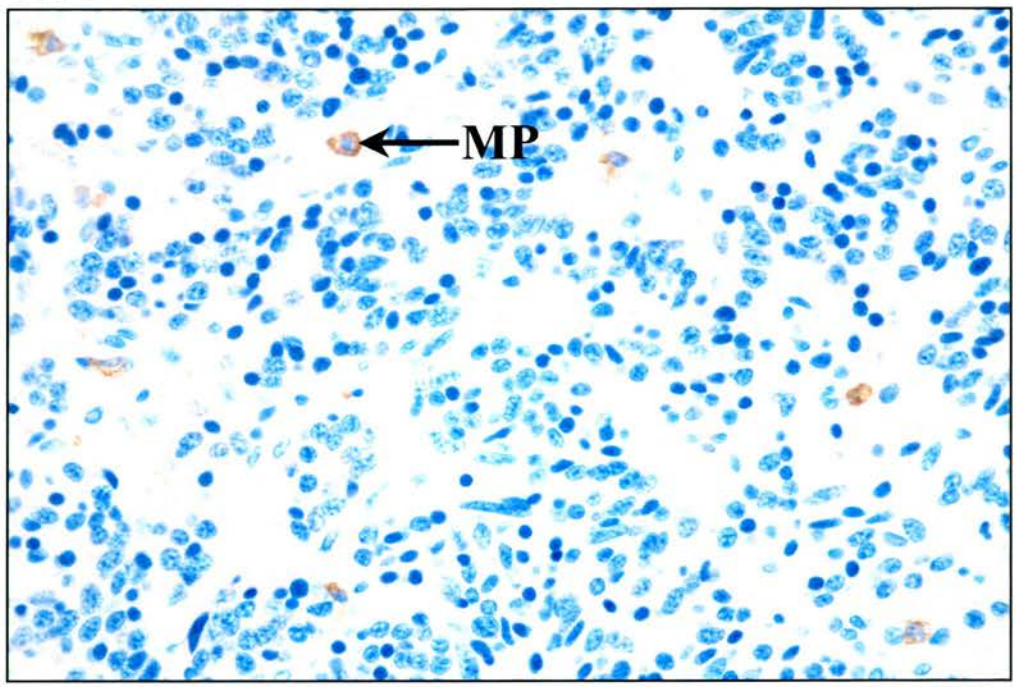


Figure 4.11: CD98 and galectin-3 expression in small cell carcinoma post-chemotherapy.
Intermediate (x200) and high (x400) power views of a SCLC sample, taken following chemotherapy. CD98 expression is membranous (*upper panel*); in contrast to other SCLC samples, galectin-3 expression is extremely strong (*lower panels*) with expression seen in cytoplasmic (C), nuclear (N) and membranous (M) compartments.

(a) CD98



(b) Galectin-3

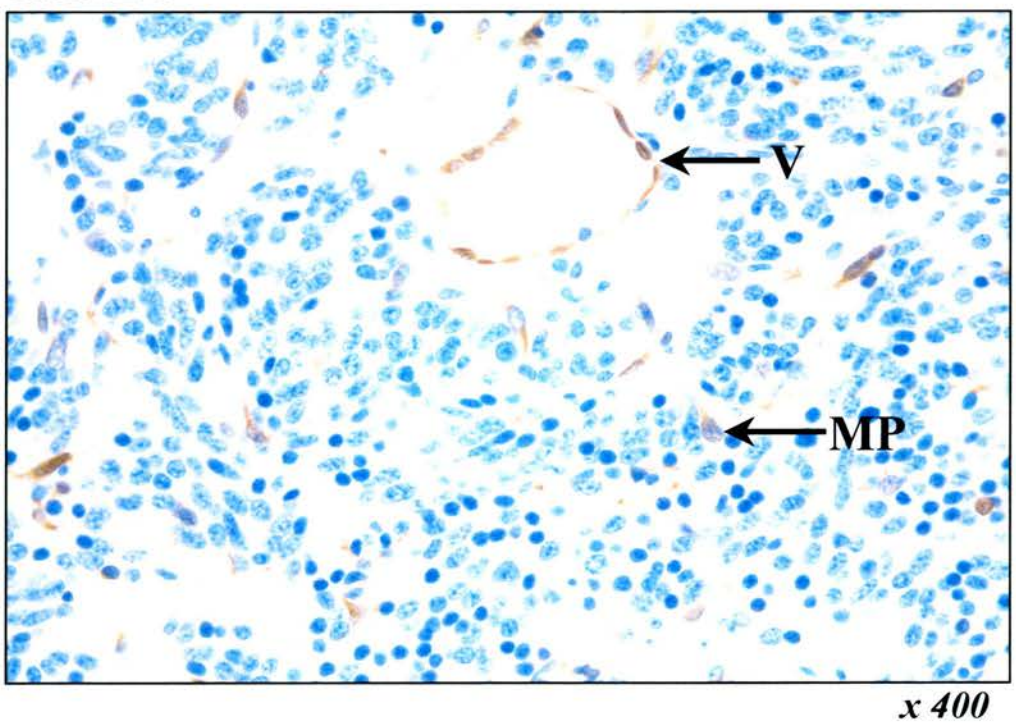


Figure 4.12: CD98 and galectin-3 expression in carcinoid tumour.
High (x400) power views of carcinoid tumour. CD98 expression was minimal or absent (a) and galectin-3 expression was absent (b). Macrophages (MP) and vascular endothelial cells (V) acted as internal positive controls for immunohistochemistry in this highly vascular tumour.

seen in SCLC ($p<0.01$) and significantly different from squamous cell carcinomas ($p<0.001$).

Galectin-3 expression by carcinoid tumour cells was negative in all cases (mean score = 0) (**Figure 4.12b**). This is significantly different from galectin-3 expression by squamous cell carcinomas ($p<0.01$) and significantly different from adenocarcinomas ($p<0.001$).

Thus, despite a neuroendocrine origin like SCLC, carcinoid tumours have a different CD98 / galectin-3 phenotype.

4.3.6 Small cell lung cancer exhibits different galectin-3 expression compared with non-small cell carcinoma

All types of aggressive lung tumour exhibited upregulated expression of CD98 to a varying degree. However, comparing galectin-3 expression between the different phenotypes of bronchogenic tumours, it was clear that there was a striking difference between SCLC and NSCLC. Whereas all NSCLC phenotypes expressed galectin-3 moderately or strongly, SCLC tumours expressed galectin-3 only very weakly, if at all. Carcinoid tumours expressed neither protein to significant levels. **Figure 4.13** summarises the strengths of expression of CD98 and galectin-3 exhibited by the different forms of bronchogenic tumour.

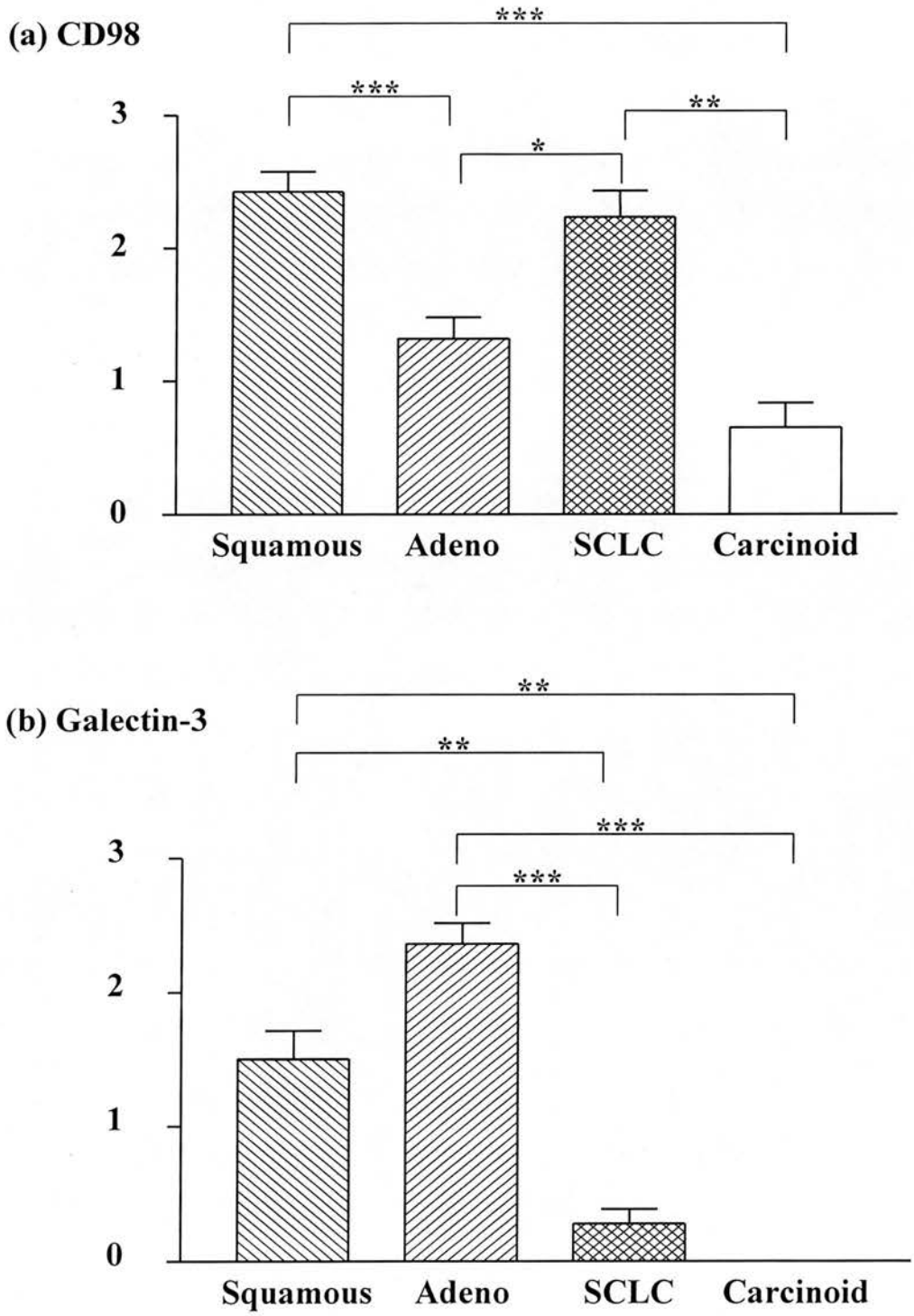


Figure 4.13: CD98 and galectin-3 expression by bronchogenic tumours.
Graphical summary of the relative strengths of (a) CD98 expression and (b) galectin-3 expression of squamous cell carcinoma, adenocarcinoma, SCLC and carcinoid tumour. Bars represent the mean +/- standard error.

4.3.7 Mesothelioma

Eleven mesothelioma samples were studied, of which 9 were of epithelioid subtype, one of spindle cell subtype and one of mixed cell type. There was no apparent difference between the different cell types in either CD98 or galectin-3 expression. CD98 expression by mesothelioma cells was weak / moderate (mean expression score = 1 ± 0.2); of those tumours expressing CD98, a variable percentage of tumour cells were expressing the protein (mean percentage score = 2 ± 0.3) (**Figure 4.14a**).

Galectin-3 expression by mesotheliomas was weak / moderate (mean expression score = 1.2 ± 0.3), although in those tumours expressing galectin-3, the majority of cells did so (mean percentage score = 2.9 ± 0.1) (**Figure 4.14b**).

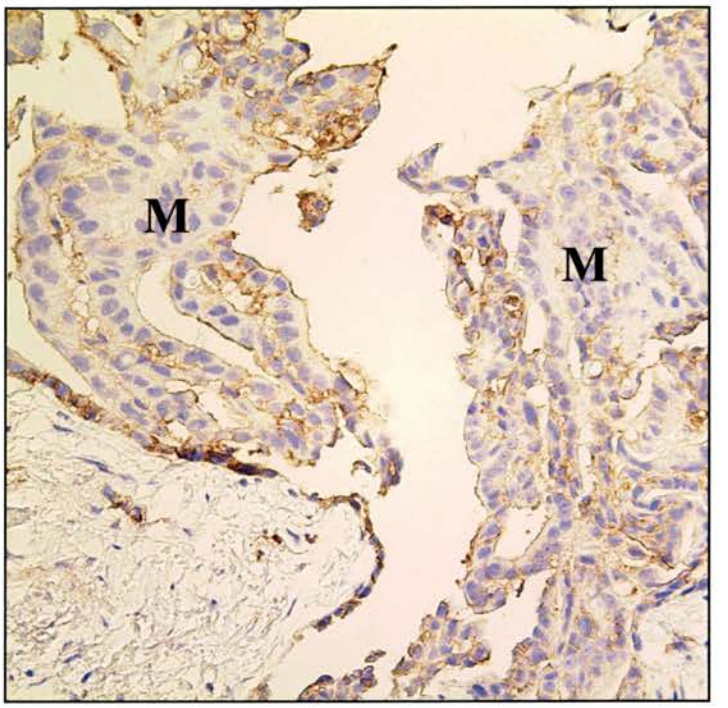
4.4 Test sections involving inflammatory lung conditions

Tumour cells were not present in the majority of these samples. Assessment of these sections therefore focused on the cells making up the inflammatory interstitium.

4.4.1 Idiopathic pulmonary fibrosis

Interstitial cells in IPF were not prominent in our histology sections. Those that were present expressed no CD98, but expressed galectin-3 to weak levels (mean score = 0.75 ± 0.3) (**Figure 4.15a and b**).

(a) CD98



(b) Galectin-3

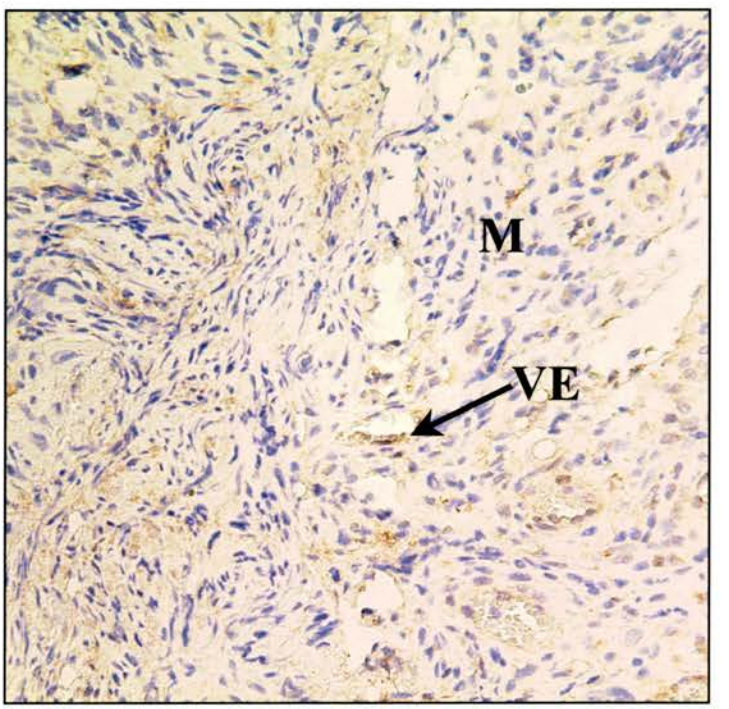
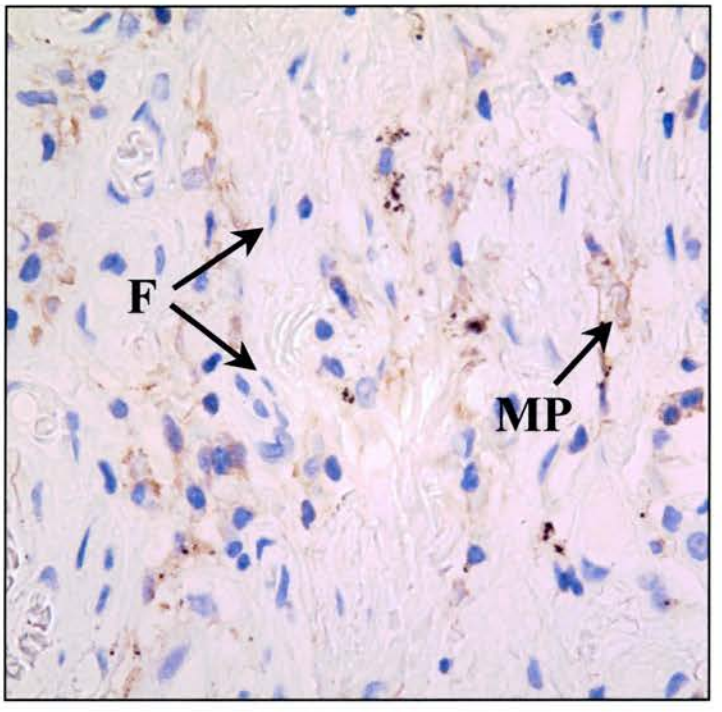


Figure 4.14: CD98 and galectin-3 expression in malignant mesothelioma.
(a) CD98 expression was membranous, but only weak/moderate in tumour cells (M).
(b) Galectin-3 expression was largely cytoplasmic and weak/moderate. Vascular endothelial cells acted as a positive internal control (VE).
Sections shown at x200 magnification.

(a) CD98



(b) Galectin-3

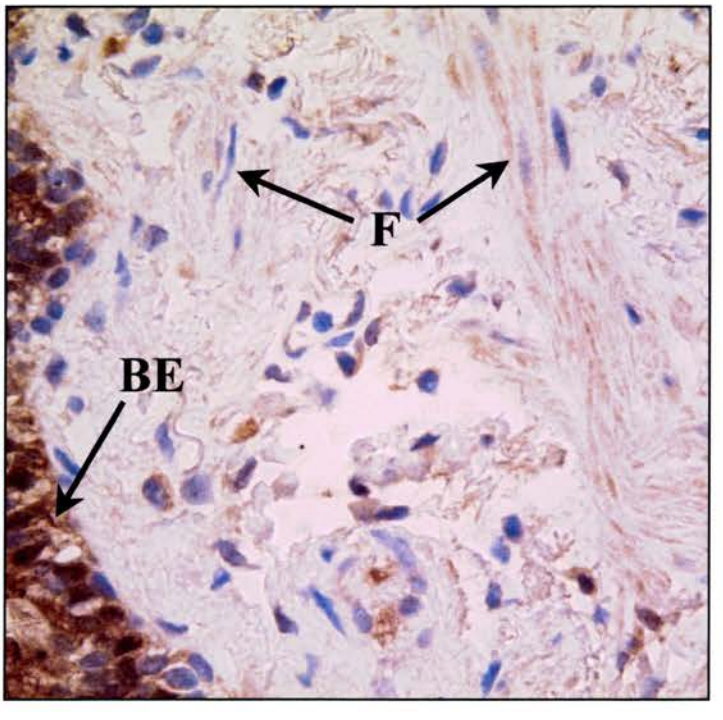


Figure 4.15: CD98 and galectin-3 expression in interstitial pulmonary fibrosis.

No tumour cells were present in these sections and assessment was made of the cells that predominated in the interstitial disease process.

(a) CD98 expression was largely absent, with no convincing expression by fibroblasts (F), the predominant cells in the interstitium. Occasional macrophages (MP) acted as internal positive controls.

(b) Galectin-3 expression was largely cytoplasmic and weakly expressed by fibroblasts. Bronchial epithelial cells (BE) expressed galectin-3 strongly in nuclear, cytoplasmic and membranous compartments and acted as positive internal controls together with vascular endothelial cells.

Sections shown at x400 magnification.

4.4.2 Sarcoidosis

The predominant interstitial changes seen in sarcoidosis were the presence of non-caseating granulomas. In all cases the epithelioid giant cells that constituted the granulomas expressed low levels of CD98 (mean score = 1 ± 0.0), whereas other interstitial cells, namely fibroblasts, were negative for CD98. All granulomatous giant cells expressed moderate levels of galectin-3 (mean score = 2 ± 0.0), but interstitial fibroblasts were negative in 2 of the three samples and not seen in the third (**Figure 4.16a and b**).

4.4.3 Bronchiolitis obliterans organizing pneumonia (BOOP)

Interstitial cells were visualized in these sections, but did not express CD98 (mean score = 0). In all sections, these interstitial fibroblasts expressed galectin-3 weakly (mean score = 1 ± 0.0) (**Figure 4.17a and b**).

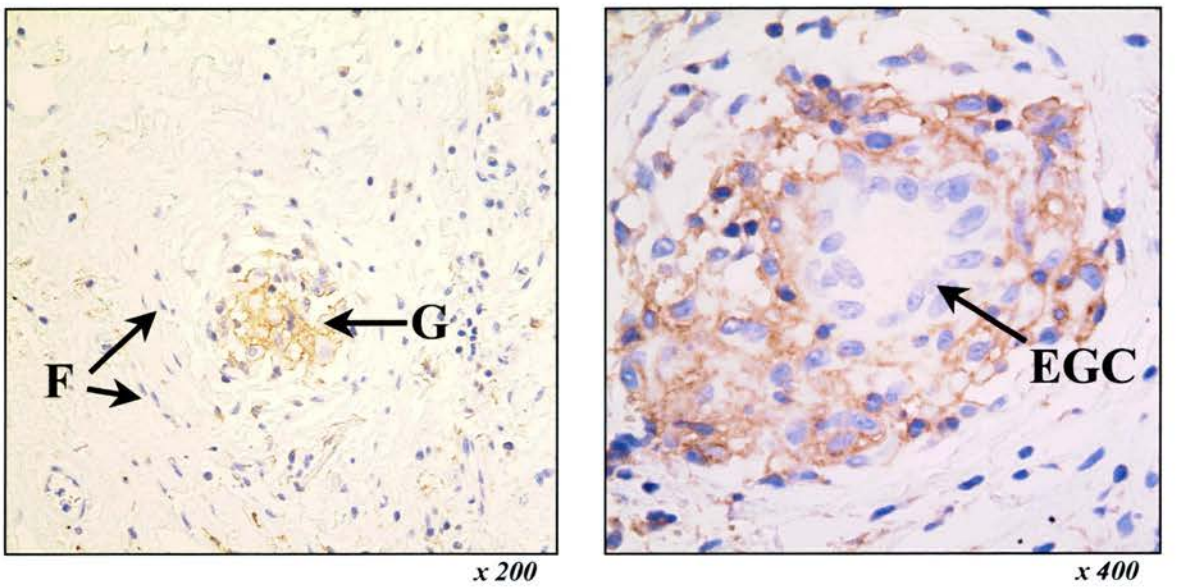
4.4.4 Extrinsic allergic alveolitis

Interstitial cells did not express CD98 in EAA, and expressed galectin-3 weakly in only 2 out of the five samples (mean score = 0.4 ± 0.2) (**Figure 4.18a and b**).

4.4.5 Langerhans cell histiocytosis

In Langerhans cell histiocytosis characteristic tumour cells were seen. In these samples there was weak/moderate expression of CD98 by the tumour cells (mean score = 1.2 ± 0.4), although the interstitial cells expressed no CD98. Galectin-3 was moderately

(a) CD98



(b) Galectin-3

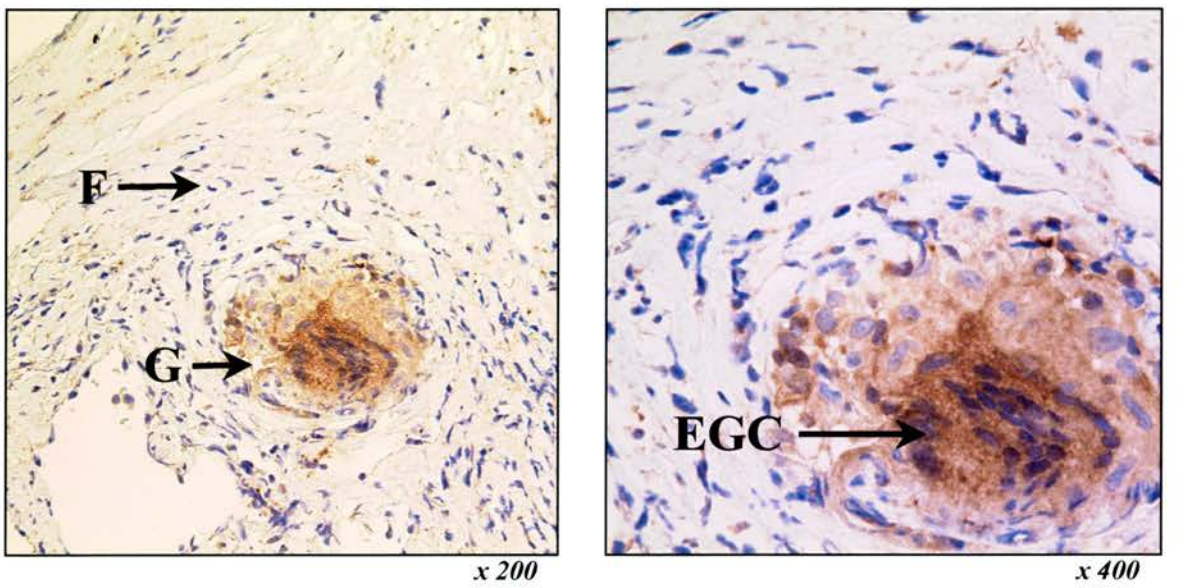


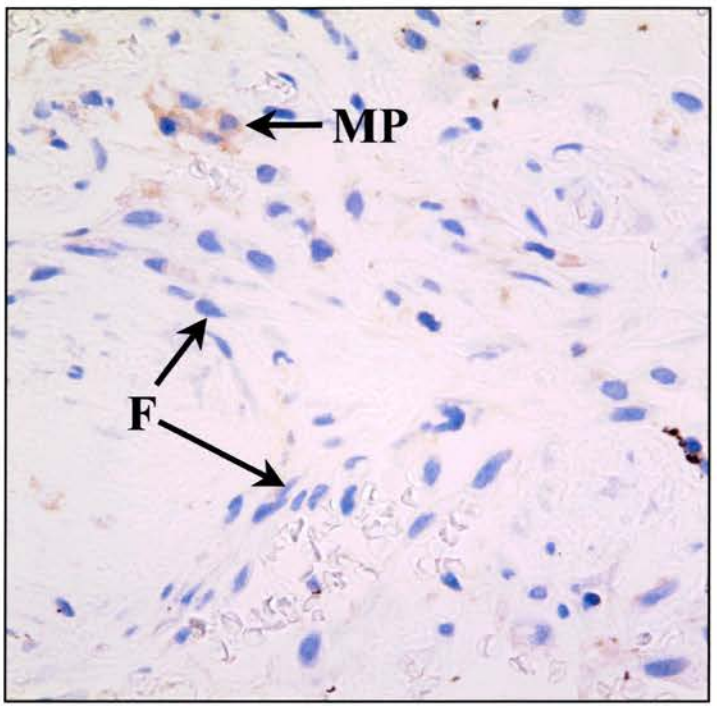
Figure 4.16: CD98 and galectin-3 expression in sarcoidosis.

No tumour cells were present in these sections and assessment was made of the cells that predominated in the interstitial disease process.

(a) CD98 expression was generally absent from fibroblasts (F), the predominant cells in the interstitium. However, non-caseating granulomas (G) with macrophages and multinucleated epithelioid giant cells (EGC) demonstrated strong membranous CD98 expression.

(b) Galectin-3 expression was absent in fibroblasts, but strong in multinucleated epithelioid giant cells.

(a) CD98



(b) Galectin-3

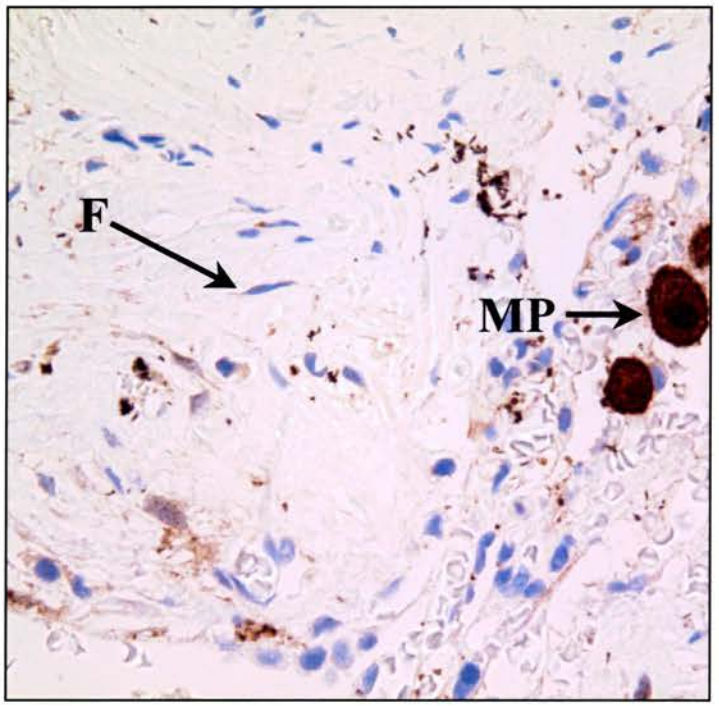
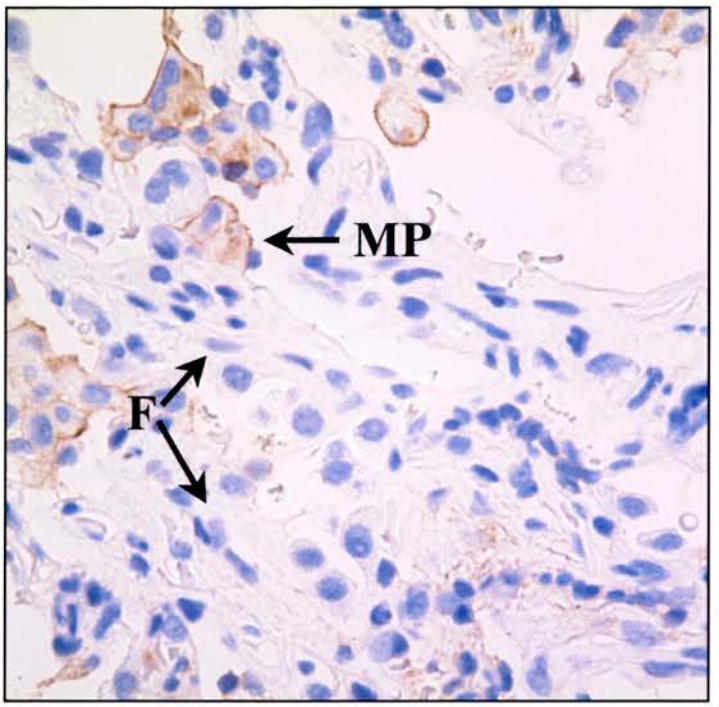


Figure 4.17: CD98 and galectin-3 expression in bronchiolitis obliterans organising pneumonia.
No tumour cells were present in these sections and assessment was made of the cells that predominated in the interstitial disease process.
(a) CD98 expression was absent from fibroblasts (F), the predominant cells in the interstitium. Macrophages (MP) provided an internal positive control.
(b) Galectin-3 expression was present at low levels in fibroblasts, but there was strong expression by macrophages.
Sections shown at x400 magnification.

(a) CD98



(b) Galectin-3

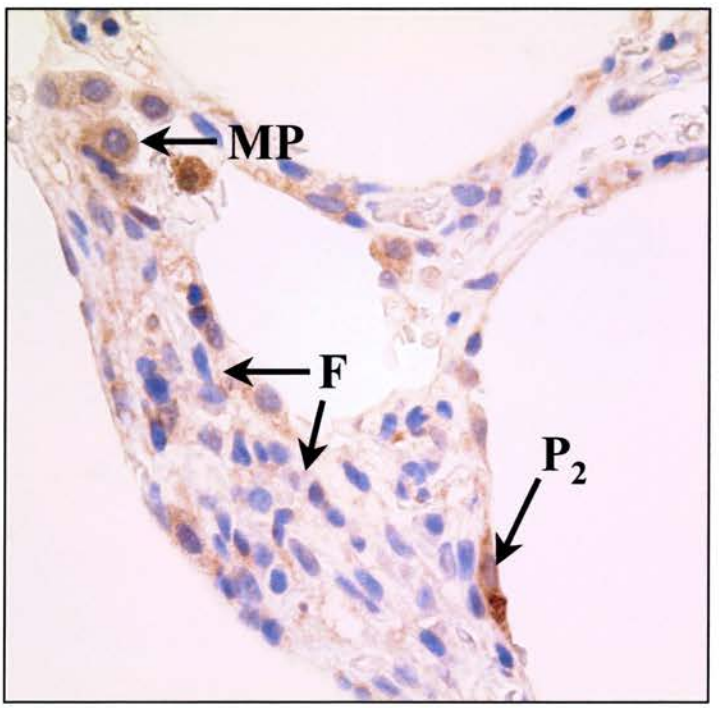


Figure 4.18: CD98 and galectin-3 expression in extrinsic allergic alveolitis.

No tumour cells were present in these sections and assessment was made of the cells that predominated in the interstitial disease process.

(a) CD98 expression was absent from fibroblasts (F), the predominant cells in the interstitium. Alveolar macrophages (MP) provided an internal positive control.

(b) Galectin-3 expression was present at low levels in fibroblasts, but there was strong expression by macrophages and moderate expression by type II pneumocytes (P_2).

Sections shown at x400 magnification.

expressed by tumour cells (mean score = 1.6 ± 0.5) but was expressed only weakly in the interstitium of 1 out of four samples (**Figure 4.19a and b**).

4.5 Discussion

The aim of experiments described in this chapter was to establish the pattern of expression of CD98 and its ligand, galectin-3, in a variety of lung conditions, particularly lung tumours. The chapter describes initial difficulties in obtaining a true assessment of CD98 expression in historical, paraffin-embedded lung tissue. It was established that the monoclonal antibody 4F2, although excellent for fresh cell work, or work on frozen sections, was inadequate for application in paraffin-embedded sections. By contrast, the commercially available goat polyclonal antibody SC-7095 proved excellent for this purpose and further work established appropriate concentrations for this and for a monoclonal antibody against galectin-3. The most likely explanation for the superiority of SC-7095 over 4F2 under these conditions would be the availability of epitopes. Presumably the epitope for 4F2 is somehow destroyed in the paraffin processing and are not recoverable, even with standard unmasking techniques as described. The epitope for SC-7095 must clearly survive these processes.

The major thrust of the chapter thereafter describes a semi-quantitative immunohistochemical assessment of the expression of CD98 and galectin-3 in a variety of lung carcinomas, carcinoid tumours, mesotheliomas, inflammatory lung conditions

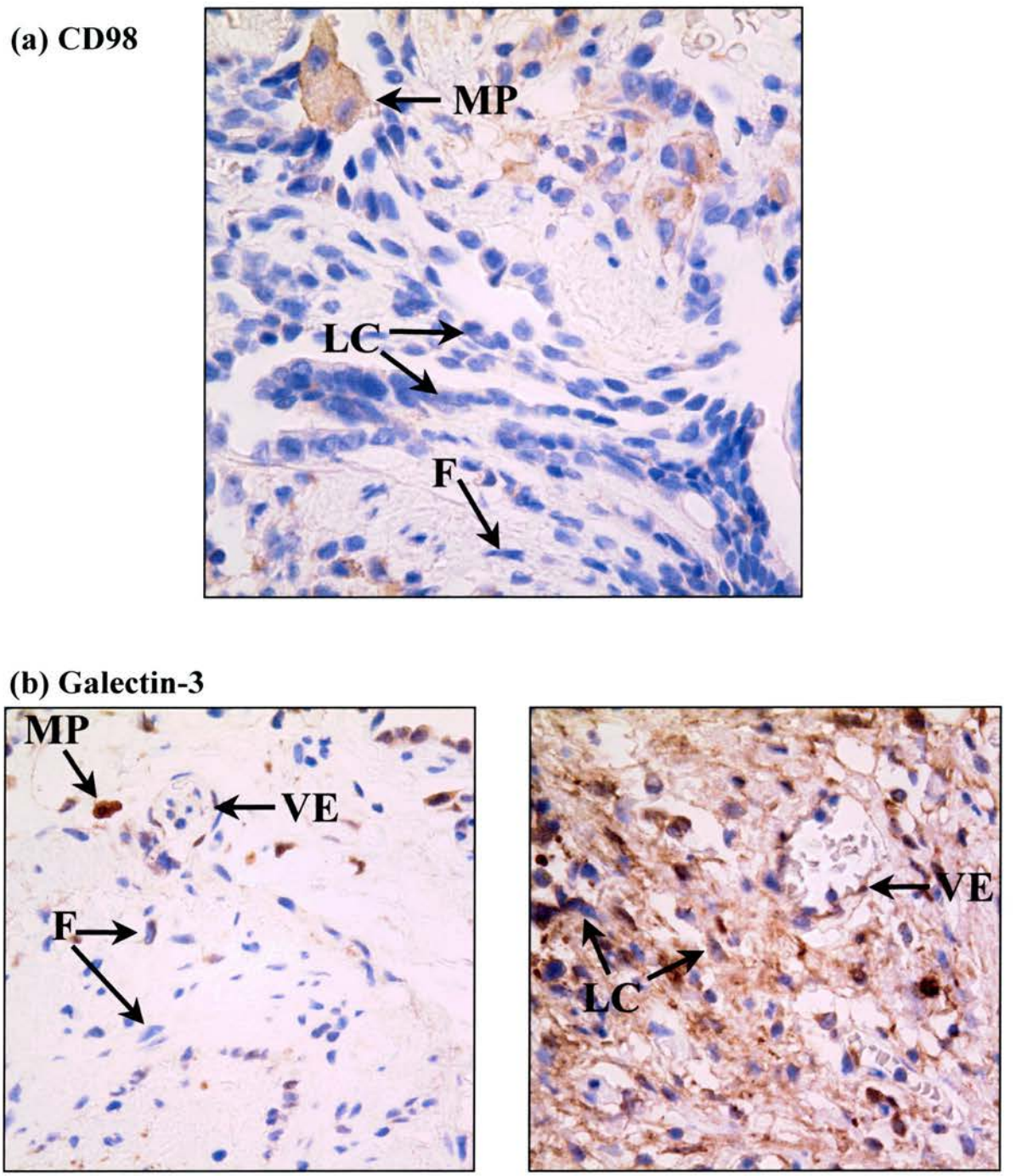


Figure 4.19: CD98 and galectin-3 expression in histiocytosis.

(a) CD98 expression was absent from fibroblasts (F), the predominant cells in the interstitium. The Langerhans cells (LC) did not express CD98. Alveolar macrophages (MP) provided an internal positive control.

(b) Galectin-3 expression was present at very low levels in fibroblasts (*left panel*); there was strong expression by macrophages and moderate expression by vascular endothelial cells (VE). Langerhans cells expressed galectin-3 to moderate levels (*right panel*).

Sections shown at x400 magnification.

and normal controls. The key findings were: (i) in NSCLC, CD98 and galectin-3 were highly expressed. In squamous cell carcinomas there appeared to be differential expression of CD98 and galectin-3 between well- and poorly differentiated carcinomas, such that increased CD98 expression was broadly associated with more poor differentiation, whereas galectin-3 expression was maximal in better differentiated tumours. (ii) In SCLC, by contrast, although CD98 was highly expressed, galectin-3 was very poorly expressed. (iii) Expression of CD98 and galectin-3 by carcinoid tumours was very low or absent.

This pattern of CD98 and galectin-3 expression may be an important feature affecting the different behaviours of these tumours, in particular chemoresistance and the development of metastatic disease. As such, the assessment of galectin-3 expression may be a useful diagnostic tool as well as a predictor of response to chemotherapy, and may therefore be important in influencing patient treatment. Additionally, assessment of galectin-3 expression may be of particular benefit in mixed tumours, where the response to chemotherapy is otherwise difficult to predict.

As previously discussed, the finding that CD98 expression was upregulated in lung carcinomas is in keeping with several studies showing that CD98 is upregulated in actively proliferating cells. It is unsurprising that slow growing, less malignant carcinoid tumours expressed only minimal CD98. There is a good correlation between the level of CD98 expression seen here and the growth rates measured by the volume

doubling time. Amongst lung carcinomas, SCLC and squamous cell carcinomas show the highest rate of growth when measured by volume doubling time (Geddes, 1979;Hasegawa *et al.*, 2000). In the present study, these were seen to express higher levels of CD98 than the more slowly growing adenocarcinomas. Carcinoid tumours are the slowest growing of the tumours studied and expressed the least amount of CD98.

Galectin-3 expression in lung tumours was examined because it has been identified as the ligand counter-receptor for CD98 (Dong and Hughes, 1997). The striking finding was that SCLC exhibited minimal or absent galectin-3 expression, in distinct contrast to all NSCLC, which expressed significant amounts of galectin-3. Additionally, one SCLC sample was histologically a mixed carcinoma. There was a clear distinction between areas of SCLC phenotype, which were galectin-3 negative, and areas of NSCLC phenotype, which expressed galectin-3 to high levels. This broadly supports the finding of differential expression of galectin-3 between SCLC and NSCLC as a whole. It offers the possibility that assessment of galectin-3 expression may be of use in differentiating SCLC from NSCLC in small tissue samples, such as core biopsies or bronchial biopsies, where confusion arises. In practice, however, this is not usually a problem, since there already exists a broad range of markers allowing classification of lung tumours between SCLC and NSCLC. Might galectin-3 expression be a useful cell marker in other ways?

Evidence from *in vitro* work suggests that galectin-3 may promote neoplastic transformation. Honjo *et al.* introduced recombinant galectin-3 into null-expressing,

non-tumourigenic BT-549 cells, resulting in the acquisition of anchorage-independent growth, one of the hallmarks of tumourigenicity (Yoshii *et al.*, 2002). In addition, human breast carcinoma BT549 cells transfected with galectin-3 and treated with genistein exhibit G2/M phase cell cycle arrest, but are protected against apoptosis, as compared with non-transfected controls (Lin *et al.*, 2000). Furthermore, *in vitro*, galectin-3 has anti-apoptotic functions. In human breast carcinoma cells, galectin-3 overexpression protects against nitric oxide-induced apoptosis in a liver ischaemia/reperfusion model (Moon *et al.*, 2001), as well as apoptosis induced by a variety of other stimuli, including loss of cell adhesion (Kim *et al.*, 1999), staurosporine and cisplatin (Matarrese *et al.*, 2000;Matarresea *et al.*, 2000). In T-cells, galectin-3 prevents apoptosis induced by anti-Fas-antibody (Yang *et al.*, 1996). Interestingly, galectin-3 shows significant homology to the anti-apoptotic protein Bcl-2 (Yang *et al.*, 1996). Overexpression studies in BT549 cells suggest that any anti-apoptotic effect of galectin-3 is not mediated through changes in Bcl-2 expression, but may be via a cysteine protease pathway (Akahani *et al.*, 1997).

In vivo, increased galectin-3 expression has been associated with neoplastic progression in a variety of tumours, including gastric (Baldus *et al.*, 2000), pancreatic (Berberat *et al.*, 2001), colonic (Irimura *et al.*, 1991) and thyroid (Cvejic *et al.*, 1998;Cvejic *et al.*, 2000) carcinomas. However, other groups have reported contrasting evidence that down-regulation of galectin-3 is associated with tumour progression in other tumour groups, such as breast and ovarian cancers (Castronovo *et al.*, 1996;van den Brule *et al.*,

1996b). Studies have also tried to examine whether subcellular localization of galectin-3 varied with tumour progression: in squamous cell tongue carcinomas, for example, galectin-3 expression was up-regulated during progression from normal to cancerous states, but there was a loss of nuclear staining compared to normal mucosa (Honjo *et al.*, 2000). Lotz *et al.* found a similar loss of nuclear expression associated with progression of colonic carcinoma, but this was in the context of an overall decrease in galectin-3 expression as measured by mRNA levels and immunohistochemistry (Lotz *et al.*, 1993).

A role for galectin-3 in the development of neoplasia has thus so far been controversial and the evidence seems to suggest that its role may be tumour-specific to some degree. Recently another group has published evidence supporting the finding that galectin-3 is differentially expressed in lung cancer. Yoshimura *et al.* reported overexpression of galectin-3 in NSCLC by comparison to SCLC, when measured by reverse transcription-polymerase chain reaction (Yoshimura *et al.*, 2003). In their study, SCLC either failed to express galectin-3, or did so at a very low level. Therefore, these two studies complement each other, with a clear differential in galectin-3 expression between NSCLC and SCLC, observed at both RNA and protein level.

No definite cellular function for galectin-3 has yet been ascribed. Localization has been demonstrated in various cellular compartments, with clear nuclear and cytoplasmic expression (Openo *et al.*, 2000); membranous expression has been documented and there is also evidence that galectin-3 may be actively secreted into the extracellular milieu

(Cherayil *et al.*, 1989;Sato and Hughes, 1994). Among its potential extracellular ligands are the ECM proteins laminin and fibronectin. Within the cell, galectin-3 has been reported to have activity as a pre-mRNA splicing factor (Dagher *et al.*, 1995) in the nucleus and to be a component of heterogeneous nuclear ribonuclear protein. If promoting oncogenic transformation, therefore, galectin-3 may be acting at a nuclear or cytoplasmic level by as yet unknown mechanisms.

Intriguingly, galectin-3 may be acting at the cell surface to cross-link CD98 and promote integrin-like signalling and hence cell survival. The quaternary structure of recombinant murine galectin-3 was recently described by sedimentation velocity and equilibrium studies, and suggested that galectin-3 exists predominantly as a monomer (Morris *et al.*, 2004). However, other lines of evidence suggest that it may self-associate into oligomeric units. Gel electrophoresis experiments have revealed dimers (Ochieng *et al.*, 1993), whilst the ability to promote haemagglutination (Frigeri *et al.*, 1990) or to co-operatively bind to laminin (Massa *et al.*, 1993) and IgE (Hsu *et al.*, 1992) suggests a protein functioning at a multivalent level. It may do this directly, through self-association of its N-terminal domains (Birdsall *et al.*, 2001), or it may do so by first binding to ECM proteins that are abundant in the local milieu. Recent work suggests galectin-3 can precipitate rapidly from a monomeric solution as pentamers, forming heterogenous, disorganised, cross-linking complexes with multivalent carbohydrate ligands (Ahmad *et al.*, 2004). Against this, the majority of galectin-3 seen in samples in

the present work was cytoplasmic or nuclear, although some membranous staining was also identified, allowing for this possibility.

The cellular role of CD98 has already been discussed in Chapter 3. Its involvement in integrin-related cell-signalling pathways raises the possibility that cross-linking CD98 by its natural ligand may profoundly affect tumour sensitivity and the induction of apoptosis by chemotherapeutic agents.

These studies therefore offer interesting insights into a possible role for galectin-3 in lung cancer. It has long been known that SCLC is, at presentation, by far the most chemosensitive histological subtype, whereas NSCLC adenocarcinomas and squamous cell carcinomas have been regarded as relatively chemoresistant. This supports the pattern of galectin-3 expression observed here, since galectin-3 negative SCLC might be expected to undergo chemotherapy-induced apoptosis and exhibit a clinical response to such chemotherapy. The galectin-3 positive NSCLC would be expected to be relatively chemoresistant, and a tumour of mixed cell type to have an intermediate response. Galectin-3 expression on initial diagnostic biopsy may therefore be of potential use as a predictor of chemotherapeutic response in tumours, rather than simply as a discriminator between SCLC and NSCLC.

In this context, the single sample of SCLC taken post-chemotherapy is intriguing, since it was strongly positive for galectin-3, in contrast to all other SCLC specimens. Since

relapsed SCLC is usually much more chemoresistant, this would be predicted from the present study, although it does not inform any further on the role, if any, that galectin-3 might play in the development of that chemoresistance.

This chapter also describes CD98 expression and galectin-3 expression in other lung conditions. These included tumours of non-bronchogenic origin – mesothelioma and Langerhans cell histiocytosis – and a number of interstitial lung diseases. There has been little research in these fields before involving either galectin-3 or CD98 and only very general conclusions may be drawn.

Since both mesothelioma and Langerhans cell histiocytosis are proliferative diseases, it was unsurprising to discover elevated CD98 expression in the tumour cells, as with bronchogenic cancers. Galectin-3 was elevated to moderate levels by both tumours. Following the discussion above, this would be in keeping with the low level of response seen in these diseases to either chemotherapy or radiotherapy. However, the numbers in these samples was low and there are no previous reports with which to compare these results.

The remaining samples broadly fall into the category of "inflammatory lung diseases", although it should be stated that for neither sarcoidosis, nor BOOP, nor IPF has the pathological trigger been clearly identified. In sarcoidosis, the characteristic pathological markers are non-caseating granulomata; in IPF there appears to be an

abnormal proliferative response from fibroblasts in the interstitium; in BOOP a chronic inflammatory cell infiltrate is found, with increased patchy fibrosis.

There is little direct evidence published on the role of CD98 in any of these diseases, although a single report looking at a variety of interstitial lung diseases found elevated CD98 expression by macrophages isolated from bronchoalveolar lavage fluid (Taylor *et al.*, 2000); along with other markers studied, this really goes no further than suggesting that the macrophages in these diseases were activated by comparison with macrophages from normal subjects. Given the chronic inflammatory response observed in these diseases, macrophages would be expected to be present; however, the present study found no striking CD98 expression elsewhere.

The role of galectin-3 is more interesting and evidence is now accumulating for a significant part in the control of inflammation. Several groups have now shown that galectin-3 is required for a normal inflammatory response. Colnot *et al.* found that galectin-3-null mice demonstrated an attenuated inflammatory cell infiltrate following peritoneal injection with thioglycollate, which was not due to increased apoptosis by the granulocytes, nor to increased phagocytosis by macrophages (Colnot *et al.*, 1998b). In a similar model, these findings were associated with a reduced NF-κB response (Hsu *et al.*, 2000). Macrophages from these animals had abnormal cell spreading and were more likely to undergo apoptosis. Conversely, gene therapy with the instillation of plasmid DNA encoding galectin-3 has been shown to reduce an eosinophilic inflammation in a

rat model of acute asthma, with a strong reduction in IL-5 mRNA levels (Del, V *et al.*, 2002). In addition, *in vitro* studies have shown that exogenous galectin-3 can both stimulate superoxide production by neutrophils (Yamaoka *et al.*, 1995) and promote the adhesion of neutrophils to laminin, both by forming lectin bridges with the laminin and by promoting other cell adhesion molecules (Kuwabara and Liu, 1996).

Taken together, these studies suggest that galectin-3 may play an important role in control of inflammatory cell responses. In the present study, therefore, it was interesting to observe that macrophages and epithelioid giant cells were universally strongly positive for galectin-3 and also that there was a low-grade expression of galectin-3 by interstitial fibroblasts in some sections.

In summary, this chapter has described important findings in the expression of CD98 and particularly galectin-3 in human lung carcinomas, with notable differences between SCLC and NSCLC. These may suggest an important role clinically for assessment of galectin-3 expression as a marker of chemoresistance and may direct further investigation into the role of galectin-3 in cell survival.

Chapter 5

Modulation of Integrin Activation Status:

R-Ras Reversal of Suppression

5.1 Introduction

One of the underlying aims of this thesis was to attempt to elucidate the mechanisms by which integrin activation status may be modulated. "Integrin activation" represents the end result of two distinct processes, affinity modulation and avidity modulation, as discussed in Chapter 1. Studies undertaken here have been directed specifically at investigating affinity modulation, which involves a conformational change in the integrin as a direct result of intracellular signalling pathways, resulting in a change in the binding affinity of the integrin towards its ligand (reviewed in (Huttenlocher *et al.*, 1995)).

Although the predominant integrins expressed on small cell lung cancer are those of the β1 family (Hirasawa *et al.*, 1994;Falcioni *et al.*, 1994), the most thoroughly investigated integrin has been the platelet integrin αIIbβ3 whose activation is required to permit fibrinogen binding and clot formation. Research has been facilitated by the use of the highly specific ligand-mimetic antibody PAC1, which recognises ligand-binding sites within the activated (but not suppressed) αIIbβ3 integrin (Shattil *et al.*, 1985). It has been shown that integrin-ligand binding is a divalant cation dependent process requiring

calcium and magnesium, such that chelation of these ions with EDTA totally inhibits PAC1 binding, whereas the presence of manganese ions increases the activation state of integrins to a maximum (Gailit and Ruoslahti, 1988). Further studies were facilitated by the use of a model system established in the Chinese hamster ovary cell line. O'Toole *et al.* showed that expression of αIIbβ3 in these cells failed to bind PAC1 even in the presence of known activation stimuli, suggesting that the mechanisms required for αIIbβ3 regulation are absent in this cell line (O'Toole *et al.*, 1990). However, if the cytoplasmic domain of the integrin was substituted with a different alpha subunit and a β1 subunit cytoplasmic change, this permitted PAC1 binding. A stable cell line was therefore established expressing the chimaeric integrin αIIbα6β3β1 (αβ-py cells), in which the extracellular domain of αIIbβ3 is constitutively active. In 1997, Hughes *et al.* published data reporting that an activated form of H-Ras (Ras G12V) inhibited PAC1 binding in a Raf-1 and MAP kinase dependent manner (Hughes *et al.*, 1997). It has been shown that the small GTP-binding protein R-Ras not only activates integrins, enhancing cell adhesion, but it is able to reverse this H-Ras mediated integrin suppression (Zhang *et al.*, 1996b;Sethi *et al.*, 1999a). However, the downstream effector pathways through which R-Ras reversal occurs are not understood. Although it is recognised that R-Ras and H-Ras can both bind the common effectors Raf1, Ral-GDS and PI 3-kinase (Urano *et al.*, 1996;Marte *et al.*, 1997;Rodriguez-Viciana *et al.*, 1997), Sethi *et al.* found that these pathways were not involved in R-Ras reversal (Sethi *et al.*, 1999a).

The use of the αβ-py CHO cell system has been established in our laboratory by Dr. Y. Lad (PhD Thesis 2001). The present chapter examines early steps that were taken in trying to identify possible cell signalling pathways that might be involved in integrin affinity modulation, with a special interest in the mechanism of R-Ras reversal. In the first instance the αβ-py CHO cell system was established as a workable test system.

It was hypothesised that possible candidates for integrin modulation might then include proteins involved in the regulation of the cell cycle. In the normal cell cycle, successive waves of cyclins and cyclin-dependent kinases (Cdks) drive progression, acting in G0 to initiate S phase and in G2 to trigger mitosis (Coqueret, 2002). Expression levels of the different cyclins vary individually across the cell cycle and are both highly regulated and rate-limiting (reviewed in Coqueret (2002)). Following serum starvation cyclin D1 may be induced early in the G1 phase in a growth factor-dependent manner, via the Ras-Raf-MAPK-ERK pathway. Its role at this point consists mainly of an association with Cdk4 to initiate retinoblastoma protein (Rb) phosphorylation (Dowdy *et al.*, 1993), relieving histone deacetylase binding and thus allowing cyclin E/Cdk2 complexes access to their targets, pushing the cell towards S phase. Using a different technique, following asynchronous cells, cyclin D1 levels have been demonstrated to be low in S phase, but elevated in G1 and G2 phases, the suggestion being that cyclin D1 initially rises in G2 and is then maintained through mitosis and G1, until suppressed in S phase (Stacey, 2003). During mitosis, cells in culture are visibly observed to "round up", becoming loosely attached to the underlying surface, enabling them to undergo cell division easily.

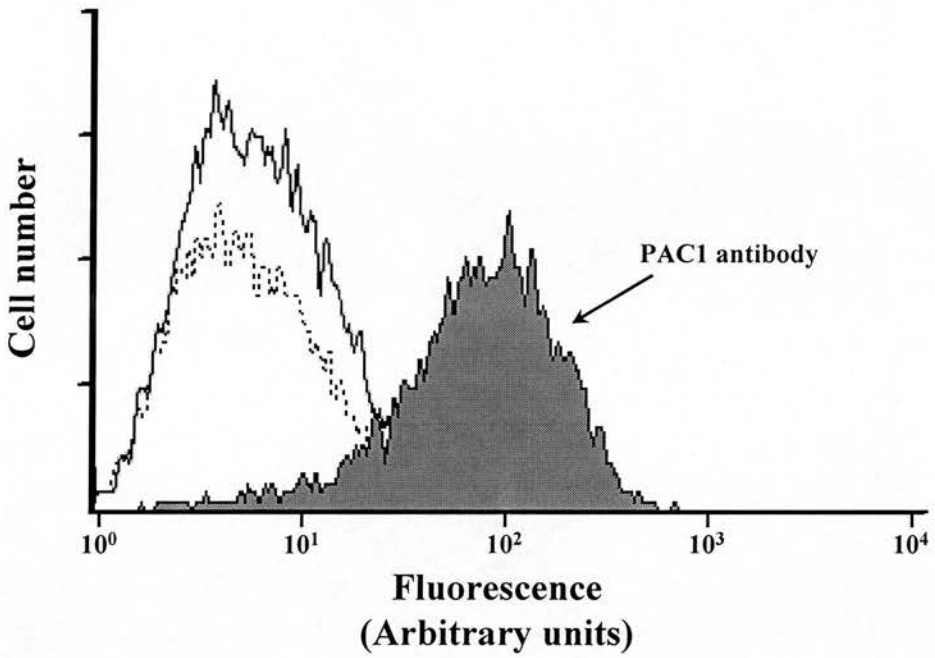
Only following mitosis do they become strongly adherent once more. This suggests an intimate relationship between the cell cycle and cell surface adhesion proteins such as integrins and it was therefore hypothesized that cyclin D1 may be involved in the promotion of integrin suppression running up to and during the mitotic phase.

An additional subject of investigation was CD98, which has been implicated in β1 integrin activation (see Chapter 3), together with a general approach to try and identify pathways involved in mediating R-Ras reversal of H-Ras mediated integrin suppression, using a range of specific enzyme inhibitors.

5.2 The αβ-py CHO cell model

5.2.1 PAC1 binding and modulation of integrin status by cations

The αβ-py system was first described by Hughes *et al.* (Hughes *et al.*, 1997) and initial experiments were used to define the binding of PAC1 antibody to αβ-py cells, as described in "Materials and Methods". Titration of PAC1 antibody for our system was performed by Dr Y Lad as described (PhD Thesis). Initial experiments for the current work confirmed that PAC1 antibody binds to αβ-py cells, in contrast to an IgM isotype control antibody. **Figure 5.1** ***(upper panel)*** shows a representative histogram of FL1-FITC Fluorescence against cell number. In αβ-py cells transfected with Tac-α5 alone, a reporter construct that does not affect integrin affinity, PAC1 antibody binds strongly, with a mean fluorescence intensity of 24.2 ± 2.5 arbitrary units (AU ± SEM), by contrast



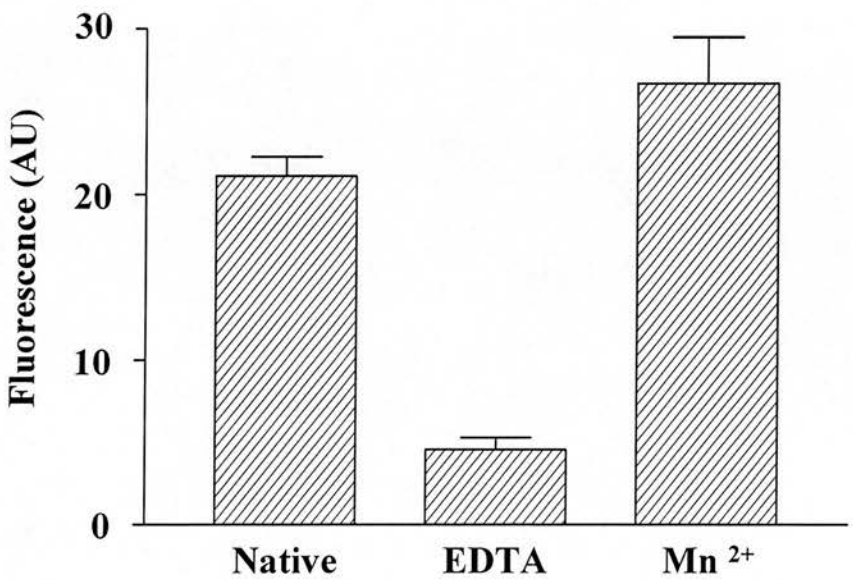


Figure 5.1: PAC1 binding to αβ-py CHO cells

Upper panel: PAC1 monoclonal antibody binds strongly to αβ-py cells (infilled line), by contrast to an IgM isotype control antibody (broken line) or carrier alone (narrow line).

Lower panel: Effect of 5mMol EDTA and 100mMol Mn^{2+} on PAC1 binding, measured by intensity of fluorescence (arbitrary units, AU).

Results are representative of at least three sets of experiments.

to binding shown with the IgM isotype-matched control antibody (4.3 ± 0.1) or unstained controls (4.2 ± 0.1). In keeping with previous work, the chimæric integrin is able to undergo conformational change by treatment with cations and **Figure 5.1** ***(lower panel)*** shows the effect of treatment with 5mM EDTA or 100mM Mn^{2+}. In the native state, without treatment, mean fluorescence intensity was 21.1 ± 1.2 AU (mean ± SEM). Addition of EDTA markedly reduced PAC1 binding to a mean fluorescence intensity of 4.6 ± 0.7 AU, whereas in the presence of exogenous Mn^{2+} there was a significant increase in PAC1 binding, to a mean fluorescence intensity of 26.7 ± 2.8 AU. This shows that the chimaeric integrin is constitutively activated and demonstrates that integrin affinity may be modulated by cations. In addition the use of EDTA and manganese permits a numerical estimate of integrin activation status, as described in "Materials and Methods". Briefly, integrin affinity in the presence of 5mM EDTA is taken to be the minimum possible (or most suppressed), whereas integrin affinity is taken to be maximum (most activated) in the presence of 100mM Mn^{2+}. Under any test conditions, therefore, the integrin activation status is represented by the activation index (AI) calculated as a percentage lying between the suppressed (0%) and activated (100%) states. In the above example, the AI for αβ–py cells in their native state is 76.1% ± 6.2, demonstrating that in this system integrins are predominantly in the activated state under native conditions.

5.2.2 H-Ras G12V suppresses integrin affinity

In order to determine the effect of Ras G12V expression on integrin affinity, the αβ-py cell system was used. Cells were transfected and integrin affinity status assessed as described in "Materials and Methods". **Figure 5.2** shows a representative dot-blot display, demonstrating the effect of Ras G12V transfection on PAC1-FITC binding. In these and subsequent experiments cDNA constructs were transfected together with cDNA for the chimæric construct Tac-α5. This contains the intracellular domain of the α5 integrin conjoined with the extracellular domain of the IL-2 receptor. Expressed at the cell surface by successfully transfected cells, the transfection efficiency may be detected by flow cytometry using an antibody against the IL-2 receptor, Tac-R-PE (R-phycoerythrin).

In **Figure 5.2** integrin affinity is represented along the X axis by increasing PAC1 binding, whilst transfection status is represented along the Y axis, with the most highly transfected cells exhibiting high Tac-α5 expression, as demonstrated by Tac-PE binding. It has been assumed that transfection of Tac-α5 corresponds to transfection of other cDNA constructs to a similar degree.

Figure 5.2 demonstrates that in the presence of 1μg cDNA for Ras G12V, integrin affinity was markedly reduced in the most transfected cells. In cells transfected with Tac-α5 alone, the majority of highly transfected cells were in the upper right hand quadrant, representing a high affinity state. In the presence of 5mM EDTA, the majority

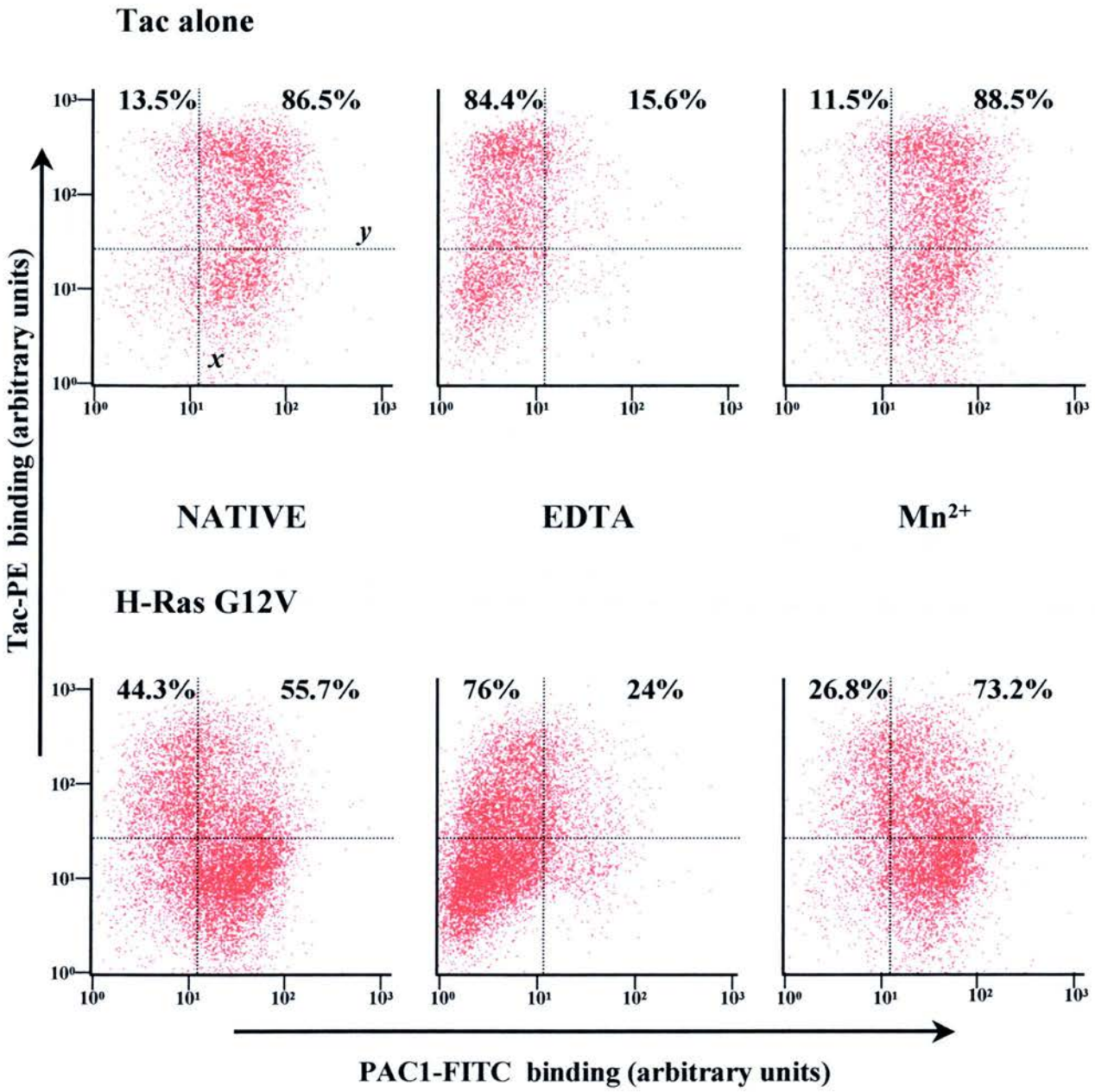


Figure 5.2: H-Ras G12V transfection suppresses integrin affinity.

Dot-blot graphs showing the effect of transfection with H-Ras G12V on PAC1 binding. Integrin affinity is reflected by PAC1-FITC binding, with elevated PAC1 binding represented by cells to the right of dashed line *x*. Transfection status is assessed by Tac-PE binding, with high transfection represented by cells above the dashed line *y*.

Upper panel, transfected with Tac-α5 alone: in the presence of 5mMol EDTA, there was an increase in percentage of transfected cells in the low affinity state (*upper left quadrant*); addition of 100mMol Mn^{2+} activated integrins, with an increased percentage of transfected cells exhibiting high PAC1 binding (*upper right quadrant*).

Lower panel, transfected with H-Ras G12V: transfection with H-Ras G12V resulted in a leftward shift in PAC1 binding in transfected cells, that was not fully recoverable even in the presence of 100mMol Mn^{2+}. Results are representative of at least three sets of experiments.

of transfected cells were in the upper left hand quadrant, representing a low affinity or suppressed integrin state, as previously described; 100mM Mn^{2+} increased integrin activation state compared to normal. However, in the presence of Ras G12V the majority of transfected cells demonstrated a reduced integrin affinity, with a leftward shift in PAC1 binding. This was exaggerated with 5mM EDTA and was not fully recoverable with 100mM Mn^{2+}. Because of this phenomenon, the AI in transfected cells was calculated by taking the maximum natural affinity state to be that represented by PAC1 binding in Tac-α5 transfected cells in the presence of 100mM Mn^{2+}.

5.2.3 Titration of H-Ras G12V

The amount of H-Ras G12V cDNA required to adequately suppress integrins was assessed by titration experiments. αβ-py cells were transfected with increasing quantities of H-Ras G12V and the integrin affinity assessed as above. **Figure 5.3a *(upper panel)*** shows the effect on AI of increasing quantities of H-Ras G12V. The AI of cells transfected with Tac-α5 alone was 84.0% ± 2.3 (mean ± SEM). Transfection of 1μg of H-Ras G12V cDNA suppressed the integrins to an AI of 38.4% ± 7.4. Increasing quantities of G12V cDNA resulted in a further reduction in AI to a minimum of 31.2% ± 3.3, with 4μg of cDNA. By contrast, transfection with 5μg of a control vector pCDNA3.1 made no difference to the AI (mean ± SEM = 83.4% ± 9.2).

In parallel with the above experiment, transfected cells were lysed as described in "Materials and Methods" and, following equilibration for total protein loading, lysates

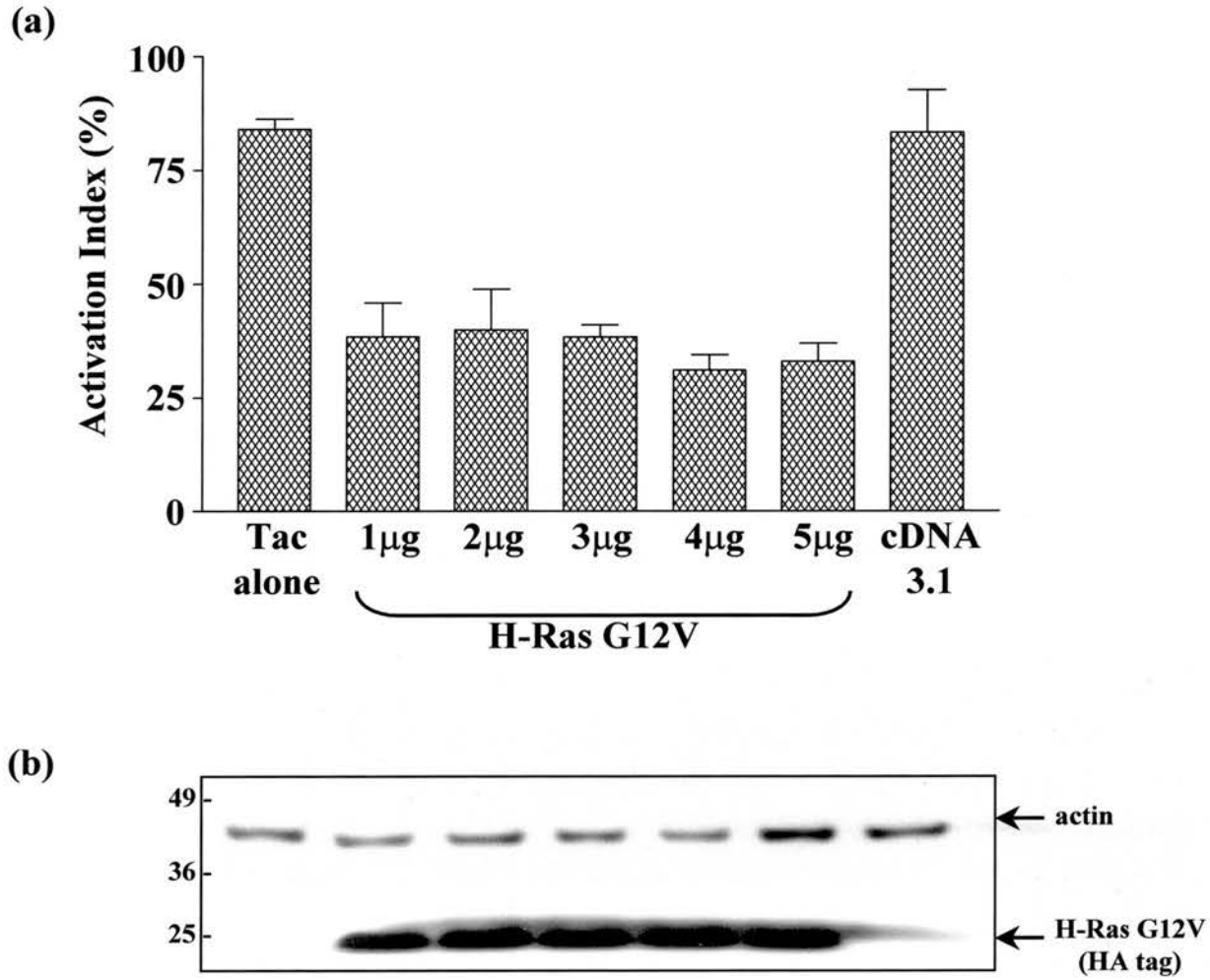


Figure 5.3: Titration of H-Ras G12V

(a) Integrin status in αβ-py cells following transfection with increasing doses of cDNA for H-Ras G12V, compared with untransfected cells or cells transfected with the vector cDNA3.1 alone. Integrin status may be represented by an Activation Index (*upper panel*) or, inversely, by a Suppression Index (*lower panel*).

(b) Western blot confirming increased expression of H-Ras, labelled via its HA tag, with increasing dose of cDNA.

Results shown are mean +/- standard error of three experiments.

were resolved on SDS-PAGE gels and probed for expression of H-Ras G12V. **Figure 5.3b** shows a representative blot demonstrating increasing expression of the Ras G12V plasmid in cells transfected with increased quantities of cDNA.

It was noted that with increasing quantities of H-Ras G12V cDNA, there was a marked increase in cell death following transfection, with a dramatic loss of recoverable cells. Since excellent suppression was achieved with 2μg cDNA, with only moderate loss of cells, this concentration of cDNA was used for future experiments.

5.2.4 R-Ras reverses H-Ras-mediated integrin suppression

It has previously been shown that the activated form of the small GTP-binding protein R-Ras G38V can reverse H-Ras G12V mediated integrin suppression. This was confirmed in transfection experiments in our αβ-py system. **Figure 5.4** ***(upper panel)*** shows that when αβ-py cells were transfected with 2μg H-Ras G12V, integrin suppression occurred in the most highly transfected cells, as indicated by reduced PAC1 binding by comparison with untransfected cells. However, in cells transfected with both 2μg H-Ras G12V and 2μg R-Ras G38V, this suppression was reversed so that the integrins were expressed in an activated state once more and PAC1 binding was restored. **Figure 5.4** ***(graph)*** confirms this graphically: the AI of cells transfected with H-Ras G12V alone was reduced (AI = 25.0% ± 2.6), but transfection with R-Ras G38V alone (115.8% ± 9.4) increased AI compared to native cells (73.3% ± 3.6) and rescued integrin suppression when co-transfected with H-Ras G12V (66.3% ± 3.6).

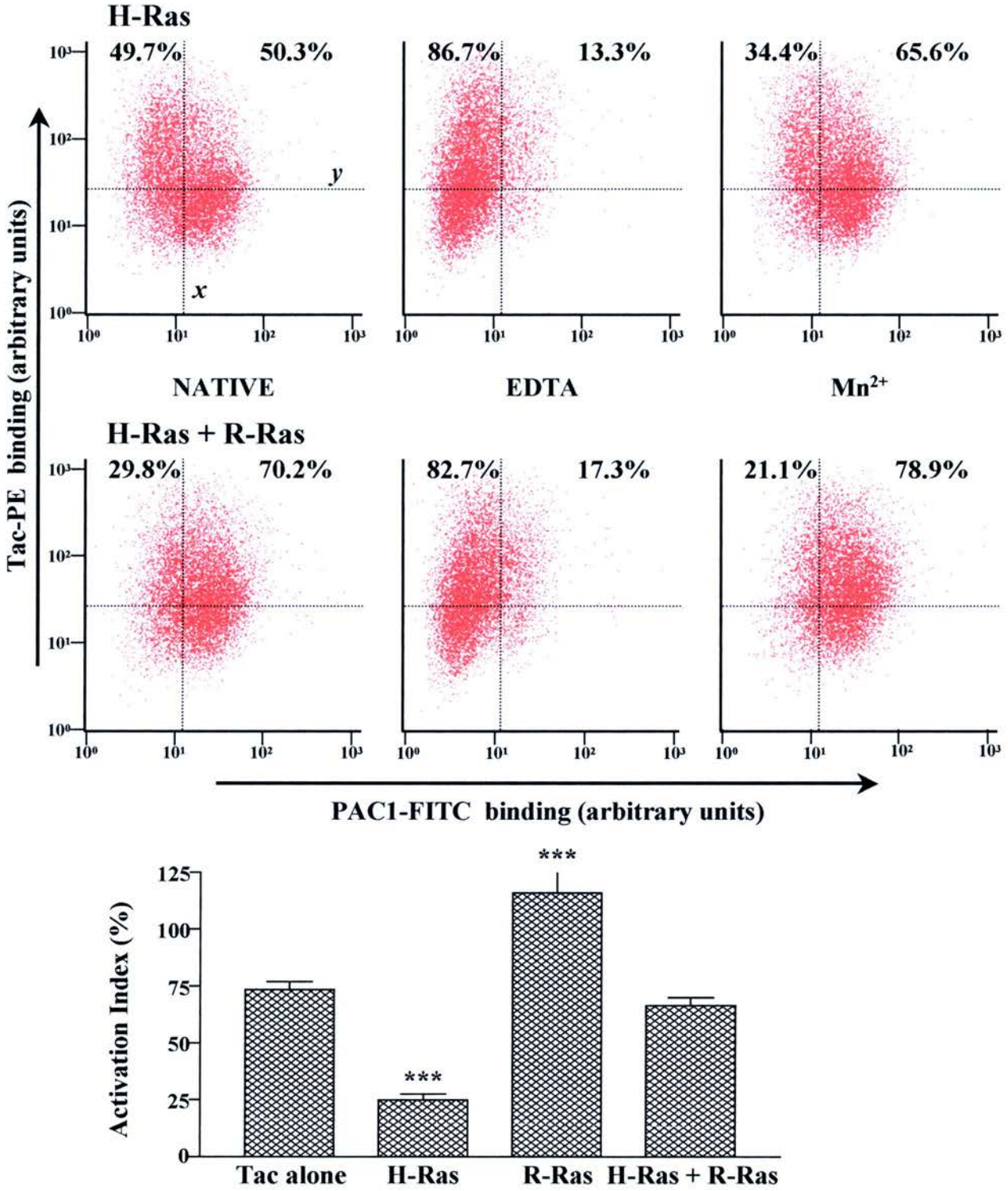


Figure 5.4: R-Ras G38V reverses H-Ras-mediated integrin suppression.

Upper panels: Dot-blots showing integrin suppression by transfection of αβ-py cells with H-Ras G12V (2μg). As previously, a greater percentage of transfected cells (*above line y*) were shifted to the left compared with control cells (*not shown*), an effect not fully retrieved by 100mMol Mn^{2+}. Transfection with H-Ras G12V (2μg) plus R-Ras G38V (2μg) rescued integrins from H-Ras-mediated suppression, increasing the percentage of transfected cells exhibiting high PAC1 binding (*right of line x*).

Lower panel: Graph demonstrating the effect on Activation Index of transfecting αβ-py cells with H-Ras G12V (2μg), R-Ras G38V (2μg), or both together, compared with Tac-α5 alone. (*** $p < 0.001$ compared with Tac-α5 alone.)

Results are representative of at least five experiments.

5.3 Cyclin D1 reduces integrin activation

It makes biological sense for integrin activation status to be modulated through the cell cycle, e.g. to allow a degree of cell detachment during mitosis when cells round up to divide. It was therefore hypothesized that proteins involved in the regulation of the cell cycle may be regulators of integrin affinity. Since cyclin D1 levels have been found to be elevated from G2, through M to G1 phase (Stacey, 2003), it was therefore hypothesized that cyclin D1 may suppress integrin activation, in order to allow rounding-up of the cell for mitosis.

Using the αβ-py cell system, a dose-response study was performed. **Figure 5.5** shows that transfection with all doses of cDNA for cyclin D1 resulted in a significant reduction in AI compared with control cells (AI = 65.4% ± 4.8), as assessed by PAC1 binding. This was most marked with 2μg cDNA of cyclin D1 (AI = 26.7% ± 3.8), but achieved significance for all doses tested ($p < 0.001$). This finding supported the suggestion that cyclin D1 might suppress integrin activation.

5.4 The effect of inhibitors

The mechanism by which R-Ras reverses H-Ras mediated integrin suppression is entirely unknown and as part of this thesis it was proposed that a number of pharmacological inhibitor agents might be assessed in the αβ-py system, to see if they

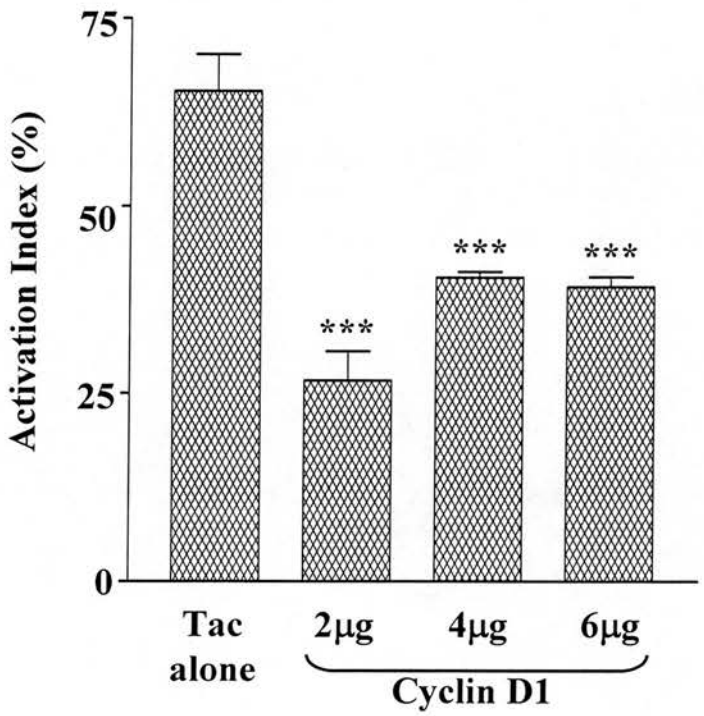


Figure 5.5: Cyclin D1 suppresses integrin activation.
Transfection of αβ-py cells with cDNA for cyclin D1 induced suppression of integrins, as measured by Activation Index. Compared with transfection with Tac-α5 alone, maximal suppression was achieved with 2μg cDNA for cyclin D1 ($p < 0.001$). Results shown are mean +/- standard error of three experiments.

influenced R-Ras reversal. For these early studies, therefore, cells transfected with both H-Ras G12V and R-Ras G38V were treated with inhibitor agents and then assessed for integrin activation as represented by PAC1 binding. The results of these early studies highlighted a problem with the αβ-py system, such that the experimental design was adjusted in all subsequent experiments, as will be discussed below.

5.4.1 Okadaic acid may cause partial inhibition of R-Ras reversal

Okadaic acid is the toxin responsible for diarrhoeic shellfish poisoning. *In vitro* work has shown it to be an inhibitor of protein phosphatases 1 and 2A and this has been confirmed by intact cell studies (Haystead *et al.*, 1989). It is useful as a specific probe for identifying cellular processes regulated by phosphorylation/ dephosphorylation events (Cohen *et al.*, 1990). **Figure 5.6** shows the influence of increasing concentrations of okadaic acid on AI in αβ-py cells transfected with both H-Ras G12V and R-Ras G38V. In these experiments, okadaic acid was added to the culture medium 16-18 hours prior to cell harvest and flow cytometry assay. By comparison to cells transfected with Tac-α5 alone (AI = 69.7% ± 7.2), H-Ras G12V alone suppressed integrin status (AI = 22.0% ± 5.8), R-Ras G38V alone activated integrins (AI = 107.3% ± 6.5) and it overcame H-Ras mediated suppression (AI = 59.8% ± 2.1), as expected. With increasing concentrations of okadaic acid there was a steady decline in AI, so that at 30nM okadaic acid the AI was reduced to 47.6% ± 2.6, but at 300nM the AI was as low as 10.5% ± 2.0. However, this was associated with a drop in the total number of

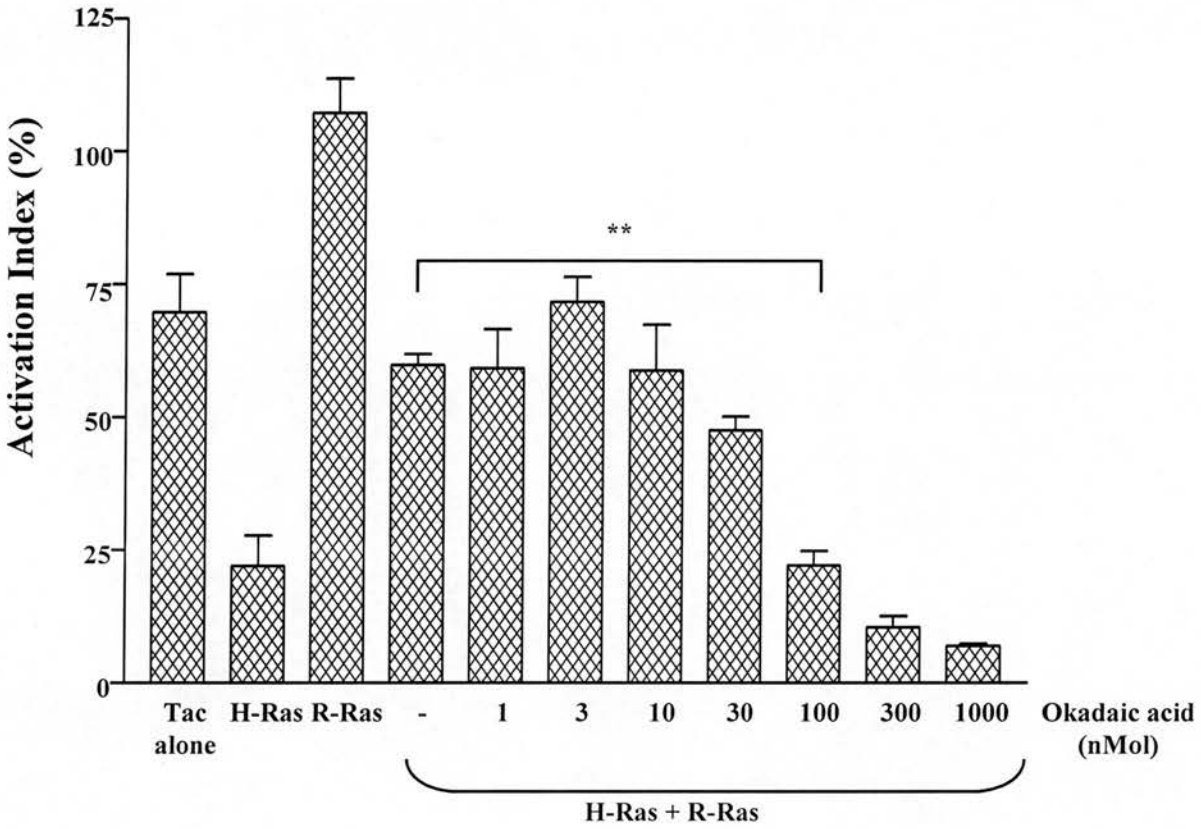


Figure 5.6: Okadaic acid may inhibit R-Ras reversal.

Effect of increasing concentrations of okadaic acid on the ability of R-Ras G38V (2μg) to reverse integrin suppression mediated by H-Ras G12V (2μg). By comparison with the reversal seen with carrier solution alone (AI = 59.8% +/- 2.1), a marked suppression of integrin activation status was observed in the presence of okadaic acid, achieving significance at a concentration of 100nM (AI = 22.1% +/- 2.7) (** $p < 0.01$). Data represent the mean +/- standard error of two experiments. A marked fall-off of cell population was noted with increased concentrations of okadaic acid (*see text*).

"events" able to be gated on flow cytometry and a shift in the cell population on the dot-blot graph.

5.4.2 Okadaic acid treatment effects are masked by cell death

The concerns raised by the above experiments were that the effect of partial inhibition of R-Ras reversal of H-Ras mediated suppression was in fact due to cell death caused by the DMSO carrier solution for the okadaic acid. This was suggested by the observation that there was a visually observable loss of adherent cells in the petri dish following treatment with okadaic acid. In addition, cell numbers detected through flow cytometry were lower and the dot-blot distribution of cells, or "events", was shifted into the region normally associated with cell debris.

In order to test this hypothesis, αβ-py cells in the same experiment above, transfected with both H-Ras G12V and R-Ras G38V, were treated with either a maximum concentration (1μM) of DMSO alone, or with the maximum concentration of okadaic acid (1μM) in DMSO (1μM). These were then subjected to analysis for cell death using an Annexin V assay. Briefly, 5 x 10^6 cells were resuspended in 90μl PBS, with 10μl Annexin V/buffer. Cells were incubated for 10 minutes at 4°C and then assessed by flow cytometry, in which Annexin V positive cells are detected by increased FL1 fluorescence. **Figure 5.7** shows that by comparison with control cells, there was an increase in Annexin V positive cells following treatment with DMSO alone. However, using the same gating analysis as had been used during the remainder of the experiment,

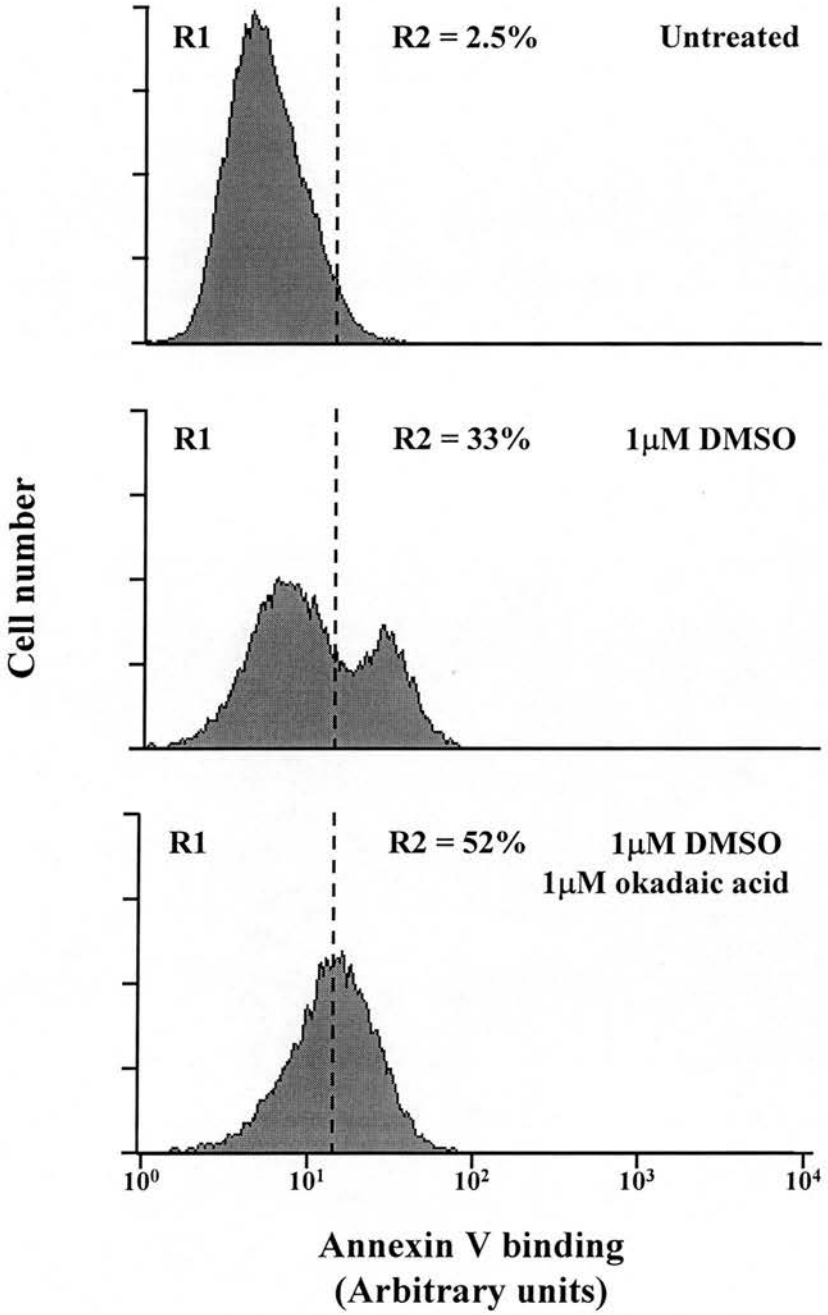


Figure 5.7: Okadaic acid increases cell death.

Histogram graphs showing the effect of treating αβ-py cells with carrier solution alone, a maximum dose of DMSO (1μM), or maximum dose of DMSO plus okadaic acid (1μM). Cell death was assessed by binding of Annexin V, measured (like FITC) in FL-1. The regions R1 and R2 were arbitrarily set for untreated cells, such that 97.5% of the total cell population fell in R1 (*upper panel*). Treatment with either DMSO alone (*middle panel*), or DMSO plus okadaic acid (*lower panel*), markedly increased the percentage of cells exhibiting positive Annexin V binding (33% and 52%, respectively), indicating that these treatments increased cell death.

there was an even greater increase in the proportion of Annexin V positive cells following treatment with okadaic acid. Since Annexin V positive cells are dead or apoptotic, this data supported the hypothesis that at least some of the effect of okadaic acid in inhibiting R-Ras reversal was in fact due to an increased cell death.

5.4.3 To-Pro-3 for live/dead analysis

The ideal agent for discrimination between live and dead cells in the αβ-py system would be detectable in a different waveband from either FITC or PE, so that there is no interference from the live/dead cell marker in assessment of FITC or PE fluorescence. The commercial product To-Pro-3 was identified (Molecular Probes) with an emission wavelength maximal at ~642nm. This is a cyanine dimer dye with high affinity for nucleic acids with which it intercalates, yet it is cell-impermeant for live cells. Dead cells are unable to exclude To-Pro-3 and fluoresce in the spectrum detected by FL-4. Following discussion with Dr. Derek Davies (Flow Cytometry Laboratory, London Research Institute), a final concentration of 100nM To-Pro-3 was used. As can be observed in **Figure 5.8**, addition of To-Pro-3 just prior to flow cytometry analysis allowed dead, To-Pro-3 positive cells to be gated out on FL-4; the remaining live cells being analysed in the usual way for FITC and PE binding.

5.4.4 Okadaic acid does not inhibit R-Ras reversal

Using the modified αβ-py system, a variety of inhibitor agents were assessed for their ability to influence R-Ras reversal of H-Ras mediated integrin suppression. Cells were

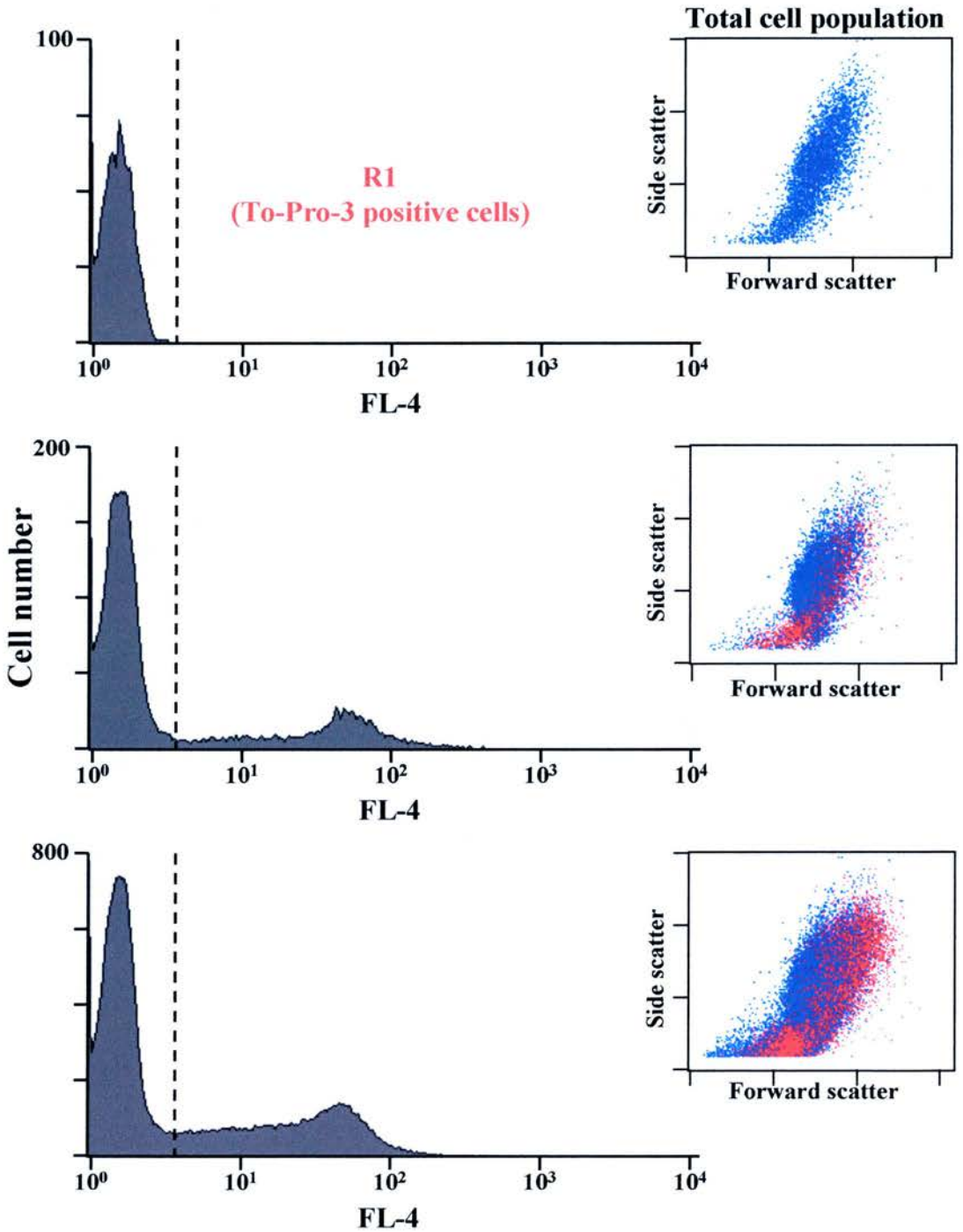


Figure 5.8: To-Pro-3 permits live/dead analysis.

Graphs showing the use of To-Pro-3 in live/dead cell gating. In the upper panel, gate R1 was set to gate out all To-Pro-3 positive cells, measured in FL-4. These would appear red in the dot-blot graph of the whole cell population (*inset*), by reverse multi-channel gating. With larger numbers of cells (*middle and lower panels, note scale of y-axis*), not only did the number of dead, To-Pro-3 positive cells become significant, but it could be seen from the dot-blot graphs (*inset*) that they were not easily separable from the general cell population in terms of size and light scatter.
Representative of more than five sets of experiments.

transfected according to standard protocol with H-Ras G12V, R-Ras G38V or both, in the presence of the transfection marker Tac-α5. Approximately 3 hours prior to harvest and analysis inhibitors were added to the culture medium to the final concentrations indicated in the table below (**Table 5a**); wortmannin was added 30 minutes prior to harvest. Cell harvest and preparation for cytometry were performed as usual, but with the addition of the live/dead cell marker To-Pro-3 (final concentration 100nM) immediately prior to flow cytometry. Dead cells were gated out using FL-4 and analysis was performed on the remaining live cells.

Table 5a: Inhibitors and concentrations

Inhibitor Agent	Final Concentration	Biochemical Target
Okadaic acid	50nM	Protein phosphatases 1 / 2
LY294002	10μM	PI 3-kinase
Wortmannin	100nM	PI 3-kinase
U-73122	1μM	Phospholipase C
PD 098059	30μM	MEK
Nocodazole	100ng/ml	Microtubule formation
DMSO	0.005%	Solvent

Figure 5.9 shows that, as baseline controls, H-Ras G12V transfection caused integrin suppression (AI = 23.6% ± 1.7), whilst R-Ras G38V produced integrin activation (AI = 134.7% ± 14.6) and was able to reverse H-Ras mediated suppression (AI = 69.2% ± 6.4), as predicted. Amongst cells transfected with both H-Ras G12V and R-Ras G38V, and incubated overnight with different inhibitor agents, only okadaic acid (50nM) resulted in any apparent reduction in AI by comparison with control cells not exposed to inhibitor.

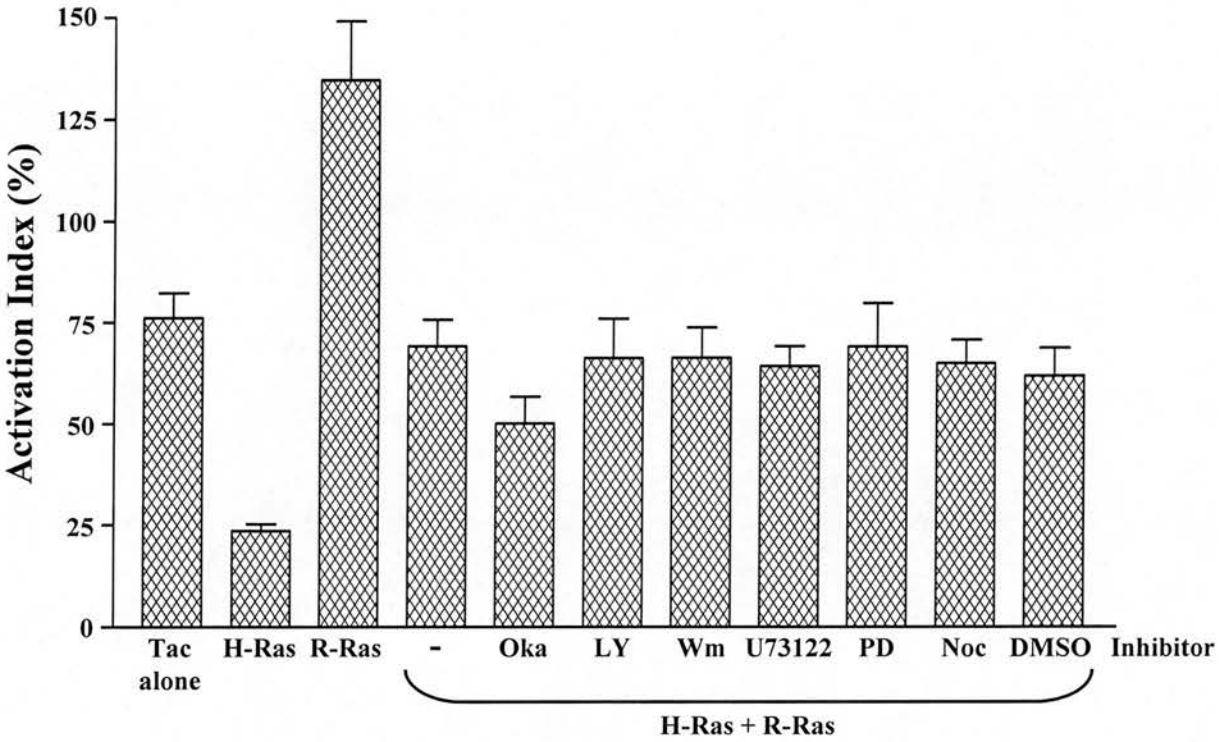


Figure 5.9: Effect of inhibitor agents on R-Ras reversal.

Summary graph of the effects of a panel of inhibitor agents on the ability of R-Ras G38V (2μg) to reverse integrin suppression mediated by H-Ras G12V (2μg). Using To-Pro-3 to screen out dead cells, the following agents were screened for any significant inhibition of R-Ras reversal: okadaic acid (Oka, 50nM); LY294002 (LY, 10μM); wortmannin (Wm, 100nM); U-73122 (1μM); PD 098059 (PD, 30μM); nocodazole (Noc, 100ng/ml). The carrier solution was DMSO (0.005%). Results shown are mean +/- standard error of three experiments.

With okadaic acid, AI was reduced to 50.1% ± 6.6; which was not significantly different from integrin activation with H-Ras G12V alone. This reduction in AI was not seen in cells incubated with DMSO carrier solution alone, suggesting that okadaic acid can partially inhibit R-Ras reversal. However, nor was it significantly different from the AI achieved with H-Ras G12V plus R-Ras G38V, so the conclusion must be that, although suggestive, there is no firm evidence that okadaic acid inhibits R-Ras reversal. The effect of incubation with other inhibitor agents was as follows: LY, AI = 66.2% ± 9.7; wortmannin, AI = 66.3% ± 7.5; U-73122, AI = 64.2% ± 5.0; PD098059, AI = 69.1% ± 10.8; nocodazole, AI = 64.9% ± 5.8 (mean ± SEM of 3 experiments). None of these agents produced any significant inhibition of R-Ras reversal.

5.5 CD98 does not reverse H-Ras suppression

Since previous work has shown that the T-cell activation antigen, CD98, is a highly specific activator of integrins, it was hypothesized that it might be able to reverse H-Ras mediated integrin suppression. However, this did not appear to be the case, at least in the αβ-py system. **Figure 5.10** shows that expression of CD98 appeared to have no influence on AI. In cells transfected with cDNA for CD98, but not with H-Ras G12V or R-Ras G38V, there was a slight increase in AI (85.9% ± 9.9) over cells tranfected with Tac-α5 alone, but this did not achieve significance. H-Ras G12V significantly reduced AI (23.6% ± 1.7), but this was not reversed to any significant degree by co-transfection

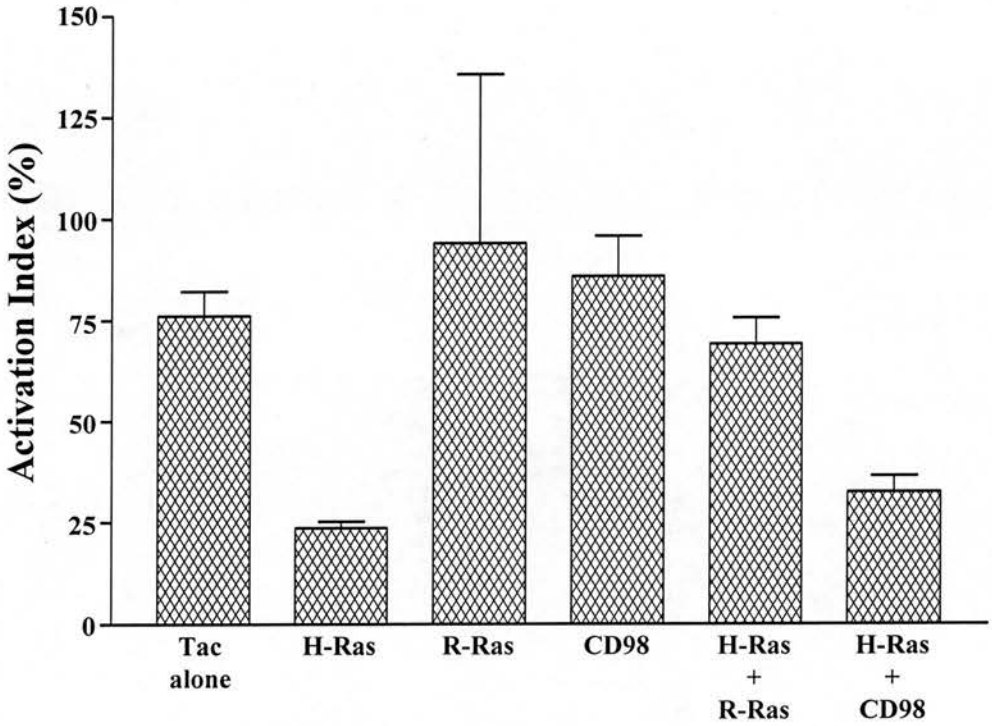


Figure 5.10: CD98 does not reverse H-Ras suppression.

Cells were transfected with cDNA for R-Ras G38V (2μg) or CD98 (2μg), in the presence or absence of H-Ras G12V (2μg). H-Ras G12V mediates integrin suppression, which was successfully reversed by R-Ras G38V; this effect was not observed with CD98. Results shown are mean +/- standard error of three experiments.

with CD98 (AI = 32.4% ± 4.0), in contrast to the reversal seen with R-Ras G38V (69.2% ± 6.4).

5.6 Discussion

This chapter describes data obtained in preliminary experiments directed at identifying candidate signalling proteins involved in R-Ras reversal of H-Ras mediated integrin suppression. The key features described are: (i) the establishment of the αβ-py CHO cell transfection system, permitting integrin activation status assessment by PAC1 binding; (ii) the influence of H-Ras G12V and R-Ras G38V on integrin activation; (iii) the effect of cyclin D1 expression; (iv) the effect of a variety of inhibitor agents on R-Ras reversal; (v) the effect of CD98. Of these, cyclin D1 appeared to reduce integrin activation status, but no other agents used here significantly influence integrin activation status in the studies shown. Finally, a significant problem was identified, namely that increased cell death might mask or exaggerate these findings. Appropriate steps were therefore taken to identify a live/dead cell marker that could be used, employing three-colour cytometry.

Baseline studies on the αβ-py CHO cell transfection system were in keeping with experience in our own and other laboratories. It has been recognised for some time that the divalent cations Mg^{2+} and Mn^{2+} promote integrin-ligand binding at a range of concentrations. They occupy co-ordinating positions in the ligand-binding sites of both

αA-domain-containing and αA-domain-lacking integrins: in the αA-domain itself, a cation is sited at the apex as a key component of the MIDAS motif (Michishita *et al.*, 1993;Lee *et al.*, 1995b); in αA-domain-lacking integrins, metal-binding sites have been identified in the αA-like domain and in the β propeller (Cierniewski *et al.*, 1994;Tuckwell and Humphries, 1997). It was therefore entirely expected that binding of the ligand-mimetic antibody PAC1 should be enhanced by Mn^{2+} but eliminated by the chelating agent EDTA. This cation-dependence and the interpretation that an "Activation Index" could be derived from the ligand binding under these different conditions, first proposed by Hughes *et al.* (Hughes *et al.*, 1997), was critical to all future work in this thesis.

Significant integrin suppression was achieved in Hughes' original report by transfection with 1μg cDNA and this has been our experience also. It is important to note that H-Ras mediated integrin suppression was not associated with integrin phosphorylation and was independent of mRNA transcription and translation (Hughes *et al.*, 1997). The pathway through which H-Ras mediates integrin suppression was shown to be through Raf-1 and MAP kinase, although more recent work by the same group has suggested that bulk activation of the ERK/MAPK pathway is not required for integrin suppression (Hughes *et al.*, 2002).

In the present work, cyclin D1 expression was associated with integrin suppression in the αβ-py system. Since a cell must be more loosely adherent to its environment in

order to undergo mitosis, cell cycle control must be intimately coordinated with integrin activation and cellular adhesion – this is clearly visible as cells "round up" *in vitro*. A corollary to this is that the protective effect brought about by integrin-mediated cell adhesion would be reduced at these times. Historically it has long been recognised that cell cycle is related to chemosensitivity, with cells in the log phase of growth much more sensitive than those in the plateau phase (Martin and McNally, 1980). Previous studies have shown that increased cyclin D1 expression is normally dependent upon Ras activation during G2 phase (Hitomi and Stacey, 1999;Sa *et al.*, 2002). Cyclin D1 transcription is stimulated through the Ras-MEK-ERK pathway in response to mitogen activation and integrin-mediated adhesion. There are further layers of control of cyclin D1 expression, due to post-transcriptional control mechanisms, since cyclin D1 accumulation occurs even in the presence of mRNA synthesis blockade (Stacey, 2003). These further layers of control include regulation of protosome-dependent proteolysis (Coqueret, 2002).

The present work does fit with this pattern of cell cycle control, in that the passage from G2 to mitosis would require coordinated integrin suppression and this is what was observed with overexpression of cyclin D1. However, it also raises the possibility that cyclin D1 contributes to the control of Ras-mediated suppression. This has not been reported before; indeed, the bulk of the literature corresponds to the flow of information the other way, i.e. control of cyclins by integrin-mediated adhesion. However, a recent report notes modulation of cellular migration (an adhesion-dependent process) in

prostate cancer cells in response to elevated levels of cdk1, associated with cyclin B2 (Manes *et al.*, 2003). In addition there is emerging evidence that the downstream effects of Ras signalling are dependent upon the cell cycle phase, such that Ras is only able to stimulate cyclin D1 in G2 phase (Stacey and Kazlauskas, 2002). Thus it may be possible that cyclins are involved in coordinating the regulation of integrin binding affinity, in an inside-out signalling process.

In the present study transfection with CD98 did not appear to significantly affect integrin affinity, although it might have been expected to do so, since it was able to rescue integrins from dominant suppression by β1 integrin cytoplasmic domains (Fenczik *et al.*, 1997b). However, CD98 did not reverse H-Ras mediated suppression in these initial studies either, so the findings presented here are thus far in agreement. There was a modest increase in integrin affinity in cells transfected with CD98 alone (compared with normal), but this did not achieve statistical significance. It is difficult to know how to interpret this. Cross-linking of CD98 has been implicated in β1 integrin activation in breast cancer cell lines (Chandrasekaran *et al.*, 1999a), and in HIV-mediated cell fusion (Ohta *et al.*, 1994) and, as discussed in Chapter 3, there is clear evidence of a direct association between CD98 and β1 integrins. Further, Fenczik *et al.* have demonstrated that the transmembrane and cytoplasmic portions of CD98 are the key domains responsible for complementation of dominant suppression (Fenczik *et al.*, 2001). CD98 is therefore intimately involved in β1 integrin function and is perhaps best thought of as a molecular facilitator within the plasma membrane.

It was a disappointment that none of the inhibitor agents appeared to influence R-Ras reversal of suppression. Of them all, the greatest effect was observed with okadaic acid. This is a potent protein phosphatase 1 and 2 inhibitor, and is the compound thought to be responsible for shellfish poisoning. Quoted IC_{50}s are 10-15nM and 0.1nM respectively for the two phosphatases (Calbiochem), although some phosphatases reveal an IC_{50} of up to 5μM (Bialojan and Takai, 1988). Since the present study used 50nM it is possible that the dose used was too low. However, this seems unlikely, since other studies on CHO cells have shown an IC_{50} of 13nM (Huynh-Delerme *et al.*, 2003). This group also demonstrated that okadaic acid induced cell death in a significant proportion of CHO cells in a time and concentration-dependent manner: at concentrations of 50nM (as for the present work), cells were engaged in apoptosis by 20 hours, but at 10nM this was delayed until 48 hours. When the initial studies with okadaic acid were performed in the present work, cells had been incubated with okadaic acid for 16-18 hours. It is unsurprising, therefore, that there was increased cell death. At the time, however, this increased cell death resulted in the false impression of inhibition of reversal of integrin suppression by okadaic acid, stimulating the search for a live/dead marker.

LY294002 is a competitive inhibitor for the ATP binding site of PI 3-kinase and has been extensively used in our group with H69 SCLC cells. Previous studies have shown an IC_{50} of 3.4 $\pm$ 1.4μM in SCLC (Rintoul, PhD thesis 2001). This compares well with published data (Vlahos *et al.*, 1994) and it would therefore appear that the concentration

used in the present study (10μM) was reasonable. Wortmannin is a non-competitive inhibitor of PI 3-kinase, extensively used by our group, with an IC_{50} of 8.0 ± 2.1 in H69 SCLC (Rintoul, PhD thesis 2001); again this is comparable to the literature (Powis *et al.*, 1994) and the dose of 100nM used in the present study would be expected to produce an effect if one existed. This concurs with the findings of other groups that the PI 3-kinase pathway is not involved in R-Ras reversal, in a variety of cell types including the $\alpha\beta$-py cell system (Sethi *et al.*, 1999a;Kinashi *et al.*, 2000;Self *et al.*, 2001).

Nocodazole disrupts microtubule formation, interfering with their function during mitosis. *In vivo* experiments on mammalian cells normally use doses of 0.04 – 10μg/ml, although higher doses have been used and the concentration used in the present study (100nM) was both within this range and sufficient to effectively block mitosis in CHO cells, as judged by an accumulation of mitotic cells (unpublished observations).

U-73122 is an inhibitor of phospholipase C, a key enzyme that modulates phosphoinositide levels, Ca^{2+} release and PKC activation. In human platelets and neutrophils its IC_{50} is in the range 1 – 2.1μM but it also acts as a potent inhibitor of neutrophils adhesion, with an IC_{50} of 50nM (Calbiochem). In the present study it was used at 1μM, which is comparable with concentrations used by Vossmeyer *et al.*, who found that $\alpha1\beta1$-mediated adhesion of PC12 cells and CHO cells (transfected with $\alpha1$ integrin subunit) to laminin, collagen and fibronectin was inhibited at doses of 0.5 – 1.0μM (Vossmeyer *et al.*, 2002). This suggests that PLC is required for $\beta1$-dependent

adhesion and the same group also showed that PLCγ coimmunpoprecipitated with α1β1 integrin, suggesting a direct physical association. In haematopoietic progenitor cells, inhibition of PLC by up to 1μM U-73122 led to reduced CD43-enhanced integrin-dependent adhesion to fibronectin (Anzai *et al.*, 1999). Thus, the weight of evidence supports an effect of PLC in promoting integrin-mediated adhesion, but the present study has not been able to observe this in the context of R-Ras reversal of suppression.

PD098059 is a MAPK inhibitor that specifically inhibits MEK1. Published data in Swiss 3T3 cells suggests an IC50 of 10μM (Dudley *et al.*, 1995); in CHO cells within our group an IC50 of 12μM was found and it was routinely used at a concentration of 30μM (Lad, PhD thesis 2002). Initial reports found no evidence that the ERK/MAPK pathway was involved in R-Ras reversal (Sethi *et al.*, 1999a) and the current work supports this, since PD098059 did not block the effect. Only weak evidence has been found to propose R-Ras activation of the ERK/MAPK pathway: for example, an R-Ras effector loop mutant was described by Osada *et al.* to have weak MAPK stimulating activity (Osada *et al.*, 1999); R-Ras has also been shown to have a synergistic effect with IGF-1 in inhibition of apoptosis, through both MAPK and PI 3-kinase (Suzuki *et al.*, 1997). It seems likely, therefore, that any MAPK activity is more important for the anti-apoptotic effects of R-Ras, rather than integrin modulation.

In summary, this chapter has described the successful establishment of the αβ-py CHO cell system for assessment of integrin activation status, together with initial assessment

of potential modulators of integrin affinity. There was some evidence of integrin suppression with cyclin D1, suggesting a role for the cell cycle machinery in controlling events at the cell surface, but a range of inhibitor agents has cast no specific light on events downstream of R-Ras in reversal of suppression. No individual key signalling protein was identified – nor, perhaps, was it likely to be using this rather broad sweep of experimental technique. A more refined technique was felt necessary to identify specific proteins, employing the αβ-py cell system as a screening device to isolate candidate proteins from a DNA library.

Chapter 6

A Genetic Screen for Reversal of H-Ras Suppression

6.1 Introduction

The method described in Chapter 5 was unlikely to bring to light a specific protein that reversed H-Ras-mediated integrin suppression – it was only ever likely to identify a general pathway through which reversal of suppression might be mediated. Proteins involved in reversal of H-Ras suppression would be expected to mediate integrin activation. In order to identify possible novel proteins that are involved in this – and in particular to try and identify mediators of R-Ras reversal of suppression – the αβ-py cell system was used in an expression cloning screening technique that has already been of proven use in this field. By this method the transmembrane protein CD98 was isolated as a protein capable of reversing β1 integrins from trans-dominant suppression induced by β1 tails (Fenczik *et al.*, 1997a); separately, PEA-15 was isolated for its ability to reverse H-Ras G12V-mediated integrin suppression (Ramos *et al.*, 1998).

To date no clear mechanism has been elucidated to explain how R-Ras is able to rescue H-Ras-mediated integrin suppression. Although it does not appear to be a direct activator of integrins, R-Ras strongly antagonized the Ras/Raf-initiated pathway involved in integrin suppression in the αβ-py cell system (Sethi *et al.*, 1999a). This was not due to simple competition between H- and R-Ras for the common effector Raf-1, since R-Ras also reversed suppression induced by the activated variant Raf-BXB, which lacks a Ras-binding domain. In addition, R-Ras had no influence on ERK2 phosphorylation by either H-Ras or Raf-BXB and nor was R-Ras reversal mediated by the PI 3-kinase or Ral pathways. Other groups have supported the findings that neither the ERK/MAPK pathway, nor the PI 3-kinase pathway are involved in R-Ras reversal, in a variety of cell types (Kinashi *et al.*, 2000;Self *et al.*, 2001). Indeed, more recently Hughes *et al.* have reported that H-Ras-mediated integrin suppression was itself not

related to bulk activation of the ERK/MAPK pathway as initially believed, although it was still mediated through Raf-1 (Hughes *et al.*, 2002). This suggests the existence of novel pathways through which integrin activation is regulated. There is, however, some evidence that both PI 3-kinase and PKC may be important for R-Ras activity in some cell types: R-Ras-induced adhesion and migration on collagen by breast epithelial cells was both PI 3-kinase- and PKC-dependent, but not MAPK-dependent (Keely *et al.*, 1999). The mechanism for R-Ras reversal may therefore be cell-type and integrin specific.

In a search for mediators of R-Ras reversal, it was postulated that cells expressing activated β1 integrins would express mRNA for proteins involved in integrin activation. A cDNA library derived from such a cell population would thus contain cDNA for candidate proteins that might be isolated with the αβ-py cell system. For this reason, a cDNA expression library (H4) derived from human promonocytic leukaemia U937 cells, activated with phorbol 12-myristate 13-acetate, was obtained from the MRC Human Genome Mapping Project and used to screen for genes capable of reversing H-Ras-mediated integrin suppression. This library had been cloned into the pCDM8 expression vector, which contains a CMV promoter and polyoma T antigen origin of replication, in order to promote autosomal replication in αβ-py cells.

6.2 Application of cDNA library

6.2.1 Library amplification

In order to produce enough cDNA for transfection, the library was amplified using commercially competent TOP10/P3 *E. coli* (Invitrogen), following the manufacturer's instructions. cDNA was recovered by use of Qiagen Midi-prep and the quality of cDNA confirmed by UV spectral analysis. This confirmed a ratio of DNA/RNA of 2.04, which was satisfactory for transfection purposes.

Following amplification, the amplified cDNA was analysed by restriction enzyme digest to ensure the amplification strategy had worked and to confirm that the amplified library still contained pCDM8 expression vector. Hind III and XhoI both have digestion sites flanking the insert cloning site for pCDM8. As shown in **Figure 6.1**, both digests confirmed the presence of pCDM8: digestion of pCDM8 empty vector produced a band of ~ 4.0kb, which is also seen in the library, together with a DNA smear of multiple bands, representing the cDNA library inserts. With XhoI these range from ~750bp – 3.0kb, whilst with HindIII they ranged from ~3.0kb – 12kb. Thus library amplification was achieved successfully, without loss of the pCDM8 vector.

6.2.2 Transfection and library sorting

Preliminary experiments had previously been conducted by Dr. Y. Lad (PhD Thesis, 2001), establishing antibody dilutions and cell numbers to be used for transfection and cell sorting. Due to the absence of an FL-4 channel on the cell sorter, the viability stain 7-AAD (observed in FL-3) replaced To-Pro-3.

Cell transfection and sorting was performed as described in "Materials and Methods". Briefly, in 100mm plates, αβ-py cells were transfected with: control pCDM8; H-Ras G12V alone; H4 library alone; or H-Ras G12V plus H4. All plates were transfected with Tac-α5 as a marker for transfection. The concentrations and quantities of cDNA were as indicated in **Table 6a**. Transfection and cell culture followed standard technique.

Table 6a: Transfection protocol for cDNA library screen

cDNA	Concentration (μg/μl)	Amount used per 100mm dish
H-Ras G12V	1	2μg
Tac-α5	1.5	2μg
H4 library	2	4μg
pCDM8	0.73	4μg

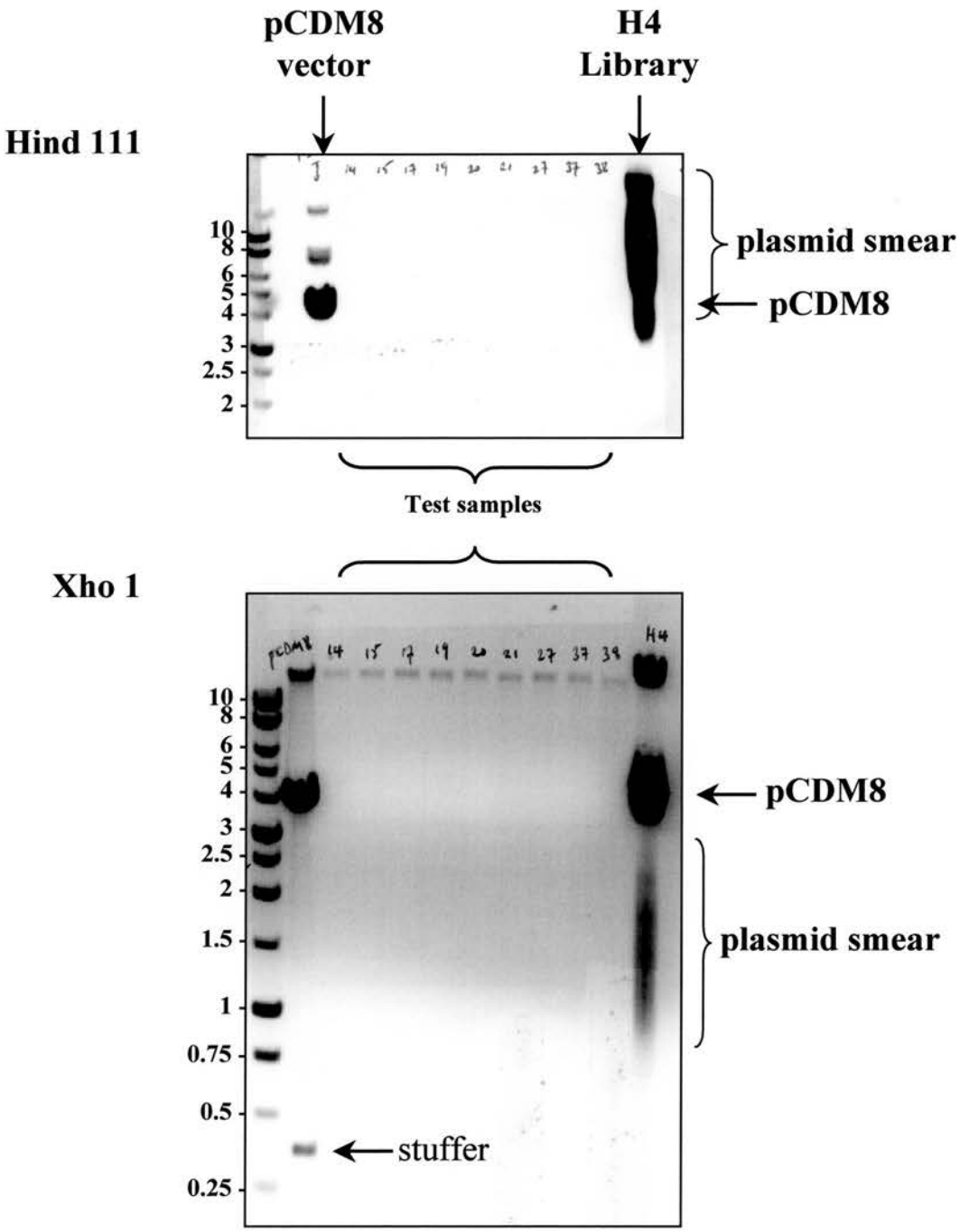


Figure 6.1: Confirmation of successful library amplification.

TAE gels following restriction enzyme digest with Hind 111 (*upper panel*) and Xho 1 (*lower panel*). A sample of positive control vector pCDM8 was digested alongside amplified H4 library (2μg) and test samples derived from *E. coli* transformation, to look for the presence of the pCDM8 vector (see later). In test samples no vector was observed, but pCDM8 vector was identified in the amplified library (last column) in each digest. Xhol digestion also revealed the blank "stuffer" of the control vector (~0.4kb) and a smear of multiple, different length plasmids in the amplified library.

During cell sorting, those cells transfected with H-Ras G12V exhibited a marked reduction in PAC1 binding compared with cells transfected with pCDM8 control vector, as expected. Using these "standards", cells transfected with H-Ras G12V plus H4 were sorted and collected from two Gates, R2 and R4. Cells in R2 exhibited exceptionally high PAC1 binding, in the face of H-Ras G12V transfection and therefore represented cells which may have been transfected with cDNA strongly promoting integrin activation. Cells in R4 also exhibited positive PAC1 binding in the face of transfection with H-Ras G12V, but were less positive than those in R2, which was thus a tighter gated population. The total number of cells (or, strictly speaking, "events") collected in Gates R2 and R4 were 97,731 and 262,232, respectively. The sorting analysis is illustrated in **Figure 6.2**.

Following cell sorting, Hirt extraction was performed on the gated cells to extract the transfected plasmids within, as described in "Materials and Methods". Plasmid DNA was digested with the Dpn I restriction enzyme, prior to transformation of TOP10/P3 *E. coli*. From Gate R2, following transformation and overnight culture on antibiotic-restricted plates, a total of 82 colonies were initially recovered. From Gate R4, a total of 622 colonies were recovered.

6.3 Analysis of Gate R2

From Gate R2 82 colonies were picked and grown up in Agar broth, with appropriate antibiotic restriction; seven failed to grow and were lost. To identify and confirm that the remaining 75 contained the correct pCDM8 plasmid, samples were miniprepped (Wizard Miniprep, as described in "Materials and Methods") and a number were subjected to DNA digest by Hind III, alongside the H4 library, with pCDM8 vector as positive control. As can be seen in **Figure 6.1**, although the pCDM8 control vector and H4 library clearly demonstrate the ~ 4.0kb band as before, no evidence of the pCDM8

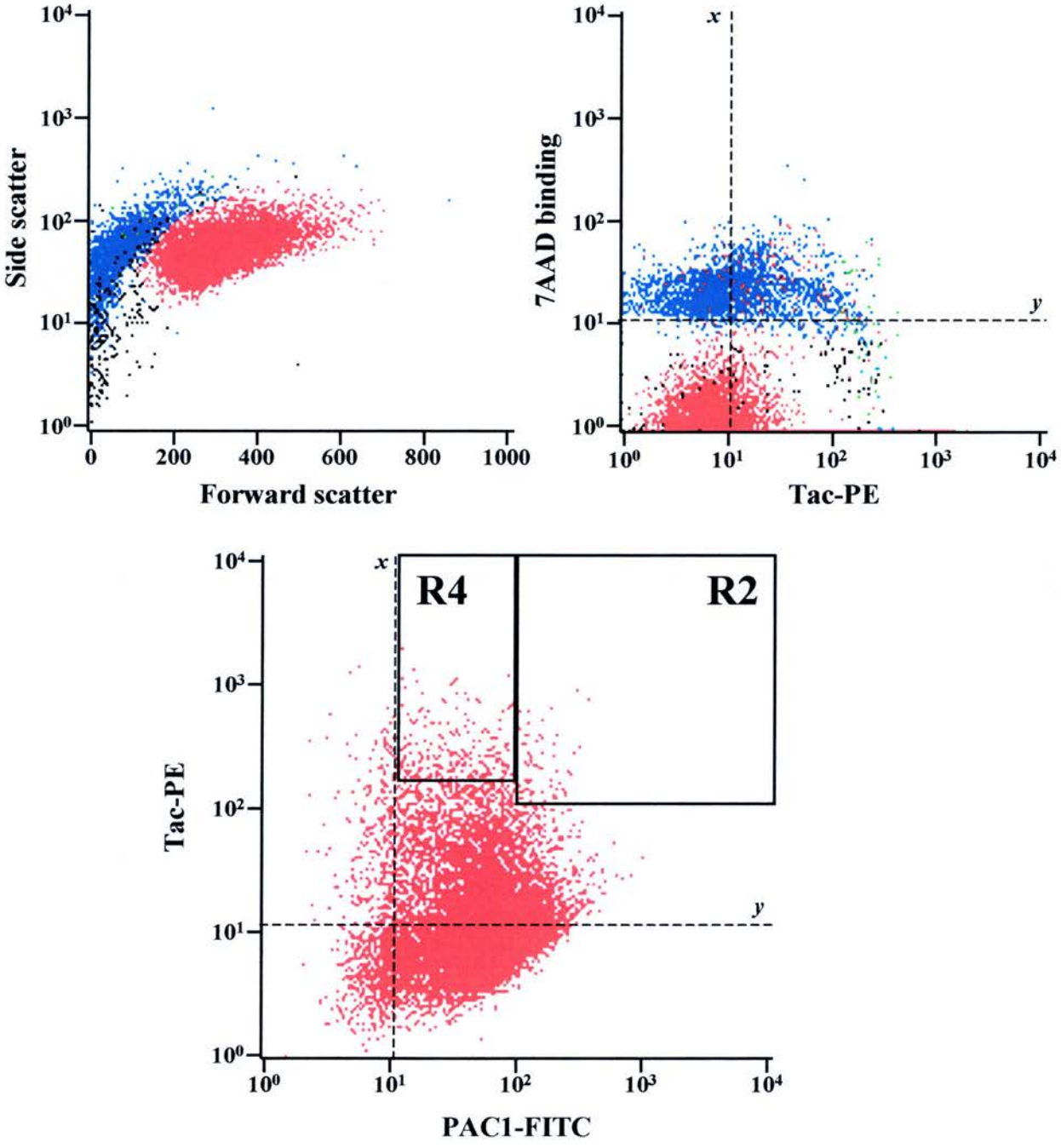


Figure 6.2: Cell sorting analysis template.

Dot-blot graphs summarising the cell sorting of transfected cells in the genetic screen. αβ-py cells transfected with Tac-α5, H-Ras G12V and the H4 cDNA library were subject to cell sorting.

The total cell population was first gated according to the viability stain 7-AAD, which was taken up by dead cells (*upper panels, blue*) and excluded by live cells (*upper panels, red*). Highly transfected cells exhibited strong Tac-PE fluorescence (*above dashed line, y*). Those that exhibited elevated PAC1-FITC binding (*right of dashed line, x*) were gated and collected in two populations, R2 and R4 (*lower panel*). Of these, R2 exhibited exceptionally high PAC1 binding and R4 positive PAC1 binding. Both populations suggested integrin activation in the face of H-Ras G12V transfection.

vector was observed in any of the test samples. This was repeated for a number of samples using XhoI, with similar results, illustrated in **Figure 6.1**. The implication from these experiments was that although the colonies had grown on antibiotic-restricted plates and LB broth, nevertheless they did not carry the pCDM8 plasmid, nor any potential candidate cDNA.

In order to confirm this as far as possible, it was determined that all R2 candidate samples should be tested for the presence of pCDM8 vector by polymerase chain reaction (PCR). 5ml cultures were prepared for all 75 colonies, under antibiotic restriction, and from these Wizard Minipreps were performed to isolate cDNA at concentrations of 45 – 85ng/μl, with DNA/RNA ratios of 1.53 – 2.63. From the known map of pCDM8 vector, forward and reverse primers were selected and ordered from MWG-Biotech. The PCR regime was as follows in **Table 6b**:

Table 6b: PCR protocol

Step	Temperature (°C)	Time (secs)
1	95	120
2	80	120
3	95	60
4	59	60
5	72	90
Repeat Steps 3-5 x 25 then stop		

A test PCR using pCDM8 vector (4ng/μl) or distilled water control confirmed that a PCR product could be identified after 25 cycles of PCR with pCDM8 vector as substrate, but not with H_2O alone (*data not shown*). PCR was therefore performed on all 75 samples, using 2μl of each sample, with positive (pCDM8 vector) and negative (distilled H_2O) controls. Following PCR, samples were separated on Southern blot in TAE gels and visualized with UV light. **Figure 6.3** shows all 75 test lanes, with controls. This demonstrates that although the pCDM8 positive control routinely produced a visible

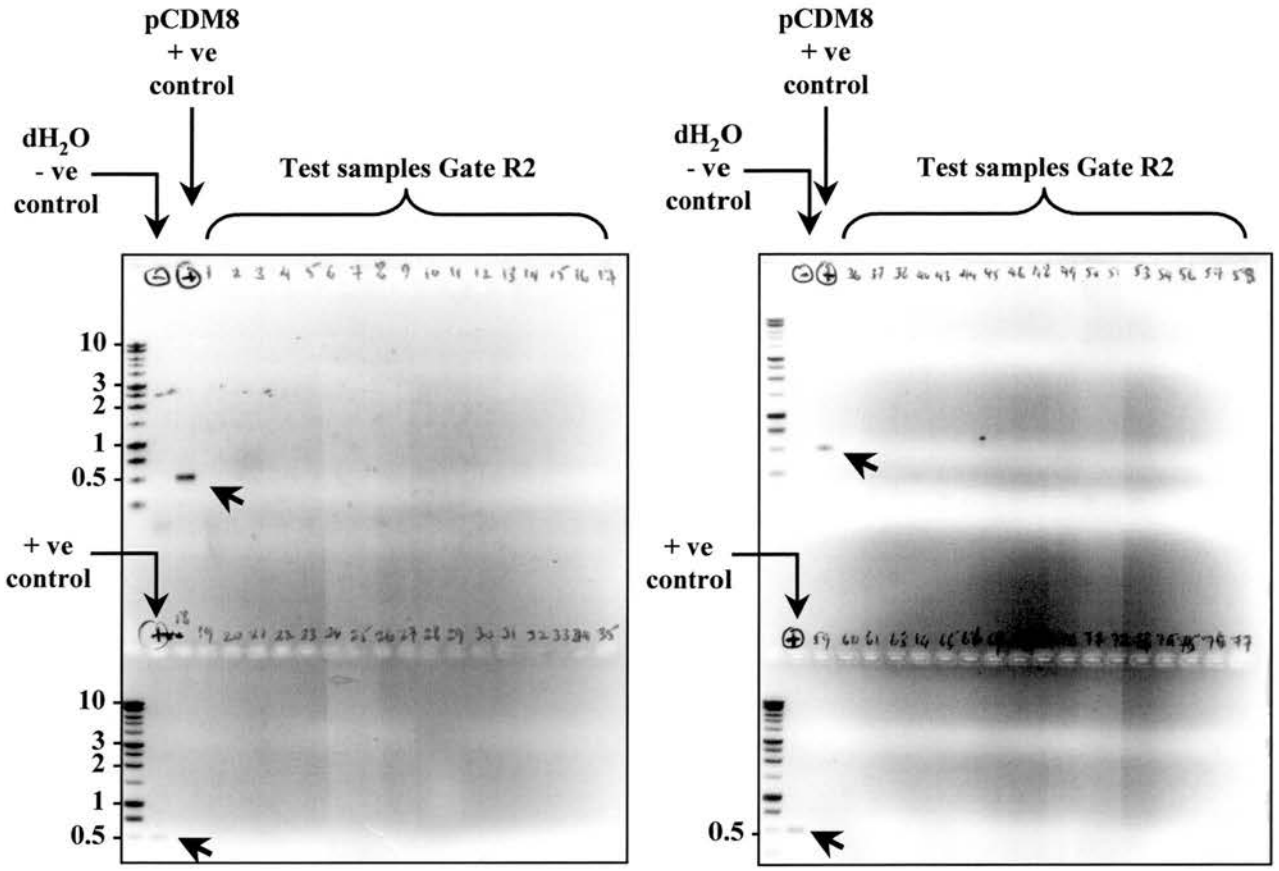


Figure 6.3: Gate R2 cells do not contain the pCDM8 vector.

TAE gel showing results of PCR, using pCDM8 vector as positive control and distilled water as negative control, following 25 cycles of PCR. The PCR product was clearly identifiable in the positive control lanes alone (*arrowed*). Successfully transformed and cultured samples from Gate R2 comprised the test samples. By comparison with the positive control pCDM8, none of the test samples (n=75) exhibited a PCR product, suggesting the absence of pCDM8 in these samples.

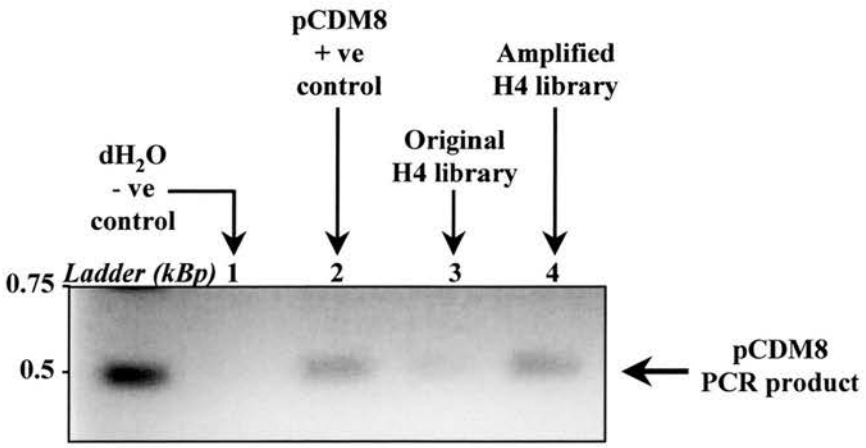


Figure 6.4: PCR confirms presence of pCDM8 in transfection library.

TAE gel showing results of PCR, using pCDM8 vector as positive control and distilled water as negative control (*lane 1*). Both the original, unamplified H4 library (*lane 3*) and the amplified H4 library used for transfection (*lane 4*) show the PCR product identified from the pCDM8 positive control lane (*lane 2*).

PCR product of ~500bp, none of the 75 test samples showed the presence of the pCDM8 vector.

To confirm that the amplified H4 library which had been used for transfection and cell sorting had definitely contained the pCDM8 vector, PCR was performed on the original H4 library and the amplified H4 library cDNA, using the above primers. Samples were run out and analysed using a TAE gel as before. **Figure 6.4** shows that the PCR product can be identified in the pCDM8 positive control (*lane 2*), the original H4 library (*lane 3*) and the amplified H4 library used for transfection (*lane 4*), but not in a negative control (*lane 1*).

The conclusion from this series of experiments was that none of the colonies grown from Gate R2 contained bacteria carrying the pCDM8 plasmid and, by inference, candidate cDNA from the H4 library.

6.4 Analysis of Gate R4

6.4.1 PCR screening of samples from cDNA for pCDM8 vector

A total of 622 colonies were recovered following transformation of the Gate R4. These were retrieved in four batches (A-D). To ensure efficiency of further studies it was felt important, given this large number of samples and the negative results in Gate R2, to use PCR as a relatively fast technique for screening Gate R4 for the presence of pCDM8 and enabling negative samples to be discarded.

Initial screening was performed on Batches A (16 samples) and B (57 samples). Qiagen Minipreps were prepared for all samples according to the manufacturer's guidelines, resulting in cDNA at concentrations of 20 – 45ng/μl, with DNA/RNA ratios of 1.75 – 2.33. Batch A samples were subject to PCR as before and samples analysed by Southern blot. **Figure 6.5a** shows that only samples 8 and 13 demonstrated a PCR product in

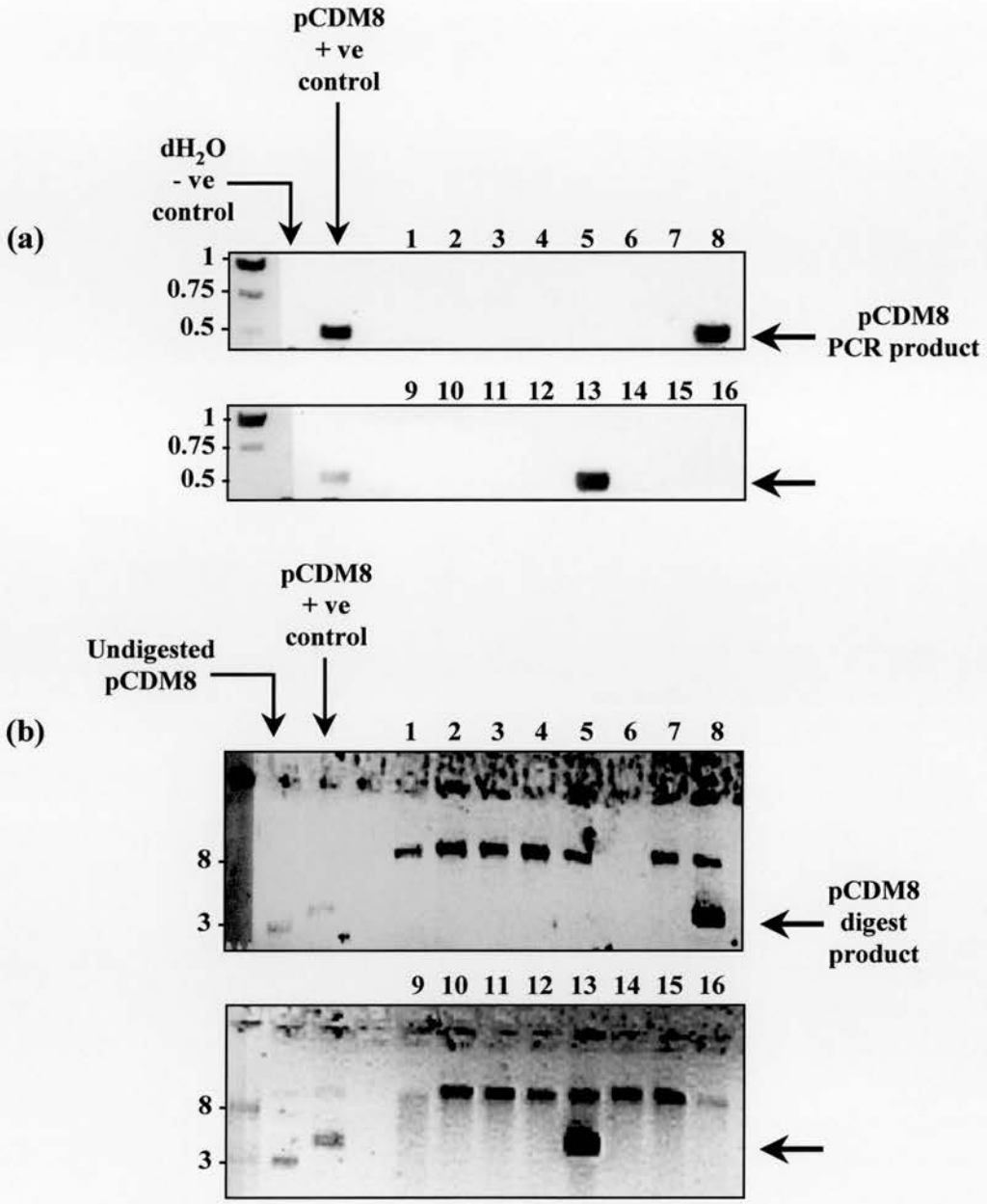


Figure 6.5: A number of Gate R4 samples contain pCDM8.

(a) TAE gel showing PCR results for the 13 samples of Batch A. Only samples 8 and 13 exhibited the PCR product corresponding to pCDM8 vector, as confirmed in the positive control lane.

(b) TAE gel showing the result of Xho 1 restriction enzyme digest for Batch A samples. Again, samples 8 and 13 were the only positive lanes, supporting the findings of PCR.

keeping with a positive control. Restriction enzyme digest with Xho I performed on the same cDNA samples confirmed this result, with all other Batch A samples exhibiting no vector (**Figure 6.5b**). From this it was concluded that the PCR was sensitive and specific; it was therefore used for Batch B. **Figure 6.6** shows that PCR identified 45 positive samples out of a possible 57 in Batch B.

6.4.2 PCR screening of samples from bacterial colonies for pCDM8 vector

Despite this success, the logistics of screening Batches C and D, which had 342 and 207 samples respectively (*i.e.* a total of 549 samples), prompted the decision to try PCR directly from bacterial samples, without the necessity for miniprepping samples of cDNA. All bacterial colonies were plated onto gridded 10cm x 10cm agarose plates for further culture and ease of management. In order to test the PCR regime for sensitivity and specificity, PCR was performed directly from colonies from Batch A, in which samples 8 and 13 had been convincingly shown to be the only positive samples. Preparation of samples for PCR was as described in "Materials and Methods", using the PCR regime in **Table 6c**.

Table 6c: PCR protocol

Step	Temperature (°C)	Time (secs)
1	95	120
2	80	120
3	95	60
4	59	60
5	72	90
Repeat Steps 3-5 x 35 then stop		

Figure 6.7 shows that once again only samples 8 and 13 were identified as carrying the pCDM8 vector, in keeping with a positive control lane (*lane 2*), suggesting the PCR regime was sensitive and specific. This was therefore used for further screening of Batches C and D samples, directly from bacterial colonies. Distilled water was used as a

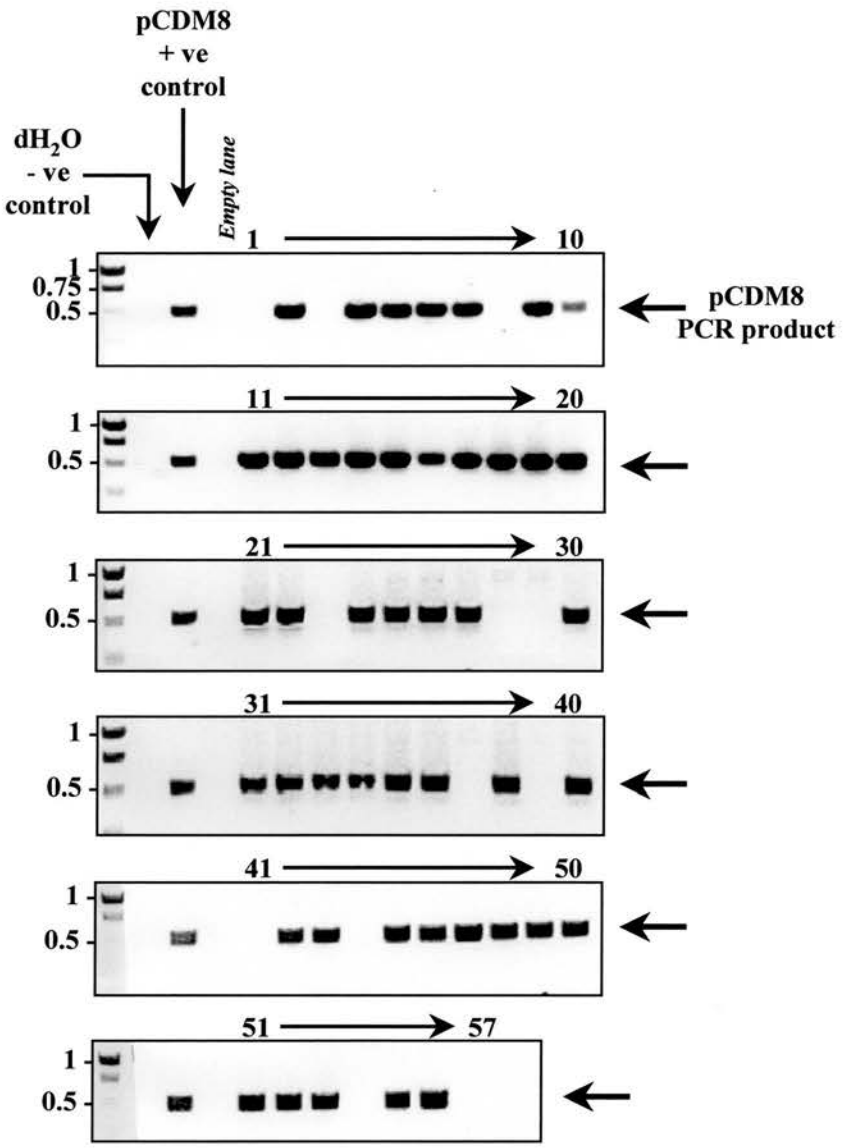


Figure 6.6: PCR results for Gate R4, Batch B.

Miniprepped cDNA from all 57 samples of Gate R4, Batch B were subjected to PCR under the conditions described. The compilation TAE gel shows that the pCDM8 PCR product was observed in the positive control lane (*lane 2*), together with 45 of the 57 test samples. A negative control (distilled water, *lane 1*) demonstrated no PCR product. Ladder shown is in kilo-basepairs.

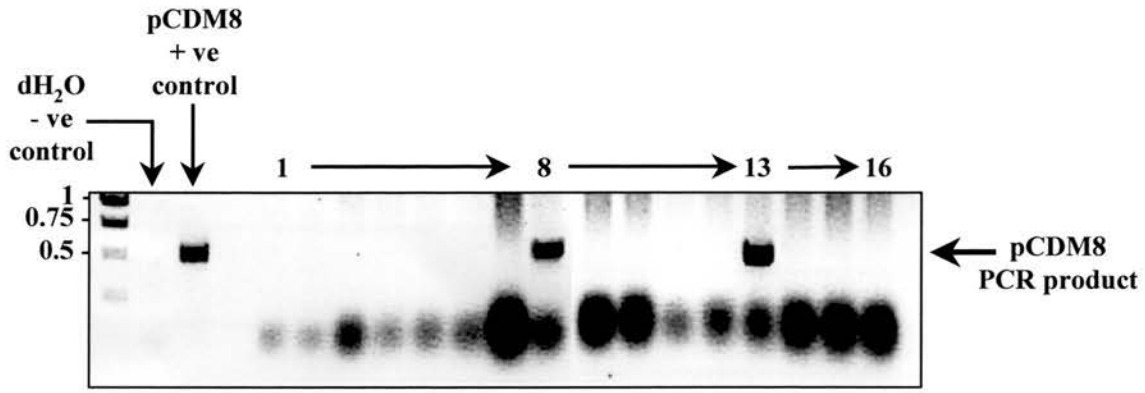


Figure 6.7: PCR directly from bacterial colonies.

Samples were taken directly from colonies of transformed bacteria for Gate R4, Batch A, grown under antibiotic restriction. The TAE gel shows that the pCDM8 PCR product was observed in the positive control lane (*lane 2*), together with samples 8 and 13. A negative control (distilled water, *lane 1*) demonstrated no PCR product. This result was identical to earlier assessments of this batch. Ladder shown in kilo-basepairs.

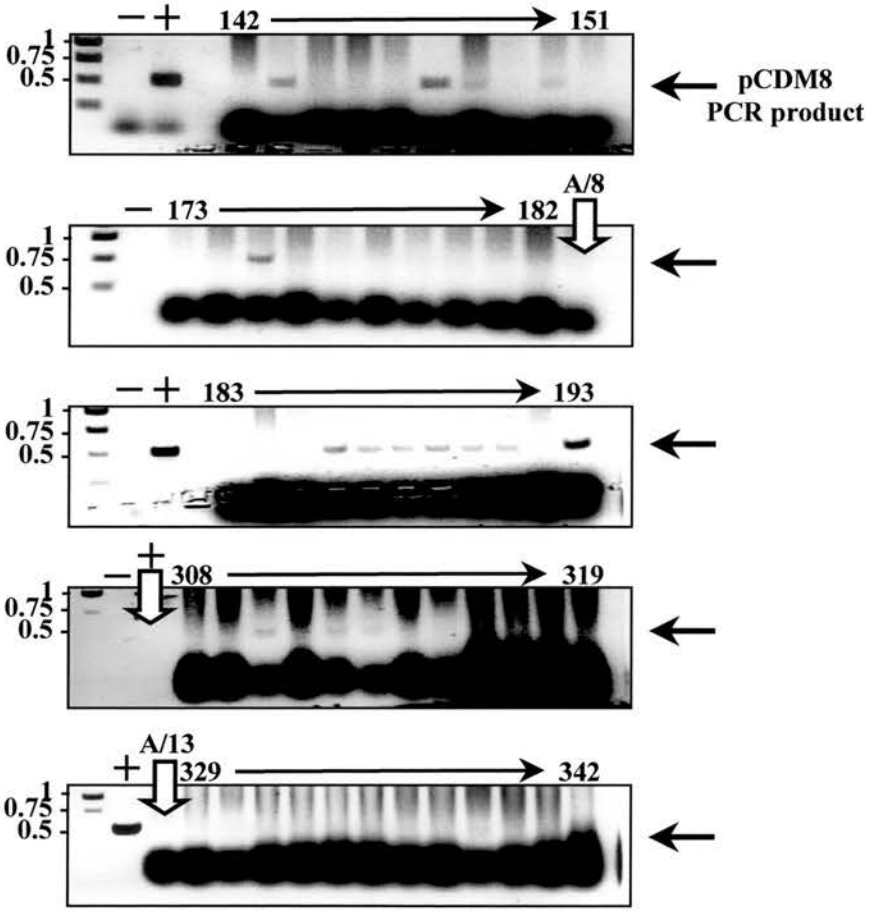


Figure 6.8: PCR for Gate R4, Batch C.

Samples were taken directly from colonies of transformed bacteria for Gate R4, Batch C, grown under antibiotic restriction. The TAE gels show representative PCR results for the 342 colonies tested in Batch C. Positive PCR products were identified in a number of samples, whilst negative results were also numerous. Notably, a number of positive controls were negative (*arrowed*); these included samples from Batch A/8 or A/13, or the pCDM8 vector itself (*positive control,* +). Distilled water was used for negative control samples (-). Ladder shown in kilo-basepairs.

negative control for this PCR regimen; for positive controls, both control pCDM8 empty vector and samples from either Batch A/8 or /13 were utilised.

The results from Batch C are illustrated in part in **Figure 6.8**. This demonstrates that although a number of positive samples were clearly identified, there were also occasions when the positive controls were *not* positive for PCR product. There were no occasions when the negative control appeared positive, however. This implied that in fact this PCR regimen was producing false negative results and might be failing to identify samples containing the pCDM8 vector.

On review of the PCR conditions, it was felt that the annealing temperature (59°C) might be too stringent for the primers, producing false negative results. Analysis of the base makeup of the primers suggested annealing temperatures of 56.6°C and 54.6°C for the forward (F2) and reverse (R1) primers, respectively. The annealing temperature was reduced and times adjusted according to the new regime (**Table 6d**).

Table 6d: PCR protocol

Step	Temperature (°C)	Time (secs)
1	95	120
2	80	120
3	95	120
4	51	120
5	72	120
6	95	60
7	51	60
8	72	90
Repeat Steps 6-8 x 33 then stop		

Batch A samples were again used for preliminary testing of the adjusted protocol, results of which demonstrated a correctly weighted PCR product for samples 8 and 13, with the remaining samples producing incorrectly sized products compared with pCDM8 control,

or no product at all (**Figure 6.9**). This test confirmed that the new protocol was, at very least, still specific for pCDM8 and suitable for further screening.

Batches C and D were subjected to PCR screening for the presence of the pCDM8 vector under the new PCR protocol. The results are shown in **Figure 6.10**, which demonstrates that multiple samples were able to produce a positive PCR product, of the correct weight, when compared to positive control pCDM8 empty vector. Of particular note, the new protocol demonstrated multiple positive samples previously deemed negative by the first protocol. These were therefore true positives, which might otherwise have been lost. The interpretation of this was that either these were true positives, containing pCDM8 vector (and therefore potential candidate cDNA), or they were false positives, despite PCR products of identical size to the pCDM8 positive control. Although the latter seemed less likely, at least retention of these samples would not result in loss of potential candidate cDNA, as might otherwise have been the case.

A total of 479 samples from Batches A – D were identified by PCR screening as carrying the pCDM8 vector. For all positive samples, fresh colonies were plated onto gridded, 10cm x 10cm, antibiotic-restricted agarose plates (A – E). Colonies were re-classified according to plate and number (A1 – 99; B1 – 99; etc) for the next stage of screening.

6.4.3 Screening of sample pools for pCDM8 vector

Further screening was performed using the αβ-py cell system to identify samples with an ability to reverse H-Ras G12V integrin suppression. Samples were first pooled into groups of 10 samples each, where A1 contained pooled cDNA from samples A1-10, A2 contained pooled cDNA from samples A11-20, etc.. For each pool, 500ml cultures were set up and cDNA prepared using Promega Maxiprep, as described in "Materials and Methods". cDNA was resuspended in nuclease-free H_2O and was of satisfactory purity for optimal transfection. To confirm that such pooled cDNA samples contained

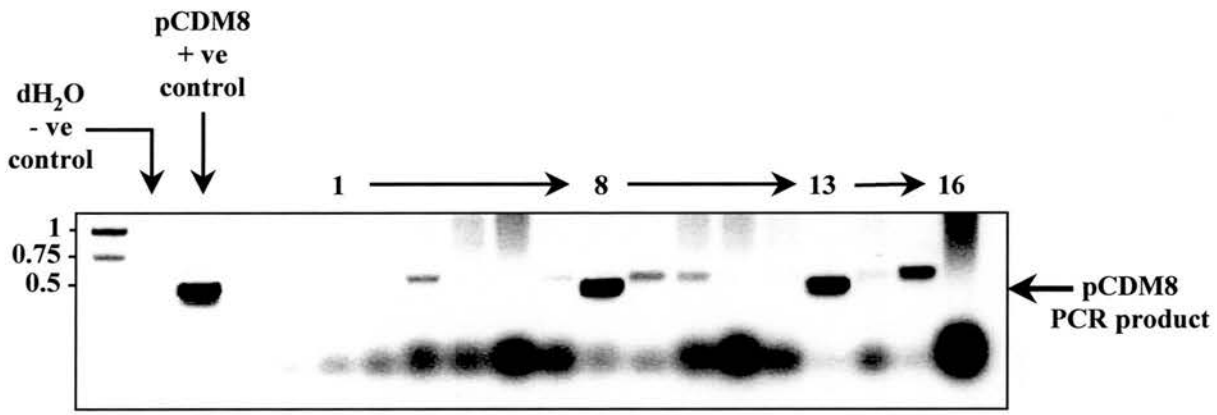


Figure 6.9: Adjusted regime for PCR directly from bacterial colonies.
Samples were taken directly from colonies of transformed bacteria for Gate R4, Batch A, grown under antibiotic restriction. The TAE gel shows that the pCDM8 PCR product was observed in the positive control lane (*lane 2*), together with samples 8 and 13. A negative control (distilled water, *lane 1*) demonstrated no PCR product. All other samples produced either a product of incorrect size, or no product at all. Ladder shown is in kilo-basepairs.

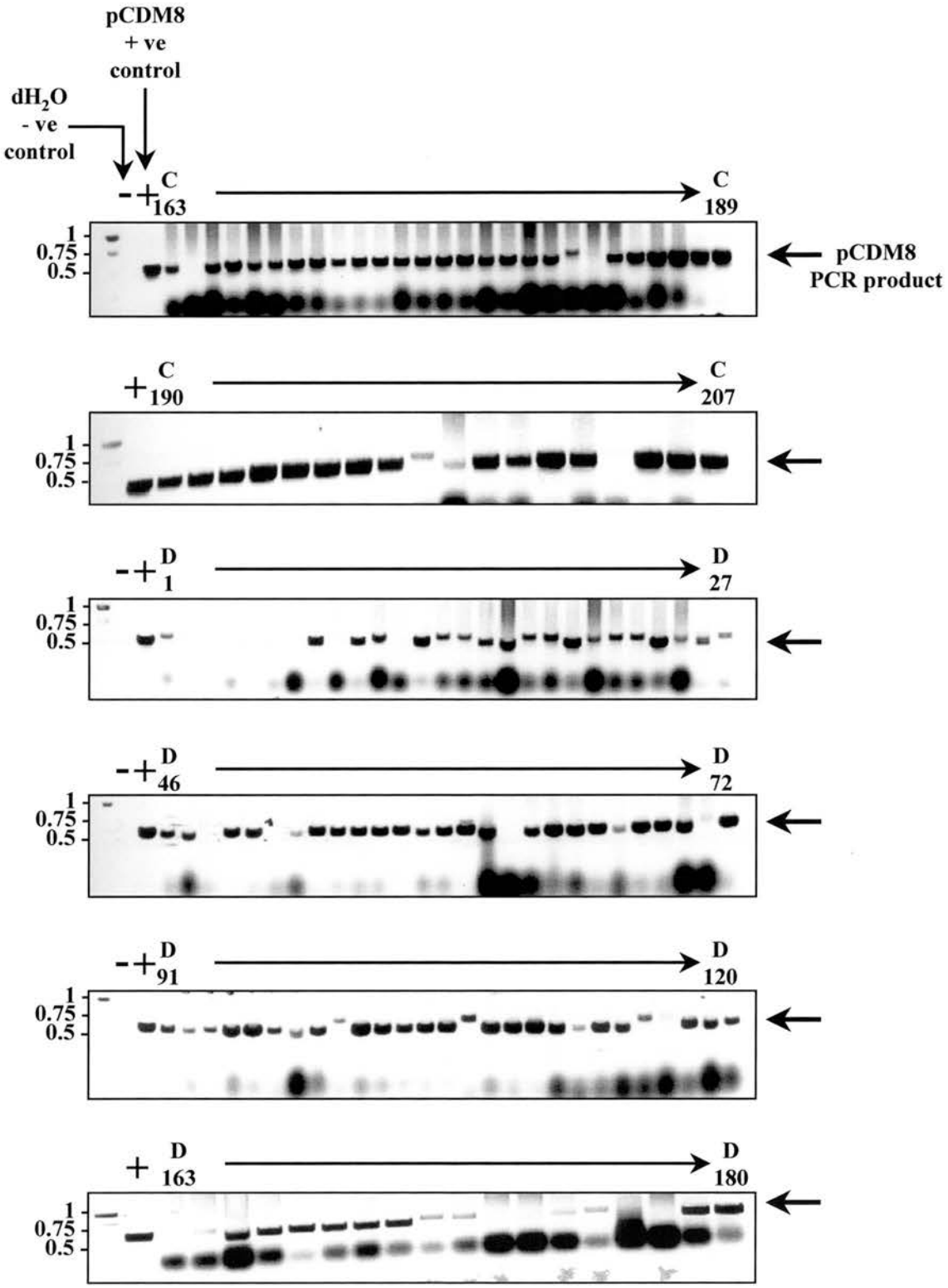


Figure 6.10: PCR identifies colonies expressing pCDM8 vector.

Samples were taken directly from colonies of transformed bacteria for Gate R4, Batches C and D, grown under antibiotic restriction. The TAE gels show representative PCR results for the 549 colonies tested. Positive PCR products were identified in a number of samples; negative results were less frequent than with the former PCR regimen. All positive controls were consistently positive (*positive control,* +). Distilled water was used for negative control samples (-). Ladder shown is in kilo-basepairs.

appropriate pCDM8 vector and cDNA inserts, sample pools were subject to Xho I restriction enzyme digest. **Figure 6.11** (*upper panel*) shows that pools A1 and A2 contain the pCDM8 vector and multiple inserts of different weights (*lanes 4 and 5*), as would be expected and as demonstrated in other single samples identified by PCR screening (above). By contrast, when not digested, pools A1 and A2 demonstrate a slightly larger band, representing the intact vector plus insert(s) (*lanes 2 and 3*). **Figure 6.11** (*lower three panels*) shows that in all 49 sample pools (A1 – E9) the pCDM8 vector was identified, together with a variety of different sized inserts. Thus all the sample pools A1 – E9 contained the pCDM8 vector and candidate cDNA.

6.4.4 Screening of sample pools for candidate cDNA

Sample pools A1 – E9 were subjected to screening using the αβ-py CHO cell system. For each experiment, 10 pools were tested. αβ-py cells underwent transfection with control or test sample cDNA as indicated in the table below (**Table 6e**).

Table 6e: Transfection protocol

	Construct	cDNA quantity
All samples transfected with 2μg Tac-α5	H-Ras G12V	2μg
	pCDM8 control	4μg
	H-Ras G12V + R-Ras G38V	2μg + 2μg
	H-Ras G12V + pCDM8 control vector	2μg + 4μg
	H-Ras G12V + pooled cDNA (A1 – E9)	2μg + 4μg

Transfection, cell harvest and preparation for flow cytometric analysis were all performed according to standard protocol, as described in "Materials and Methods".

The results are shown in **Figure 6.12a – e**. This shows that for all experiments, control samples behaved as predicted: H-Ras G12V induced integrin suppression; pCDM8 control vector alone had no effect in integrin status, nor did it reverse integrin

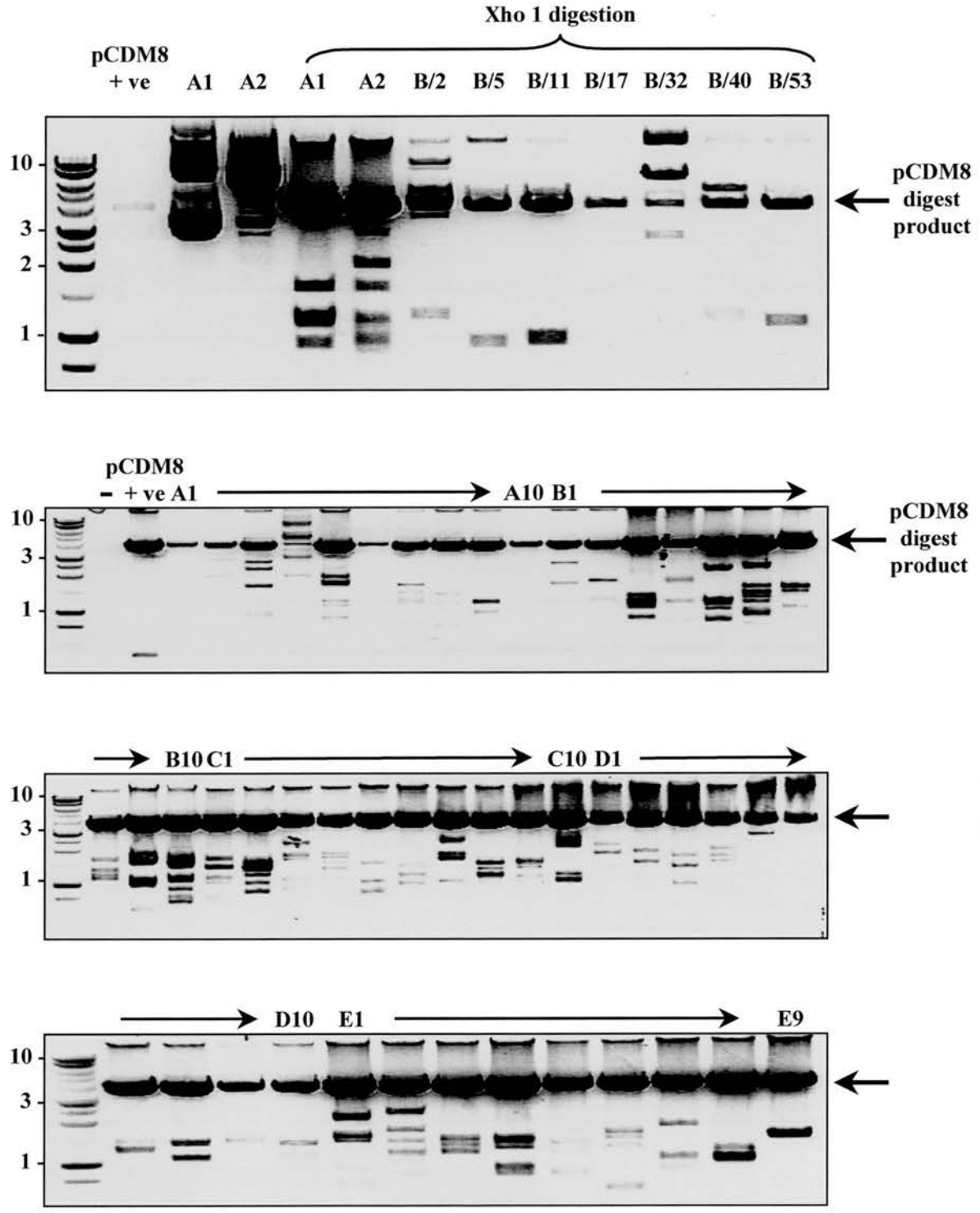


Figure 6.11: Sample pools contain pCDM8 vector and candidate cDNA inserts.

Upper panel: TAE gel confirming the presence of vector pCDM8. Individual samples from Gate R4, Batch B were subject to Xho 1 restriction enzyme digest, together with sample pools A1 and A2; sample pools A1 and A2 were also run in the undigested state. Undigested sample pools A1 and A2 (*lanes 2 and 3*) ran at a greater size than when digested (*lanes 4 and 5*); digested pools revealed inserts of multiple size. All digested samples confirmed the presence of pCDM8 vector, corresponding to the positive control (*lane 1*).

Lower three panels: TAE gel showing the results of Xho 1 restriction enzyme digest for all pooled samples A1 – E9. This confirms the presence of pCDM8 and multiple candidate inserts for all pooled samples. Distilled water was used as negative control (-). Ladders shown in kilo-basepairs.

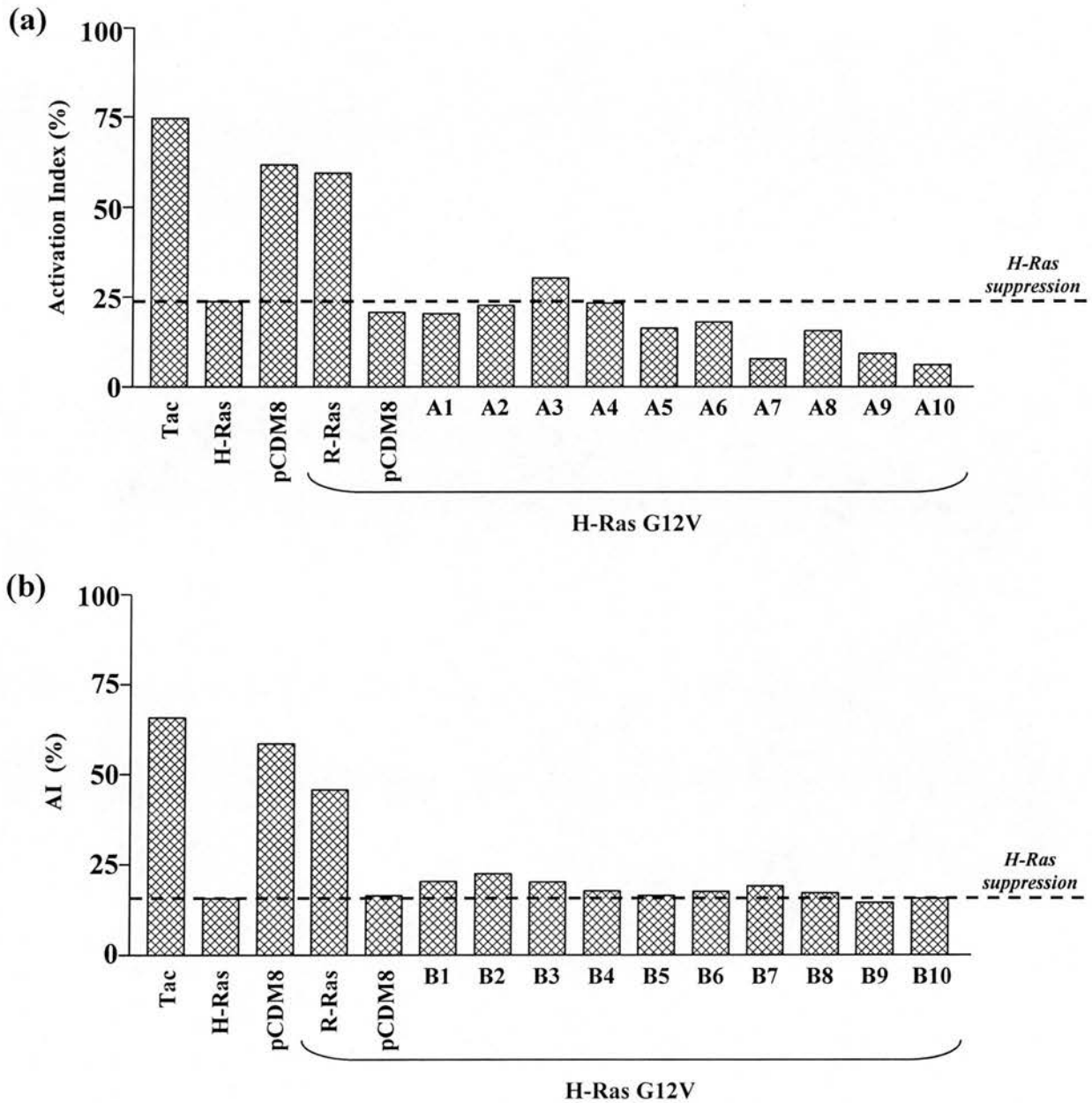
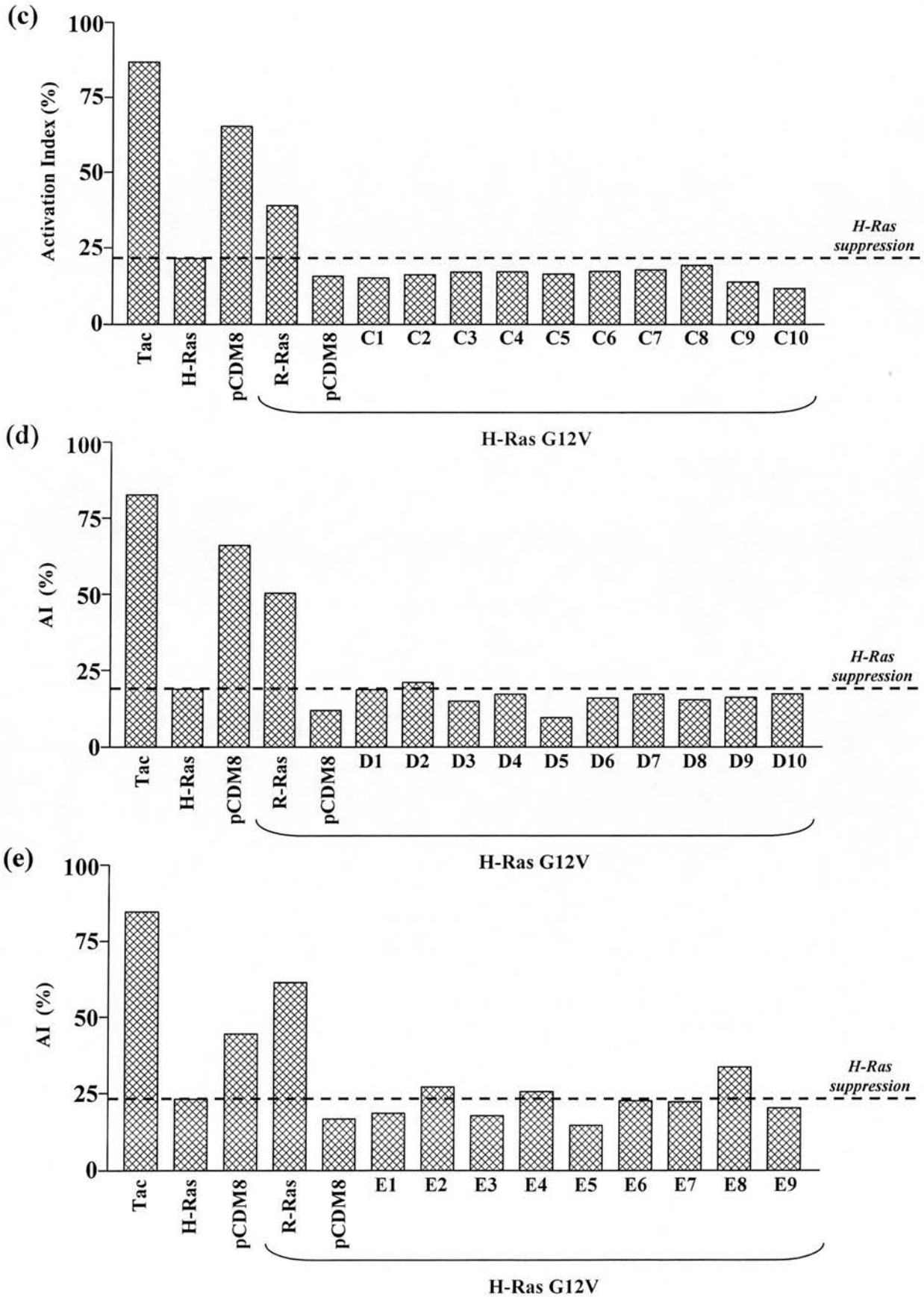


Figure 6.12 (a-e): Screening of sample pools for reversal of H-Ras suppression.
Using the αβ-py CHO cell system, sample pools A1 – E9 were screened for their ability to reverse H-Ras G12V-mediated integrin suppression. In the presence of Tac-α5, cells were transfected with H-Ras alone or H-Ras plus pooled cDNA, as indicated. The Activation Index was compared with the suppression achieved by transfection with H-Ras alone (*dashed line*). Reversal by R-Ras G38V was taken as a positive control in each batched experiment. Control pCDM8 vector had no influence on H-Ras-mediated suppression.


(c)
Activation Index (%)
100
75
50
25
0
H-Ras suppression
Tac
H-Ras
pCDM8
R-Ras
pCDM8
C1
C2
C3
C4
C5
C6
C7
C8
C9
C10
H-Ras G12V
(d)
AI (%)
100
75
50
25
0
H-Ras suppression
Tac
H-Ras
pCDM8
R-Ras
pCDM8
D1
D2
D3
D4
D5
D6
D7
D8
D9
D10
H-Ras G12V
(e)
AI (%)
100
75
50
25
0
H-Ras suppression
Tac
H-Ras
pCDM8
R-Ras
pCDM8
E1
E2
E3
E4
E5
E6
E7
E8
E9
H-Ras G12V

suppression; R-Ras G38V significantly reversed H-Ras G12V-mediated suppression. The effect of pooled sample cDNA on integrin activation status was compared with the degree of integrin suppression achieved by H-Ras G12V in each experiment. The majority of pools demonstrated no ability to reverse H-Ras-mediated suppression. Those three pools that demonstrated the best ability to reverse H-Ras suppression (Pools A3, B2 and E8) were deemed most likely to contain candidate cDNA for proteins able to activate integrins.

6.4.5 Screening of individual samples for candidate cDNA

For all samples in the candidate pools A3, B2 and E8, individual cDNA samples were prepared from 10ml cultures of bacterial colonies in antibiotic-restricted LB broth, followed by Wizard Miniprep. These individual cDNA were then tested in the αβ-py system under the same protocol as for the pooled samples (Section 6.4.4). The results are shown in **Figure 6.13a - c**. From this screen, 11 candidate cDNA were identified that appeared to demonstrate some ability to reverse H-Ras-mediated integrin suppression. To confirm that these candidate cDNA still contained the pCDM8 vector, they were subject to restriction enzyme digest with Xho I. **Figure 6.14** shows that all 11 candidate cDNA contained the pCDM8 vector, as seen in the control lane (*lane 1*). In addition, each carried an insert, which was of variable size, ranging from ~750kb – 3.5kb.

These 11 candidate cDNA were then subjected to a further round of screening under the same protocol as before, confirming that several of these cDNAs appeared to reverse H-Ras mediated suppression (*data not shown*). Of these, sample 480 appeared to give consistent reversal and this was therefore taken forward for detailed studies.

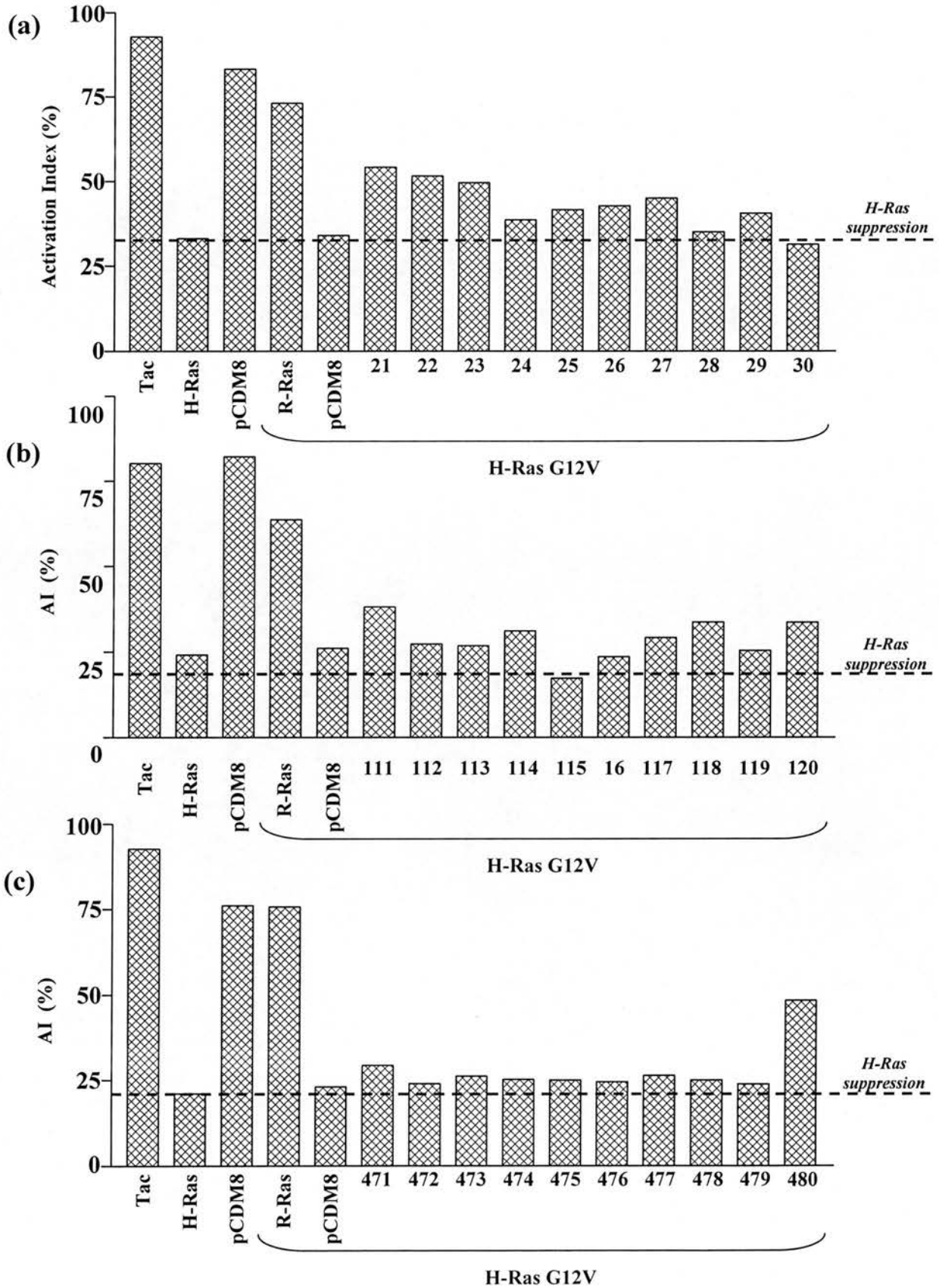


Figure 6.13 (a-c): Screening of individual cDNAs for reversal of H-Ras suppression.
Individual cDNA samples from sample pools A3 (21-30), B2 (111-120) and E9 (471-480) were tested in the αβ-py CHO cell system for their ability to reverse H-Ras G12V-mediated integrin suppression, as previously. Reversal by R-Ras G38V was taken as a positive control in each batched experiment. Control pCDM8 vector had no influence on H-Ras-mediated suppression.

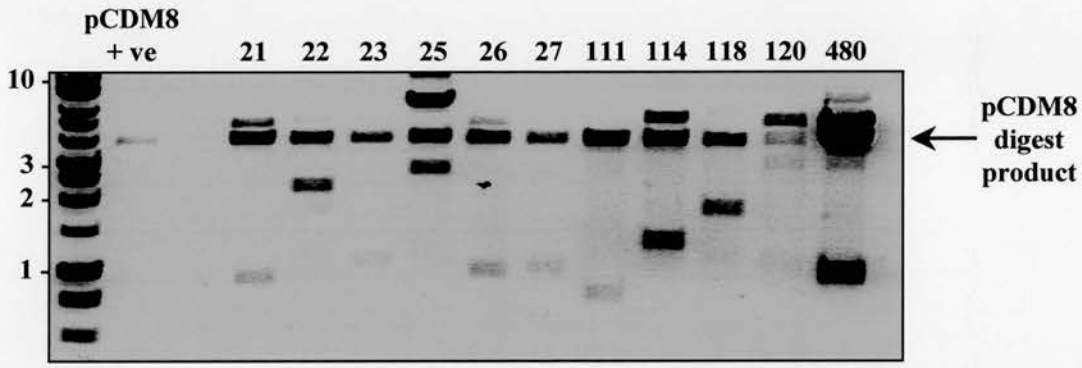


Figure 6.14: Screening of best candidate cDNAs for presence of pCDM8 vector.
Xho 1 restriction enzyme digest confirmed that all candidate samples still retained expression of pCDM8, as compared with empty vector alone (*lane 1*). In addition, each candidate sample exhibited an additional band consistent with a cDNA insert.

6.5. Studies on Sample 480

6.5.1 Sample 480 demonstrates reversibility of H-Ras suppression

A number of early studies were conducted to assess the ability of 480 to reverse H-Ras mediated suppression. All these studies used the αβ-py cell system and in some the standard flow cytometric analysis of PAC1 binding was refined to enable cells to be sorted and collected at analysis, so that further protein analysis could be performed.

The result of a pilot dose-response study is shown in **Figure 6.15**, comparing the effect of different doses of 480 cDNA on reversing H-Ras suppression. It can be seen that, whether H-Ras was used at 1μg or 2μg/10cm plate, increasing the dose of 480 to 4μg or 10μg (for 1μg H-Ras only) resulted in an increase in integrin activation; the reversal appeared more marked if only 1μg H-Ras G12V was used for transfection. The use of 4μg of 480 alone did not obviously induce integrin activation above the resting state. In order to make the assay as sensitive as possible, it was concluded that 1μg H-Ras G12V should be used for transfection in further experiments.

A further series of experiments was conducted to observe the ability of 480 to reverse integrin suppression mediated by 1μg H-Ras, the results of which are shown in **Figure 6.16**. This demonstrates that cells transfected with increasing doses of 480 exhibited a progressively increasing Activation Index. Cells transfected with H-Ras G12V and 5μg of control pCDM8 exhibited a mean AI (± SEM) of 38.6% (± 0.8). By comparison, H-Ras G12V plus 0.5μg 480 exhibited a degree of reversal, with an increased AI of 55.0% (± 3.9), rising to an AI of 64.5% (± 4.4) with 1μg ($p < 0.05$) and 72.9% (± 2.8) with 5μg 480 ($p < 0.01$); this contrasts with the reversal seen with 1μg of R-Ras G38V, to an AI of 92.7% (± 8.1).

These results demonstrated that, in the presence of a constant amount of cDNA for H-Ras G12V, transfection with increasing amounts of cDNA for 480 resulted in a reversal

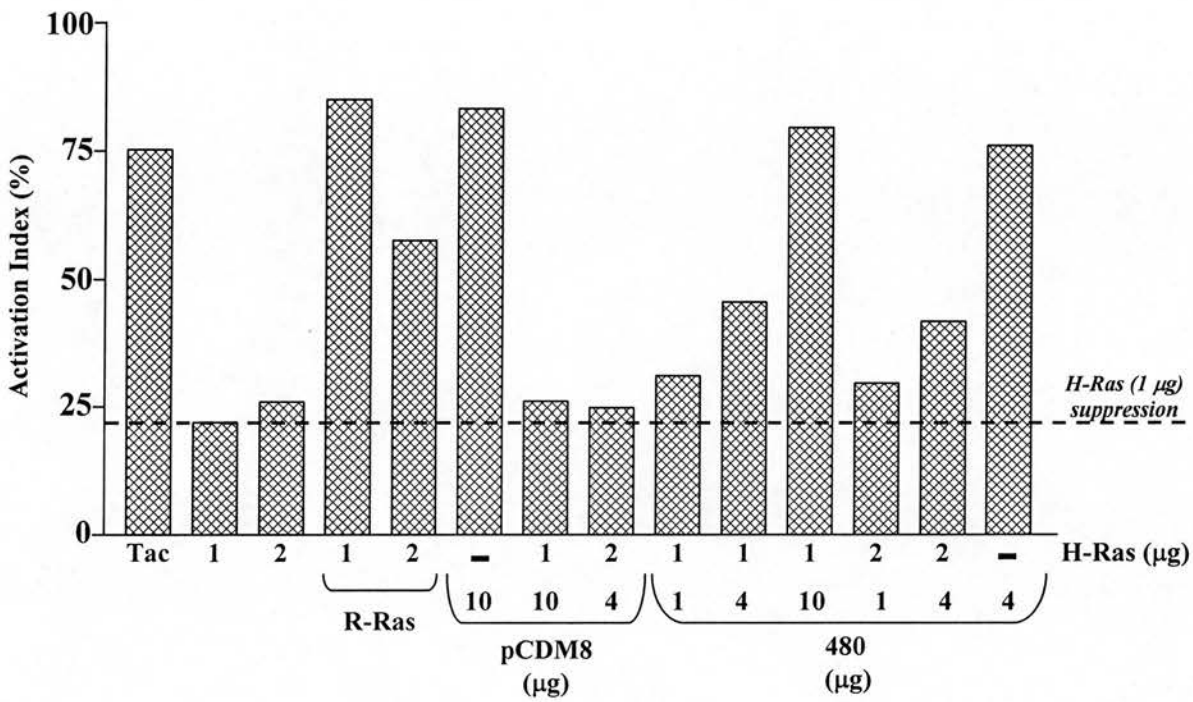


Figure 6.15: Pilot study of Clone 480 and reversal of H-Ras.

Initial titration experiment to assess the ability of cDNA 480 to reverse H-Ras-mediated integrin suppression. Increasing doses of cDNA 480 were transfected into αβ-py CHO cells in the presence of 1μg or 2μg of H-Ras G12V. An apparent dose-response curve was noted. Reversal by R-Ras G38V was taken as a positive control. Maximum doses of control pCDM8 vector had no influence on Activation Index.

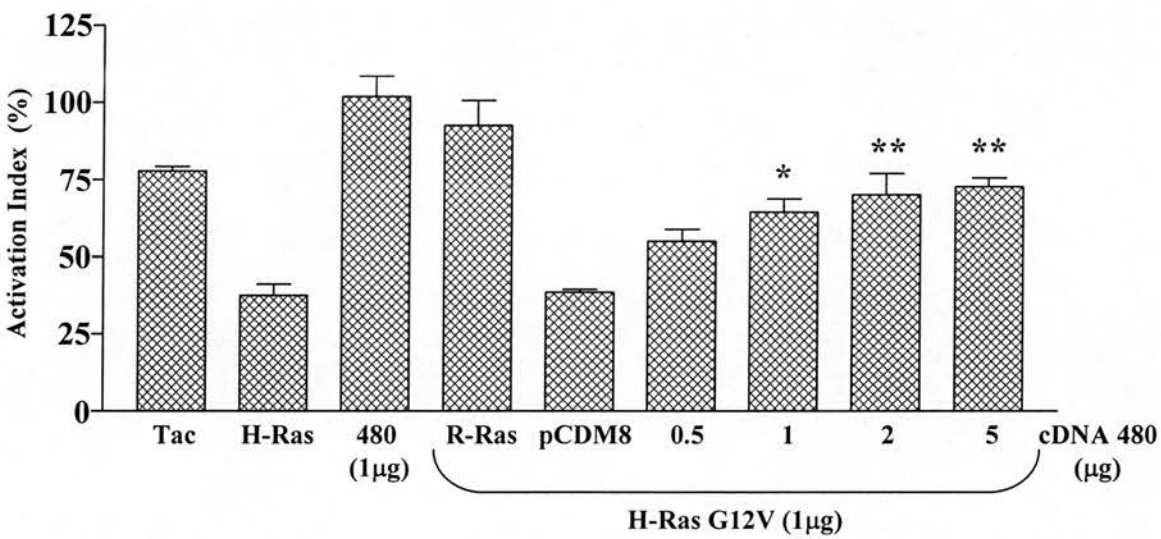


Figure 6.16: Clone 480 reverses H-Ras-mediated integrin suppression.
Increasing doses of cDNA 480 were transfected into αβ-py CHO cells in the presence of 1μg of H-Ras G12V. A dose-response curve was noted, with significant reversal achieved at 1μg of 480. Reversal by R-Ras G38V was taken as a positive control. Maximum doses of control pCDM8 vector (5μg) had no influence on Activation Index. Graph represents the mean of three experiments. (*, $p < 0.05$; **, $p < 0.001$).

of H-Ras-mediated suppression. This may have been a true effect, or the result of reduced transcription of H-Ras G12V.

6.5.2 Analysis of protein expression

To establish whether transfection with increased quantities of 480 was reducing H-Ras G12V expression, cell populations were probed for the expression of H-Ras G12V as denoted by the presence of the HA tag. Flow cytometry of transfected cells was refined so that cells underwent sorting and collection at the same time. Cells that exhibited significant transfection, defined by elevated Tac-α5 expression, were collected in HBSS at 4°C for each experimental condition. Samples were pelleted at 1000rpm for 1 minute and excess HBSS aspirated. The cells were then lysed in 100μl MAPK lysis buffer, prior to protein assay. Alternatively, for some experiments, parallel samples of transfected cells were harvested by direct lysis from culture plates, as described in "Materials and Methods", and assessed for protein concentration.

Figure 6.17 shows the results of a titration experiment, in which lysates of sorted cells, having been equilibrated for protein loading, were run out on Western blots and probed for the presence of the HA tag for H-Ras G12V expression. Increasing doses of cDNA 480 resulted in reversal of H-Ras suppression (**Figure 6.17, *graph***). In the sorted cells expression of H-Ras diminished with increasing dose of 480 cDNA. However, when this Western blot was stripped and re-probed for actin and Tac-α5, expression of both these proteins also declined in parallel (**Figure 6.17, *Western blots, upper panel***). This suggested that although higher doses induced loss of H-Ras expression, this might simply be a result of higher cell death in these samples, not in itself surprising. To try and confirm this, attempts were made to equilibrate the loading of samples for cell number. According to the cell sorting data, the following numbers of events were collected for each experimental group, as shown in **Table 6f**.

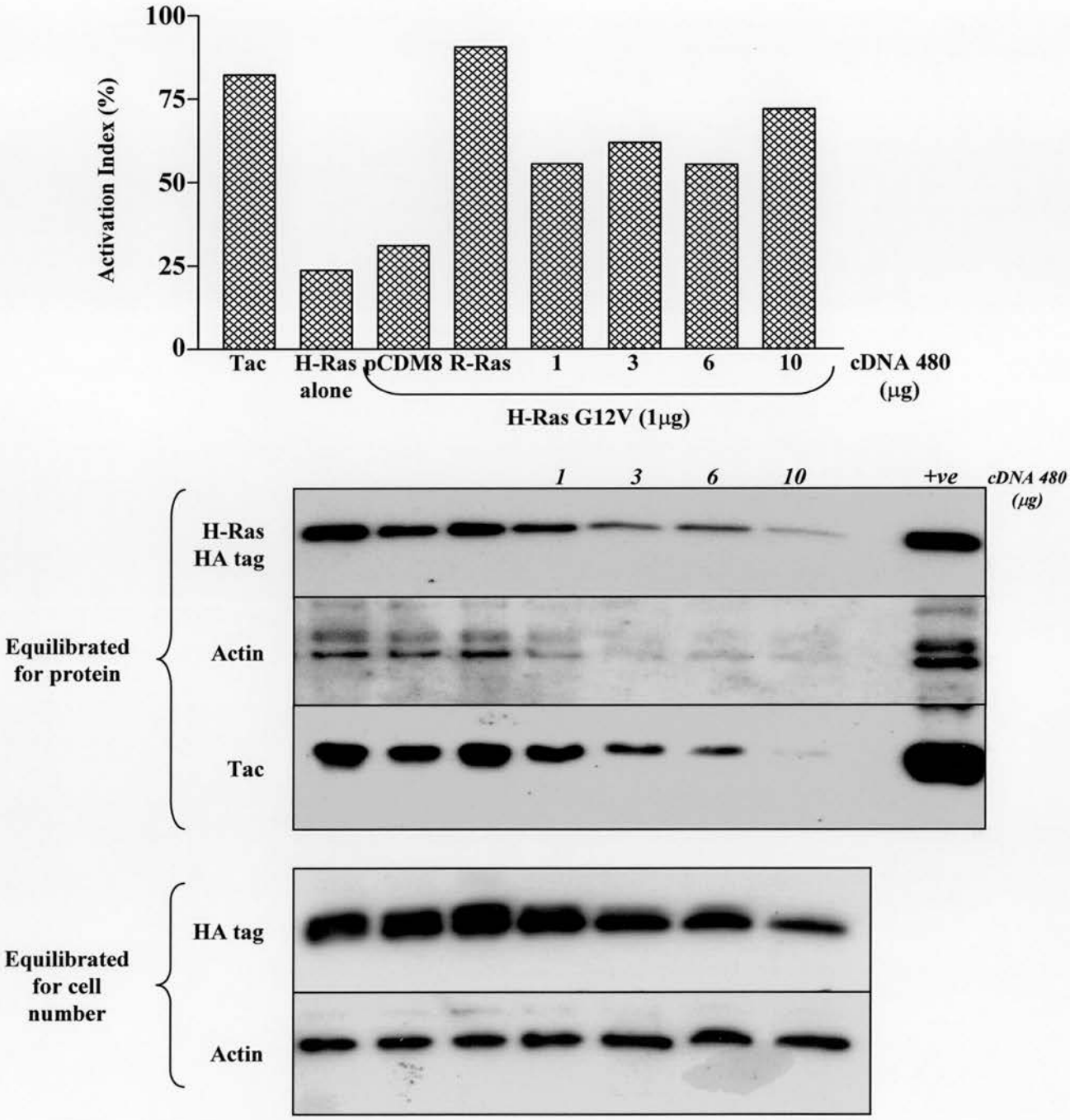


Figure 6.17: Clone 480 reverses integrin suppression in the face of H-Ras expression.

Graph: Representative graph showing effect of increasing doses of clone 480 on the AI, in the face of H-Ras transfection (1μg). Cells were sorted concurrently with flow cytometric analysis and lysates used for protein analysis.

Western blots: *(Upper panel)* When loaded according to protein concentration, increased doses of clone 480 resulted in an apparent decrease in expression of H-Ras, labelled by the HA-tag. However, this was parallelled by decreased actin and Tac-α5 expression. *(Lower panel)* When loaded according to estimated cell number, supported by equivalence in actin expression, the decline in H-Ras expression was much less marked.

Table 6f: Events sorted for each condition

Sample	"Events" sorted
Tac alone	1277323
Tac, H-Ras G12V	598557
H-Ras + pCDM8 control	350378
H-Ras + R-Ras	450667
H-Ras + 1μg 480	208262
H-Ras + 3μg 480	96538
H-Ras + 6μg 480	106885
H-Ras + 10μg 480	97183

When samples were re-probed, having been loaded according to the number of events collected at sorting, the results were improved (**Figure 6.17, *Western blots, lower panel***). More even loading of actin was apparent, but when stripped and re-probed for HA-tag / H-Ras G12V expression, some decline in H-Ras expression was still observed with the highest doses of 480 (*lanes 6 and 7*). Nevertheless, it can be seen that an effect of 480 on reversing integrin suppression was observed *at least in the face of* H-Ras G12V expression (**Figure 6.17, *graph***).

In samples from parallel plates, equilibrated for protein, it appeared that even with good equilibration of actin loading, H-Ras expression diminished with higher doses of 480 (**Figure 6.18**). However, this was not the case with transfection with the maximum dose of pCDM8 control vector, in which H-Ras expression remained robust (*lane 5*). This was difficult to interpret. It remains possible that higher doses of 480 were indeed suppressing H-Ras G12V expression, thereby producing an apparent reversal of integrin suppression. An alternative explanation, accounting for the preservation of H-Ras expression in the presence of maximum doses of pCDM8 control vector, is that higher doses of 480 (but not control vector) produced increased cell death. The most highly

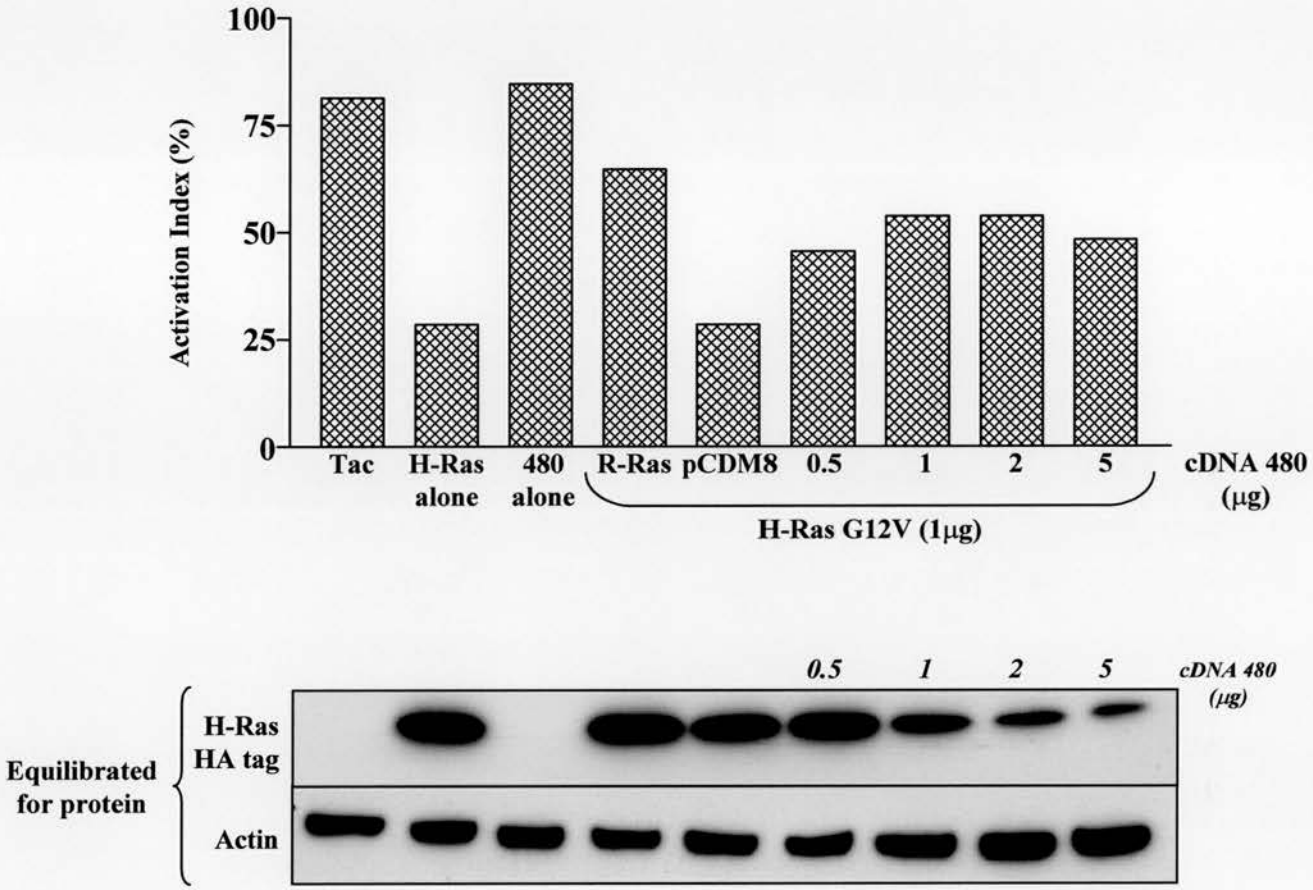


Figure 6.18: Clone 480 reverses integrin suppression in the face of H-Ras expression.
Graph: Representative graph showing effect of increasing doses of clone 480 on the AI, in the face of H-Ras transfection (1μg). Parallel transfection plates were lysed and equilibrated for protein, then used for protein analysis.
Western blots: When loaded according to protein concentration, as evidenced by equivalence in actin expression (*upper panel*), increased doses of clone 480 resulted in a decrease in expression of H-Ras, labelled by the HA-tag (*lower panel*). This was not observed with transfection with pCDM8 (10 μg) (*lane 5*).

transfected cells died, leaving a greater proportion of untransfected or less transfected cells from which lysates were obtained. This would particularly be the case in those Western blots derived from the parallel plates, where the cell populations were unsorted.

6.5.3 Analysis of sequence

The sequence of 480 was obtained by sending a sample of cDNA to a commercial company (Genetix), with forward and reverse primers. From earlier studies (**Figure 6.14**), it was known that Xho I restriction enzyme digest resulted in an insert of approximately 1kb. When the results of forward and reverse sequencing were analysed, it confirmed that there was an overlap between the two and the contiguous sequence for 480 was derived. This is shown in **Figure 6.19**, with Xho I digest sites highlighted. Also highlighted are potential transcription start sites, designated by the sequence ATG (methionine). Using BLAST software (www.ncbi.nlm.nih.gov/BLAST/), the sequence was analysed for the most probable reading frame from which a candidate protein could be translated. As indicated in **Figure 6.19**, the only reading frame to result in a protein of any length was the 5'3' frame 3. This yielded the following cDNA and corresponding protein sequence:

Proposed 480 cDNA Sequence

ATGGGTGGCCTCATCATCCTCTTCCTCATCGCCATCATCTGGTTCCCGCTGCT
CTTCATGTCGCTGGTGCGCTCCGTGGTTGGGGTTGTCAACCAGCCCATCGAT
GTCACCGTCACCCTCAAGCTGGGCGGCTATGAGCCGCTGTTCACCATGAGCG
CCCAGCAGCCGTCCATCATCCCCTTCACGGCCCAGGCCTATGAGGAGCTGTC
CCGGCAGTTTGACCCCCAGCCGCTGGCCATGCAGTTCATCAGCCAGTACAGC
CCTGAGGACATCGTCACGGCGCAGATTGAGGGCAGGCTCCNGGGGCGATGT
GGCGCAATCAGTCCCCCCAGCCGTGCCCAGATNGAAGCGGGAGCTCTACAA
CNGGCACGGCCGACATCACCCTGCGCTTCACCTGGAACTTCCAGAGGGACCT
GGCGAAGGGAGGCACTGTGGAGTATGCCAACGAGAAGCACATGCTGGCNCC
TGGCCCCCCAACAGCACTGCACGGCGGCAGCTGGCCAGCCTGCTCGAGGGC

Contiguous sequence for clone 480

ATCCCTCGACCTCGAGATCCATTGTGCTCTAAAGCAAAGGGCAGAAGAAGAAGAAGATCG
TCAAGTACGGCATGGGTGGCCTCATCATCCTCTTCCTCATCGCCATCATCTGGTTCCCGCT
GCTCTTCATGTCGCTGGTGCGCTCCGTGGTTGGGGTTGTCAACCAGCCCATCGATGTCACC
GTCACCCTCAAGCTGGGCGGCTATGAGCCGCTGTTCACCATGAGCGCCCAGCAGCCGTCC
ATCATCCCCTTCACGGCCCAGGCCTATGAGGAGCTGTCCCGGCAGTTTGACCCCCAGCCG
CTGGCCATGCAGTTCATCAGCCAGTACAGCCCTGAGGACATCGTCACGGCGCAGATTGAG
GGCAGGCTCCNGGGGCGATGTGGCGCAATCAGTCCCCCCAGCCGTGCCCAGATNGAAGC
GGGAGCTCTACAACNGGCACGGCCGACATCACCCTGCGCTTCACCTGGAACTTCCAGAGG
GACCTGGCGAAGGGAGGCACTGTGGAGTATGCCAACGAGAAGCACATGCTGGCNCCTGG
CCCCCCAACAGCACTGCACGGCGGCAGCTGGCCAGCCTGCTCGAGGGCACCCTCGGAAAC
CAGTCTGTGGTCATCCCCAATCTCTTTCCCCAAGTACATCCGTGCCCCCAACGGGCCCGAA
GCCAACCCTGTGAAGCAGCTGCAGCCCAATGAGGAGGCCGACTACCTCGGCGTGCGTATC
CAGCTGCGGAGGGAGCAGGGTGCGGGGGCCACCGGCTTCCTCGAATGGTGGGTCATCGA
GCTGCAGGAGTGCCGGACCGACTGCAACCTGCTGCCCATGGTCATTTTCAGTGACAAGGT
CAGCCCACCGAGCCTCGGCTTCCTGGCTGGCTACGGCATCATGGAACTTGCCGATGACCA
GCACGATGGACACGTACAGCCCTTTAGAG

Presumptive reading frame for clone 480

P S T S R S I V L Stop S K G Q K K K K I V K Y G Met G G L I I L F L I A I I W F P L L F Met S L
V R S V V G V V N Q P I D V T V T L K L G G Y E P L F T Met S A Q Q P S I I P F T A Q A Y E E
L S R Q F D P Q P L A Met Q F I S Q Y S P E D I V T A Q I E G R L X G R C G A I S P P S R A Q X
E A G A L Q X A R P T S P C A S P G T S R G T W R R E A L W S Met P T R S T C W X L A P Q Q
H C T A A A G Q P A R G H P R K P V C G H P Q S L S P S T S V P P T G P K P T L Stop S S C S P
Met R R P T T S A C V S S C G G S R V R G P P A S S N G G S S S C R S A G P T A T C C P W S F
S V T R S A H R A S A S W L A T A S W N L P Met T S T Met D T Y S P L E

Figure 6.19: Clone 480 sequences.

Upper panel: Contiguous sequence of the plasmid cDNA for clone 480, derived from overlapping forward and reverse sequences. Putative start sites are marked by methionine ATG sequences (*green*); Xho 1 restriction enzyme sites TCGAC were also noted (*underlined*), including one within the 480 sequence. *Lower panel*: Best-fit reading frame for clone 480. Translation start sites are indicated by methionine (*Met, green*); stop sites are indicated (*red*). All other potential reading frames would produce only very short sequences.

ACCCTCGGAAACCAGTCTGTGGTCATCCCCAATCTCTTTCCCCAAGTACATCC
GTGCCCCCAACGGGCCCGAAGCCAACCCTGTGA

Proposed 480 Protein Sequence

MGGLIILFLIAIIWFPLLFMSLVRSVVGVVNQPIDVTVTLKLGGYEPLFTMSAQQP
SIIPFTAQAYEELSRQFDPQPLAMQFISQYSPEDIVTAQIEGRLXGRCGAISPPSRA
QXEAGALQXARPTSPCASPGTSRGTWRREALWSMPTRSTCWXLAPQQHCTAA
AGQPARGHPRKPVCGHPQSLSPSTSVPPTGPKPTL

The Clone 480 cDNA sequence was analysed by use of a BLAST search and corresponds to the C-terminal part of a large gene termed KIAA0233, which has previously been sequenced but not fully characterized (Nagase *et al.*, 1996) (**Figure 6.20**). Clone 480 exhibits 98% identity with an extensive region of KIA0233, covering base pairs 5067 – 5656, with only 11/603 gaps (1%). The full length of the gene is ~6kb, with a locus of 16q24.3 and it codes for a predicted 230kDa protein. The predicted amino acid sequence of the first 98 residues from Clone 480 is identical to residues 1689 – 1786 of KIA0233 protein. Using a web-based Simple Modular Architecture Research Tool (SMART) protein analysis (www.smart.embl-heidelberg.de/), the amino acid sequence of KIA0233 predicts a protein having 22 (possibly 23) transmembrane domains (**Figure 6.21**). Colloquially, it has thus been christened "Nessie". The region corresponding to Clone 480 commences within the predicted 21st transmembrane region (residues 1684 – 1706). The Nessie protein is apparently evolutionarily conserved since it has been identified in a variety of species other than *Homo sapiens*; these include *M. musculus*, *R. norvegiens*, *F. rubripes*, *D. melanogaster*, *A. gambiae*, *C. elegans* and *C. briggsae* (www.smart.embl-heidelberg.de/).

Origin

```
1    ccatggacct gcgccctgag ctgcccacca ccctgggccc cgtcagcctg cgccagctgg
61   ggctggagca cacccgctac ccctgtctgg accttggtgc catgttgctc tacaccctga
121  ccttctggct cctgctgcgc cagtttgtga aagagaagct gctgaagtgg gcagagtctc
181  cagctgcgct gacggaggtc accgtggcag acacagagcc tacgcggacg cagacgctgt
241  tgcagagcct gggggagctg gtgaagggcg tgtacgccaa gtactggatc tatgtgtgtg
301  ctggcatgtt catcgtggtc agcttcgccg gccgcctcgt ggtctacaag attgtctaca
361  tgttcctctt cctgctctgc ctcaccctct tccaggtcta ctacagcctg tggcggaagc
421  tgctcaaggc cttctggtgg ctcgtggtgg cctacaccat gctggtcctc atcgccgtct
481  acaccttcca gttccaggac ttccctgcct actggcgcaa cctcactggc ttcaccgacg
541  agcagctggg ggacctgggc ctggagcagt tcagcgtatc cgagctcttc tccagcatcc
601  tggtgcccgg cttcttcctc ctggcctgca tcctgcagct gcactacttc cacaggccct
661  tcatgcagct caccgacatg gagcacgtgt ccctgcctgg cacgcgcctc ccgcgctggg
721  ctcacaggca ggatgcagtg agtgggaccc cactgctgcg ggaggagcag caggagcatc
781  agcagcagca gcaggaggag gaggaggagg aggactccag ggacgagggg ctgggcgtgg
841  ccactcccca ccaggccacg caggtgcctg aaggggcagc caagtggggc ctggtggctg
901  agcgcctgct ggagctggca gccggcttct cggacgtcct ctcacgcgtg caggtgttcc
961  tgcggcggct gctggagctt cacgttttca agctggtggc cctgtacacc gtctgggtgg
1021 ccctgaagga ggtgtcggtg atgaacctgc tgctggtggt gctgtgggcc ttcgccctgc
1081 cctacccacg cttccggccc atggcctcct gcctgtccac cgtgtggacc tgcgtcatca
1141 tcgtgtgtaa gatgctgtac cagctcaagg ttgtcaaccc ccaggagtat tccagcaact
1201 gcaccgagcc cttccccaac agcaccaact tgctgcccac ggagatcagc cagtccctgc
1261 tgtaccgggg gcccgtggac cctgccaact ggtttggggt gcggaaaggg ttccccaacc
1321 tgggctacat ccagaaccac ctgcaagtgc tgctgctgct ggtattcgag gccatcgtgt
1381 accggcgcca ggagcactac cgccggcagc accagctggc cccgctgcct gcccaggccg
1441 tgtttgccag cggcacccgc cagcagctgg accaggatct gctcggctgc ctcaagtact
1501 tcatcaactt cttcttctac aaattcgggc tggagatctg cttcctgatg gccgtgaacg
1561 tgatcgggca gcgcatgaac tttctggtga ccctgcacgg ttgctggctg gtggccatcc
1621 tcacccgcag gcaccgccag gccattgccc gcctctggcc caactactgc ctcttcctgg
1681 cgctgttcct gctgtaccag tacctgctgt gcctggggat gcccccggcc ctgtgcattg
1741 attatccctg gcgctggagc cgggccgtcc ccatgaactc cgcactcatc aagtggctgt
1801 acctgcctga tttcttccgg gcccccaact ccaccaacct catcagcgac tttctcctgc
1861 tgctgtgcgc ctcccagcag tggcaggtgt tctcagctga gcgcacagag gagtggcagc
1921 gcatggctgg cgtcaacacc gaccgcctgg agccgctgcg gggggagccc aaccccgtgc
1981 ccaactttat ccactgcagg tcctaccttg acatgctgaa ggtggccgtc ttccgatacc
2041 tgttctggct ggtgctggtg gtggtgtttg tcacgggggc cacccgcatc agcatcttcg
2101 ggctgggcta cctgctggcc tgcttctacc tgctgctctt cggcacggcc ctgctgcaga
2161 gggacacacg ggcccgcctc gtgctgtggg actgcctcat tctgtacaac gtcaccgtca
2221 tcatctccaa gaacatgctg tcgctcctgg cctgcgtctt cgtggagcag atgcagaccg
2281 gcttctgctg ggtcatccag ctcttcagcc ttgtatgcac cgtcaagggc tactatgacc
2341 ccaaggagat gatggacaga gaccaggact gcctgctgcc tgtggaggag gctggcatca
2401 tctgggacag cgtctgcttc ttcttcctgc tgctgcagcg ccgcgtcttc cttagccatt
2461 actacctgca cgtcagggcc gacctccagg ccaccgccct gctagcctcc aggggcttcg
2521 ccctctacaa cgctgccaac ctcaagagca ttgacttcca ccgcaggata gaggagaagt
2581 ccctggccca gctgaaaaga cagatggagc gtatccgtgc caagcaggag aagcacaggc
2641 agggccgggt ggaccgcagt cgcccccagg acaccctggg ccccaaggac cccggcctgg
2701 agccagggcc cgacagtcca gggggctcct ccccgccacg gaggcagtgg tggcggccct
2761 ggctggacca cgccacagtc atccactccg gggactactt cctgtttgag tccgacagtg
2821 aggaagagga ggaggctgtt cctgaagacc cgaggccgtc ggcacagagt gccttccagc
2881 tggcgtacca ggcatgggtg accaacgccc aggcggtgct gaggcggcgg cagcaggagc
2941 aggagcaggc aaggcaggaa caggcaggac agctacccac aggaggtggt cccagccagg
3001 aggtggagcc agcagagggc cccgaggagg cagcggcagg ccggagccat gtggtgcaga
3061 gggtgctgag cacggcgcag ttcctgtgga tgctggggca ggcgctagtg gatgagctga
3121 cacgctggct gcaggagttc acccggcacc acggcaccat gagcgacgtg ctgcgggcag
3181 agcgctacct cctcacacag gagctcctgc agggcggcga agtgcacagg ggcgtgctgg
3241 atcagctgta cacaagccag gccgaggcca cgctgccagg ccccaccgag gcccccaatg
3301 ccccaagcac cgtgtccagt gggctgggcg cggaggagcc actcagcagc atgacagacg
```

```
3361 acatgggcag ccccctgagc accggctacc acacgcgcag tggcagtgag gaggcagtca
3421 ccgaccccgg ggagcgtgag gctggtgcct ctctgtacca gggactgatg cggacggcca
3481 gcgagctgct cctggacagg cgcctgcgca tcccagagct ggaggaggca gagctgtttg
3541 cggaggggca gggccgggcg ctgcggctgc tgcgggccgt gtaccagtgt gtggccgccc
3601 actcggagct gctctgctac ttcatcatca tcctcaacca catggtcacg gcctccgccg
3661 gctccctggt gctgcccgtg ctcgtcttcc tgtgggccat gctgtcgatc ccgaggccca
3721 gcaagcgctt ctggatgacg gccatcgtct tcaccgagat cgcggtggtc gtcaagtacc
3781 tgttccagtt tgggttcttc ccctggaaca gccacgtggt gctgcggcgc tacgagaaca
3841 agccctactt cccgccccgc atcctgggcc tggagaagac tgacggctac atcaagtacg
3901 acctggtgca gctcatggcc cttttcttcc accgctccca gctgctgtgc tatggcctct
3961 gggaccatga ggaggactca ccatccaagg agcatgacaa gagcggcgag gaggagcagg
4021 gagccgagga ggggccaggg gtgcctgcgg ccaccaccga agaccacatt caggtggaag
4081 cgagggtcgg acccacggac gggaccccag aaccccaagt ggagctcagg ccccgtgata
4141 cgaggcgcat cagtctacgt tttagaagaa ggaagaagga gggcccagca cggaaaggag
4201 cggcagccat cgaagctgag gacagggagg aagaagaggg ggaggaagag aaagaggccc
4261 ccacggggag agagaagagg ccaagccgct ctggaggaag agtaagggcg gccgggcggc
4321 ggctgcaggg cttctgcctg tccctggccc agggcacata tcggccgcta cggcgcttct
4381 tccacgacat cctgcacacc aagtaccgcg cagccaccga cgtctatgcc ctcatgttcc
4441 tggctgatgt tgtcgacttc atcatcatca tttttggctt ctgggccttt gggaagcact
4501 cggcggccac agacatcacg tcctccctat cagacgacca ggtacccgag gctttcctgg
4561 tcatgctgct gatccagttc agtaccatgg tggttgaccg cgccctctac ctgcgcaaga
4621 ccgtgctggg caagctggcc ttccaggtgg cgctggtgct ggccatccac ctatggatgt
4681 tcttcatcct gcccgccgtc actgagagga tgttcaacca gaatgtggtg gcccagctct
4741 ggtacttcgt gaagtgcatc tacttcgccc tgtccgccta ccagatccgc tgcggctacc
4801 ccacccgcat cctcggcaac ttcctcacca agaagtacaa tcatctcaac ctcttcctct
4861 tccaggggtt ccggctggtg ccgttcctgg tggagctgcg ggcagtgatg gactgggtgt
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5041 agaagaagaa gatcgtcaag tacggcatgg gtggcctcat catcctcttc ctcatcgcca
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5161 agcccatcga tgtcaccgtc accctcaagc tgggcggcta tgagccgctg ttcaccatga
5221 gcgcccagca gccgtccatc atccccttca cggcccaggc ctatgaggag ctgtcccggc
5281 agtttgaccc ccagccgctg gccatgcagt tcatcagcca gtacagccct gaggacatcg
5341 tcacggcgca gattgagggc agctccgggg cgctgtggcg catcagtccc cccagccgtg
5401 cccagatgaa gcgggagctc tacaacggca cggccgacat caccctgcgc ttcacctgga
5461 acttccagag ggacctggcg aagggaggca ctgtggagta tgccaacgag aagcacatgc
5521 tggccctggc ccccaacagc actgcacggc ggcagctggc cagcctgctc gagggcacct
5581 cggaccagtc tgtggtcatc cccaatctct tccccaagta catccgtgcc cccaacgggc
5641 ccgaagccaa ccctgtgaag cagctgcagc ccaatgagga ggccgactac ctcggcgtgc
5701 gtatccagct gcggagggag cagggtgcgg gggccaccgg cttcctcgaa tggtgggtca
5761 tcgagctgca ggagtgccgg accgactgca acctgctgcc catggtcatt ttcagtgaca
5821 aggtcagccc accgagcctc ggcttcctgg ctggctacgg catcatgggg ctgtacgtgt
5881 ccatcgtgct ggtcatcggc aagttcgtgc gcggattctt cagcgagatc tcgcactcca
5941 ttatgttcga ggagctgccg tgcgtggacc gcatcctcaa gctctgccag gacatcttcc
6001 tggtgcggga gactcgggag ctggagctgg aggaggagtt gtacgccaag ctcatcttcc
6061 tctaccgctc accggagacc atgatcaagt ggactcgtga gaaggagtag gagctgctgc
6121 tggcgcccga gagggaagga gccggcctgc tgggcagcgt ggccacaagg ggcggcactc
6181 ctcaggccgg gggagccact gccccgtcca aggccgccag ctgtgatgca tcctcccggc
6241 ctgcctgagc cctgatgctg ctgtcagaga aggacactgc gtccccacgg cctgcgtggc
6301 gctgccgtcc cccacgtgta ctgtagagtt tttttttaa ttaaaaaatg ttttatttat
6361 acaaatgg
```
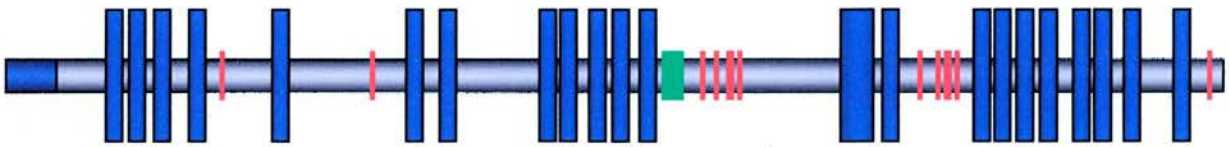

Figure 6.20 Published sequence of KIA0233

Full nucleotide sequence for KIA0233, taken from Nagase T, (1996). Clone 480 represents the region highlighted in red, bp5067 – 5658.



Name	Start residue	End	Name	Start residue	End
Signal peptide	1	48	Low complexity	969	990
Transmembrane	94	113	Low complexity	1002	1013
Tm	117	136	Tm	1203	1234
Tm	143	165	Tm	1244	1266
Tm	195	217	Low complexity	1335	1346
Low complexity	254	270	Low complexity	1378	1390
Tm	337	359	Low complexity	1400	1419
Low complexity	460	471	Low complexity	1426	1440
Tm	499	521	Tm	1475	1497
Tm	551	573	Tm	1517	1537
Tm	674	691	Tm	1544	1566
Tm	698	717	Tm	1576	1593
Tm	732	754	Tm	1614	1636
Tm	761	778	Tm	1646	1663
Tm	798	817	Tm	1684	1706
Coiled coil	853	882	Tm	1944	1966
Low complexity	897	912	Low complexity	2006	2013
Low complexity	936	950			

Figure 6.21 Predicted structure of KIA0233 protein.
Protein structure for KIA0233 was predicted using SMART protein analysis and predicts for a protein containing 22 transmembrane domains, shown schematically (*above*). Transmembrane domains are shown in blue; a coiled coil region is shown in green; multiple segments of low compositional complexity are shown in red. There is an initial signal peptide (dark grey).
Lower table: Amino acid residue map of confidently predicted domains. Key: transmembrane domain (*Tm*).

6.6 Appendix to Chapter 6: further studies on Clone 480

Substantial further work has been done on the nature and relevance of Clone 480 since the end of the period allotted for this thesis. This work has direct relevance to the present thesis and as such a brief summary of the current findings is presented here. For taking on this work, the author is grateful for the efforts of Dr. Yatish Lad and Dr. Brian McHugh.

In the first instance, the above dose-response tests were successfully repeated by Dr. Lad to ensure that the results quoted were not investigator-dependent. It was clearly important that some means of localizing Clone 480 expression should be established and therefore Clone 480 was successfully tagged with Myc on the 3' end and tested once again for activity. It was again found that 1μg of 480-Myc could reverse H-Ras G12V-mediated suppression. By probing for Myc expression on Western blotting it was established that Clone 480 was being expressed. Immunofluorescence experiments in CHO $\alpha\beta$-py cells also demonstrated that this expression appeared to be localized around the nucleus, possibly in the endoplasmic reticulum.

The full-length form of KIAA0233/Nessie was obtained from a Japanese group (The Kazusa DNA Research Institute,) and tagged with Myc at the C-terminus for analysis. Full length Nessie-Myc also rescues integrins from H-Ras G12V-mediated suppression, although there is some suppression of H-Ras expression in these experiments. The Nessie protein is expressed and is detectable at greater than 250kDa on Western blots; in immunofluorescence experiments, the full-length protein, like the Clone 480, localizes to the endoplasmic reticulum. There does not appear to be any plasma membrane expression.

In addition to rescuing H-Ras G12V suppression, Nessie appears to rescue suppression induced by the effector mutants H-Ras T35S and H-Ras D38N, but only partially rescues

the activated Raf mutant Raf(BxBCAAX). On its own, Nessie activates integrins and enhances activation observed with R-Ras. None of these effects appear to be related to activation or suppression of the MAPK/ERK pathway, since Nessie alone does not influence ERK phosphorylation, nor does it inhibit ERK phosphorylation by H-Ras G12V.

6.7 Discussion

This chapter describes the application of a genetic screen to isolate potential regulators of integrin activation. Thus the screen was performed in the αβ-py CHO cell system, with cells transfected with H-Ras and gating out those cells with activated integrins despite H-Ras expression. This work builds on that of the previous chapter. In particular, the use of To-Pro-3 as a live/dead cell marker has become standard technique and played a role in filtering out spurious "noise" from the dead cell population. The result of this screening process has been the identification of a novel gene, now termed Nessie, which appears to reverse H-Ras G12V-mediated integrin suppression.

It was disappointing to discover that there were no positive clones in the more tightly gated population of cells defined by Gate R2, since this would have provided a rapid focus for further efforts. None of these 82 colonies appeared to contain the pCDM8 vector or, therefore, candidate cDNA. This appears surprising in the first instance, since they all grew under strict antibiotic restriction, which should have identified only pCDM8-expressing bacteria. However, discussion with others who have performed such genetic screens suggests that false positive colonies will appear and that this is presumably due to acquisition of antibiotic resistance, either from the pCDM8 vector or *de novo* (*personal communication,* Dr. L. Vernon-Wilson). This was supported by the finding that a significant proportion (23%) of Gate R4 colonies apparently contained no pCDM8 vector either.

This αβ-py CHO cell system has already been used to identify a number of proteins related to integrin activation. In 1997, Fenczik *et al.* used it to identify CD98 as a specific activator of integrins (Fenczik *et al.*, 1997a). In that study, CD98 was the only protein isolated for its ability to rescue integrins from dominant suppression induced by overexpression of β1 integrin cytoplasmic domains, so-called complementation of dominant suppression (CODS). The same expression cloning technique identified PEA-15 as a protein able to reverse H-Ras-mediated integrin suppression, in a strategy similar to the one taken in the current work (Ramos *et al.*, 1998). PEA-15 is a death effector domain-containing protein, found in a family of proteins that normally regulate apoptosis through this death effector domain (DED). In PEA-15, however, the DED is required for regulation of integrin function. PEA-15 also appeared to be downstream of R-Ras G38V, since a dominant negative R-Ras prevented PEA-15 reversal of integrin suppression.

In the case of PEA-15 it was demonstrated that H-Ras expression levels in PEA-15 transfected cells remained comparable to cells transfected with H-Ras alone. This was clearly critical if it was to be argued that reversal of H-Ras-mediated suppression by PEA-15 was not due simply to a loss of H-Ras expression. Within the limits of the initial studies on Clone 480, it was felt that there was good evidence that reversal of suppression was observed despite reasonable preservation of H-Ras expression. However, these experiments were limited by the lack of an expression marker for Clone 480 itself and therefore cannot conclusively rule out an effect of overexpression. Without such a marker it proved difficult to satisfactorily differentiate between the two possibilities of (a) a true reversal of H-Ras suppression and (b) a loss of H-Ras G12V expression. Although analysis of protein expression suggested that H-Ras expression was diminishing as a result of diminishing cell survival, an alternative possibility might also be that Clone 480 was targeting H-Ras to the ER, from which it was not being adequately released during preparation of cell lysates. Such an effect would certainly compound any loss of H-Ras expression due to decreased cell survival. In fact,

subsequent experiments with 480-Myc and Nessie-Myc have strengthened the view that the reversal of suppression is a true phenomenon, rather than simply the effect of expressing a large protein that localises to the ER (Dr. B. McHugh, *personal communication*). Current studies using truncated forms of Nessie, lacking the 480 region, should further establish whether the effect is true reversal.

It was noted that there were differences between the predicted amino acid sequences of Clone 480 and the corresponding region of KIA0233 – only the first 98 amino acid residues for Clone 480 concur with the predicted sequence of KIA0233 (**Figure 6.22**). This is relatively easily explained. If the two DNA sequences are aligned using BLAST software, there is an extra guanine in Clone 480 that results in a frameshift. Thereafter, the reading frame will differ between the two sequences, and hence also the amino acid sequence (**Figure 6.23**).

Nessie does not immediately appear to be a candidate for a link between R-Ras and reversal of suppression. A number of recent papers have begun to pull apart the mechanism of R-Ras reversal. Analysing the closely related H-Ras and R-Ras proteins, it appears that the C-terminal 53 amino acid residues are sufficient to confer upon R-Ras its specific biological properties, in terms of morphological changes, reactive oxygen species production and reversal of suppression (Hansen *et al.*, 2002). R-Ras reversal has been shown to be dependent upon the presence of a proline-rich motif, similar to an SH3 domain-binding site (Wang *et al.*, 2000). Additionally, the tyrosine 66 residue, located in the effector domain, is a point of critical phosphorylation/dephosphorylation events controlling R-Ras activity. This is a target for the ephrin receptor tyrosine kinase, EphB2: transfection with activated EphB2 causes reduced cellular adhesion, associated with phosphorylation at this site (Zou *et al.*, 1999). Interestingly, this is also the site of phosphorylation for an activated form of Src (Src527), which has been found in human colon cancers (Zou *et al.*, 2002).

Origin

1 MDLRPELPTTLGPVSLRQLGLEHTRYPCLDLGAMLLYTLTFWLLLRQFVKEKLLKWAESP
61 AALTEVTVADTEPTRTQTLLQSLGELVKGVYAKYWIYVCAGMFIVVSFAGRLVVYKIVYM
121 FLFLLCLTLFQVYYSLWRKLLKAFWWLVVAYTMLVLIAVYTFQFQDFPAYWRNLTGFTDE
181 QLGDLGLEQFSVSELFSSILVPGFFLLACILQLHYFHRPFMQLTDMEHVSLPGTRLPRWA
241 HRQDAVSGTPLLREEQQEHQQQQQEEEEEEDSRDEGLGVATPHQATQVPEGAAKWGLVAE
301 RLLELAAGFSDVLSRVQVFLRRLLELHVFKLVALYTVWVALKEVSVMNLLLVVLWAFALP
361 YPRFRPMASCLSTVWTCVIIVCKMLYQLKVVNPQEYSSNCTEPFPNSTNLLPTEISQSLL
421 YRGPVDPANWFGVRKGFPNLGYIQNHLQVLLLLVFEAIVYRRQEHYRRQHQLAPLPAQAV
481 FASGTRQQLDQDLLGCLKYFINFFFYKFGLEICFLMAVNVIGQRMNFLVTLHGCWLVAIL
541 TRRHRQAIARLWPNYCLFLALFLLYQYLLCLGMPPALCIDYPWRWSRAVPMNSALIKWLY
601 LPDFFRAPNSTNLISDFLLLLCASQQWQVFSAERTEEWQRMAGVNTDRLEPLRGEPNPVP
661 NFIHCRSYLDMLKVAVFRYLFWLVLVVVFVTGATRISIFGLGYLLACFYLLLFGTALLQR
721 DTRARLVLWDCLILYNVTVIISKNMLSLLACVFVEQMQTGFCWVIQLFSLVCTVKGYYDP
781 KEMMDRDQDCLLPVEEAGIIWDSVCFFFLLLQRRVFLSHYYLHVRADLQATALLASRGFA
841 LYNAANLKSIDFHRRIEEKSLAQLKRQMERIRAKQEKHRQGRVDRSRPQDTLGPKDPGLE
901 PGPDSPGGSSPPRRQWWRPWLDHATVIHSGDYFLFESDSEEEEEAVPEDPRPSAQSAFQL
961 AYQAWVTNAQAVLRRRQQEQEQARQEQAGQLPTGGGPSQEVEPAEGPEEAAAGRSHVVQR
1021 VLSTAQFLWMLGQALVDELTRWLQEFTRHHGTMSDVLRAERYLLTQELLQGGEVHRGVLD
1081 QLYTSQAEATLPGPTEAPNAPSTVSSGLGAEEPLSSMTDDMGSPLSTGYHTRSGSEEAVT
1141 DPGEREAGASLYQGLMRTASELLLDRRLRIPELEEAELFAEGQGRALRLLRAVYQCVAAH
1201 SELLCYFIIILNHMVTASAGSLVLPVLVFLWAMLSIPRPSKRFWMTAIVFTEIAVVVKYL
1261 FQFGFFPWNSHVVLRRYENKPYFPPRILGLEKTDGYIKYDLVQLMALFFHRSQLLCYGLW
1321 DHEEDSPSKEHDKSGEEEQGAEEGPGVPAATTEDHIQVEARVGPTDGTPEPQVELRPRDT
1381 RRISLRFRRRKKEGPARKGAAAIEAEDREEEEGEEEKEAPTGREKRPSRSGGRVRAAGRR
1441 LQGFCLSLAQGTYRPLRRFFHDILHTKYRAATDVYALMFLADVVDFIIIIFGFWAFGKHS
1501 AATDITSSLSDDQVPEAFLVMLLIQFSTMVVDRALYLRKTVLGKLAFQVALVLAIHLWMF
1561 FILPAVTERMFNQNVVAQLWYFVKCIYFALSAYQIRCGYPTRILGNFLTKKYNHLNLFLF
1621 QGFRLVPFLVELRAVMDWVWTDTTLSLSSWMCVEDIYANIFIIKCSRETEKKYPQPKGQK
1681 KKKIVKYG**MGGLIILFLIAIIWFPLLFMSLVRSVVGVVNQPIDVTVTLKLGGYEPLFTMS**
1741 **AQQPSIIPFTAQAYEELSRQFDPQPLAMQFISQYSPEDIVTAQIEG**SSGALWRISPPSRA
1801 QMKRELYNGTADITLRFTWNFQRDLAKGGTVEYANEKHMLALAPNSTARRQLASLLEGTS
1861 DQSVVIPNLFPKYIRAPNGPEANPVKQLQPNEEADYLGVRIQLRREQGAGATGFLEWWVI
1921 ELQECRTDCNLLPMVIFSDKVSPPSLGFLAGYGIMGLYVSIVLVIGKFVRGFFSEISHSI
1981 MFEELPCVDRILKLCQDIFLVRETRELELEEELYAKLIFLYRSPETMIKWTREKE

Figure 6.22 Full predicted sequence of KIA0233 protein.

The full amino acid sequence predicted for the protein coded by KIA0233 (taken from Nagase T, 1996), showing the region represented by Clone 480 in red. Only the first 98 amino acid residues predicted for Clone 480 concur with those within the predicted sequence for KIA0233 (residues 1689 – 1786, in bold). Thereafter the predicted sequence for Clone 480 differs from that of KIA0233 (see text and Figure 6.23).

```
Query: 1     atgggtggcctcatcatcctcttcctcatcgccatcatctggttcccgctgctcttcatg 60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5067  atgggtggcctcatcatcctcttcctcatcgccatcatctggttcccgctgctcttcatg 5126

Query: 61    tcgctggtgcgctccgtggttggggttgtcaaccagcccatcgatgtcaccgtcaccctc 120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5127  tcgctggtgcgctccgtggttggggttgtcaaccagcccatcgatgtcaccgtcaccctc 5186

Query: 121   aagctgggcggctatgagccgctgttcaccatgagcgcccagcagccgtccatcatcccc 180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5187  aagctgggcggctatgagccgctgttcaccatgagcgcccagcagccgtccatcatcccc 5246

Query: 181   ttcacggcccaggcctatgaggagctgtcccggcagtttgacccccagccgctggccatg 240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5247  ttcacggcccaggcctatgaggagctgtcccggcagtttgacccccagccgctggccatg 5306

Query: 241   cagttcatcagccagtacagccctgaggacatcgtcacggcgcagattgagggcaggctc 300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct: 5307  cagttcatcagccagtacagccctgaggacatcgtcacggcgcagattgagggca-gctc 5365

Query: 301   cnggggcgatgtggcgcaatcagtccccccagccgtgcccagatngaagcgggagctcta 360
              | |||||| |||||||| |||||||||||||||||||||||| ||||||||||||||
Sbjct: 5366  c-ggggcgctgtggcgc-atcagtccccccagccgtgcccagat-gaagcgggagctcta 5422

Query: 361   caacnggcacggccgacatcaccctgcgcttcacctggaacttccagagggacctggcga 420
              |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5423  caac-ggcacggccgacatcaccctgcgcttcacctggaacttccagagggacctggcga 5481

Query: 421   agggaggcactgtggagtatgccaacgagaagcacatgctggcncctggcccccccaacag 480
              |||||||||||||||||||||||||||||||||||||||| ||||| ||||||||||
Sbjct: 5482  agggaggcactgtggagtatgccaacgagaagcacatgctggc-cctgg-cccccaacag 5539

Query: 481   cactgcacggcggcagctggccagcctgctcgagggcaccctcggaaaccagtctgtggt 540
              |||||||||||||||||||||||||||||||||||||||| ||||||  ||||||||||||
Sbjct: 5540  cactgcacggcggcagctggccagcctgctcgagggca-cctcgg--accagtctgtggt 5596

Query: 541   catccccaatctctttccccaagtacatccgtgcccccaacgggcccgaagccaaccctg 600
              |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 5597  catccccaatctc-ttccccaagtacatccgtgcccccaacgggcccgaagccaaccctg 5655

Query: 601   tga 603
              |||
Sbjct: 5656  tga 5658
```

Figure 6.23 Mismatching between Clone 480 and KIA0233.
Alignment of the nucleotide sequences for Clone 480 (Query) and KIA0233 (Sbjct), commencing at the first ATG/methionine start site in Clone 480, shows that the two sequences are identical as far as base pair 296 of Clone 480 (highlighted). Here an extra guanine base is found in Clone 480, not present in KIA0233. As a consequence of this, there would be a frame-shift in the reading frame of Clone 480, if working from the original predicted sequence: the predicted amino acid sequences would therefore differ from this point on.

Signalling events downstream of R-Ras remain poorly understood. R-Ras can activate the PI 3-kinase pathway, but although it binds to the common effector Raf-1, it does not activate Raf-1 or the ERK/MAPK pathway, nor the JNK pathway (Marte *et al.*, 1997;Self *et al.*, 2001). The PI 3-kinase pathway has recently been demonstrated to be important in controlling monocyte adhesion and migration in the context of atherosclerosis (Cole *et al.*, 2003). Microinjection of R-Ras into a macrophage cell line has shown activation of αMβ2 integrins via Rap1 (Self *et al.*, 2001). Recent studies have shown that R-Ras localises to focal adhesions in the lipid membrane and that the C-terminal residues confer this property (Furuhjelm and Peranen, 2003); unlike H-Ras, which migrates out of lipid rafts upon activation, activated R-Ras remains localised (Hansen *et al.*, 2003), where it has been shown to promote adhesion through phosphorylation of FAK and p130(Cas) (Kwong *et al.*, 2003).

The protein identified in this chapter, Nessie, appears to promote reversal of integrin suppression, yet how 480/Nessie might achieve this is quite unclear. The parent protein is extremely large, with multiple transmembrane domains. To date, those proteins recognised to be involved in regulation of integrin affinity have either been small signalling molecules, such as the Ras family of GTPases, or proteins that localise to the plasma membrane, where they could potentially regulate affinity in the context of a membrane lipid rafts, such as CD98, β3-endonexin or, now most prominently, R-Ras itself. However, so far both 480-Myc and Nessie-Myc have only ever localised to the ER, not to the plasma membrane where Nessie might have been expected to influence integrin affinity.

Potential mechanisms by which Nessie might regulate integrin affinity might include sequestration of important signalling molecules, such as H-Ras or its mediators, such that they are unavailable to cause integrin suppression. The author is unaware of any evidence to support this. Intriguingly, however, a recent report by Werner *et al.* has linked the functional effects of R-Ras with the ER (Koopman *et al.*, 2003). This group

has observed that activated R-Ras alters the handling of Ca^{2+} by the ER, with increased leak of Ca^{2+} from the ER and a decrease in frequency of cholecystokinin-induced Ca^{2+} oscillations. Observing that this reduction in ER Ca^{2+} stores resembles the effect of Bcl-2, it has been suggested that these effects may underlie both the antiapoptotic effect of R-Ras and its modulatory role on integrin affinity, through alterations in the activity of downstream effector molecules. Thus it is now interesting to speculate whether Nessie, as a large transmembrane protein situated in the ER, may be involved in the regulation of ion exchange, with downstream effects on integrins as a result. In addition, the large number of transmembrane domains predicted in Nessie might be in keeping with a function as an ion channel. This need not necessarily be influenced by R-Ras in any way.

In summary, this chapter has presented the results of an expression cloning screen designed to identify proteins involved in the reversal of integrin suppression. In particular, it was hoped to identify potential downstream effectors of R-Ras. Preliminary findings appear promising: a cDNA clone demonstrated reversal of H-Ras-mediated suppression in the face of continued H-Ras expression. This appears to be a fragment of a larger, novel gene encoding a large protein with multiple transmembrane domains. Further studies by colleagues have reproduced the author's findings and confirmed them with the whole protein; outstanding issues still require clarification, in particular confirmation that this is not simply an overexpression effect.

Chapter 7

Conclusions and Future Directions

This thesis has covered three main areas of study relating to integrins and lung cancer: the association between β1 integrin and the transmembrane protein CD98; the expression of CD98 and its ligand galectin-3 within a variety of lung cancers; and a search for regulatory pathways involved in the modulation of integrin affinity in a model cell system. Briefly summarised, this work has confirmed the physical association of CD98 and β1 integrin, as well as demonstrating that cross-linking CD98 results in integrin-like signalling and functional effects. Histochemically, there was a striking differential expression of galectin-3 between SCLC and NSCLC samples. Finally, a novel gene and protein – now termed *Nessie* because of multiple predicted transmembrane domains – have been identified with an apparent ability to rescue H-Ras-mediated integrin suppression.

The setting of this work is the field of lung cancer, in particular small cell lung cancer. Survival figures for lung cancer as a whole are poor. Primarily the reason for this is the late presentation of the disease, such that even for non-small cell lung cancer only a minority of patients have the chance of potentially curative surgery. Additionally, until recently NSCLC was deemed insensitive to chemotherapy and it is only over the last decade or so that more aggressive chemotherapy regimes are being used for good performance status patients with inoperable NSCLC. By comparison, SCLC, described even by politicians as "the most aggressive form" of lung cancer, is initially chemosensitive. Perhaps this is just as well, since the vast majority of patients present with metastatic disease and it is very rarely operable. The tragedy remains, however, that even with chemotherapy, SCLC almost inevitably relapses with more resistant disease. Behind this thesis lies the proposition that a better understanding of the cell

biology of lung cancer, the reasons for early metastasis and for the development of resistance to chemotherapy, will lead to more rational and effective treatments.

Key to this is an understanding of integrin function and the way in which they integrins link a cell to its local milieu. Extracellular matrix-mediated protection is now increasingly understood to confer protection from chemotherapeutic agents and has been demonstrated in a number of cell types (Boudreau *et al.*, 1995;Hoyt *et al.*, 1996;Sethi *et al.*, 1999c;Hazlehurst *et al.*, 2000). A cytoprotective effect, mediated through β1 integrins, prevents chemotherapy-induced caspase-3 activation and apoptosis, despite persistent DNA damage. Hence, SCLC cells adhered to ECM may escape chemotherapy-induced cell death and subsequent drug-resistant clones are selected with an increasing burden of genetic damage. This is an excellent model to explain why partial responses and local recurrence of SCLC are often seen after chemotherapy and why there is no unifying genetic change causing chemoresistance in SCLC cells.

The work presented here contributes to our understanding of how a specific regulator of integrin affinity, CD98, may be involved in the augmentation of integrin-ECM binding. This would be predicted to have a direct effect on protection for chemotherapeutic agents, which indeed is confirmed *in vitro*. Furthermore, it supports other work suggesting that integrin-mediated PI 3-kinase signalling is a key signalling pathway involved in ECM-mediated protection. The intracellular signalling mechanisms mediating β1 integrin-induced drug resistance are beginning to be defined. Tyrphostin-25, a selective inhibitor of protein tyrosine kinase (PTK), blocks adhesion-mediated drug resistance in SCLC cells. PI 3-kinase acts as a direct biochemical link between a phosphoinositide pathway and a number of proteins containing tyrosine kinase activity. PI 3-kinase requires tyrosine phosphorylation for full activation and would thus be a target for PTK activity. Both PI 3-kinase and PKB suppress anoikis and the induction of chemotherapy-induced chemoresistance (Schmidt *et al.*, 2002). The introduction of a constitutively active form of PKB to A549 lung non-small cell lung cancer (NSCLC)

cells resulted in profoundly decreased chemosensitivity to mitoxantrone and cisplatin. PKB has been shown by other groups to be constitutively activated in NSCLC and to confer resistance to both radiation- and chemotherapy-induced apoptosis (Brognard *et al.*, 2001).

It is exciting to hypothesize how differential expression of galectin-3 between SCLC and NSCLC may be linked with the different cellular phenotypes, the former so chemosensitive and of high metastatic potential. In particular, although it is hard to put much weight on a single sample, it is striking that the one sample of SCLC taken after chemotherapy demonstrated dramatically positive galectin-3 expression – quite opposite to all other SCLC samples, yet akin to NSCLC samples. Given that relapsed SCLC becomes increasingly chemoresistant, like NSCLC, this suggests that this change may be accompanied by increased galectin-3 expression.

Clearly this is too far to extend these results at present. Nevertheless, it opens up a whole series of relatively simple studies that may be highly elucidating. These would include an assessment of a range of cell lines *in vitro* for endogenous galectin-3 expression, including NSCLC, SCLC and known chemoresistant SCLC. It would also be interesting to assess whether recurrent chemotherapeutic treatment could induce galectin-3 expression and, more importantly, whether transfection with an expression vector for galectin-3 conferred chemoresistance. At a clinical level, it has now become important to assess galectin-3 expression in a larger series of lung cancer patients, both SCLC and NSCLC. Such a study would include samples before treatment with chemotherapy and preferably at a later time point of relapse, which may be death; the objective would be to see whether galectin-3 expression is a useful marker clinically for predicting response to chemotherapy and progression of disease. Additionally, the relationship between galectin-3 expression and metastatic potential should be examined. This may of particular importance in NSCLC, where metastasis prevents curative surgery and may occur post-surgery, as relapsed disease. If galectin-3 expression were

to predict the likelihood of metastatic relapse following surgery, this would be an important tool in targeting adjuvant therapies such as aggressive chemotherapy or cranial radiotherapy.

Little interest has yet been paid in the literature to the fact that galectin-3 is a ligand for CD98, but if galectin-3 is indeed inducible at the cell surface or secreted, then it would appear to have the ability to cross-link CD98, augmenting integrin survival signalling. Chemoresistance is a far more complex entity than this conveniently tidy model, but it would be an interesting model to test, nevertheless, and the creation of a galectin-3 knockout mouse would be a useful adjunct for these studies (Colnot *et al.*, 1998a;Pugliese *et al.*, 2001).

The discovery of a novel gene involved in modulating integrin affinity is scientifically exciting. However, at the time of completion of this thesis much work was still required to ensure this was a valid interpretation of the results. Some of this work has been done and supports the findings of the original Clone 480: the results have been replicated and the use of Myc tagging has enabled clarification of protein expression levels; additionally, the results seem to hold true for the parent – and much larger – protein, full-length Nessie. Providing overexpression effects have been satisfactorily excluded, this work will have isolated a novel protein to stand with the relatively small band already described as activators of integrins. However, it appears to differ from those others most markedly in its large size and structure, as well as its apparent location in the endoplasmic reticulum. As already discussed in Chapter 6, further work is ongoing to exclude overexpression effects. It will then be interesting to establish exactly how Nessie is influencing integrin affinity. As an early step it would seem prudent to develop an antibody to the native protein, so that its cellular location can be pinpointed under normal cellular conditions. If it indeed localises solely to the ER and not the plasma membrane, further studies might include assessments of Ca^{2+} control, as well as

co-immunoprecipitation experiments to look for associations between Nessie and Ras-family signalling proteins.

The regulation of integrins therefore lies at the heart of this work. At first glance, the accumulating evidence for ECM-mediated protection from apoptosis sits somewhat paradoxically with the clinical finding of highly metastatic disease, as found in SCLC. However, integrin-ligand binding is a highly dynamic process, with rapid changes in integrin affinity and avidity. Biologically this is intuitive, since the requirement for cellular adhesion to a basement membrane *must* vary in different phases of cellular behaviour: for example, during mitosis, cells are seen to "round up", becoming largely detached from underlying matrix so that cellular division can occur satisfactorily. Data from this thesis support the proposition that cyclin D1, a key driver of the cell cycle, promotes integrin suppression. A link from the cell cycle back to the cell surface and adhesion – effectively, inside-out signalling – requires further corroboration and detailed study.

Integrin-ligand binding must also be highly dynamic for active migration to occur. An optimum degree of cell-matrix adhesion must exist and be alterable both over very short distances (from the leading edge of a cell to the trailing edge) and over very short time-frames. Lauffenburger and Horwitz argue that cell motility is a balance between contractile forces pulling the cell forward over a surface and adhesive forces between the cell and substratum. If adhesive forces are too strong the cell becomes unable to move, but if there is too little adhesion no tensile force can be generated to transmit to the cytoskeleton and allow movement (Lauffenburger and Horwitz, 1996). These models predict a bell-shaped curve for the rate of migration against the strength of cell adhesion.

We can therefore picture SCLC cells in more than one phase of cellular behaviour. At times they may be strongly adherent to ECM, with an integrin-mediated survival advantage in the face of chemotherapy. When less strongly adhered to ECM proteins,

they will be more mobile and have a greater propensity to metastasize – during which process they have a survival advantage due to their autocrine/paracrine growth loops, but may be more susceptible to chemotherapy-induced apoptosis. Chemotherapy would selectively spare those cells most adherent and most protected. This may explain why clinical recurrence of SCLC tends to occur in the same sites as the initial disease, where there would be the highest likelihood of a subpopulation of cells with strong cell-ECM contacts. On a cellular level, increased cellular adhesion to ECM broadly correlates with increased chemoresistance, although clearly at very high doses of chemotherapy all cells would succumb. The converse is that the less adherent a cell is, the more susceptible it is to chemotherapy. The relationship of adhesion to cell motility, however, would exhibit a peak of cell motility limited by excessive adhesion in one direction, but insufficient tensile force in the other. These relationships of cell motility and cellular chemoresistance against the strength of cell adhesion are represented schematically in **Figure 7.1**.

There is now some direct evidence to support this model in SCLC. Kraus *et al.* have shown that spontaneously adherent sublines of SCLC are phenotypically different from the parent line: they exhibit upregulation of cyclin D1 and of integrin subunits $\alpha 2$, $\beta 3$ and $\beta 4$. Moreover, they were significantly more resistant to either chemotherapy or ionising radiation compared with the parent lines, in a PKB-dependent manner (Kraus *et al.*, 2002). In squamous cell carcinomas, alterations in integrin expression have also been observed to influence susceptibility to anoikis. Using retroviral vectors to introduce αv integrins to αv-negative cells, it has recently been shown that a switch from $\alpha v\beta 5$ expression to $\alpha v\beta 6$ expression resulted in protection from anoikis, again associated with PKB activation (Janes and Watt, 2004). These results point to the critical importance of tight regulation of integrin expression and indicate how loss of integrin regulation may promote neoplasia.

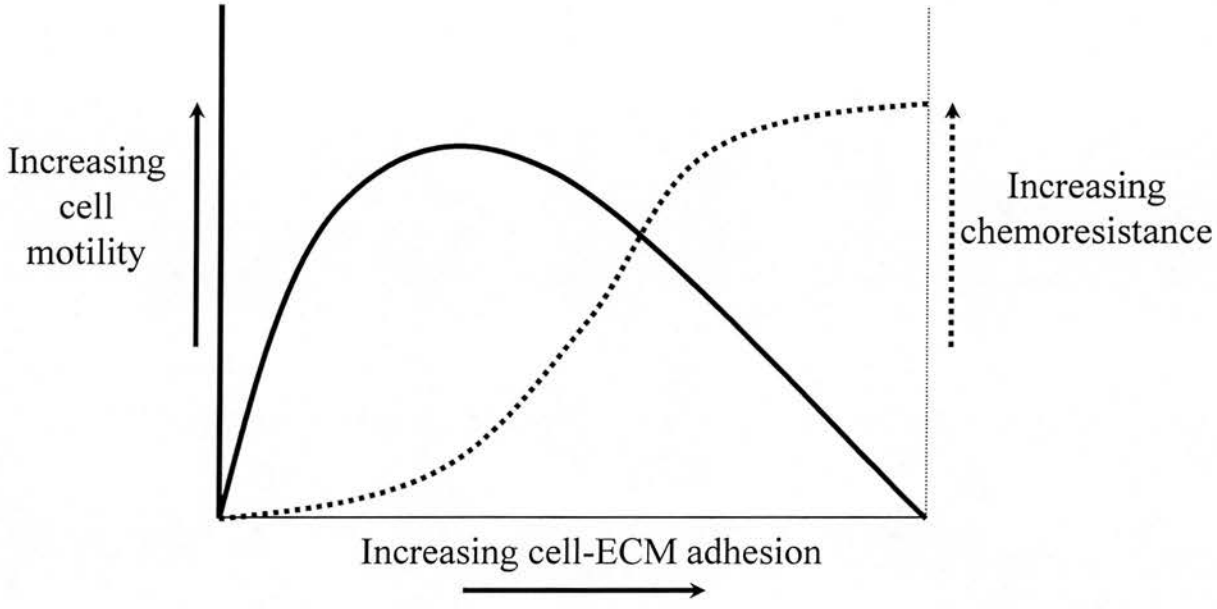


Figure 7.1: Relationship between adhesion and motility or chemoresistance

Schematic graph representing theoretical relationship between cellular adhesion to underlying matrix and both cellular motility and chemoresistance. Cells that are weakly adherent to ECM (*extreme left of graph*) may be unable to generate the tensile forces necessary for migration and will receive little by way of integrin-mediated survival signals – they will therefore be most vulnerable to chemotherapy. As adherence to underlying matrix increases, tensile forces are generated, resulting in increased motility, until such a point as adhesive forces become more difficult to overcome. Increasing adherence results in increased integrin-mediated cell-survival signalling and therefore increasing chemoresistance. Cells that are most strongly adherent to ECM (*extreme right of graph*) will be unable to overcome adhesive forces and will be immotile; however, they will receive strong integrin-mediated survival signals and be the most chemoresistant.

In conclusion, this work has answered some questions regarding integrins in the context of lung cancer. Inevitably, it has opened up more avenues of enquiry. Some of these have immediate clinical application to lung cancer; others are less directly accessible to clinical medicine, but remain important areas of study if we are to fully comprehend mechanisms of cellular control in normality and disease. Such basic scientific studies will ultimately result in more intelligent therapies for diseases, whether neoplastic or degenerative. It is upon such foundations that medicine should be constructed.

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