Aspects of the regulation and role of Focal adhesion kinase and Src in oncogenic transformation

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This thesis is submitted to the University of Glasgow in accordance with the requirements for the degree of Doctor of Philosophy in the faculty of Medicine.

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March 2000.

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Declaration

Unless stated, all the work in this thesis is my own. The work undertaken was performed under the supervision of Dr. Margaret Frame. No part of this work has been submitted for consideration for any other degree or award.

'OM'

For my parents and my brother

Abstract

Focal adhesion kinase (FAK) is a non-receptor tyosime kinase present in cell-ECM focal adhesions. As a part of the integrin signalling complex, FAK is implicated to play a role in the mediation of integrin induced cell growth, motility and survival-processes that are also required for tumour development and progression. A role for FAK has thus been proposed in the process of tumorigenesis. In support of this, FAK is found to be upregulated in various different tumours and tumour cell lines. However, the mechanisms that lead to the upregulation of FAK in cancer cells have not been investigated thus far. The first part of this study focused on establishing some of the genetic alterations adopted by cancer cells to elevate the level of FAK protein. We used fluorescent *in situ* hybridisation (FISH) to first confirm that *fak* localised to human chromosome 8q in normal cells. Elevated FAK protein levels in cell lines derived from invasive squamous cell carcinomas of the head and neck were accompanied by gain in *fak* gene copy number in all the cases examined. Moreover, increased *fak* gene dosage, including genetic alterations like amplification and isochromosome formation, were observed in cell lines derived from human tumours of lung, breast and colon. In addition, conversion from adenoma to carcinoma, in an *in vitro* model for human colon cancer progression, was accompanied by an elevation in FAK protein level and gain in *fak* gene copy number. Although, other genes like *c-myc*, lying near the *fak* locus, were also found to be co-amplified or increased in copy number, given the biological functions FAK is proposed to play a role, it may contribute in exerting the selective pressure for the retention of the whole region in increased number of copies.

In order to further investigate the role of FAK in tumour development, we studied its importance in cell survival. For this we used an *in vitro* model of apoptosis, wherein, apoptosis was induced in v-Src transformed Rat-1 cells after serum-deprivation and inhibition of v-Src activity. In particular, we examined the importance of FAK proteolysis in the induction of apoptosis. We found that although induction of apoptosis was accompanied by FAK cleavage, inhibition of FAK proteolysis was unable to promote cell survival.

The *in vitro* model of apoptosis using v-Src transformed Rat-1 cells was also characterised further in this thesis. Like FAK, the v-Src oncoprotein also resides in the focal adhesions where its constitutive kinase activity induces disruption of focal adhesions and cell transformation. Unlike other oncoproteins like c-Myc, and v-Jun, v-Src does not induce cell death under low serum conditions. However, the data described in this thesis suggested that v-Src primes the cells to undergo apoptosis, partly through disruption of integrin signalling, while providing them with a survival signal at the same time. Removal of this survival signal, thus, induced the cells to undergo apoptosis. Further investigations indicated that the surrogate integrin survival signal provided by v-Src required the activation of the PI 3-kinase/Akt pathway, while the MAP kinase pathway did not play any role in the mediation of the survival signalling.

In addition, the apoptotic response induced in v-Src transformed Rat-1 cells was shown to be accompanied by activation of caspases, and stress-activated kinase, p38. Cell death was inhibited by overexpression of the anti-apoptotic protein Bcl-2 or by the inhibtion of caspase and p38 activity simultaneously.

Acknowledgements

I would like to thank my supervisor, Dr. Margaret Frame, for her support and help during the entire course of my PhD, specially for teaching me the importance of doing nothing less than my best, and the Cancer Research Campaign for providing me with a studentship. A very special thanks to Val Fincham for teaching me all that I know about lab work and to Anne Wyke for proof-reading my thesis at such a short notice. I am very grateful to Ian Morgan, my advisor for his valuable suggestions and great support through some stressful times.

Thanks also to all the members of R1 and R11, specially Dr. Debbie Riley for her helpful suggestions and co-operation. Thanks to all the PhD students, past and present, specially Mary, Rob, Paul and for making my three years at the Beatson really enjoyable and to Andy for helping me cope with 'writing-up stress' and also for helping me out in printing this thesis.

I am also grateful to all the staff members at the Beatson, specially, Iain White, Peter McHardy, and Tom McGuire for their technical help.

Finally, a very special thanks to my parents and my brother for their continuous help and support, without which, this thesis would not have become a reality.

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Abbreviations

ALV	Avian Leukosis virus
AIF	Apoptosis inducing factor
AP-1	Activaintg protein-1
BAD	Bcl-XL/Bcl-2 associated death factor
CAD	Caspases activated deoxyribonuclease
CAS	Crk associated substrate
CDKs	Cyclin dependent kinases
CE	Chick embryos
CEF	Chick embryo fibroblasts
CHO	Chinese hamster ovary cells
CSF-1	Colony stimulating factor 1
CSK	C terminal Src kinase
DAPI	Diamidino-2-phenylindole hydrochloride
DIG	Digoxigenin
DISC	Death inducing signaling complex
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Dioxyribonucleic acid
EC	Endothelial cells
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERK	Extracellular regulated kinases
FADD	Fas-associating protein with a death domain
FAK	Focal adhesion kinase
FITC	Fluorescein isothiocyanate
FRNK	FAK related non kinase
GAP	GTPase activating protein

Gly	Glysine
GRAF	GTPase regulator associated with FAK
GSK-3	Glycogen synthase kinae 3
GST	Glutathione S Transferase
HLA	Histocompatibility antigen
ICAD	Inhibitor of CAD
ICE	Interleukin 1β converting enzyme
IGF	Insulin like growth factor
IL-2	Interleukin 2
ILK	Integrin linked kinase
IRS-1	Insulin receptor substrate 1
JNK	Jun N terminal kinase
LPA	Lysophosphatidic acid
MAP Kinase	Mitogen activated protein kinase
MEK	MAP kinase kinase/ERK kinase
MEKK	MEK kinase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
NGF	Nerve growth factor
OHT	4-hydroxy-tamoxifen
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PAK	p21-activated kinases
PARP	Poly (ADP-ribose) polymerase
Pca	Prostate cancer
PDGF	Platelet derived growth factor
PDT	Photodynamic therapy
PH	Plekstrin homology domain
PI 3-kinase	Phosphatidyl inositol 3-kinase
PIP	Phosphatidylinositol monophosphate
PIP2	Phosphatidylinositol-4,5 bisphosphate

PIP3	Phosphatidylinositol tri phosphate
PKB	Protein kinase B
PKC	Protein kinase C
PTEN	Paxillin and tensin homologue deleted on chromosome 10
Rb	Retinoblastoma protein
REF	Rat embryo fibroblasts
RGD	Arg-Gly-Asp
RNA	Ribonucleic acid
RSV	Rous Sarcoma Virus
SAPK	Stress activated protein kinase
SCC	Squamous cell carcinomas
SEK-1	SAP-ERK kinase-1
Ser	Serine
SH2	Src homology domain 2
PTB	Phosphotyrosine binding domain
SH3	Src homology domain 3
SRE	Serum regulatory element
SREBPs	Sterol-regulatory element binding proteins
TdT	terminal deoxynucleotidyl transferase
Thr	Threonine
TNF	Tumour necrosis factor
TRAIL	TNF- related apoptosis inducing ligand
TUNEL	TdT-mediated dUTP nick end labelling
Ts	Temperature sensitive
Tyr	Tyrosine
UV	Ultra violet
VCAM-1	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor

Aim

The study detailed in this report is essentially divided into two parts. The first half, Chapters 1, 3, and 4, concentrates on the importance and role of focal adhesion kinase (FAK) in tumour cells. FAK, as is discussed later in detail, is upregulated at protein level in several tumours and tumour cell lines and is also known to be important in integrin mediated cell survival and migration- both of which are essential for tumour development and progression. Chapter 1 (Introduction 1) outlines the role of integrins in focal adhesion signalling, and the importance of Src and FAK, two of the kinases known to localise at the focal adhesions, in its mediation. Based on studies reporting overexpression of FAK in tumours, initial experiments were carried out to determine whether overexpression at protein level was accompanied by genetic alterations, which could account for the upregulation at protein level (Chapter 3). At the same time, role played by FAK in protecting transformed cells from undergoing apoptosis was also investigated (Chapter 4). Results indicated that in the model system being studied, FAK proteolysis was not required for the induction of apoptosis. Therefore, the focus of the study shifted to characterisation of the apoptotic pathway activated in the system being studied, and also to investigate the survival pathway impinging on it to protect the transformed cells from dying. Chapter 5 reviews the role of some of the key molecules involved in mediation of apoptosis and cell survival. Results of the characterisation study are outlined in Chapter 6 and Chapter 7. The final chapter discusses the findings of this study in a general way and outlines some of the possibilities for future work.

Chapter 1: Introduction 1

1.1 Signalling at the focal adhesions: A role for integrins

Cultured cells attach to the underlying substrate with the help of specialised membrane associated structures known as focal adhesions. They were first identified in electron microscopic studies of cultured fibroblasts when it was noted that some regions of the ventral surface were closer to the substrate than others (Abercrombie *et al.*, 1971). They appear as tear shaped plaques at the ends of actin filament stress fibres (reviewed in Burridge *et al.*, 1988). Besides providing sites of attachment for the membrane to the substrate, focal adhesions also serve the important structural role of anchoring and stabilization of the actin cytoskeleton and in mediating signals to the cell interior which regulate cell proliferation, differentiation, motility and survival (reviewed in Juliano and Haskill, 1993).

Focal adhesions have a complex molecular organisation. On the outside of the membrane lies the extracellular matrix (ECM). ECM is composed of glycoproteins, proteoglycans and glycosaminoglycans that are secreted and assembled locally to which cells adhere (reviewed in Hay, 1982). Thus, fibronectin, vitronectin, laminin, collagen, tenascin-C, thrombospondin and fibrinogen, all go to make up the ECM (reviewed in Otey, 1996; Boudreau and Jones, 1999). The primary transmembrane components of focal adhesions are the integrins (reviewed in Hynes, 1987; Hynes, 1992). Integrins act as transmembrane receptors for ECM components on the outside of the cell, and interact with cytoskeletal and cytoplasmic components of the focal adhesions on the inside of the cell (Sastry and Horwitz, 1993; Pavalko and Otey, 1994). Actin binding proteins that co-localize with integrins at focal adhesions include, α-actinin, talin, tensin, paxillin, vinculin and tensin along with a diverse range of cytoskeletal proteins, protein kinases like focal adhesion kinase (FAK), c-Src, protein kinase C and integrin linked kinase (ILK) (reviewed in Kumar, 1998), and phosphatases, other signalling molecules, and proteins of unknown functions (reviewed in Craig and Johnson, 1996). Integrins interact with the cytoskeleton on the inside of the cell through their cytoplasmic domains. Thus, integrins form a physical link between the outside environment of the cell, like the ECM or other

cells, and the cytoskeleton, and other cytoplasmic proteins found to be concentrated at the focal adhesions (Figure 1).

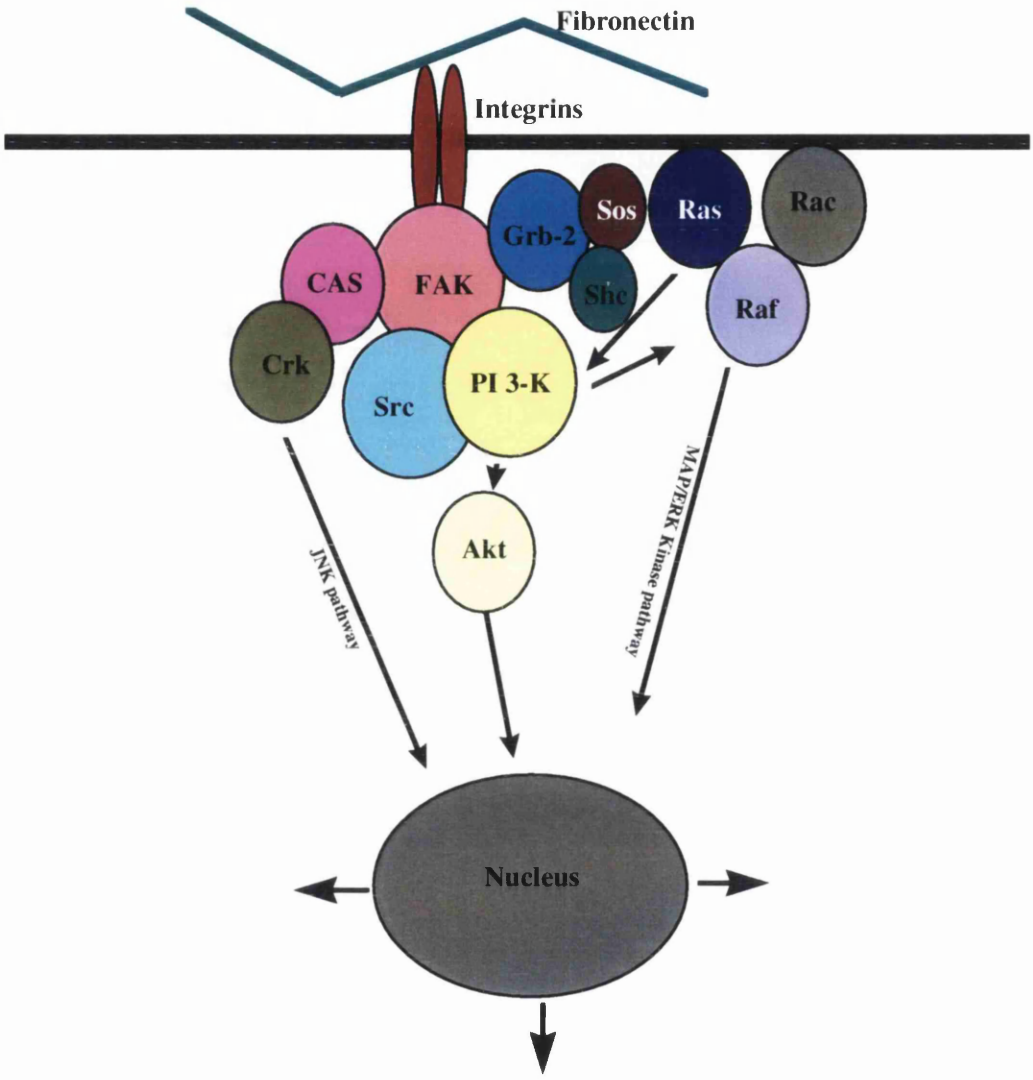


Figure 1 Signalling at the integrins: The diagram outlines some of the pathways involved in mediating integrin signalling to bring about cell proliferation, migration and survival.

1.1.1 Integrins

Integrins are heterodimers of α and β subunits (reviewed in Hynes, 1992). The subunits associate non-covalently to produce 22 different receptors (reviewed in Kumar, 1998; Aplin *et al.*, 1998). Many integrins are expressed in specific cell types. For example, $\alpha_{IIb}\beta_3$ is expressed in platelets and $\alpha_6\beta_4$ in epithelial cells. Integrins contain a large extracellular domain formed by the α and β subunits, a transmembrane segment from each of the subunits and two short cytoplasmic carboxy terminal domains (Hogervorst *et al.*, 1990; Suzuki and Naitoh, 1990). Extracellular domains bind to proteins of the insoluble ECM or to counter-receptors on other cells. Some integrins can also bind to soluble ligands such as fibrinogen. Most of the integrins recognise the RGD (Arg-Gly-Asp) sequence in their matrix ligands (reviewed in Ruoslahti and Pierschbacher, 1987; Ruoslahti, 1996). They are also capable of distinguishing between the different RGD containing ligands such that some bind, for example, to fibronectin while others do not (Kumar, 1998).

The interaction of integrin heterodimers with the ECM on the outside and the cytoskeleton on the inside, facilitates bi-directional signaling. The binding affinity of the integrins for the ligands can be altered by various physiological changes accompanying transduction of signals within the cell. For example, soluble agonists, such as thrombin or adenine nucleotides, activate platelet and leukocyte integrins not through direct interaction with the integrins but by binding to cellular receptors, which lead to engagement of classical signal transduction pathways mediated by G proteins and tyrosine kinases (Dustin and Springer, 1989). This process adds flexibility to integrin function and is indispensable for many of their functions (Schwartz *et al.*, 1995).

The assembly of soluble, dimeric fibronectin into soluble fibrils is probably important in formation of the ECM (Schwartz *et al.*, 1995). Several lines of evidence indicate that integrins also participate in matrix assembly. For example, chinese hamster ovary (CHO) cells lacking $\alpha_5\beta_1$ assemble a matrix only after reconstitution of the integrin by transfection (Wu *et al.*, 1993). Also, overexpression of $\alpha_5\beta_1$ promotes matrix assembly and suppresses the transformed phenotype of CHO cells

(Giancotti and Ruoslahti, 1990). However, certain mesodermal structures form and cells can assemble a matrix in the absence of $\alpha_5\beta_1$ (Yang *et al.*, 1993). This indicates that although matrix assembly may require integrins in some cases, other receptors are able to substitute for particular integrins in other cases. Also, $\alpha_5\beta_1$ are the only integrins so far implicated in matrix assembly (Schwartz *et al.*, 1995). The finding that a number of cell lines expressing only low affinity $\alpha_5\beta_1$ did not assemble a fibronectin matrix (O'Toole *et al.*, 1994), suggested that fibrillogenesis may be a function of high-affinity $\alpha_5\beta_1$. A study by Faull *et al.*, (1994) established that cytochalasins, which had been earlier found to reduce the process of fibrillogenesis, did not profoundly diminish the affinity of fibronectin binding to $\alpha_5\beta_1$. Since cytochalasins also inhibit actin polymerisation (Schliwa, 1982), this raised the possibility of the involvement of the cytoskeleton in the process (Schwartz *et al.*, 1995).

1.1.2 Signal transduction through ECM-integrin interaction

Adhesive interactions between cells and the ECM have long been known to play a vital role in embryonic development and in regulation of gene expression in adults (reviewed in Juliano and Haskill, 1993). Most of these responses are mediated by integrins (reviewed in Juliano and Haskill, 1993). Interest in the role of integrins in signalling was raised by studies reporting increased levels of tyrosine phosphorylation associated with integrin clustering at the focal adhesions. Specifically, clustering of integrins causes enhanced tyrosine phosphorylation of a 120-130 kd protein complex (Kornberg *et al.*, 1991). Another study on mouse fibroblasts reported the phosphorylation of ~120 kd protein, now known to be focal adhesion kinase (see section 1.3), after adhesion to fibronectin coated substrate (Guan *et al.*, 1991). This change in tyrosine phosphorylation of a cellular protein was not evident on adhesion to non-specific ligands such as poly-L-lysine.

The ECM, through interaction with integrins, is now known to play an important role in regulating growth, differentiation, migration and survival in cells, *via* signals induced at the focal adhesions (reviewed in Hynes, 1992; Ruoslahti and Reed, 1994; Vuori and Ruoslahti, 1994).

1.1.3 Evidence for role of ECM-integrin signalling in cell growth

Much of the evidence for the involvement of integrins in cell proliferation has come from studies showing activation of the mitogen-activated protein kinase (MAP kinase) pathway (Chen *et al.*, 1994; Miyamoto *et al.*, 1996; Zhu and Assoian, 1995; Schlaepfer *et al.*, 1994; Wary *et al.*, 1996). MAP kinase is a major cellular signalling pathway that mediates effects of growth factor on cell cycle progression (reviewed below; reviewed in Marshall, 1994).

Chen *et al.*, (1994) showed that adhesion of cells to substrates coated with ECM proteins, or with synthetic peptide containing the RGD sequence, causes activation of MAP kinases in 3T3 or REF52 fibroblasts. This activation of MAP kinases was specific to the binding of the integrins to fibronectin or laminin, and not to poly-D-lysine, a non-specific adhesion promoting peptide. This suggested that activation of MAP kinases was dependent on integrin engagement rather than just cell adhesion (Chen *et al.*, 1994). ERK 1 and ERK2, two conventional MAP kinases, were found to be activated by long term adhesion of NIH 3T3 cells to fibronectin, vitronectin or type IV collagen coated dishes (Zhu and Assoian, 1995). The stimulation of activity was gradual and associated with cell spreading rather than cell attachment.

Studies have also shown that activation of the MAP kinase cascade by some growth factors like epidermal growth factor (EGF) and platelet derived growth factor, is impaired when cells are held in suspension as compared with cells adhered to a fibronectin substratum (Lin *et al.*, 1997). When NIH 3T3 cells held in suspension were treated with either EGF or PDGF, there was only a very modest activation of MAP kinases. However, after about 10min of cell adhesion to fibronectin-coated substrata, generic MAP kinases were found to be strongly activated (Lin *et al.*, 1997). Also, in the absence of EGF, adherent cells were again not able to activate MAP kinases fully. Specifically, experiments suggested that in non-adherent cells, the MAP kinase pathway is attenuated between Ras and Raf kinases (Lin *et al.*, 1997). Another study also showed that activation of the MAP kinase pathway by serum or growth factors is strongly dependent on integrin mediated cell adhesion to ECM

(Renshaw *et al.*, 1997). However, unlike Lin *et al.*, (1997), this study showed that this effect occurs at the level of activation of MAP kinase/ERK kinase (MEK) by Raf, since endogenous Ras and Raf were found to be maximally activated by serum in non-adherent cells, while MEK was not (Renshaw *et al.*, 1997). Integrins have also been shown to associate with growth factor receptors directly (Vuori and Ruoslahti, 1994). Insulin stimulation was found to promote association of the $\alpha_v\beta_3$ integrin (a vitronectin receptor) with insulin receptor substrate-1 (IRS-1) (Vuori and Ruoslahti, 1994). IRS-1 is phosphorylated by insulin and insulin like growth factor (IGF) and associates with other signaling molecules. The interaction between $\alpha_v\beta_3$ and IRS-1 substantially enhances the growth stimulating effects of insulin and IGF and is specific to integrin $\alpha_v\beta_3$ (Vuori and Ruoslahti, 1994).

Rho family GTPases like Rac and Rho, which are important regulators of the actin cytoskeleton, are now known to cooperate with Raf-1 to activate the ERK pathway (Frost *et al.*, 1997). These small G proteins have also been implicated downstream of integrin signaling as integrins were found to stimulate Rho mediated production of PIP2 (Giancotti, 1997).

Some groups have also reported that the activation of cyclins and cyclin-dependent kinases (cdk) requires cell adhesion (Fang *et al.*, 1996; Zhu *et al.*,1996). The cyclin E-cdk2 complex, which is required for the G1-S transition of the cell cycle, was activated in the late G1 phase in attached human fibroblasts, but not in fibroblasts maintained in suspension (Fang *et al.*, 1996). Cell adhesion regulates the activity of cyclin E-cdk2 by determining the extent of its association with the cdk inhibitors p21 and p27 (Zhu *et al.*, 1996). Cell adhesion is also required for the induction and translation of cyclin D1 mRNA and the expression of cyclin A mRNA and protein (Zhu *et al.*, 1996).

1.1.4 Mechanism of integrin-mediated activation of MAP kinase pathway

Integrin mediated activation of MAP kinase pathway requires the activity of two main kinases known to localise to the focal adhesions (FAK) (Hanks *et al.*, 1992; Schaller *et al.*, 1992), and Src, which localises at the focal adhesions upon integrin

activation (Horvath *et al.*, 1992; Clark and Brugge, 1993). Clustering of integrins causes activation of FAK through phosphorylation (Miyamoto *et al.*, 1995). Autophosphorylation of FAK at Tyr 397 (Schaller *et al.*, 1994) facilitates the binding of Src by a mechanism involving the SH2 and SH3 domains (Cobb *et al.*, 1994; Thomas *et al.*, 1998). Subsequent phosphorylation of FAK Tyr 925 by Src creates a binding site for Grb2 to FAK *via* the SH2 domain (Schlaepfer *et al.*, 1994). Binding of FAK to Grb2 provides a link to the activation of the Ras signalling pathway (Schlaepfer and Hunter, 1996). Grb2 forms a complex with Shc, a SH2-PTB domain adaptor protein that links various tyrosine phosphorylated signal transducers to Ras (Pawson, 1995), and Sos, a Ras specific nucleotide exchange protein which activates Ras by converting it to its GTP-bound form (Bowtell *et al.*, 1992). Shc is also a Src substrate (McGlade *et al.*, 1992). Once converted to its GTP-bound form, Ras then binds to c-Raf and leads to its localisation to the plasma membrane, followed by activation (Jelinek *et al.*, 1996). Activated Raf then phosphorylates and activates MEK, that can directly activate ERK1 and ERK2, the conventional MAP kinases (Peraldi *et al.*, 1995). These kinases phosphorylate and a number of substrates that participate in cell cycle progression, including Elk-1, phospholipase A_2, and p90RSK (Renshaw *et al.*, 1997). Phosphorylation of the ternary complex factors Elk-1 and SAP-1 regulates the transcription from the *fos* serum response element (SRE) and thus contribute to cell cycle progression (Wary *et al.*, 1996).

In addition, integrin mediated activation of ERK2 can also be mediated by PI 3-kinase (King *et al.*, 1997). PI 3-kinase is an enzyme that catalyses the phosphorylation of the inositol lipids and associates with FAK through the SH2 domain of its p85 subunit (Chen and Guan, 1994). PI 3-kinase association with FAK is dependent on the autophosphorylation of FAK at Tyr 397 (Chen *et al.*, 1996a). Integrin-mediated activation of ERK2, MEK and Raf-1 were about 80% inhibited by PI 3-kinase inhibitors LY294002 and wortmannin (King *et al.*, 1997). PI 3-kinase was shown to function upstream of Raf-1, but downstream of Ras during integrin mediated activation of ERK2 (king *et al.*, 1996).

Other studies have shown FAK to regulate cell proliferation through CAS (Crk associated substrate) (Oktay *et al.*, 1999). Binding of CAS to FAK (Polte and Hanks,

1995) and its phosphorylation by Src (Polte and Hanks, 1997; Vuori *et al.*, 1996) leads to the subsequent recruitment of Crk, a SH3 domain-containing adaptor protein (Mayer *et al.*, 1988) to the complex, followed by activation of the JNK pathway (Oktay *et al.*, 1999). Activation of the JNK pathway was also found to be necessary for proper progression through the G1 phase of the cell cycle and thus cell proliferation (Oktay *et al.*, 1999).

Inspite of all the evidence implicating FAK in the mediation of MAP kinase pathway, a study has shown that in some cases, integrin-mediated activation of MAP kinase pathway can be independent of FAK (Lin *et al.*, 1997). Expression of FRNK, a FAK related non-kinase, discussed in section 1.3, as a dominant negative inhibitor of FAK phosphorylation, was shown to have no effect on integrin mediated activation of MAP kinase suggesting the existence of at least two distinct integrin signalling pathways in fibroblasts (Lin *et al.*, 1997).

1.1.5 Role of integrins in cell migration

Cellular migration plays an important role in development, wound healing, immune defense and metastasis. It is generally accepted that the driving force for cell movement requires re-organisation of the actin cytoskeleton, directing protrusions at the front of the cell and retraction at the trailing edge (reviewed in Nobes and Hall, 1999). Dynamic regulation of focal adhesions is required for making new points of attachments at the front and detachment of the old ones at the rear. Also, the ability of a cell to form focal adhesions correlates with its ability to assemble an organised cytoskeleton, which, in turn, is a prerequisite for cell spreading and migration (Dejana *et al.*, 1987).

Binding of the integrins to the ECM components is essential for cell spreading and migration (Leavesly *et al.*, 1992). However, binding alone is not enough and the integrins are required to cluster at the focal adhesions in order to mediate cell spreading and migration (Leavesly *et al.*, 1992). FG human carcinoma cells failed to induce migration on vitronectin substrate in spite of expressing one of the vitronectin receptors, $\alpha_v\beta_5$ integrin, the defect being in failure to localise to the focal adhesions.

However, when these cells were transfected with a cDNA encoding the human β_3 integrin subunit and were thus able to express $\alpha_v\beta_3$, an integrin that binds to vitronectin and also clusters at the focal adhesions, the cells were able to migrate on a vitronectin matrix (Leavesly *et al.*, 1992). Thus, the β subunit seems to play an important role in cell migration. Mutational analysis of the β_1 subunit showed that the structural basis for integrin/focal contact formation might depend on three domains within the cytoplasmic tail of β_1 (Reszka *et al.*, 1992). β_1 and β_3 are extremely well conserved in each of these regions (Leavesly *et al.*, 1992).

1.1.6 Mechanisms of integrin-mediated cell migration

Recent studies have shed more light on the molecular mechanisms of cell migration. Strength of adhesions between a cell and its matrix, as regulated by receptor affinity, organization of focal adhesions and substrate concentration have been implicated in cell migration (Huttenlocher *et al.*, 1996). Increase in ligand affinity, decreases cell movement. Cells in which the integrins were locked in high affinity state showed maximal migration at lower substrate concentrations than cells expressing low affinity receptors (Huttenlocher *et al.*, 1996).

MAP kinase pathways have also been shown to be involved cell migration. MAP kinase (ERKs) was found to influence cell movement by phosphorylating and, thereby, enhancing myosin light chain kinase (MLCK) activity leading to phosphorylation of myosin light chains (MLC) (Klemke *et al.*, 1997). MLC, in turn, promotes cytoskeletal contraction necessary for cell movement. Inhibition of MAP kinase activity decreased MLCK activity, MLC phosphorylation and cell migration on ECM proteins (Klemke *et al.*, 1997). Thus, integrin-induced MAP kinase activity may contribute to integrin-dependent cell motility.

Stress fiber formation and membrane ruffling have been associated with the activation of small G proteins like Rho, Cdc42 and Rac (Nobes and Hall, 1994; Parsons, 1996). Analysis of Swiss 3T3 fibroblasts has also implicated Rac, Rho and Cdc42 in cell motility. Studies found Rac to be essential for the protrusion of lamaellipodia and forward movement, Cdc42 was required to maintain cell polarity

and Rho was required to maintain cell adhesion during movement (Nobes and Hall, 1999). The Rho family G proteins are also well known for their ability to induce rearrangement of filamentous actin, a prerequisite for cell motility (Sells *et al.*, 1999). Cells expressing wild type or constitutively active PAK, an effector protein of Rho GTPases, were more motile than their normal counterparts (Sells *et al.*, 1999). In addition, cells expressing the kinase-defective form of PAK showed reduced persistence of movement. Although highly motile, they had defects in directed motility (Sells *et al.*, 1999). Expression of constitutively activated PAK was accompanied by increased phosphorylation of MLC (Sells *et al.*, 1999).

Two of the most likely regulators of cell motility are the non-receptor tyrosine kinases of the Src family and FAK. Rapid turnover of focal adhesion contacts, induced by these tyrosine kinases, therefore seems to play a major role in cell migration. Transformation of cells by v-Src stimulates not only the phosphorylation of FAK but also its degradation in chicken embryo fibroblasts (CEF), causing disruption of focal adhesions resulting in cell rounding and detachment (Fincham *et al.*, 1995). In this study, impaired morphological transformation was linked to inefficient degradation of FAK suggesting that phosphorylation of FAK by v-Src stimulates turnover and loss of focal adhesions. More recently, v-Src induced FA turnover was shown to be a critical component of motility (Fincham and Frame, 1998). CAS and its adapter protein Crk, also seem to be involved in mediating cell migration (Cary *et al.*, 1998; Klemke *et al.*, 1998). As stated before, CAS binds to FAK (Polte and Hanks, 1995) and is phosphorylated by Src (Vuori *et al.*, 1996). Expression of CAS or Crk in FG cells was sufficient to promote cell migration which depended on its anchorage dependent phosphorylation facilitating an SH2-mediated complex with Crk (Klemke *et al.*, 1998). Resulting migration could be disrupted by a dominant negative form of Rac but not Ras (Klemke *et al.*, 1998).

1.1.7 Role of integrins in cell survival

The role of integrins in cell survival was suggested by a study carried out by Meredith *et al.*, (1993). These workers investigated the potential role of ECM as a survival factor for human endothelial cells and found that they underwent apoptosis

in the absence of ECM interactions. Induction of apoptosis was specific to integrin-ECM interactions since it was prevented by plating the cells on an immobilized integrin β_1 antibody but not by antibodies against either class 1 histocompatibility antigen (HLA) or vascular cell adhesion molecule-1 (VCAM-1) (Meredith *et al.*, 1993). A later study termed this type of detachment induced apoptosis as 'anoikis' (Frisch and Francis, 1994). Disruption of cell-matrix interactions induced apoptosis in MDCK epithelial cells. However, normal fibroblasts did not die under similar conditions. Myc/Ras or E1A/Ras transformed REF (Rat embryo fibroblasts), however, did undergo apoptosis in the absence of matrix adhesion (McGill *et al.*, 1997). Also, c-Myc induced apoptosis in CEF on serum-withdrawal was suppressed by plating the cells on ECM components, collagen and fibronectin (Crouch *et al.*, 1996). Apoptosis was also prevented by a β1 specific integrin antibody (Crouch *et al.*, 1996). The role of different kinases and signaling pathways which lie downstream of the integrins in mediating cell survival are introduced in sections 1.2 and 1.3. Epithelial cells isolated from pregnant mouse mammary gland die by apoptosis in culture, but the process of cell death is suppressed by integrin mediated cell adhesion to the basement membrane (Pullan *et al.*, 1996). In addition, a hormonal signal from insulin or insulin like growth factor was found to be necessary to suppress mammary cell apoptosis, indicating an ECM and soluble factor cooperation in providing the survival signal (Farrelly *et al.*, 1999).

1.2 Src

c-Src, one of the enzymatic components of the integrin-linked focal adhesions, was first discovered as the normal cellular homologue of v-Src, the transforming gene of Rous Sarcoma Virus (RSV), an acutely transforming avian retrovirus which induces sarcomas in chickens (Stehelin *et al.*, 1976). The isolation of c-Src led to the concept of the existence of cellular *proto-oncogenes* from which *oncogenes* are derived.

Src is a member of non-receptor tyrosine kinases defined by a common modular structure (Neet and Hunter, 1996). The Src family is the largest and the best studied family of cytoplasmic protein tyrosine kinases. 9 members have so far been placed in this group. They are: c-Src, c-Yes, Fyn, c-Frg, Yrk, Lck, Lyn, Hck and Blk (Neet and Hunter, 1996). Like Src, Yes and Frg were first described as transforming proteins of retroviruses (Kitamura *et al.*, 1982; Naharro *et al.*, 1983). Lck was discovered as an overexpression protein in a transformed cell line (Martha *et al.*, 1985). The other members of the family were identified by the screening of cDNA libraries. Src, Yes, and Fyn are widely expressed proteins whereas, expression of rest of the members of the family is restricted, predominantly to the immune system.

1.2.1 Structure of Src

The basic structure of Src is outlined in Figure 2. The first 16 N-terminal amino acids form the myristylation domain. The myristylation group is added to the glycine residue at position 2 of the protein. Myristylation is a fatty acid modification that is determined by the first seven amino acids of the molecule and causes association with the plasma membrane (Resh, 1994). Myristylation-defective mutants of v-Src do not associate with the cellular membranes and fail to induce cell transformation (Cross *et al.*, 1984; Kamps *et al.*, 1986). Myristylation of Src is necessary but not sufficient for membrane localisation as there are myristylated variants of v-Src which fail to associate with cellular membranes (Stoker *et al.*, 1986). Besides the myristylation domain, amino acids 38-111 also seem to be important in membrane

localisation. Fusion of this region to pyruvate kinase, a cytoplasmic protein resulted in its association with the plasma membrane (Kaplan *et al.*, 1990). In addition, several of the other Src family members, such as Lck and Fyn undergo palmitylation of the cysteine residues near their amino terminus (Resh, 1994; Koegl *et al.*, 1994). Unlike myristylation, palmitylation is reversible and is likely to increase membrane binding efficiency (Alland *et al.*, 1994).

Carboxy terminal to the myristylation domain lies a 50-80 amino acid sequence that varies highly among the various family members and is called the unique domain (Figure 2). Deletions within this domain have little effect on c-Src kinase activity but the deletion of the whole domain can result in kinase activation (Nemeth *et al.*, 1989). This domain contains a number of phosphorylation sites. Association of cellular proteins with the unique domains of Lck and Lyn has been reported (Shaw *et al.*, 1989; Turner *et al.*, 1990; Pleiman *et al.*, 1993), perhaps suggesting a role for this domain in substrate recognition. In support of this idea, recent studies have found that phosphorylation of the NMDA (N-methy D-aspartate) receptor by Src is dependent on the Src unique region (Yu *et al.*, 1997). Also, mutation of certain Ser and Thr residues within the unique domain, which are phosphorylated upon Src activation during M phase, was found to reduce Src activity but not eliminate it (Shalloway *et al.*, 1992).

Carboxy terminal to the unique domain is a Src homology 3 (SH3) domain (amino acid residues 88-139) followed by a Src homology 2 (SH2) domain (amino acid residues 148-250). These domains are shared not only with the members of the Src family but also with a variety of unrelated proteins involved in signal transduction and mediate specific protein-protein interactions (reviewed in Pawson and Gish, 1992).

In c-Src, the ligand binding sites of the SH3 domain have been identified by chemical shift perturbation studies (Yu *et al.*, 1992). The domain comprises of a hydrophobic binding site on the surface of the protein that is lined with the side chains of conserved aromatic amino acids (Yu *et al.*, 1992). It has been shown to preferentially

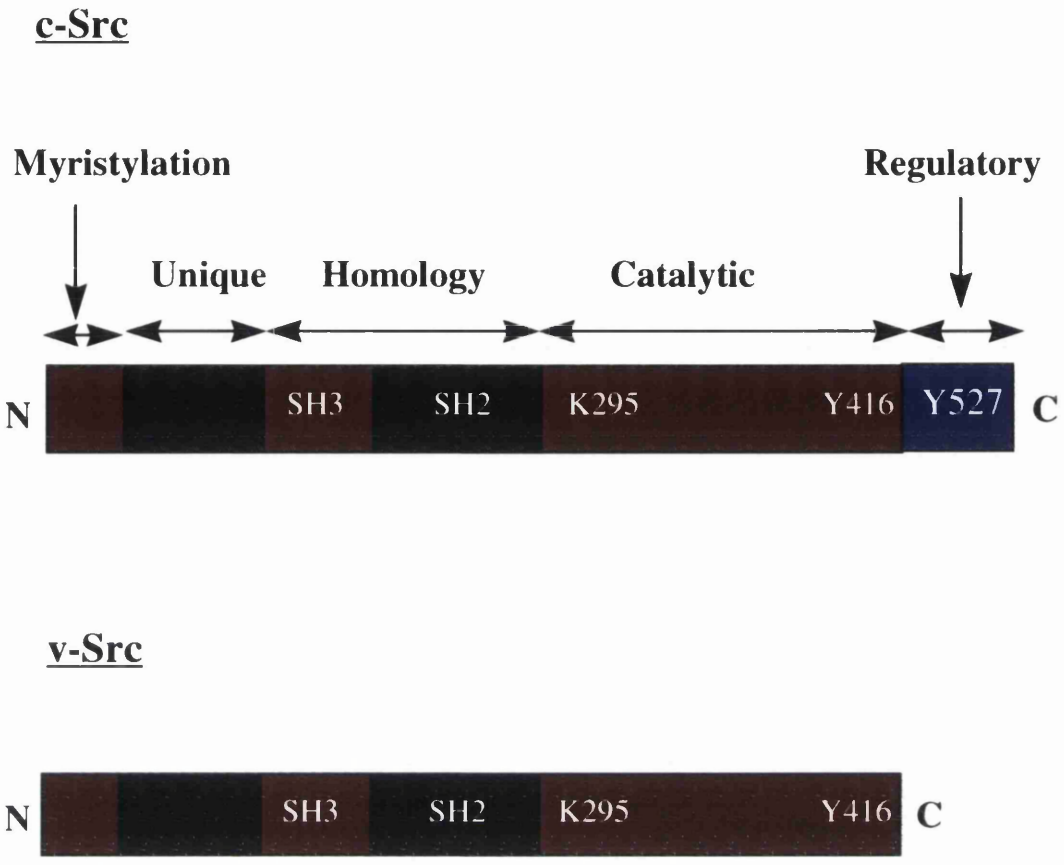


Figure 2 Structures of c-Src and v-Src: Domain structures of c-Src and v-Src are shown. Myristylation domain is necessary for membrane attachment of the protein. Myristylation group is added to the Gly residue at poisition 2. The SH2 and SH3 domains are required for protein-protein interactions and also regulate c-Src activity. Mutation of Lys295 results in a catalytically inactive protein. Tyr416 is a major autophosphorylation site in Src and its phoshorylation is required for tyrosine kinase function. Apart from a number of other point mutations, v-Src differs from c-Src in lacking the regulatory domain, the lack of which renders it constitutively active.

bind peptides with the consensus sequence RPLPXXP (Alexandropoulos *et al.*, 1995).

The SH2 domain of v-Src was originally identified as a 100 amino acid noncatalytic domain (Sadowski *et al.*, 1986). Crystal structure analysis of v-Src SH2 domain complexed with a high affinity phosphopeptide has revealed two binding pockets (Waksman *et al.*, 1992 and 1993). One of the two binding pockets interacts with phosphotyrosine and contains a highly conserved FLVRES sequence spanning from amino acid residues 172 to 177.

The catalytic or the kinase domain of Src family protein tyrosine kinases lies towards the carboxy-terminus. The domain encompasses amino acid residues 250 to roughly 516. It is also known as the SH1 domain and is the most highly conserved region among Src family members. This domain shows a high degree of homology to the catalytic domains of other protein tyrosine and serine/threonine kinases. These strictly conserved residues are thought to be important for the binding of ATP and the phosphotransfer reaction (Hunter and Cooper, 1985). An invariant lysine residue at position 295 in Src is believed to be involved in proton exchange during the phosphotransfer reaction (Kamps and Sefton, 1986). Substitution of this residue with methionone, glutamic acid, arginine, or histidine results in a kinase dead form of the protein (Kamps and Sefton, 1986).

A major site of auto-phosphorylation lies within the kinase domain in a region called the activation loop at position 416 (Smart *et al.*, 1981). Autophosphorylation appears to occur mainly by an intermolecular mechanism (Cooper and MacAuley, 1988). Mutation of the autophosphorylation site leads to decreased kinase activity (Schwartzberg, 1998). It has also been speculated that this site might play an important role in the regulation of the interaction of Src with its substrates (Superti-Furga and Courtneidge, 1995).

At the extreme carboxy terminus of the c-Src molecule lies a regulatory domain. This domain corresponds to residues 517 and 531 in c-Src. Phosphorylation of tyrosine

residue 527 is critical for the regulation of Src family protein tyrosine kinase catalytic activity (Cooper *et al.*, 1986).

1.2.2 Regulation of c-Src activity

Evidence for the involvement of phosphorylation in regulation of c-Src came from the study which showed that c-Src activity is reduced in the presence of phosphotyrosine protein phosphatase inhibitor, sodium vanadate (Courtneidge, 1985). A later study suggested that the difference in the transforming ability and kinase activity of c-Src and v-Src was due to the presence of Tyr527 in c-Src, whose phosphorylation was responsible for the negative regulation of the molecule (Cooper *et al.*, 1986). Substitution of this residue with phenylalanine results in activation of the c-Src kinase domain (Kmiecik and Shalloway, 1987). Phosphorylation of Tyr527 is carried out by another tyrosine kinase, Csk (cellular Src kinase) (Nada *et al.*, 1991; Okada *et al.*, 1991). Csk-deficient mice, generated by gene targeting in embryonic stem cells were found to exhibit a complex phenotype including neural tube defects and these mice die during gestation (Imamoto and Soriano, 1993). Cells derived from these embryos showing increase in Src kinase activity and a reduction, but not elimination of Tyr527 phosphorylation. This suggests that other protein kinases can substitute for Csk or that, as it does to a limited extent *in vitro* (Cooper and King, 1986), autophosphorylation of this residue takes place *in vivo*.

The SH2 and SH3 domains are also important in regulating c-Src activity, as mutations of either of these domains can lead to c-Src activation (Parsons and Weber, 1989). These observations lead to the proposal of model in which phosphorylated Tyr-527 interacts intramolecularly with Src's own SH2 domain, leading to the distortion or closing of the kinase domain and thus inactivation of the protein. According to this model, dephosphorylation of the regulatory phosphotyrosine residue releases the SH2 domain and makes c-Src adopt an 'open', catalytically active conformation. The role of the SH3 domain was though to be aiding in the binding of the SH2 domain to Tyr527 by binding to some proline rich sequence (Courtneidge *et al.*, 1993b). Recent crystallographic studies of structure of c-Src and other members of the Src family, such as Hck (haematopoietic cell kinase) and Lck,

provide a more detailed understanding of these interactions (Yamaguchi and Hendrickson, 1996; Sicheri *et al.*, 1997; Xu *et al.*, 1997). It is now clear that the kinase domain remains exposed during interaction between the SH2 domain and Tyr527. However, the SH3 domain was shown to interact with the linker connecting the SH2 domain to the catalytic domain (Sicheri *et al.*, 1997; Xu *et al.*, 1997), as shown in Figure 3. The role of the SH2 domain appears to be to position the SH3 binding site, and it is the SH3 interaction that provides a direct connection to the regulation of kinase activity (Sicheri *et al.*, 1997). It was also shown that elimination of the SH3 domain-linker interaction, caused a higher increase in Hck activity than did displacement of SH2 domain interaction with phosphorylated Tyr527 (Moarefi *et al.*, 1997). Inactivation of the kinase may therefore result from torsional constraint where the dual binding of the SH2 and SH3 domains prevents free movement within the kinase domain (Schwartzberg, 1998).

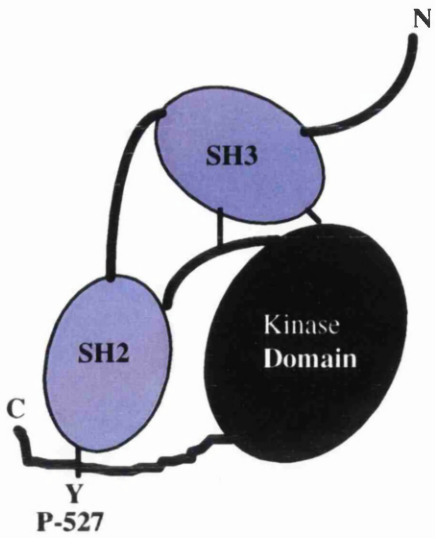
In addition to Tyr527, Tyr416, the presumed autophosphorylation site of Src, may also play a role in regulation of Src activity. Autophosphorylation of tyr416 has been shown to be required for the efficient transformation by host range mutants of v-Src (DeClue and Martin, 1989; Woods and Verderame, 1994). Though, c-Src is only phosphorylated at Tyr416 *in vitro*, and not *in vivo* (Smart *et al.*, 1981; Patschinsky *et al.*, 1982), mutation of Try416 to phenylalanine in c-Src was able to eliminate its partial transforming activity, suggesting that transient or otherwise restricted phosphorylation of Tyr416 is important for c-Src function (Kmiecik and Shalloway, 1987). Also, the mutation did not significantly alter the kinase activity of c-Src.

1.2.3 From c-Src to v-Src

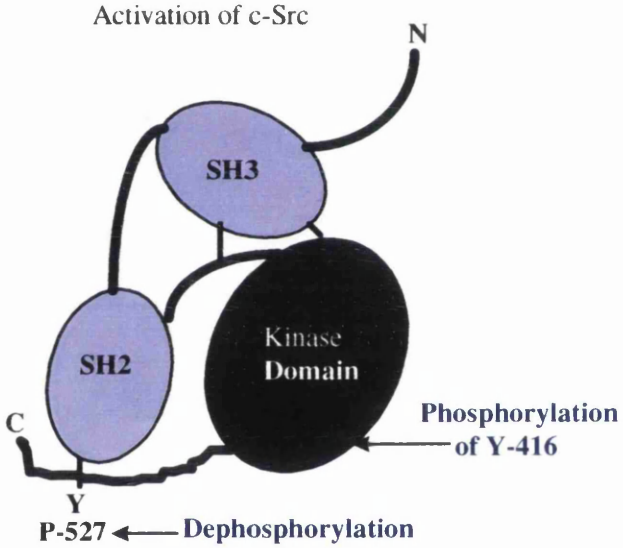
The *v-src* oncoprotein was created by transduction of *c-src* by avian leukosis virus (ALV), which became RSV (Figure 2). (Swanstrom *et al.*, 1983). The transduction event was accompanied by 19 C-terminus amino acids of c-Src being replaced by 12 novel amino acids in v-Src (Takeya and Hanafusa, 1983). This included tyrosine residue 527, which, as mentioned above, is critical for the regulation of the protein. The substitution generated a constitutively activated form of the Src protein. In addition, *v-src* differs from its normal cellular counterpart by a number of point

Figure 3 Regulation of c-Src activity: (a) (adapted from Thomas and Brugge, 1997) In its inactive state, c-Src is phosphorylated at Tyr527 which lead to its binding to the SH2 domain. Also, SH3 domain interacts with the linker domain, which lies between the kinase domain and the SH2 domain, and regions of the catalytic domain. These interactions create conformational changes that prevent the interaction of the substrate with the kinase domain, thus regulating the activity of the protein. (b) Activation of c-Src requires dephosphorylation of Tyr527 and phosphorylation of Tyr416, (c) which results in an 'open' conformation, allowing the kinase activity of the protein.

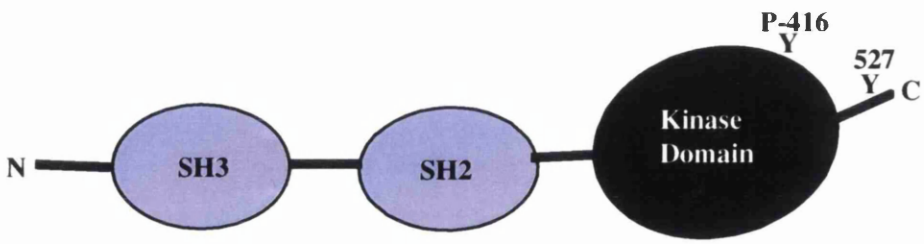
a Inactive c-Src



b Activation of c-Src



c Active c-Src



mutations which it accumulated after the transduction event (Reviewed in Jove and Hanafusa, 1987).

Expression of v-Src induces transformation of cells. v-Src localises at the plasma membrane (Levinson *et al.*, 1981). Transformation is accompanied by three major phenotypic features (reviewed in Jove and Hanafusa, 1987). These include, a change in cell morphology to a round, more refractile type, proliferation of cells in low serum conditions and anchorage independence. The actin cytoskeleton undergoes gross intracellular alteration upon cell transformation (David-Pfeuty and Singer, 1980) and there is a dramatic reduction in the production of fibronectin matrix (Olden and Yamada, 1977). The transforming ability of v-Src, in part, depends on its mitogenic action. Cell transformation by v-Src is accompanied by the constitutive activation of mitogenic signaling from pathways such as the MAP kinase pathway, which is permanently active in fibroblasts transformed by v-Src (Gupta *et al.*, 1992). This is thought to lead to the activation of transcription factors like AP-1 (Catling et al., 1993), and thus contribute to cell cycle progression.

Temperature-sensitive (*ts*) mutants of v-Src have proven valuable in many studies investigating the signal transduction pathways modulated by v-Src to confer transformation. Many of these mutants were obtained by random isolation of single foci of transformation produced at the permissive temperature by mutagenised virus (Wyke, 1973; Wyke, 1975; Wyke, 1976). The mutants are generally classed into two groups, C and T. Class T mutants are defective only in transforming properties at the non permissive temperature while class C mutants have a coordinate lesion in both cell transformation and viral replication. All the mutant were found to be transforming at 35^{O}C and selected for inability to transform at 41^{O}C (Wyke and Linial, 1973). One of these mutants, *ts LA29 v-src*, was used in the experiments reported in this thesis and is described in detail in the following section. Generation of a c-Src temperature-sensitive mutant has also been described (Koegl *et al.*, 1993). Mutation of Gly 478 to Asp rendered both wild type c-Src and its activated allele c-Src (Y527F) temperature sensitive. Rat-1 fibroblasts infected with the temperature-sensitive mutant of c-Src (Y527F) displayed a transformed phenotype when grown at 34^{O}C but not at 39^{O}C (Koegl *et al.*, 1993).

1.2.4 *ts LA29*: A v-Src mutant

ts LA29 v-src belongs to the T class of temperature sensitive mutants and thus, as mentioned before, its defective only in transformation ability at the non permissive temperature (Wyke, 1973). The kinase activity of the protein was found to decrease more than five folds on being shifted from the permissive to the non permissive temperature (Stoker *et al.*, 1984).

ts LA 29 v-src differs from the wild type v-Src at three amino acid residues. (Welham and Wyke, 1988). All three of these mutations lie within the kinase domain. The alterations include a change at residue 351 from lys to arg, at 375 from ala to thr and at 507 from pro to ala. A mutation in the 3' terminal 100 nucleotides of the coding region for ts LA29 v-Src had been shown to confer temperature sensitivity (Fincham *et al.*, 1982). Studies with chimeric proteins showed that only one of the mutations at residue 507 accounts for all the temperature sensitive characteristics (Welham and Wyke, 1988). However, cells infected with the chimeric virus encoding only the alanine substitution at 507 were found to have a fusiform morphology suggesting that this mutation also has other effects on the function of v-Src and that other mutations somehow compensate for these effects (Welham and Wyke, 1988). A mutation at residue 427 was found to restore the wild type phenotype. The mechanism by which temperature sensitivity is conferred is not known. It can be speculated that upon shift to non permissive temperature, a conformational change (perhaps analogous to that found in c-Src) mediated by residue 507, restricts the kinase activity of the protein.

The transformed phenotypes of ts LA29 v-Src is similar to that wild type v-Src (Wyke and Linial, 1973; Stoker *et al.*, 1984). At the permissive temperature, 50% of ts LA29 v-Src protein was found in the membrane in contrast to 80% of the wild type protein but was sufficient for cell transformation (Stoker *et al.*, 1986). Shift to non permissive temperature did not change the ability of the mutant protein to incorporate myristic acid but only 15% of the protein localises to the membrane (Stoker *et al.*, 1986). Also, like the wild type protein, transformation by ts LA29 v-Src mutant also reduces production of fibronectin matrix and causes redistribution of actin and α-actinin.

Studies using ts LA29 v-Src mutant have revealed the importance of mitogenesis in v-Src transformation of cells (Wyke *et al.*, 1993; Johnson *et al.*, 1998). Experiments with ts v-Src mutant have shown that v-Src activity is required at two points in the cell cycle- at the G0/G1 transition and at the G1/S transition (Wyke *et al.*, 1993). Activity of AP-1 transcription factor is required for the mitogenic function of tsLA29 v-Src (Frame *et al.*, 1994; Wyke *et al.*, 1995). The transformed Rat-1 cells traverse the G1 phase of the cell cycle more rapidly and fail to exit cycle efficiently in response to serum starvation and cell confluence (Johnson *et al.*, 1998).

1.2.5 Role of c-Src in Growth factor signalling

The protein tyrosine kinase activity of c-Src has been found to be important in signaling from certain tyrosine kinase growth factor receptors, like platelet derived growth factor (PDGF) and colony stimulating factor-1 (CSF-1) (Gould and Hunter, 1988; Courtneidge *et al.*, 1993a). On binding to their respective ligands, the receptors activate their own intrinsic tyrosine kinase activity. This leads to the autophosphorylation of tyrosine residues within the molecule. One of these phosphorylated tyrosines then binds to the SH2 domain of Src and activate it transiently (Ralston and Bishop, 1985; Kypta *et al.*, 1990). Binding and activation of c-Src by the PDGF and the CSF-1 receptors is important for their function. Also, microinjection of kinase-defective c-Src or injection of antibodies specific for c-Src were able to inhibit PDGF induced entry into S-phase of NIH 3T3 cells after serum-starvation (Twamley-Stein *et al.*, 1993). There is also evidence that that c-Src may be involved in epidermal growth factor (EGF) receptor signaling. Overexpression of c-Src in murine fibroblasts results in enhanced incorporation of [^{3}H] thymidine into DNA in response to EGF stimulation (Luttrell *et al.*, 1988). c-Src associates with phosphorylated EGF receptor via its SH2 domain (Luttrell *et al.*, 1994). However, a recent study has reported that c-Src and other members of the Src family kinases are dispensable for PDGF induced signalling (Klinghoffer *et al.*, 1999). Cells lacking functional Src, Yes and Fyn were found to be fully competent to respond to PDGF in assays for substrate phoaphorylation, *myc* induction, entry into S phase, cell proliferation and chemotaxis (Klinghoffer *et al.*, 1999).

1.2.6 Signal-Transduction pathways downstream of Src in mediation of growth factor signalling

A study of augmented mitogenic response to EGF in c-Src overexpressing murine fibroblasts suggested that increased level of tyrosine phosphorylation of GAP-associated proteins, p62 and p190, resulting in higher levels of active Ras, lie downstream of c-Src (Chang *et al.*, 1993). Other studies have also shown requirement of Ras activity for v-Src transformation (Smith *et al.*, 1986). The connection between Src and Ras is an SH2 containing protein called Shc, which is a Src substrate (McGlade *et al.*, 1992). Phosphorylation of Shc on tyrosine promotes formation of a complex of Shc, Grb2 (a SH2 and SH3 domain containing adaptor protein) and Sos (a Ras specific nucleotide exchange protein), which activates Ras by converting it to its GTP-bound form (Egan *et al.*, 1993; reviewed in Courtneidge, 1994).

c-Myc, another transcription factor activated and growth factor signalling (Armelin *et al.*, 1984) and is important in cell proliferation (Kelly *et al.*, 1983; Biro *et al.*, 1993). c-Myc has been implicated in c-Src-mediated growth factor signalling (Barone and Courtneidge, 1995). The block in PDGF signalling elicited by dominant negative Src in NIH 3T3 cells were rescued by injection of c-Myc into the nucleus (Barone and Courtneidge, 1995).

Several studies have confirmed the critical role played by its kinase domain in transducing mitogenic signals (Wyke *et al.*, 1993; Johnson *et al.*, 1998). Overexpression of c-Src has also been reported to play a role in regulating mitosis in fibroblasts (Chackalaparampil and Shalloway, 1988). The stimulation of the kinase activity of c-Src during mitosis was accompanied by phosphorylation of serine and threonine residues on c-Src by Cdc2 kinase (Shenoy *et al.*, 1989; Morgan *et al.*, 1989). A 68 kd protein, also known as SAM68 (Src associated during mitosis), has also been identified as a protein which is phosphorylated and bound by c-Src activated during mitosis (Fumagalli *et al.*, 1994; Taylor and Shalloway, 1994). A study suggesting a role for c-Src in G2/M transition during the cell cycle have

reported that SAM68 is unlikely to participate at this stage but may be an important effector later in mitosis (Roche *et al.*, 1995).

1.2.7 Role of c-Src in integrin-mediated signalling

v-Src localisation to the focal adhesions first suggested the role of v-Src in integrin signalling (Shriver and Rohrschneider, 1981; Stoker *et al.*, 1986). Also, translocation of c-Src to the cytoskeleton during platelet activation is dependent on the integrins, again suggesting functional connection between integrins and c-Src (Horvath *et al.*, 1992; Clark and Brugge, 1993). Transformation of cells by v-Src results in tyrosine phosphorylation of several components of the cortical cytoskeleton and focal adhesions (Hirst *et al.*, 1986; reviewed in Thomas and Brugge, 1997). Fibronectin stimulated activation of ERK was reduced in c-Src null fibroblasts (Schlaepfer *et al.*, 1997). However, a truncated Src molecule lacking the kinase domain has been demonstrated to restore partial activation of ERK, thus suggesting that at least in this case, the role played by Src seems to be more of an adaptor protein rather than that of a kinase (Schlaepfer *et al.*, 1997).

c-Src has been implicated in regulation of cell adhesion (Kaplan *et al.*, 1995). Fibroblasts derived from *src*-/- mice were found to be defective in initial cell spreading (Kaplan *et al.*, 1995). Defects in phosphorylation of CAS and a decrease in MAP kinase activation in response to plating on fibronectin were linked to this defect, indicating that Src is critical in pathways downstream of integrin crosslinking (Kaplan *et al.*, 1995). However, in the case of regulation of cell adhesion, the kinase domain of Src does not seem to be important. In fact, defects in integrin signaling in *src*-/- cells could be complemented, in part, by kinase inactive molecules, generated either through point mutations or the truncation of the entire kinase domain (Kaplan *et al.*, 1995).

1.2.8 Role of c-Src in integrin-mediated cell migration

Although there have been reports that c-Src is not involved in cell motility (Kundra *et al.*, 1994; Rodier *et al.*, 1995), most studies carried out using both v-Src and c-Src

function have shown its importance in integrin mediated cell migration (Hall *et al.*, 1996; Fincham and Frame, 1998; Klinghoffer *et al.*, 1999). c-Src is implicated in hyaluron receptor RHAMM-induced motility of mouse cells, an effect requiring Src catalytic activity (Hall *et al.*, 1996). Cells derived from mice null for c-Src, Yes and Fyn were found to have reduced motility *in vitro* (Klinghoffer *et al.*, 1999). Fibronectin-induced tyrosine phosphorylation of focal adhesion proteins, including FAK, was nearly eliminated in the absence of Src, Fyn and Yes (Klinghoffer *et al.*, 1999). Both the kinase domain and the myristylation domain are required for the mediation of this function. Binding of Src to FAK through the SH2 and SH3 domains (Schaller *et al.*, 1994; Thomas *et al.*, 1998), followed by phosphorylation and degradation of FAK by v-Src is though to be important during cell migration (Fincham and Frame, 1998).

1.2.9 Role of Src in integrin-mediated cell survival

v-Src has also been implicated in integrin mediated survival signaling. Frisch and Francis, 1994, showed that v-Src transformation was able to protect MDCK cells from detachment induced apoptosis. Other studies have also linked v-Src to survival signaling. Transformation by activated c-Src rendered Myc/Ras or E1A/Ras transformed REF cells selectively resistant to adhesion-dependent apoptosis (McGill *et al.*, 1997). It has been recently shown that inhibtion of c-Src kinases expression activity results in the induction of cell death in DHER14 cells (Karni *et al.*, 1999). STAT3, a protein known to play a role in protection from apoptosis, has been shown to act downstream of v-Src and is required for cellular transformation by it (Bromberg, 1998).

1.3 Focal Adhesion Kinase

Focal adhesion kinase or FAK was first identified in 1992 by two groups of workers at the same time (Schaller *et al.*, 1992; Hanks *et al.*, 1992). It was identified as a v-Src substrate required in oncogenic transformation of chick embryo (CE) cells (Schaller *et al.*, 1992). Using a PCR based technique, it was isolated as a cDNA encoding a protein, pp125, which was found to be a major phosphotyrosine containing protein in untransformed cells that exhibited an increase in phosphotyrosine in v-Src transformed CE cells (Schaller *et al.*, 1992). They proposed the name focal adhesion kinase because it was found to localise to the focal adhesions. At the same time, it was identified by a homology based cDNA cloning strategy (Hanks *et al.*, 1992).

1.3.1 Structure of FAK

Immunoflurescence localisation experiments showed that FAK is a component of focal adhesions (Hanks *et al.*, 1992). FAK is a non-receptor tyrosine kinase (Figure 4). The molecular structure of FAK is different from other non-receptor tyrosine kinases like c-Src in that it lacks SH2 and SH3 domains. The kinase domain lies in the middle portion of FAK and comprises about one third of the FAK protein. A region has been identified in the carboxy terminus of FAK, specifically in residues 856- 1012, that is essential for efficient localization of FAK to adhesion sites (Hildebrand *et al.,* 1993). This was determined by the use of FAK variants which contained mutations in this region and failed to localise properly to the focal adhesions. Also, when this sequence was added to other non-FAK related proteins they localised to the focal adhesions. These results were further supported by work which showed that FRNK, an alternatively spliced product transcribed from *fak* gene, contains only the carboxy terminus region of FAK and localises efficiently to the focal adhesions (Schaller *et al.,* 1993). The carboxy-terminal domain is also involved in forming complexes with other focal adhesion proteins such as paxillin and talin (Hildebrand *et al.*, 1995; Chen *et al.*, 1995). It has been suggested that the paxillin-

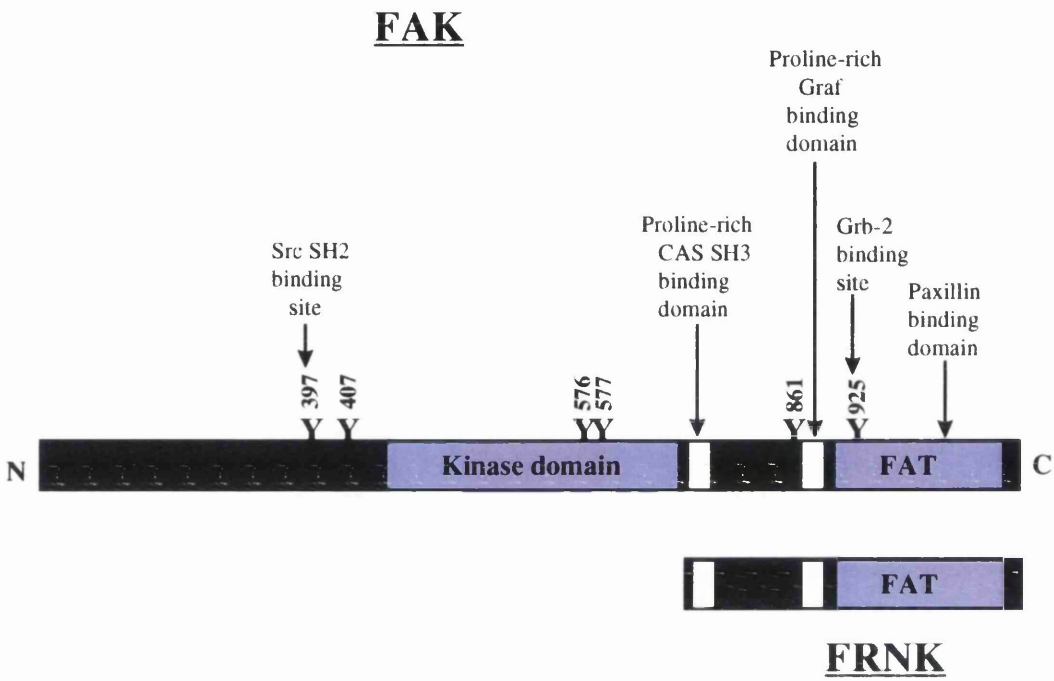


Figure 4 Structure of FAK: Figure shows FAK structural domains and phosphorylation sites (adapted from Hanks and Polte, 1997). Tyr397 is a major autophosphorylation site of FAK. Phosphorylation of Tyr397 fascilitates its binding with Src SH2 domain. Binding of Src to FAK leads to phosphorylation of Tyr925, which is a binding site for Grb-2. FAK is also known to bind to CAS and Graf through the proline rich regions. The kinase activity of FAK is elevated by phosphorylation of Tyr576 and Tyr577, which lies within the kinase domain. The focal adhesion targetting (FAT) domain is required for the localisation of the protein at the focal adhesions. Within the FAT domain also lies the paxillin binding site. FRNK, an alternatively spliced form of FAK, is homologous to the carboxy terminus region of FAK and is also targetted to the focal adhesions.

binding site appears to be distinct from the focal adhesion targeting sequence in FAK and is therefore unlikely to be responsible for recruiting FAK to the focal adhesion (Otey, 1996). Another study, however, reports that glutathione S- tranferase (GST)-FAK fusion proteins, which specifically bind to paxillin, localize to focal adhesions. This correlation suggests that FAK is localized to focal adhesions by the direct association with paxillin (Tachibana *et al.,* 1995). This controversy still remains to be resolved.

The amino-terminal domain of FAK appears to be important for the binding of FAK to integrins (Schaller *et al.*, 1995). FAK was found to precipitate efficiently with a synthetic peptide, that mimics the cytoplasmic domain of β1 integrin (Schaller *et al.*, 1995). Furthermore, when expressed in isolation, the amino terminal domain of FAK co-precipitated with the integrin peptide, whereas the carboxy terminal domain of FAK did not (Schaller *et al.*, 1995). These results suggest that FAK may bind directly, through its amino-terminal domain, to a membrane- proximal site in the cytoplasmic tail of β1 integrin (Schaller *et al.*, 1995), although such an interaction *in vivo* is still uncertain.

1.3.2 FRNK: An alternatively spliced form of FAK

Isolation of a cDNA encoding a carboxy-terminal, non-catalytic domain of FAK has been reported (Schaller *et al.*, 1993). The protein has been called FRNK (for FAK related non kinase). FRNK is identical in amino acid sequence to the carboxy-terminal of FAK and localizes to the focal adhesion (Figure 4). It has been proposed that FRNK is generated by alternative splicing and/or utilization of alternative transcriptional promoters (Schaller *et al.,* 1993). Two isoforms of FRNK, p41 and p43 are observed. It is believed that p41 is phosphorylated on serine and threonine residues to generate the p43 isoform (Schaller *et al.*, 1993). FRNK also becomes phosphorylated in an ECM-dependent manner with the major sites of phosphorylation corresponding to the ones on FAK. Two of the sites of phosphorylation are Ser148 and Ser151 (Richardson *et al.*, 1997). Studies have implicated FRNK in negative regulation of FAK (Schaller *et al.*, 1993; Richardson and Parsons, 1996). This will be discussed in more detail in the following section.

1.3.3 Phosphorylation sites in FAK and their importance in substrate binding

FAK activity is regulated by phosphorylation (Guan and Shalloway, 1992). Immunoprecipitated FAK from fibronectin-adhered cells, with high phosphotyrosine content, is 2.5 times catalytically more active as compared to FAK from cells plated out on polylysine, which has lower phosphotyrosine content (Guan and Shalloway, 1992). Six tyrosine residues, 397, 407, 576, 577, 925 and 861 have been identified which are phosphorylated (Schaller *et al.*,1994; Schlaepfer *et al.*, 1994; Calalb *et al.*, 1995; Schlaepfer and Hunter, 1996; Calalb *et al.*, 1996). Of these, Tyr- 397 is thought to be a major site of FAK autophosphorylation, both *in vivo* and *in vitro* (Schaller *et al.*, 1994) and is a strong binding site for the Src SH2 domain (Schaller *et al.*, 1994). Both v-Src and c-Src have been shown to bind to FAK through the SH2 domain (Cobb *et al.*, 1994; Schaller *et al.*, 1994; Xing *et al.*, 1994). Mutation of this site does not abolish the kinase activity of FAK *in vitro*, but dramatically reduces the tyrosine phosphorylation of FAK cytoskeletal substrate paxillin *in vivo* (Otey, 1996). Taken together, these facts raise the possibility that complex formation between c-Src and FAK is essential for the phosphorylation and activity of FAK.

Phosphorylation of Tyr 397 is also required for binding of FAK to PI 3-kinase (Chen and Guan, 1994; Chen *et al.*, 1996a). Three other phosphorylation sites, Tyr-407, Tyr-576, and Tyr-577 are phosphorylated by c-Src *in vitro* and to have elevated phosphorylation levels in v-Src transformed cells (Calalb *et al.*, 1995). Tyr-576 and Tyr-577 lie within the catalytic domain and their mutation significantly lowers the kinase activity of FAK immune complexes (Calalb *et al.*, 1995). Phosphorylation of Tyr-925 by Src facilitates binding of Grb2 to FAK thorough the SH2 domain (Schlaepfer *et al.*,1994). In the case of c-Src, this phosphorylation is dependent on adhesion of cells to fibronectin while in v-Src transformed cells, phosphorylation is independent of cell adhesion to fibronectin (Schlaepfer *et al.*,1994). Mutation of FAK Tyr-925 disrupts Grb2 binding whilst mutation of Tyr-397 disrupts both c-Src and Grb2 binding to FAK *in vivo* (Schlaepfer and Hunter, 1996). In view of the fact that FAK is also recognised as a possible substrate for c-Src (discussed earlier), a model was proposed whereby Src kinase binding and phosphorylation of FAK at Tyr-925 creates a Grb2-SH2 domain binding site and provides a link to the activation of

the Ras signal transduction pathway (Schlaepfer and Hunter, 1996). Tyr-861 is thought to be another major site of phosphorylation by c-Src which may function in additional interactions between FAK and SH2-containing proteins (Calalb *et al,* 1996).

FAK is also phosphorylated *in vivo* on a number of serine residues (Calalb *et al.*, 1995; Schlaepfer and Hunter, 1996), but the functional significance of these is yet to be determined (Hanks and Polte, 1997).

1.3.4 Regulation of FAK phosphorylation and activation

Integrins have been implicated in the activation of FAK by stimulating its phosphorylation. In 1991, Guan *et al.,* reported that the phosphotyrosine levels in FAK increased when integrins were bound to their ECM ligands. Since then, a number of groups have confirmed that when cells are grown in suspension, or on substrates such as polylysine, phosphotyrosine content of FAK remains low, but if cells are allowed to spread on fibronectin or other matrix proteins, the phosphotyrosine content of FAK increases dramatically (Burridge *et al.*, 1992; Guan and Shalloway, 1992; Hanks *et al.,* 1992; Kornberg *et al.*, 1992). Other studies have shown that integrin clustering, and not merely their occupation, is involved in integrin-dependent phosphorylation of FAK (Guan *et al.,* 1991; Miyamoto *et al.,* 1995). Expression of constitutively acivated form of integrin receptors, in which the putative binding site for FAK is altered, does not lead to FAK phosphorylation unless occupied by their ligand (Lyman *et al.*, 1997). Not only the clustering but also the conformational state of the integrin might be important in FAK activation (Pelletier *et al.,* 1995). Activation of FAK by integrins is independent of focal adhesion and stress fibre assembly or the association of integrins with them (Lyman *et al.*, 1997).

Platelet-activating factor (PAF) induces early phosphorylation in tyrosine residues of focal adhesion kinase and paxillin in endothelial cells (Soldi *et al.,* 1996). FAK is also activated by the binding of PDGF to its tyrosine-kinase receptor (Rankin and Rozengurt, 1994; Knight *et al.*, 1995; Abedi *et al.*, 1995). Vascular endothelial growth factor (VEGF) caused a rapid increase in tyrosine phosphorylation of FAK in

human umbilical vein endothelial cells (Abedi and Zachary, 1997). VEGF phosphorylation of FAK was completely inhibited by the actin filament-disrupting agent cytochalasin D, showing that it is cytoskeltal dependent (Abedi and Zachary, 1997). Insulin and IGF-1 have also been shown to induce FAK phosphorylation in non-attached cells (Knight *et al.*, 1995; Baron *et al.*, 1998). Thrombin and collagen activation of platelets caused an induction of FAK tyrosine phosphorylation (Lipfert *et al.*, 1992). However, FAK was not phosphorylated in platelets deficient in the fibronectin receptor and in platelets that were not allowed to aggregate. Also, in another study, enhanced tyrosine phosphorylation of FAK by clustering of tetraspanins (proteins from the transmembrane 4 superfamily TM4SF which include CD9, CD63 etc.) required cell attachment (Berditchevski and Odintsova, 1999). Other mitogenic peptides and growth factors such as lysoposphatidic acid (Seufferlein and Rozengurt, 1994), bombesin (Sinnet-Smith *et al.*, 1993, Zachary *et al.*, 1993), vasopressin (Zachary *et al.*, 1993), endothelin (Zachary *et al.*, 1993), bradykinin (Leeb-Lundberg *et al.*, 1994), and angiotensin ll (Polte *et al.*, 1994) have also been shown to mediate FAK and paxillin phosphorylation (reviewed in Otey, 1996). In some cases, like that of bombesin, stimulation of phosphorylation requires an intact cytoskeleton (Sinnett-Smith *et al.*, 1993).

Apart from Src itself, another member of the Src family of protein tyrosine kinases, Fyn has also been shown to bind directly to FAK and phosphorylate it (Grant, 1996). Furthermore, Fyn is responsible for the engagement of CD146 (a transmembrane receptor belonging to a group of cell adhesion molecules) in human endothelial cells resulting in tyrosine phosphorylation of FAK (Anfosso *et al.*, 1998).

FAK phosphorylation was found to accompany increase in Ca++ following integrin binding to ECM or mechanical deformation by cyclic stretch/ relaxation (Sinnett-Smith, 1993). The small GTP-binding protein Rho p21 is thought to play a role in cyclic strain mediated phosphorylation of FAK since preincubating the cells with C3, a specific inhibitor for rho p21, inhibited tyrosine phosphorylation of FAK (Yano *et al.*, 1996b).

Other studies have focused on the regulation of FAK dephosphorylation. It has been reported that overexpression of Csk, enhanced and prolonged the insulin-induced de-phosphorylation of FAK (Tobe *et al.*, 1996). Recent studies have also shown that PTEN, a known tumor suppressor and a phosphatase, interacts with FAK and reduces its tyrosine phosphorylation (Tamura *et al.*, 1998). A decrease in the tyrosine-phosphorylation level of FAK by overexpression of FRNK has also been reported. It was first suggested by Schaller *et al.*, (1993) that FRNK could function to regulate the activity of FAK in cells. A later study then confirmed that FRNK acts as an inhibitor of FAK by transiently blocking the formation of focal adhesions on fibronectin (Richardson and Parsons, 1996). The inhibitory effects of FRNK were found to be reversed by co-expression of FAK suggesting that FAK and FRNK compete for a common binding protein whose association with FAK is important for signaling by FAK (Richardson and Parsons, 1996). FRNK therefore might compete with FAK for localization at the focal adhesions to induce its inhibitory effect. A recent study showed that FAK dephosphorylation at Tyr-397 and blocking of FAK-mediated cell-migration by expression of FRNK is dependent on its localisation to the focal adhesions as the inhibitory effects of FRNK could be reversed by a point mutation (Leu-1034 to Ser) which prevented its localisation to the focal adhesions (Sieg *et al.*, 1999).

1.3.5 Potential substrates of FAK

Tensin is one of the potential substrates of FAK. Tensin is a 215 Kda protein that binds to actin filaments and is concentrated both in cell- matrix and cell- cell adhesions (Bockholt *et al.*, 1992; Lo *et al.*, 1994). Tensin is not known to bind to FAK directly but it binds to vinculin and α-avtinin, both of which are focal adhesion proteins. The evidence supporting the phosphorylation of tensin by FAK came when it was shown that the conditions which promote tensin phosphorylation, *i.e.* an intact actin cytoskeleton, clustering of integrins and cell spreading, are the same as that required to promote FAK activation (Bockholt and Burridge, 1993). However, this does not provide conclusive proof. On the other hand, much more data is available in support of FAK mediated activation of paxillin. As mentioned earlier, FAK and paxillin have been shown to be directly associated through the carboxy-terminal

domain of FAK (Tachibana *et al.,* 1995). Furthermore, overexpression of wild type FAK in fibroblasts treated with vandate (to inhibit tyrosine phoshatases), lead to a significant increase in the level of tyrosine phosphorylation of paxillin (Schaller and Parsons, 1995). It was also found that FAK mutants which failed to localize to focal adhesions, also failed to induce paxillin phosphorylation (Schaller and Parsons, 1995). In addition, mutation of Tyr-397, the major autophosphorylation site of FAK, dramatically reduces the tyrosine phosphorylation of paxillin and tensin *in vivo* (Coorey *et al.,* 1996).

In another approach, expression cloning was used recently to identify cDNAs that encode potential FAK binding proteins (Hildebrand *et al.,* 1996). The study found Graf (GTPase regulator associated with FAK), a member of the GAP family of GTPase regulators, to bind to the C-terminal domain of FAK in a SH3-dependent manner. Graf preferentially stimulates the GTPase activity of RhoA and cdc42 (Hildebrand *et al.,* 1996). This finding provides another possible candidate through which FAK might mediate integrin signaling. However, *in vivo* interaction between FAK and Graf has not yet been definitively established (Hildebrand *et al.,* 1996).

Another study has shown the interaction between FAK and CAS (Polte and Hanks, 1995). CAS was first identified as a FAK-interacting protein by two-hybrid screens; subsequently, co-immunoprecipitation experiments confirmed that CAS and FAK are associated in mouse fibroblasts (Harte *et al.,* 1996). Association of FAK and CAS is mediated by the binding of the CAS SH3 domain to a proline rich sequence at the carboxy-terminus of FAK (Polte and Hanks, 1995; Harte *et al.,* 1996). A region of FAK spanning the amino acids 712-718 has been mapped as the binding site (Cary *et al.,* 1998). CAS has been shown to localize to focal adhesions and also bind to FRNK (Harte *et al.,* 1996), and is implicated in FAK-mediated cell motility (discussed below).

1.3.6 Functions of FAK

As stated earlier, the phosphorylation and activation of FAK induced by integrins implies that FAK might play an important role in integrin mediated cell-signaling which is responsible for regulating cell proliferation, migration and survival.

1.3.7 Role of FAK in cell motility

In normal cells, migration plays a critical role in many biological processes such as embryonic development, wound healing and tumour metastasis. The most compelling evidence for involvement of FAK in cell motility comes from a study in which cells derived from FAK deficient mouse embryos showed reduced mobility *in vitro* (Ilic *et al.*, 1995). A recent study showed that re-expression of epitope tagged FAK is able to reverse the morphlogical defects of the FAK-null cells and the cells display indistinguishable fibronectin receptor-stimulated migration properties when compared to normal fbroblasts (Sieg *et al.*, 1999). In another study, overexpression of FAK in CHO cells increased their migration on fibronectin (Cary *et al.*, 1996). Loading of glutathione-s-transferase fusion protein (GST-Cterm) containing the FAK focal adhesion targeting domain into cells decreased phosphotyrosine content of focal adhesions and reduced cell motility (Gilmore and Romer, 1996). However, no decrease in the number of focal adhesions was observed implying that FAK activity is not required for the formation of focal adhesions (Gilmore and Romer, 1996).

Evidence of FAK involvement in cell migration is also provided by studies using malignant tumour cells. FAK expression correlates significantly with mean migration rate in the six melanoma lines tested (Akasaka *et al.*, 1995). A study carried out to look at FAK mRNA in 49 human tissue samples, including paired normal and neoplastic samples, found increased levels of FAK in 1 out of 8 adenomatous tissues, 17 out of 20 invasive tumours and in all 15 metastatic tumours (Weiner *et al.*, 1993). Owens *et al.*, (1995) have also reported the correlation of increased levels of FAK with the invasive potential of tumour cells. They found FAK levels significantly elevated in 17 out of 17 invasive and metastatic colonic lesions and in 22 out of 25 invasive and metastatic breast tumours compared with normal tissue from the same

patient. Furthermore, a study by Tremblay *et al.,* (1996) reported that higher levels of FAK protein and mRNA were observed in Pca, prostate carcinoma cancer tissues from patients with metastasis, whereas, normal, hyperplastic prostates and localised Pca tissues showed undetectable or low levels of both. Additionally, they found that FAK associated preferentially with paxillin and Csk in metastatic tissues compared to the non-metastatic tissues (Tremblay *et al.*, 1996).

Some work has also been carried out to determine the molecular signaling pathways involved downstream of FAK which play a role in cell migration. It was shown that overexpression of kinase-defective FAK, phosphorylated at Tyr397 by endogenous FAK, was able to increase migration in CHO cells to the same extent as the overexpression of the wild type FAK (Cary *et al.*, 1996), suggesting that FAK catalytic activity was not required for its mediation in cell motility. However, another study using FAK mutants has shown that FAK kinase activity is required to promote cell migration in FAK-null fibroblasts (Sieg *et al.*, 1999). Phosphorylation of Tyr-397 is important in FAK-mediated cell migration (Cary *et al.*, 1996; Sieg *et al.,* 1999). It was also shown that mutation of Tyr-397 abolished its ability to stimulate cell migration, whereas its phosphorylation in the kinase defective variant of FAK by endogenous FAK led to increased migration (Cary *et al.*, 1996). A recent study involving the expression of FAK mutants with impaired activation loop phosphorylation, resulting in lack of catalytic activity of the protein in FAK null cells, suggests that FAK activation loop phosphorylation plays an important role in mediating activation of FAK/Src complexes by stimulating intermolecular FAK autophosphorylation at Tyr-397 (Owen *et al.*, 1999). Also, the SH3 domain binding proline rich region in the carboxy-terminus of FAK, which has also been implicated in Src binding to FAK (Thomas *et al.,* 1998), is also required for FAK mediated migration (Sieg *et al.*, 1999).

CAS seems to play an important role in mediating cell motility as a result of association with FAK. Cells expressing a FAK mutant, which significantly reduced CAS association with FAK, were unable to enhance cell migration (Cary *et al.*, 1998). Association of FAK with Grb2, on the other hand, is not required in FAK-promoted cell migration (Cary *et al.,* 1998). MAP kinases were found to play no

significant role in migration as no increase in activation of ERKs in the cells showing increased cell motility due to the expression of wild type FAK, was observed (Cary *et al.*, 1998). Also, MEK inhibitor, PD98059, did not decrease FAK promoted cell migration. Association of FAK with PI 3-kinase is also required for its role in migration (Reiske *et al.*, 1999).

1.3.8 Role of FAK in cell proliferation

The first evidence of FAKs role in cell growth came from the study carried out by Gilmore and Romer (1996). Cells loaded with GST-Cterm fusion protein of FAK decreased association of FAK with the focal adhesions and decreased DNA synthesis compared with the control cells (Gilmore and Romer, 1996). Binding of FAK to Grb2 and PI 3-kinase (Schlaepfer and Hunter, 1996; Chen and Guan, 1994), two proteins implicated in the activation of the MAP kinase pathway through Ras (Schlaepfer and Hunter, 1996; King *et al.*, 1997). Combined activity of c-Src and FAK, after fibronectin stimulation, promotes signalling *via* the MAP kinase pathway (Schlaepfer *et al.*, 1998).

A recent study has proposed a role for FAK in the activation of another MAP kinase pathway- JNK (Jun NH2-terminal kinase; Oktay *et al.*, 1999). Integrin mediated stimulation of JNK required the association of FAK with c-Src and CAS, and also phosphorylation of CAS and the subsequent recruitment of Crk, the adapter protein. Activation of the JNK pathway is necessary for proper progression through the G1 phase of the cell cycle and thus regulates cell proliferation (Okay *et al.*, 1999).

1.3.9 Role of FAK in survival

FAK undergoes degradation during c-Myc induced apoptosis in CEF after serum withdrawal, and has thus been implicated in this process (Crouch *et al.,* 1996). Furthermore, integrin signalling has been shown to suppress apoptosis induced by c-Myc transformation (Frisch *et al.,* 1996b) and was found to suppress FAK cleavage (Crouch *et al.*, 1996). Constitutively activated forms of FAK were able to rescue two established epithelial cell lines from anoikis (Frisch *et al.*, 1996b). It was also found

that both the Tyr-397 site and the kinase activity of FAK were required for this effect (Frisch *et al.*, 1996b). This suggested that once initiated, apoptosis requires the inactivation of FAK, possibly through degradation. Another study suggested that calpain mediated FAK cleavage and the subsequent down regulation of its autokinase activity and cytoskeletal localization may be responsible for its signal termination (Cooray *et al.*, 1996). Furthermore, when a FAK peptide, containing the integrin binding sequence of FAK was micro-injected into CEF, these cells underwent apoptosis (Hungerford *et al.*, 1996). The peptide most likely acted as a dominant negative and presumably did not allow FAK's association with the β1 integrin, again suggesting that FAK-integrin association is required for the suppression of apoptosis. Treatment of tumour cell lines known to express high levels of FAK with different antisense oligonucleotides to FAK in order to attenuate FAK expression, caused the cells to undergo apoptosis (Xu *et al.*, 1996). This study provides further evidence of FAK's importance in cell survival. However, this effect of attenuation of FAK expression was found to be specific to overexpressing tumour cell lines. Normal cells did not undergo apoptosis on being treated similarly (Xu *et al.*, 1996).

FAK is also upstream of the PI 3-kinase-Akt survival pathway in hydrogen peroxide induced apoptosis in T98G cells, a glioblastoma cell line (Sonoda *et al.*, 1999). Hydrogen peroxide causes oxidative stress *in vitro*. FAK is phosphorylated and associated with PI 3-kinase after hydrogen peroxide stimulation. Inhibition of PI 3-kinase with wortmannin accelerated apoptosis in the cells suggesting that signal transduction from FAK to PI 3-kinase and Akt exerts an anti-apoptotic effect (Sonoda *et al.*, 1999). In another recent study, expression of exogenous PTEN, a phosphatase for FAK, in glioblastoma and breast cancer cells lacking PTEN caused the cells to undergo apoptosis (Tamura *et al.*, 1999). Overexpression of FAK in these PTEN transfected cells was able to partially rescue PTEN induced apoptosis, again suggesting FAK's role in PI 3-kinase mediated cell survival pathway (Tamura *et al.*, 1999).

1.3.10 PYK2: A FAK homologue

Another protein tyrosine kinase related to FAK, with a molecular weight of 113 kd, has also been identified. Its also referred to as CAKβ, RAFTK, FAK2 or PYK2 (Sasaki *et al.*, 1995; Avraham *et al.*, 1995; Herzog *et al.*, 1995; Lev *et al.*, 1995). PYK2 shows an overall identity of about 42% to the amino acid sequence of FAK (Herzog *et al.*, 1996). PYK2 isolated from the rat brain is about 60% identical to the catalytic domains of mouse and human FAK (Sasaki *et al.*, 1995). Comparison of the amino acid sequences of human and murine PYK2 revealed 95% homology (Avraham *et al.*, 1995). It has all the characteristic of a non-receptor protein tyrosine kinase (Sasaki *et al.*, 1995). PYK2 does not have myristoylation sites, a transmembrane region or SH3, SH2 domains (Avraham *et al.*, 1995). Comparison of the amino acid sequence of FAK and PYK2 also reveals that there exists a 60-70% homology in the FAT region.

However, despite belonging to the same family of protein tyrosine kinases, FAK and PYK2 show different pattern of expression. PYK2 is found to be expressed mainly in brain, intestine, kidney, spleen and epididymis and is only weakly expressed in the cerebellum, testis, and adrenal glands- organs where FAK is expressed in abundance (Sasaki *et al.*, 1995). Confocal analysis showed PYK2 at the cell-cell contacts instead of the focal adhesions (Sasaki *et al.*, 1995). Other studies have reported PYK2 to localize to the perinuclear region (Sieg *et al.*, 1998) and diffusely in the cytoplasm (Zheng *et al.*, 1998). There have also been conflicting reports as to the regulation of its phosphorylation or activation. Studies have suggested that PYK2 is not found to be activated in response to cell interaction with fibronectin (Sasaki *et al.*, 1995). In contrast, PYK2 has been shown to display integrin-dependent phosphorylation in B lymphocytes, CMK cells, and transfected COS cells (Zheng *et al.*, 1998). In cells overexpressing endogenous PYK2, integrin mediated cell adhesion produced only a small increase in PYK2 phosphorylation. A significantly higher increase in PYK2 phosphorylation was observed when cells were stimulated with soluble factors such as angiotensin II, PDGF and sorbitol (Zheng *et al.*, 1998). PYK2 has also been shown to be rapidly phosphorylated on tyrosine residues in response to various stimuli that

elevate intracellular calcium concentration as well as protein kinase C activation (Lev *et al.*, 1995).

Like FAK, PYK2 has been found to interact with paxillin, that is also known to bind to FAK (Zheng *et al.*, 1998). A recent study has shown that PYK2 associates with active Src family members after plating on fibronectin in murine fibroblasts lacking FAK (Sieg *et al.*, 1998). PYK2 tyrosine phosphorylation is also enhanced by fibronectin stimulation in these cells. However, PYK2 was unable to fully restore the migrating capacity of the *fak* -/- cells (Sieg *et al.*, 1998). PYK2 has also been reported to have an opposing effect to FAK during the process of apoptosis (Xiong and Parsons, 1997). Overexpression of PYK2, unlike FAK, was found to induce apoptosis in rat and mouse fibroblasts (Xiong and Parsons, 1997).

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 General Chemicals and Reagents

Chemical/Reagent	Suppliers Name
β Glycerophosphate	Fisher
λ HindIII	GIBCO
ϕXHaeIII markers	GIBCO
10% Block solution	Boehringer Manneheim/Roche
2 mercapto ethanol	Sigma
Acrylamide (30:0.8)	Severn Biotech Limited
Agar	Beckton Dickinson
Agarose	GIBCO
ALLN	Sigma
Ammonium per sulphate (APS)	Fisher
Ampicillin	Sigma
Aprotinin	Sigma
Anti-sheep donkey texas red	Jackson Immuno research
Avidin-FITC	Sigma
BamHI	GIBCO
Benzamidine	Sigma
Biotin labelling nick translation kit	Boehringer Manneheim/ Roche
Biotinylated anti-avian D	Vecta Labs
Biotinylated dUTP	Roche
Boric acid	Fisher
Bromophenol blue	Sigma/BDH
BSA	Sigma
BSA (For TUNEL analysis)	Sigma
Cacodylic acid	Sigma
Calcium chloride ($CaCl_2$)	Fisher
Chick Serum	GIBCO
Chick tryptose phosphate	GIBCO

Chromosome Medium A	GIBCO
Cobalt chloride	Sigma
Colcemid	GIBCO
Cow Gum	BDH
DAPI	Sigma
Dextran sulphate	Sigma/Pharmacia
DH5α competent cells	GIBCO
Diaminethanetetra-acetic acid (EDTA)	Fisher
DIG labelled centromeric probe for chromosome 8	Appligene Oncor
DIG labelled c-Myc FISH probe	Appligene Oncor
Dimethyl Sulfoxide (DMSO)	Fisher
Disodium phosphate ($Na_2PO_4.2H_2O$)	Fisher
Dithiothreitol (DTT)	Sigma
DNA ladder 1kb	GIBCO
Donor Calf Serum	Sigma
DOTAP	Roche
Dulbecco's Medium (10XDMEM)	Sigma
ECL reagents	Amersham
EcoRI	GIBCO
Ethanol	Hayman Limited
Ethidium bromide	Sigma
Ethylene glycol tetraacetic acid (EGTA)	Fisher
Fetal Calf Serum	Seromed
FICOLL (Type 400)	Sigma
FITC-Avidin DCS	Vecta Labs
Formaldehyde	Fisher
Formamide	Sigma
G-418 (Geneticin)	GIBCO
Glacial acetic acid	Fisher
Glycerol	Fisher
Glycine	Fisher

Hepes	BDH
Hexanucleotide mix (10X)	Boehringer Mannheim
HindIII	GIBCO
Human Cot-1 DNA	GIBCO
Hydrochloric acid (HCl S.G.I.16 32%)	Fisher
Hydrocortisone	Sigma
Insulin 200u/ml	Sigma
Isopropanol	Fisher
Kanamycin	Sigma
Klenow polymerase	Kramel
Leupeptin	Sigma
L-Glutamine	GIBCO
LY294002	Calbiochem
Maleic acid	Sigma
Methanol	Fisher
Milk	Marvel
New Born Calf serum	GIBCO
NP-40	Calbiochem
Nucleotides (dNTPs)	Promega
PBS tablets	Oxoid
Penicillin	Sigma
Pepsin	Sigma
Phenol	Rathburn
Phenyl methyl sufonyl fluoride (PMSF)	Sigma
Plasmid Prep kits	Qiagen
Polyvinyl pyrolidine (PVP)	Sigma
Potassium acetate	Fisher
Potassium chloride (KCl)	Fisher
Potassium hydroxide	Sigma
Propidium iodide	Sigma
Protein rainbow molecular weight markers	Amersham
Proteinase K	Roche

Rnase A	Sigma/Kramel
RPMI medium	GIBCO (Cat no. 31870-025)
Salmon Sperm DNA	Sigma
SB 203580	Calbiochem
Sheep anti-digoxigenin	Boehringer Manneheim
Sodium acetate (NaAc)	Fisher
Sodium bicarbonate	Sigma
Sodium chloride (NaCl)	Fisher
Sodium dodecyl sulphate (SDS)	BDH
Sodium fluoride (NaF)	Sigma
Sodium pyrophosphate	Fisher
Sodium pyruvate	Sigma
STF (Post fix)	Alpha labs/MBF
Streptomycin	Sigma
Taq	Bioline
TdT	Roche
TEMED	Sigma
Tris base	GIBCO
Tris-Sodium Acetate	Fisher
Triton X100	Sigma
Trypsin	GIBCO
Tween 20	Sigma
Vecta shield	Vector Labs
ZVAD-FMK	Calbiochem

2.1.2 List of Antibodies

Antibodies	**Suppliers**
FAK (Monoclonal)	Transduction Labs
c-Myc	Provided by David Gillspie, Beatson Institute, Glasgow, UK
ERK	Provided by Anne Wyke, Beatson Institute,

	Glasgow, UK
PYK2	Affinity, Cambridge
Phospho ERK	Biolabs
P38	New England Biolabs
Phospho p38 (Rabbit) (Against Thr180, Tyr182	New England Biolabs
Akt	New England Biolabs
Phospho Akt (Ser473)	New England Biolabs

2.1.3 Solutions

2.1.3.1 General Solutions

Blocking buffer with BSA

3% BSA

0.2% Tween 20

Make up with PBS or 1X TBS

Blocking buffer with milk

5% Milk

0.1% Tween 20

Make up with PBS or 1X TBS

Block Solution 10% (For FISH)

!0% Boehringer Mannheim blocking reagent in maleic acid buffer

Microwave until solution becomes yellow and turbid and then autoclave to dissolve

Cacodylate buffer

For 10 tubes

200µl (0.2M) 0.5M Potassium cacodylate (pH 7)

50µl (2.5mM) 25mM Tris-Hcl pH6.6

50µl (2.5mM) 25mM Cobalt Chloride

50µl (0.25mg/ml) 2.5mg/ml BSA

150µl dH_2O

2µl TdT

5µl b-dUTP

Calpain Buffer

50mM Hepes pH 7.5 (with NaOH)

2mM EDTA

3mM $CaCl_2$

Denhardts solution (50X)

1% FICOLL (Type 400)

1% PVP

1% BSA

Make up in dH_2O

FDE

100mM EDTA

20% FICOLL

0.05% Bromophenol blue

Fix Solution (For FISH)

3 volumes methanol: 1 volume acetic acid

High stringency wash

0.2XSSC

0.1% SDS

Hybridisation solution (50%)

50% Formamide

2XSSC

!0% dextran sulphate

500ug/ml salmon sperm DNA

Hypotonic solution

0.075M KCL

ICE Buffer

25mM Hepes

10% Glycerol

5mM DTT

L Broth-Agar

500ml L Broth

2% Agar

Autoclave to dissolve agar

Add 1mg/ml Ampicillin after cooling the Broth down to less than $50^{O}C$

Pour in petri dishes (Sterlin) and allow to set

Low stringency wash

2XSSC

0.1% SDS

Malaic acid buffer

0.1M Malaic acid

0.15M NaCl

0.35M NaOH

pH to 7.5 with NaOH

NP40 Buffer (without inhibitors)

For 500ml:

1% TritonX100

0.5% NP40

150mM NaCl

10mM Tris pH 7.5

1mM EDTA

1mM EGTA

10mM NaPPi (add solid)

(Store at $4^{O}C$)

NP40 +Inhibitors

To10ml NP40 buffer:

10µl 200mM PMSF

10µl 100mM Na Vanadate

10µl 10mg/ml Leupeptin

10µl 10mg/ml Benzamidine

10µl 0.5M NaF

10µl Aprotinin

10mM β Glycerophosphate (add solid)

10µl 1M DTT

(Store at $-20^{O}C$)

200mM PMSF was made up in propanol. For Sodium vanadate, it was first converted into pervanadate by dissolving in 100ml of water and adjusting the pH to 10. At this point, the solution (orange in colour) was boiled in the microwave till it was colourless. It was then cooled and aliquots stored at $-20^{O}C$.

P1 (For PAC clone isolation)

15mM Tris, pH 8

10mM EDTA

100µg/ml RNaseA

Filter sterilise and store at $4^{O}C$

P2 (For PAC clone isolation)

0.2N NaOH

1% SDS

Filter sterilise and store at room temperature

P3 (For PAC clone isolation)

3M KOAc, pH 5.5

PBS

For 2 L of distilled water add 20 tablets of PBS (Dulbecco 'A') formulated tablets

Pepsin solution

0.01% Pepsin

10mM HCl

Prehyb solution

For 500ml:

125ml 20XSSC

12.5ml 20% SDS or 12.5ml of 10% SDS

50ml of 50X Denhardts solution

500mg Total Yeast RNA

Make up with dH_2O

Sample Buffer (2X)

1.3ml dH_2O

1.3ml Tris pH 6.8

2ml Glycerol

5ml 10% SDS

800µl 2 mercapto-ethanol (add this in the fume hood)

Bromophenol blue

SDS-PAGE gels

15%	9%	10%	7.5%	12%	Stacking

Acrylamide (30:0.8)	20ml	12ml	13.3ml	10ml	16ml	3.2ml
1M Tris pH 8.8	15ml	15ml	15ml	15ml	15ml	-
dH_2O	4-2ml	12.3ml	10.9ml	15ml	8.2ml	14ml
10% SDS	0.4ml	0.4ml	0.4ml	0.4ml	0.4ml	0.2ml
1M Tris pH 6.8	-	-	-	-	-	2.5ml
10% APS	375µl	375µl	375µl	375µl	375µl	200µl
TEMED	20µl	20µl	20µl	20µl	20µl	20µl

SDS-PAGE gel running buffer (10X)

For 2L:

60g Tris

288g Glycine

20g SDS

pH should be 8.3

SSB (10X)

50% Gycerol

0.25% Bromophenol blue

(make up in water)

SSC (10X)

3M NaCl

0.3M Tris-sodium citrate

pH to 7.0 with NaOH

SSC-TB (4X)

4XSSC

0.05% Tween 20

0.5% Block solution

4XSSC-T Wash Solution

4XSSC

0.05% Tween 20

SSPE (20X)

175.3g NaCl

31.2g $Na_2PO_4.2H_2O$ (or monohydrate 27.6g)

7.4g EDTA

pH should be around 7.4 or else pH with 10M NaOH and make up to 1L with dH_2O

Stain buffer (for TUNEL analysis)

For 10 tubes:
1ml 4XSSC+ 0.1%Triton X-100

50mg (5%) Milk

1µl (5µg/ml) 5mg/ml Avidin-FITC

Strip Buffer (for western blots)

1% SDS

0.2M Glycine (pH 2.5)

TBE (1X)

89mM Tris

89mM Boric acid

2.5mM EDTA

TE

10mM Tris pH 8

1mM EDTA pH 8

TNE

10mM Tris pH 8.2

400mM NaCl

2mM EDTA pH 8

Tris Acetate gel running buffer (10X)

For 1L:

60.5g Tris Base

16.4g Sodium Acetate

7.4g EDTA

5.8g NaCl

Tris Borate (10X)

For 1L:

55g Boric Acid

108g Tris Base

9.3g EDTA

pH should be about 8.5

Western Blot transfer Buffer (10X)

600mM Tris

500mM Glycine

16mM SDS

For use (500ml):

100ml Methanol

50ml 10X Western Blot Buffer

350ml dH_2O

2.1.3.2Tissue Culture Solutions and Medium

Solutions made by the central services

Distilled H_2O

PBS

PE (PBS+EDTA)

L Broth

DMEM- Medium for Rat-1, *ts* v-Src transformed Rat-1, BT474, LS277, HT29, CALU3

For 1L: To 800ml dH_2O add

10X DMEM	100ml
200mM L-Glutamine	10ml
Sodium Bicarbonate 7.5%	50ml
100mM Sodium Pyruvate	10ml
New Born Calf Serum	100ml (10% serum for BT474, LS277, HT29, CALU3)
New Born Calf Serum	50ml (5% serum for Rat-1 fibroblasts)
New Born Calf Serum	2ml (For 0.2% serum)
1.12M HCl	6ml
Streptomycin 10,000 μg/ml	5ml
Penicillin 10,000 unit/ml	5ml

(Antibiotics made up in PBS)

10H- Medium for BICR and HEK cell lines

For 1L: To 800ml dH_2O

Penicillin	5ml
Streptomycin	1ml
200mM L-Glutamine	10ml
1N HCl	4ml
100mM Sodium Pyruvate	10ml
Sodium Bicarbonate	40ml

Top up to 900ml with dH_2O. Then add

10X DMEM	100ml
Remove 100ml of 1XDMEM and add	
Fetal Calf Serum	100ml
Hydrocortisone (Diluted)	1ml

10C- Medium for Swiss 3T3 feeder cells

For 1L:

Same as for 10H except add 30ml Sodium Bicarbonate, 100ml of Donor calf serum instead of Fetal Calf serum, and no hydrocortisone.

Standard Chicken Medium

For 500ml

dH_2O	450ml
10X DMEM	50ml
Na Pyruvate	5ml
L-Glutamine	5ml
New Born Calf Serum	25ml
Chick serum	5ml
Chick Tryptose phosphate	50ml
Penicillin	5ml
Streptomycin	5ml

Medium for AA/C1 and AA/C1/SB10 cells

For 500ml:

1XDMEM	500ml
Fetal Bovine Serum	100ml
L-Glutamine	5ml
Insulin 200units/ml	0.5ml
Hydrocortisone 100μg/ml	5ml
Streptomycin	5ml
Penicillin	5ml

Freezing medium for Rat-1 fibroblasts

For 100ml:

10XDMEM	10ml
L-Glutamine	1ml
Na Pyruvate	1ml
Na Bicarbonate	5ml
New Born Calf Serum	20ml
DMSO	7.5ml
dH_2O	54.9ml
HCl	0.6ml

(Store at -20^{O}C in 10ml aliquots)

RPMI- Medium for COLO320, ALT1, MDA231

1X RPMI	500ml
L-Glutamine	5ml
Fetal Calf Serum	50ml
Penicillin	5ml
Streptomycin	5ml

Hydrocortisone (diluted)

5mg/ml stock:

0.1g Hydrocortisone

20ml 95% EtOH

Store at 4^{O}C

Diluted:

1ml stock

11.5ml 10C medium

Filter and store 1ml aliquots at -20^{O}C

Trypsin

1/10 0.25% trypsin in PBS

2.2 Methods

2.2.1 Isolation of *fak* PAC clone

2.2.1.1 Random primed labelling of FAK cDNA

20μg of FAK cDNA (A gift from Allan Richardson and Tom Parsons) was restriction digested using HINDIII restrictive enzyme to generate a 558bp fragment. The DNA fragment was isolated from 1% agarose gel using the protocol outlined in the manufacturers protocol (Qiaquick gel extraction kit, Qiagen). The approximate yield was calculated to be about 1μg using the following reasoning:

Total length of plasmid = 6200bp
Total length of fragment = 558bp
Expected Yield (if 100%) = 558/6200 = 1/12 of 20μg = 1.6μg
Taking into account loss during extraction procedure, approximate yield = 1μg

200ng of the isolated DNA was used for radioactive labelling. DNA was diluted in dH_2O to give a total volume of 8μl and transferred to a screw cap microfuge tube. In order to denature the DNA, the tube was boiled for 10min, buried in ice for 5min, and then flashspun in the microfuge. To the denatured DNA, 5μl of random hexamers/cold dNTPs (2μl Hexamers, 1μl of 10mM dATP (0.5mM), 1μl of 10mM dGTP, 1μl of 10mM dTTP), 5μl of α^{32}PdCTP (1.85 Mbq/50μC), and 2μl of Klenow polymerase were added quickly to prevent DNA from re-annealing. The tube was then incubated for 30min at 35^{O}C. Following incubation, labelled DNA was separated from unincorporated label using Biospin columns (Biorad) following manufacturer's protocol. Prior to use, the probe was denatured by boiling for 10min and then placed in ice for another 5min.

2.2.1.2 Probing a library

The DNA library filters (obtained from UK HGMPR) were prehybridised in 200ml of prehyb solution at 65^{O}C overnight. For this the 7 filters were placed in a plastic box, which was then put in a 65^{O}C waterbath (Grant) and weighed down using heavy

lead blocks. Next day, the prehyb solution was poured off and replaced by 70ml of fresh prehyb containing 200ng of the denatured, radioactively labelled probe. Again, probing was carried out overnight at 65^{O}C. Following incubation, the filters were washed once at high stringency for 10min and then at low stringency for 20min. They were dried on 3mm paper, wrapped in DOW Saran wrap, and exposed to Fuji RX medical X-ray film overnight in large film cassettes with intensifying screens at -70^{O}C. The film was developed in Kodak X-OMAT 480 RA processor. For the purpose of orientation, tape with radioactive markings was stuck on the cassette.

2.2.1.3 Confirmation of the *fak* PAC clone

To select out the right clone, PCR was carried out on the clones using primers designed from the 5' and 3' UTR (untranslated region) of the known *fak* mRNA sequence. The primers were designed using the chick *fak* mRNA sequence that has >90% homology to the human sequence. They were chosen to be from the untranslated region since it is the most conserved amongst different species. Following were the sequences of the primers used:

From the 5' UTR-
5a (6) 5'- ACTGTGAGCCCGCGGCGTGA-3'
5b (229) 5'- AATAATGGCAGCTGCTTACCTTGA-3'
Primer- 5'- TCAAGGTAAGCAGCTGCCATTATT-3'
5c (187) 5'- TAACGGAAGGGAGAATATGACAGA-3'
Primer- 5'- TCTGTCATATTCTCCCTTCCGTTA-3'

From the 3' UTR-
3a (3717) 5'- GGGATGGCCAAGGGGTGACATCTT-3'
Primer- 5'- AAGATGTCACCCCTTGGCCATCCC-3'
3b (3369) 5'- TTGGGCAGACGAGACCACAC-3'
3c (3444) 5'- TTCCACCAGCAGCGAGGAATTAAC-3'

Concentration of the primers was worked out using the OD reading. Following formula was used:

(Concentration X OD at 260 X 40)/ 1000= x μg/ul

Final concentrations of the primers were:

3a- 3.67μg/μl

3b- 2.12μg/μl

3c- 1.5μg/μl

5a- 1.83μg/μl

5c- 1.7μg/μl

PCR reactions were carried out using the primers to determine the PAC clone for *fak*. Following PCR reagents and conditions were used.

PCR reaction mix:

Total volume- 50μl

10XNH_4 buffer- 5μl

50mM Mg^{++}- 2.5μl

100ng/μl forward primer- 5μl

100ng/μl reverse primer- 5μl

Taq- 0.2-0.5μl

DH_2O- 26μl

Target DNA- 1μl

PCR conditions:

5min- 95^{O}C

Cycle X 30:
1min- 91^{O}C

1min- 55^{O}C

1min- 72^{O}C

5min- 72^{O}C

Soak- 4^{O}C

The completed reactions were run on a 1.5% agarose gel.

2.2.1.4 Isolation of DNA from PAC clones

Clones obtained were streaked onto LB agar plates containing 25μg/ml kanamycin and incubated overnight at 37^{O}C. A single bacterial colony was isolated the next day and inoculated into 2ml of L Broth supplemented with 25μg kanamycin. It was left overnight at 37^{0}C for incubation with shaking (225-300rpm). Following incubation, the bacteria were spun down at 3000rpm in a bench top centrifuge (MSE) for 10min at room temperature. After discarding the supernatant, the bacterial pellet was re-suspended in 0.3ml of P1 solution. 0.3ml of P2 solution was added to the re-suspended pellet and the tube was shaken gently to mix the contents and left for 5min at room temperature. 0.3ml of P3 was slowly added to the mix with gentle shaking and then the tube was placed on ice for 5min. To remove the thick white precipitate formed after the addition of P3, the tube was centrifuged at 10,000rpm for 10min at 4^{O}C in Sorvall RC-5B refrigerated superspeed centrifuge. The supernatant was transferred to a 1.5ml eppendorf tube containing 0.8ml of ice cold isopropanol. The tube was inverted to mix the contents and left on ice for 5min. To precipitate the DNA, tube was centrifuged at maximum speed in a microfuge for 15min at 4^{O}C. Supernatant was removed, taking care not to dislodge the pellet. The pellet was washed with 0.5ml of 70% ethanol, spun for 5min and then left to air dry. The pellet was re-suspended in 40μl of TE.

2.2.2 Fluorescent *in situ* hybridisation (FISH)

2.2.2.1 Preparation of chromosomes from Lymphocytes

Chromosomes were prepared using a small amount of fresh blood from a volunteer and 'chromosome medium 1A'. 200μl of blood was added to each 5ml tube of medium and incubated for 72 hours at 37^{O}C. Tubes were agitated daily to prevent setting of the contents. 71 hours after the blood had been seeded, colcemid was added to each tube to a final concentration of 0.1g/ml. Tubes were then agitated gently and left for 1 hour at 37^{O}C. Following this incubation, the blood was transferred to 15ml Falcon tubes, spun down in benchtop centrifuge (MSE) (2500rpm for 5min), and the supernatant was poured off. Cells were re-suspended in small amount of remaining

medium and 10ml of warmed (37^{O}C) hypotonic solution added slowly. Tubes were incubated at 37^{O}C for 10min before being spun, as detailed before. The hypotonic solution was poured off, leaving a very small amount remaining above the cell pellet in which the cells were re-suspended. At this stage it was crucial that cells were well suspended and not clumped. 10ml of fix was added to the cell suspension very slowly drop-wise, with the tube constantly being agitated with the fingers. Once the fix was all added, the cells were left at room temperature for 15min. Tubes were then spun as before. The fix was poured off, leaving a small amount remaining, in which the cells were re-suspended and 10ml of the fix solution was added for the second time. Cells were spun immediately this time.

This process of fixing and spinning samples was continued till the re-suspended solution became clear and there was no trace of haemoglobin from the lysed red blood cells, usually 5-6 repetitions. A sample of the fixed cells was then dropped onto a slide to check for the presence of metaphase spreads and also for determination of cell density. Final lymphocyte preparations were stored in fix at -20^{O}C.

2.2.2.2 Preparation of chromosomes from the BICR cell lines and HEK cells

Since these cells were grown on feeders, the first step was to remove the feeders a day before preparing the chromosomes from the keratinocytes. To do this, medium was removed and 4ml of warmed up (37^{O}C) PE was added to the 90mm tissue culture dish (Falcon) and left for about 30 seconds. The feeder cells were removed by vigorous pipetting. After removing the PE, the remaining keratinocytes in the dish were washed three times with 5ml of PBS and the medium replaced.

Chromosomes were prepared from 90mm tissue culture dishes of sub-confluent (70%) cell lines. Colcemid was added at a concentration of 1/100 to the medium and left for 3 hours. After the incubation period, medium was removed from the plate into a 15ml falcon tube. The cells were washed with 2-3ml of PBS and added to the falcon tube. They were trypsinised as usual and neutralised using the medium poured out earlier into the falcon tube. They were then spun down at 600rpm for 10min and the supernatant was poured off. The tube was flicked gently to loosen the pellet and 2ml of the warmed up (37^{O}C) hypotonic solution was added dropwise with constant flicking of the tube using the fingers. The re-suspended cells were incubated at 37^{O}C.

Following incubation, 2ml of fresh fixative was added and mixed by inverting the tube a few times. The tube was spun at 600rpm for 5min in a benchtop centrifuge. After the spin, supernatant was poured off and 2ml of fresh fixative was added dropwise as described before. This time, the tube was left at room temperature for 30min and then spun down as before. The re-suspension, incubation at room temperature and the spinning down steps were repeated once more before finally re-suspending the cells in a volume of fixative which would not result in highly concentrated spreads.

2.2.2.3 Preparation of chromosomes from other cell lines

Chromosomes were prepared from 75cm^2 tissue culture flasks (NUNC) of sub-confluent (70%) cell lines. 1/100 concentration of colcemid was added to the medium and then incubated for 3 hours at 37^{O}C. Medium was then removed, cells were rinsed with PBS and trypsinised. Cells were washed in PBS, spun down (2500rpm for 5min) in a benchtop centrifuge and the PBS poured off, leaving a small amount covering the cells. The pellet was re-suspended in this small amount of PBS and 10ml of warmed hypotonic solution added as for the lymphocyte and keratinocyte preparations. All cell lines were incubated for 15min with hypotonic solution at 37^{O}C and then spun, as before. The chromosome preparation was continued as outlined for lymphocytes with fix and spin steps being repeated 4-5 times.

2.2.2.4 Fixing chromosomes on slides

A small volume of chromosomes in the fixing solution were dropped onto a slide from a height and the area of spread marked on the slide using a diamond pen. The slide was fixed for an hour at room temperature and air-dried. The dried slide was incubated for an hour in 100μg/ml RNase in 2XSSC at 37^{O}C. After rinsing the slide in 2XSSC, it was incubated in pepsin solution for 10min at 37^{O}C. The slide was rinsed in water once and post-fixed for 10min in STF at room temperature. Finally, it was dehydrated 2X2min in 70% ethanol and then 2X2min in 100% ethanol.

2.2.2.5 Biotin labelling for *fak* probe

Nick translation reaction was carried out using 1μg of isolated FAK fragment and 4μl of biotin labelling mix, made upto 20μl with dH_2O and incubating for one and a half

hours at 16^{O}C. 5µl of sodium acetate, 100µl Human Cot-1 DNA, and 300µl of 100% ethanol were added to the mix and then kept at -20^{O}C overnight. The next morning, the tube was spun at 13,000- 15,000rpm in a microfuge for 15min and the supernatant was removed. Precipitated DNA was washed in 100µl of 70% ethanol and then re-spun for 5min at 13,000- 15,000rpm. Ethanol was removed and the DNA was left to air dry before being re-suspended in 60µl of 50% hybridization mix.

2.2.2.6 Chromosome denaturation

In order to denature the chromosomes after they have been being fixed onto the slide (as described in 'chromosome preparation'), 30µl of 70% formamide (made up in 2XSSC) were pipetted on the marked section on the slide and covered with 22mm X 32mm glass cover slip. The chromosomes were then denatured for 5min at 80^{O}C using the Hybaid Omnislide system, dehydrated first in 70% and then 100% ethanol, and were finally dried by placing on a slide drying rack.

2.2.2.7 Probe denaturation

<u>For FAK probe</u>

The labelled probe was heated at 75^{O}C for 5min, placed briefly on ice and then spun down. After spinning down, the probe was placed at 37^{O}C for half an hour.

<u>For centromeric probe</u>

The digoxigenin labelled probe was heated at 75^{O}C for 5min.

<u>For c-Myc probe</u>

The digoxigenin labelled probe was incubated for 5-10min at 37^{O}C.

2.2.2.8 Hybridisation of slides

After denaturing the chromosomes and the probe, 12µl of denatured probe was added to the slide, covered with a coverslip making sure that there were no bubbles, and then the edges of the coverslip were sealed with Cow Gum, rubber adhesive using a 1 or 2ml syringe. The slide was incubated in the Hybaid omnislide system overnight at 37^{O}C. Where double hybridisations were performed, using a centromeric probe and a single copy (like FAK) probe, 3µl of the centromeric probe and 9µl of single copy

probe were added. For doubles with two single copy probes (FAK and c-Myc), 5μl of each was applied.

2.2.2.9 Probe detection

After the overnight incubation, the slides were first washed and blocked before proceeding to the detection steps. The wash and block procedure was the same for single or double hybridisations. For washing, the coverslips were removed by pouring a small volume of 2XSSC over them and then peeling off the Cow Gum. The slides were then washed first in 50% formamide in 1XSSC at 42^{O}C for 20min followed by a wash in 2XSSC at 42^{O}C for 20min in Hybaid wash module. After washing, the slides were rinsed in 4XSSC-T for 3min in a Hybaid wash sleeve and then blocked in 4XSSC-TB by squirting it onto the slides and covering with parafilm for 10min.

The following is the procedure for the detection of both biotin and digoxigenin labelled probes in a double hybridisation reaction. For single label detections, biotin or digoxigenin, only antibodies against either of the labels were used at the 2nd and 3rd layer detection step.

For the 1st layer detection, FITC-Avidin DCS was used at a concentration of 1:200 in 4XSSC-TB. 100μl of the dilution were added to each slide, covered with parafilm and the slides were then incubated at room temperature in a dark Hybaid humidity chamber for 1 hour. Following incubation, the slides were washed in 4XSSC-T at room temperature for 10min using Hybaid wash sleeve.

For the 2nd layer detection, biotinylated anti-avian D was used 1:100 and sheep anti-digoxigenin was used at 1:200. Both were diluted in 4XSSC-TB and 100μl of the dilution of each was applied to the slides and covered with parafilm. As before, the slides were incubated at room temperature for an hour in the dark Hybaid humidity chamber and then washed in 4XSSC-T for 10min at room temperature.

3rd layer detection involved incubating the slides with FITC-avidin DCS (1:200) and anti-sheep donkey texas red (1:300). 100μl each of the dilutions (made in 4XSSC-TB) were applied to the slides and covered with parafilm. Following incubation at room temperature for an hour in the dark Hybaid humidity chamber, the slides were washed with 4XSSC-T for 20min. They were then dehydrated first in 70% ethanol and then 100% ethanol and dried on a slide drying rack. A drop of vectashield with

DAPI was added to each slide, covered with a glass coverslip and sealed with nail varnish. Slide were viewed using Axioskop (Zeiss) fluorescent microscope and photographed with the fitted camera and detector controller (Princeton instruments Ltd).

2.2.2.10 Cutting and storage of frozen sections

5mm thick frozen sections from tissue blocks were removed from liquid nitrogen and placed on aminopropyltriethoxysilane (APES)-treated slides and air-dried. Each slide was then wrapped in parafilm to protect from condensation and stored at -20^{O}C until use.

2.2.2.11 Preparation of frozen sections

The slides were allowed to come down to room temperature before removing the parafilm. The sample region was marked using a diamond pen and the slides were rinsed in PBS. After being rinsed, they were fixed for 10-20min and then incubated for 1 hour in 100μg/ml RNase in 2XSSC at 37^{O}C. Following this, the slides were incubated with 0.02% pepsin in 10mM HCl at 37^{O}C for 20min. They were then post-fixed in STF for 10min at room temperature after being rinsed in water. Finally, the slides were dehydrated twice in ethanol for 2min each and then air-dried.

2.2.2.12 Probe detection for frozen sections

Probe detection for the frozen sections was carried out in the same way as outlined before for chromosomal preparations from cell lines.

2.2.3 Protein analysis

2.2.3.1 Cell Lysis

For immunoprecipitation or western blot analysis requiring protein quantification, cells were lysed in NP40 buffer (with inhibitors). For this purpose, an appropriate volume of the buffer was added to the plates that had been washed with PBS, and spread around so that all the cells were covered. Frozen plates were first allowed to thaw on ice (These had been washed twice in PBS, snap frozen on dry ice and then stored at –70^{O}C). The plates were left for 15-30min on ice to let the cells lyse. After

the incubation, cells were scraped off using a cell scraper (Costar) and the lysate transferred to a microfuge tube. The tubes were spun at 14,000rpm for 20min in the Sorvall RC-5B centrifuge and the supernatant was transferred to fresh eppendorf tubes. The lysate was then either used straight away or stored at -70^{O}C.

For all other western blot analysis, cells were lysed straight in appropriate volume of 2XSB and scraped off using cell scrapers. The viscous lysate was sonicated giving 4, 5-10 second pulses. The lysate was then either loaded on to a gel or stored at -70^{O}C.

2.2.3.2 Protein assay

Protein assays were carried out using Micro BCA protein assay reagent kit. For calibration, 7 standards containing 0, 5, 10, 15, 20, 25 and 30μg of BSA were prepared in 1ml of dH_2O and 1ml of assay reagent mix (prepared according to manufacturers guidelines). 7.5μl of the lysis buffer were also added. For the samples, 7.5μl of the lysate were added to 1ml of dH_2O and 1ml of assay reagent mix. The standards and the samples were incubated for 45min at 60^{O}C in a waterbath. After incubation, the contents were transferred to plastic cuvettes (Elkay). Protein was quantified using Beckman DU 650 Spectrophotometer at the wavelength of 562λ. Standards were then plotted as a graph and protein concentration for the samples determined.

2.2.3.3 Immunoprecipitation

Protein A-sepharose beads were re-hydrated by incubating with PBS on ice for 1 hour, washed twice with PBS and taken up in the same buffer to give a 50% w/v protein A sepharose slurry.

Aliquots of cell lysate containing about 1mg of protein (as determined by protein assays) were incubated with 10μl of normal rabbit serum on ice for 15min to pre-clear. 50μl of protein A sepharose slurry was added to each of the aliquots and the tubes were rotated on a spiramix (Denley) at 4^{O}C for half an hour. They were then flashspun in the microfuge to precipitate the beads. The supernatant from each tube was removed and incubated with 5μl of anti-FAK antibody on ice overnight. As before, 50μl of protein A sepharose slurry was added and the tubes rotated on the spiromix for half an hour to precipitate the protein. After precipitation, the beads

were spun down at 14000rpm in a microfuge, washed 3 times with NP40 buffer (without inhibitors), and kept on ice for western blot analysis or cleavage assays.

2.2.3.4 Calpain and ICE cleavage assays

Immunoprecipitated FAK was washed with either 1ml of calpain or ICE buffer without any DTT. After spinning at 14000rpm, the supernatant was removed and the beads re-suspended in 30µl of either calpain or ICE buffer, this time containing DTT. For calpain cleavage assays, 1µl of 1:10 diluted calpain I was added to the re-suspended immunoprecipitates and the reaction carried out in a 30^{O}C waterbath. For ICE cleavage assays, 1µl of S/M extracts (a gift from Prof. Earnshaw, Edinburgh; Lazebnik *et al.*, 1994) was added to each immunoprecipitate and the reaction carried out in a 37^{O}C waterbath.

2.2.3.5 SDS polyacrylamide gel electrophoresis

SDS-PAGE gels were set using ATTO gel casters and formers. Protein samples were mixed with equal volumes of 2XSB (sample buffer) first or in cases where the cells had been lysed in it, they were boiled directly for 10min on the heating block before loading on the gel. Gels were run using Pharmacia electrophoresis power supply-EDS 600. Protein molecular weight standards were used for determination or verification of the molecular weight of the proteins investigated.

2.2.3.6 Western blotting analysis

Proteins separated in SDS polyacrylamide gels were transferred to a nitrocellulose membrane by semi- dry blotting. For this purpose 12 pieces of 3mm Whatman chromatography paper and a piece of nitrcellulose membrane, all the same size as the gel, were soaked in transfer buffer. The membrane and the gel were placed between stacks of six pieces of soaking paper on either side after being soaked themselves, and put on the blotting apparatus. After transfer for 1 hour, the membrane was incubated for one hour or overnight with blocking buffer. Incubations with primary antibodies, and subsequent washes were carried out as per the instructions supplied with each antibody. For anti-FAK antibody, the blocking buffer was made up with BSA, incubation with primary antibody was for 1 hour and three 15mins washes were done with PBS+0.2% Tween-20. For other antibodies, overnight incubation

with the primary antibody was carried out at 4°C. After the washes following incubation with primary antibody, the membrane was incubated with 1:3000-5000 dilution of horseradish peroxidase-linked sheep anti-mouse or anti-rabbit antibody in blocking buffer for 1 hour at room temperature on the shaker. After three more washes with the wash buffer, the proteins were detected by ECL following manufacturer's instructions. The nitrocellulose membranes were dried briefly on Whatman chromatography paper, wrapped in DOW Saran wrap and put into a film cassette with intensifying screens. Chemiluminescence was recorded on Fuji RX medical X-ray film. Films were developed in Kodak X-OMAT 480 RA processor. In some cases the amount of staining was quantified using a pdi Arcus II scanner and pdi Quantity one software. To reprobe the blot with another antibody, membrane was stripped in strip buffer for 30min at room temperature. After stripping, the membrane was washed twice in wash buffer for 10min each followed by repetition of the western blot protocol starting from blocking.

2.2.4 DNA analysis

2.2.4.1 Bacterial transformations

20µl of competent DH5α bacterial cells were aliquoted into chilled microfuge tubes. 1µl of diluted DNA was added to the cells and shaken to mix. The tube was then placed on ice for 30min, heat shocked for 40 seconds in a 42°C water bath, then placed back on ice. 80µl of L Broth was added to the cells and the microfuge tubes were left to shake at 225 rpm for 1 hour at 37°C. Afterwards, the cells were all plated onto a LB agar plate containing the antibiotic for which the resistance gene is carried by the transfected DNA.

2.2.4.2 Extraction of Plasmid DNA

DNA extraction was carried out using Qiagen plasmid prep kits.

2.2.4.3 Calculation of DNA concentration

The concentration and purity of solutions of isolated DNA fragments or plasmids was determined spectrophotometrically as described in Sambrook *et al.*, 1989 using a Beckman DU 650 Spectrophotometer.

2.2.4.4 DNA agarose gel electrophoresis and isolation of DNA fragments from agarose gel

Agarose gels were prepared by boiling a solution of 50ml 1XTris acetate buffer mixed with the appropriate percentage of agarose (normally ranging from 0.8 to 1.2%). 5μl of 10mg/ml ethidium bromide solution was added to the gel mix before poring it into an LKB agarose gel former. Before loading into the set gel, equal volume of 2XSSB buffer was added to the samples and the gel run at 100mA in a LKB 2012 Maxiphor electrophoresis unit using a Consort Flowgen electrphoresis power supply. 1μl of λHindIII or ϕX Hae III DNA markers were run alongside the samples after adding 2XSSB. The DNA bands were visualised on a UV light box and video image prints of the gels were taken using an Appligene Imager video camera and control unit and Seikosha VP-1500 printer.

DNA fragments were isolated from the agarose gel using the Qiaquick gel extraction kit (Qiagen) following manufacturer's instructions.

2.2.4.5 DNA extraction from cells for DNA laddering analysis

Trypsinized cells were collected in 50ml Falcon tubes and washed in PBS by being spun for 5min at 1000rpm and then re-suspended in PBS 2-3 times. After the final spin, PBS was removed and the cells re-suspended in 200μl of TNE and transferred to microfuge tubes. 10μl of 10% SDS (1/10th volume) was added to the re-suspended cells and mixed gently but thoroughly in order to lyse them. The viscous mix was then incubated with 5μl of 10mg/ml proteinase K at $37^{O}C$ overnight. Following incubation, an equal volume of water saturated phenol was added to the tubes and mixed gently. The tubes were spun for 5min at 13000rpm in a microfuge and the upper layer was removed using a broad tip as the mix is very viscous at this stage. Addition of water saturated phenol and removal of the upper layer was repeated again. After this, an equal volume of chloroform was added, the mix shaken gently and the spun as before. The upper layer that contains the DNA, was removed to a fresh tube. To this, added 1.5 to 2 volumes of 100% ethanol and mixed. The precipitated DNA was spun for 5mins in a microfuge at room temperature and the supernatant poured off. The DNA pellet was then washed in 1ml of 70% ethanol, vortexed, and re-spun for 2min at room temperature. All ethanol was removed and

the pellet re-disolved in 50-100µl of TE. 1ul of 10mg/ml DNase free RNase A was added and heated at 37^{O}C for 30min with the cap open to get rid of all ethanol. To run the DNA on a 2% tris-borate gel, an equal volume of gel loading buffer (FDE) was added and the mix heated for 10-15min at 65^{O}C, leaving the cap open. The gel was run at about 50V, 100mA for 2-3 hours.

2.2.4.6 Sub-cloning the constitutively active form of p110

The construct for the constitutively active form of p110 was originally contained within the pEF-BOS vector which does not carry the gene for neomycin resistance. Since neomycin selection was required for stable transfections, the p110 construct was sub-cloned into another vector, pcDNA3.1 (+), which is resistant to neomycin. Restriction digests were carried out in order to cut out the p110 construct from pEF-BOS. BamH1 and Sal1 were used for restricting 5µg of DNA in a total reaction volume of 50µl. Digests were run on a 0.8% agarose gel and the required DNA fragment, of 4kb length, was extracted. 3µg of pcDNA3.1 (+) was also cleaved using BamH1 and Xho1. Following the restriction digestion of pcDNA3.1 (+), phosphatase reaction was carried out to prevent the restricted fragments from ligating back. For the ligation of p110 into the pcDNA3.1 vector, ligation reactions with approximately 100ng of vector DNA and 200ng of p110 DNA were carried out at 4^{O}C overnight, in a total reaction volume of 20µl. Ligation reactions with just p110DNA and vector DNA were also set up as controls. Following ligation, the reaction mix was used for bacterial transfection, and plated out on ampicillin minus agar plates. After incubation overnight at 37^{O}C, ampicillin resistant clones were picked up and DNA was extracted. Restriction digests were carried out on extracted DNA to confirm that the required construct comprised of pcDNA3.1 with p110 as the insert.

2.2.5 TUNEL assay

2.2.5.1 Fixing the cells

Medium from the tissue culture dishes was transferred to universal tubes. The cells were washed once in PBS and then trypsinised. Medium in the tubes was used to neutralise trypsin and the cells transferred back to the tubes. The tubes were spun at 1100rpm for 5min in a benchtop centrifuge. After removing the medium, the cells

were fixed in 1ml 1% formaldehyde in PBS (stored at $4^{O}C$) for 15min on ice. Following fixation, they were centrifuged at 2000rpm for 5min. Formaldehyde was removed and the cells were re-suspended in 200µl of PBS and then 1ml of 70% ethanol in PBS (stored at $-20^{O}C$) was added and the cells transferred to eppendorf tubes. The cells were stored at $4^{O}C$ till ready to stain.

2.2.5.2 Staining the cells

Fixed cells were centrifuged at 5000rpm for 5min in a microfuge and re-hydrated in PBS on ice for 30min. After re-hydration, they were again centrifuged for 5min at 5000rpm, resuspended in 50µl of cacodylate buffer and incubated at $37^{O}C$ for 30min. Following incubation, 1ml of PBS was added to wash the cells and then the tubes were centrifuged at 5000rpm for 5min. The cells were re-suspended in 100µl of stain buffer and incubated in the dark for 30min at room temperature. For the final wash, 1ml of PBS containing 0.1% Triton X-100 was added and the cells were spun as before. They were then re-suspended in 500µl of PBS containing 10mg/ml propidium iodide.

2.2.5.3 Flow cytometric analysis

Flow cytometric analysis was carried out on Becton Dickinson FACScan using the Cell Quest programme. The cells were collected at 0% compensation for FITC labelling because the analysis was done on two dimension plots where the propidium iodide labelled red cells were distinguishable. The forward scatter (FSC) was set at E-1. Green fluorescent cells (FL1) were collected in the log phase at the voltage of 528. The voltage for the collection of cells labelled red (FL2) was set at 430. These values were adjusted from time to time depending on the intensity of signals from sample to sample. Labelled cells were visualised using a dot plot of FL2-W (width of the signal) against FL2-H (height or the intensity of the signal) to gate single cell population. Percentage of FITC labelled cells was determined from this gated cell population.

2.2.6 Caspase assay

2.2.6.1 Cell preparation

Medium from the tissue culture dishes was transferred to 50ml Falcon tubes. The cells were washed once in PBS and then kept at $37^{O}C$ for a few minutes after addition of 10ml PE. Attached cells were then detached by vigorous pipetting of PE and then transferred to the Falcon tubes. Tubes were centrifuged at 1100rpm for 5min. The supernatant was removed and the cells washed three times in 1ml of PBS and finally re-suspended in 100µl of PBS. The cell suspension was transferred directly to eppendorf tubes chilled in liquid nitrogen for snap freezing.

2.2.6.2 Caspase assay

The assay to detect the presence of active caspases was carried out using a biotin labelled peptide called zEKD-amok which binds to the active site of the caspases and was detected in a western blot analysis using streptavidin conjugated horse radish peroxidase antibody. The assay was carried out as described in Martins *et al.,* 1997.

2.2.7 Tissue Culture

2.2.7.1 Recovering frozen cells

Cells contained in freezing ampules (NUNC) were recovered from liquid nitrogen and thawed in warm water ($37^{O}C$). The cells were then transferred to universals containing fresh medium and centrifuged for 1100rpm for 5min. They were re-suspended in fresh medium and added to tissue culture flasks for culturing.

2.2.7.2 Preparing CEF from chick embryos

Primary CEF were prepared from 10 day old embryos obtained from Wickham Laboratories Ltd., Wickham, Hampshire, England by the method outlined in Tato *et al.*, 1978. Cells were counted using a Neuber haemocytometer and the cell suspension diluted to a cell density of 10^{7} cells/ml. 1ml were then stored in liquid nitrogen as described below or cultured in tissue culture flasks.

2.2.7.3 Cell culture

All cells were cultured in tissue culture flasks and dishes and cultured at appropriate temperatures in humid incubators with 5%CO_2 levels. Confluent cultures were trypsinised and split for passaging. All manipulations were carried out in Class 2 Microbial Safety Cabinets.

2.2.7.4 Trypsinisation

For Rat-1 fibroblasts:

Medium was removed from confluent culture flasks or dishes. 10ml of diluted trypsin (in PE) was added, spread around to wash all the cells and then all but 1ml of it was removed. The cells were left for a couple of minutes at room temperature to let them detach and then 10ml of fresh medium was added to neutralise trypsin. Cells were then spun at 1100rpm for 5min, the medium replaced with fresh medium and then appropriately plated out in flasks or dishes containing medium.

For CEF:

Medium was removed from the flasks and the cells were washed once with PBS. 10ml of trypsin diluted in PBS was added to the flask and spread around till the cells started to detach. Trypsin was then removed immediately and 10ml of fresh medium was added to neutralise trypsin and collect the detached cells. The cells were then spun down at 1100rpm for 5min, and plated out in new flasks containing fresh medium.

For Feeder cells:

Cells were washed in 7ml of PBS before adding 1ml of diluted trypsin (in PBS). They were then left for a few minutes at 37^{O}C to allow them to detach from the base. Trypsin was neutralised by adding 4ml of medium, collected in a universal tube (Sterilin) and spun down at 1100rpm for 5min. They were then counted and 10^5 cells were plated out in each dish. The rest were irradiated as described below.

For the BICR cell lines grown on feeders:

Medium was removed and the cells washed with PBS. Then to remove the feeders, about 5ml of PE was added and pipetted vigorously a few times. After removing PE, 10ml of PBS were again added to remove all feeder cells. The keratinocytes were themselves trypsinised by adding 1ml of diluted trypsin (in PE). Trypsin was

neutralised by 4ml of fresh medium, the cells were spun down at 1100rpm for 5min and counted as described for the feeder cells. 3X 10^5 cells were then mixed with 10^6 of feeder cells and re-plated.

For all other cell lines:

Same as the BICR cell lines after the removal of feeders.

2.2.7.5 Freezing the cells

Following trypsinisation, the cells were cenrifuged for 5min at 1100rpm and re-suspended in freezing medium. 1ml aliquots were transferred to freezing ampules (NUNC). The freezing ampules were kept for a few days at $-70^{O}C$ and then stored in liquid nitrogen.

2.2.7.6 Irradiating feeder cells

Feeder cells were irradiated using the Alcon II Teletherapy unit containing a 222 TBq (6000 Ci) Co-60 source.

2.2.7.7 Counting cells

400µl of the cell suspension was added to 19.6ml of PBS and the number of cells per ml of the solution determined by using Casy1 cell counter and analyser systems (Scharfe system).

2.2.7.8 Cell transfection

Semi-confluent (50%-70%) cell cultures plated out in $80mm^2$ tissue culture flasks or 60mm tissue culture dishes, were transfected with plasmid DNA using DOTAP transfection reagent (Boehringer Mannheim) following manufacturer's protocol.

For transfections using c-Myc RCAN (Provided by David Gillespie, Beatson Institute, Glasgow, UK), fresh medium was added the day following the transfection. The cells were then cultured and split like normal cells.

For transfection with vectors carrying the *neo* gene for G418 resistance, the cells were trypsinised upon reaching confluence. Two days following trypsinisation, fresh medium containing 1mg/ml G418 was added to select for the transfected cells. Fresh medium containing G418 was added every other day to remove the dead cells. When single transfected clones had grown to a visible size, they were picked up and plated

out in 30mm tissue culture dishes. To pick up clones, each colony was marked using a marker pen. Medium was then removed and the colonies picked up by detaching the cells through vigorous pipetting of a very small amount of medium. Care was taken to pick up well isolated colonies so that cells from neighbouring colonies did not get picked up. Each clone was maintained in medium containing G418.

Chapter 3: Results 1

Increased dosage and amplification of FAK in human cancer cells

3.1 Introduction

There are certain characteristics that differentiate cancer cells from their normal counter parts. Tumour cells need to be able to proliferate in a deregulated way, they need to be able to invade surrounding tissues and then finally be able to survive in an anchorage-independent manner to colonize distant organs. Tumour development is a multistage process and may result from the action of any one or a combination of chemical, physical, biologic or genetic insults to the cell (Weinberg, 1989; Pitot, 1993). The development of a tumour involves a number of complex genomic changes. Two predominant categories of genes influencing this process are, the oncogenes and the tumour suppresser genes. Proto-oncogenes (cellular genes having a viral counterpart) or cellular oncogenes (genes that have the capacity to cause cancer but no viral counterpart has been found) require to be activated in order to drive the unrestricted growth or survival of a tumour cell (Pitot, 1993). On the other hand, the growth promoting effects of oncogenes are counter balanced during normal cell proliferation by the effects of tumour suppressor genes. Development of a tumour thus requires the silencing of such genes.

3.1.1 Importance of overexpression of proteins in tumours

Tumour progression in spontaneous or experimentally induced cancers is accompanied by genomic instability. Multiple chromosomal abnormalities have been reported in a variety of human cancers including small cell lung carcinoma (Birrer and Minna, 1989), and colonic adenocarcinoma (Vogelstein *et al.*, 1988). These genomic alterations can lead to overexpression of oncogenes and reduced expression of the tumour suppresser genes involved in tumour progression.

Of particular relevance to this study is the overexpression of some known oncogenes in tumour cells, and the genetic changes which cause overexpression. In a study done to analyse global profiles of gene expression in human cancer cells, 108 out of a total of 289 differentially expressed transcripts, were expressed at higher levels in the colon cancers than in normal colon tissue (Zhang *et al.*, 1997). The study also

suggested that the genes exhibiting the greatest differences in expression are likely to be the most biologically important. Two examples of proteins that are often upregulated during tumour development are given below.

The *myc* family of cellular oncogenes is an example of the oncogenic effects being projected through overexpression. The *myc* family consists of three protein members, c-Myc, N-myc , and L-myc, all of which encode related nuclear phosphoproteins (reviewed in Prins *et al.*, 1993). The proteins appear to play a central role in the control of cell proliferation and differentiation. c-Myc is a DNA binding transcription factor. Tumour cells expressing higher levels of the protein show a reduced growth factor requirement and a shorter G1-phase of the cell cycle. In some cases, c-Myc has also been known to promote immortalization of cells (reviewed in Prins *et al.*, 1993). Overexpression of L-myc is observed in SCLC specimens or cell lines and in some human leukaemia cell lines (reviewed in Prins *et al.*, 1993).

Telomerase, an enzyme required to maintain the length of the chromosomal ends, known as telomeres, is another example of a protein whose altered expression has been implicated as having a role in tumour formation (Soder *et al.*, 1997). Telomerase is inactivated before or soon after birth, as a result of which telomeric attrition is experienced by all normal cells and is thought to be a mechanism by which cells limit their clonal proliferation (Soder *et al.*, 1997). Thus, reactivation of the enzyme is thought to contribute to immortality in tumour cells. Telomerase is often, but not always, found to be overexpressed in immortal human cells and tumours.

3.1.2 Mechanisms of protein upregulation

The mechanism/s by which overexpression of any gene is achieved in a cell are varied. Generally, they can be divided into two categories:

1. **Genetic:** These changes occur in the genome and lead to an increase in the copy number of the gene in question. More copies of the gene could, in theory, lead to more protein. For example, a study has shown the correlation between the gene

copy number of DNA topoisomerase 1, an enzyme that catalyses the breakage and rejoining of DNA during replication, and protein expression (McLeod and Keith, 1996). This analysis was carried out in breast and colon cancer cell lines, which showed an altered gene copy number for the enzyme that correlated with altered protein expression. Gene amplification, isochromosome formation and gain of whole chromosomes are genomic changes which could lead to an increase in the gene copy number. Increase in protein level can also be achieved through deregulation of transcription control. Point mutations in the promoter region or translocation of the gene to another region in the genome can facilitate more efficient transcription. For example, *c-myc* activation in B cells involves its translocation to the Ig locus which lies on chromosome 14 (Haluska *et al.*, 1986).

2. **Post transcriptional and post translational:** Increased mRNA or protein stability can lead to more efficient translation of the protein and thus to increased protein levels. An increase in protein stability can also enhance the level of detectable protein in the cell.

Since this study deals with the genetic mechanisms that might contribute to increased levels of proteins, the process that leads to the development of these changes is described.

3.1.3 How amplicons arise

Amplification is defined as the production of additional copies of a chromosomal region occurring as tandem repeats. Numerous hamster cell line models have been used to investigate the mechanism involved in the creation of these regions. Some of these models have been reviewed by Stark (1993). The primary events of amplification do not involve over replication of the DNA region, but are based on recombination followed by unequal distribution of the recombined DNA into the two daughter cells. How an initial duplication may eventually give rise to an amplicon containing several copies of that region is outlined in Figure 5.

Three possible ways by which recombination between two sister chromatids can lead to duplication of a region of DNA are shown in Figure 5. The process involves unequal sister chromatid exchange followed by bridge-break-fusion cycles (Wintersberger, 1994). As is shown, two of the possible mechanisms involved, a and b, require the creation of a dicentric chromosome which will break at the next round of replication to produce one chromosome with two copies of the defined DNA region (in black). The third mechanism, c, involves the formation of the duplication following non-homologous recombination. This mechanism does not require any subsequent breakage as both the chromatids are monocentric.

3.1.4 Mechanism of formation of isochromosomes

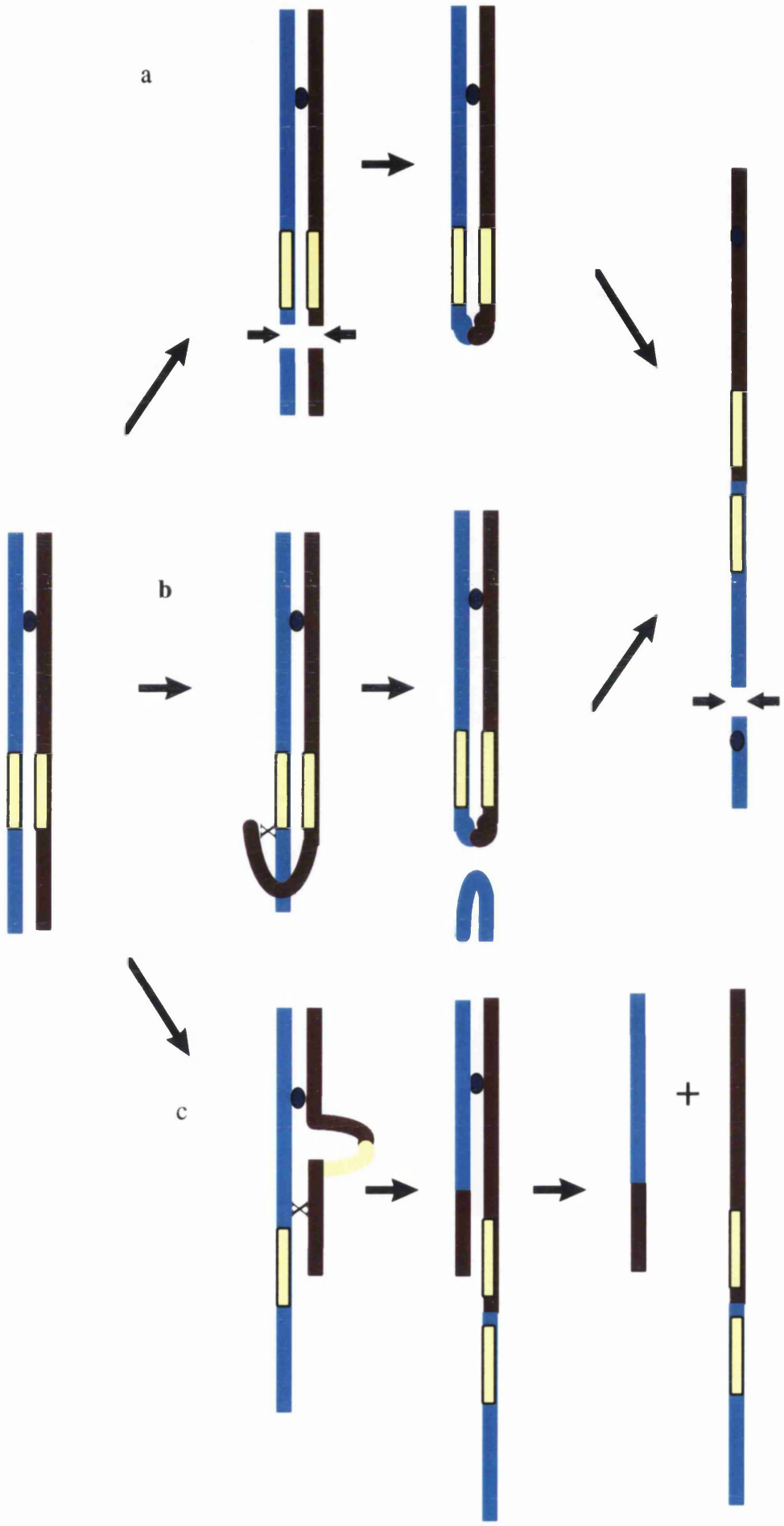
Isochromosomes are defined as chromosomes in which the two arms, on either side of the centromere, are mirror images of each other. In other words, isochromosomes carry the duplication of one of the chromosome arms. A possible mechanism behind the formation of isochromosomes is shown in Figure 6. The process is thought to initiate during DNA replication as the chromosomes line up at the equator to segregate to the daughter cells. The process starts when the chromosome in question (shown in red), fails to line up correctly at the equator, *i.e.* instead of lining up along the plane of the equator, it lines up perpendicular to the plane. This would lead to the segregation of the two parts of the chromosome at the centromere, but the daughter cells would have only one arm of the chromosome each. An isochromosome would thus be formed.

3.1.5 Mechanism for gain of chromosomal copies

Most cancer cells are aneuploid, *i.e.*, they carry extra or are missing chromosomes compared to the normal complement. Non-disjunction of sister chromatids during replication can be one of the mechanisms giving rise to aneuploidy. Non-disjunction of sister chromatids during anaphase, involving separation of the centromeres, leads to the gain of a chromosome by one of the daughter cells, leaving the other without a full complement. Expectedly, this means that the cell gaining an extra copy of the chromosome would gain extra copies of the genes on this chromosome, and might

Figure 5 Mechanism for regional duplication: (adapted from Wintersberger, 1994) The diagram shows the currently accepted model for gene/regional duplication on a chromosome. The two sister chromatids are represented in red and light blue, dark blue circle represents the centromere and the region to be amplified is coloured yellow. There are three possible ways by which the depicted region can be amplified. According to pathway a, the process can start by a break at the telomere creating frayed chromosome ends followed by sister chromatid fusion. As a result, a dicentric chromosome is formed which breaks during mitosis to produce one chromosome with two copies of the region while the other ends up without any copies. The second pathway, b, again involves fusion of the sister chromatids at the telomere following unequal sister chromatid exchange. Thus resulting in a dicentric chromosome leading to duplication of the region. Pathway c starts by non-homologous sister chromatid exchange, resulting in the formation of two chromosome, one with no copy of the region and the other with a duplication of the region.

Three different mechanisms for regional duplication



Mechanism for amplification of an initial duplication

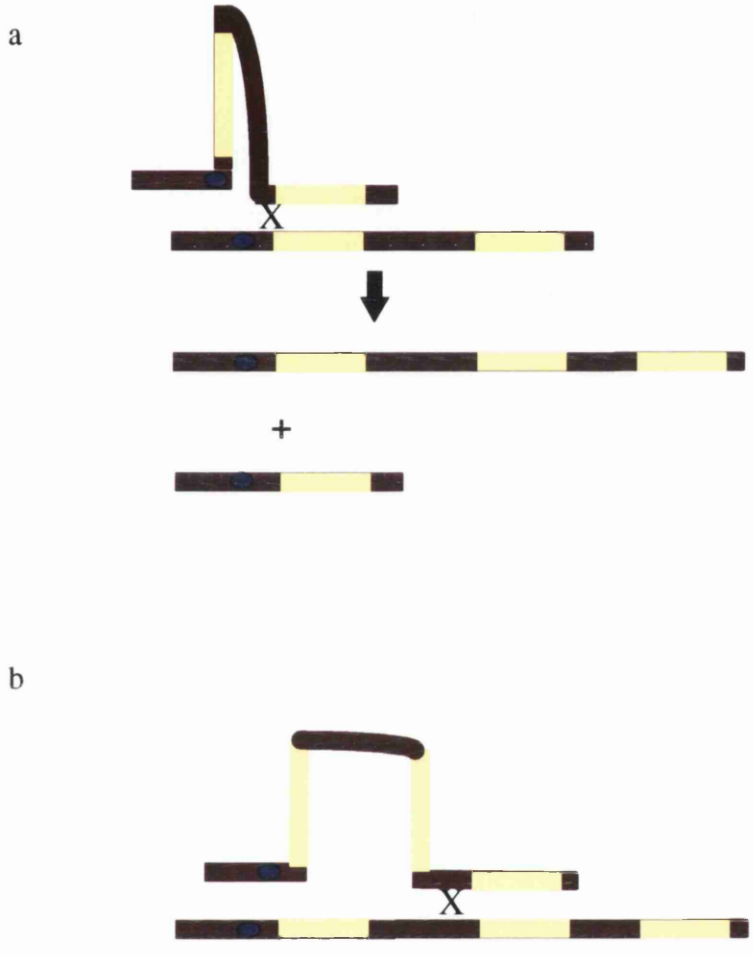


Figure 6 From duplication to amplification: (a) Following initial duplication of a region on the chromosome, subsequent amplification can arise in successive replication cycles. As is shown in the figure, homologous, but unequal sister shromatid exchange during mitosis can result in one chromosome aquiring 3 copies of the region while the other being left with just one.
(b) Repeated unequal sister chromatid exchanges can ultimately lead to the amplification of the region.

thus express a higher level of the proteins encoded by genes on the gained chromosomes.

As stated earlier, FAK has been implicated to play a role in cell migration and cell survival. Since the expression level of FAK maybe a determinant of the rate of cell movement, and since elevated FAK may also enhance tumour cell survival, it is perhaps not surprising that a number of groups have found FAK to be up-regulated at the protein level in cancer cells. In particular, FAK is elevated in cell lines derived from the human melanomas, with FAK levels correlating with the rate of migration on fibronectin (Akasaka *et al.,* 1995), in cervical carcinoma cell lines (McCormack *et al.*, 1997); in prostatic carcinoma tumours and cell lines (Tremblay *et al.*, 1996), and in colon and breast tumours and cell lines (Owens *et al.*, 1995). The latter study also inferred a link between FAK expression and tumour invasiveness. However, despite the mounting evidence that the expression of FAK is elevated in tumour-derived cell lines, the mechanism of upregulation has not been addressed.

The work now presented in the following section identifies genetic changes associated with FAK upregulation. An important factor which influenced this study was the finding that the tip of the q-arm of chromosome 8, where the *fak* gene is thought to localize, was amplified in the lung adenocarcinoma cell line, CALU3 (Hoare *et al.*, 1997). The study found *c-myc* to be one of the oncogenes amplified within the region, but the length of the amplicon suggested that other genes within the region might be co-amplified.

3.2 Isolation of a *fak* genetic probe

To isolate a PAC clone containing the sequence coding for *fak* protein, nylon filters supporting a total of human genomic DNA library were obtained from UK MRC Human Genome Mapping Project Resource (UK HGMPR).

In order to screen the library, chicken *fak* cDNA sequence contained within the Bluescript vector was used. 20μg of the cDNA was digested using Hind III restriction enzyme. The enzyme cuts the cDNA at four sites; 0, 1782, 2340, and 3211 bases

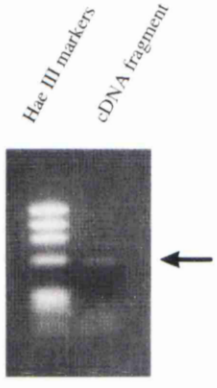
generating three fragments sized 1782 bp, 558 bp and 871 bp respectively. Since the 558 bp fragment lies within the ideal size range for a probe to screen a genomic library, it was selected as the optimal probe. The fragment was recovered by excising from the gel and isolating it from agarose using the gel recovery kit (Qiagen). The approximate yield was calculated to be about 1μg using the reasoning described in Materials and Methods. The recovered DNA was resuspended into 10μl of TE to give a final concentration of 0.1μg or 100ng/μl. 1ul of this was then run on a 1% agarose gel alongside 0.5μg of the Hae III markers to check the concentration and the size of the recovered fragment. The size of the fragment was confirmed to be 558 bp long and the yield to be approximately correct (Figure 7a).

The *fak* cDNA fragment was then radiolabelled with [α^{32}P]- dCTP using the random priming procedure. The nylon filters supporting the human PAC library were then incubated overnight with the radiolabelled probe for hybridization. Filters were washed and exposed to film. Hybridization of the probe to clones containing corresponding sequence generated positive signals (Figure 7b). True positive signals appear as doublets since each clone is fixed twice onto the filter (Figure 7b). 22 positive signals were picked up in all from the library. Identity of the clones was determined by using the manufacturers guidelines supplied along with the filters. 17 of these signals were confirmed as false positives on checking up against a list of false positives provided by the UK HGMPR. 5 clones, 4 I 16, 231 C 7, 305 A 3, 307 J 23, and 316 G 19 were finally selected.

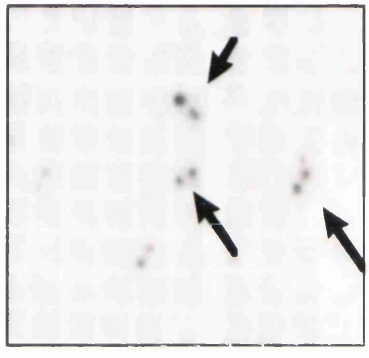
To confirm that the selected clones were indeed positive for *fak*, PCR reactions required to be carried out using primers designed from the 5' and 3' UTR (untranslated region) of the known *fak* mRNA sequence. The primers were designed using the chick *fak* mRNA sequence that has >90% homology to the human sequence. They were chosen from the untranslated region since it is the most conserved amongst different species. To check if the primers were functioning as expected, a test reaction was carried out using genomic DNA isolated from the human fibroblast cell line, SUSM-1 (1mg/ml). 4 reactions were set up with different combinations of the primers, *i.e.* 3ab, 3ac and 5ac, and a negative control reaction with no DNA added. The completed reactions were run on a 1.5% agarose gel.

Figure7 Isolation of *fak* PAC clone: (a) A 558bp fragment was isolated by restrictive digestion of FAK cDNA and run alongside ϕHaeIII markers to confirm size and yield (the product runs between markers sized 603bp and 3210bp). (b) The isolated fragment was radiolabelled and used as a probe to isolate PAC clones positive for *fak* from a human PAC library (provided by the UKHGMPR). The positive signals appeared as duplicates on the filter and their identity was determined following the guidelines provided along with the filters. (c) Primers generated from the 3' or 5' untranslated region (UTR) were first tested on DNA from whole genome to confirm the size of the products (run alongside ϕHaeIII markers; products run between markers sized 603bp and 310bp) and to see if they work (described in more detail in Materials and Methods). (d) Two of the primer pairs from the 3' region, 3ab and 3ac, were found to be suitable for the task of identifying the PAC clone containing *fak*. DNA was isolated from 5 possible PAC clones and following PCR analysis using the primer pairs from 3'UTR, clone 307 J 23 was identified to be positive for *fak*.

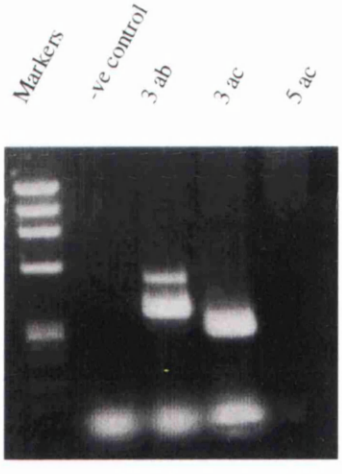
a Isolation of *fak* cDNA fragment



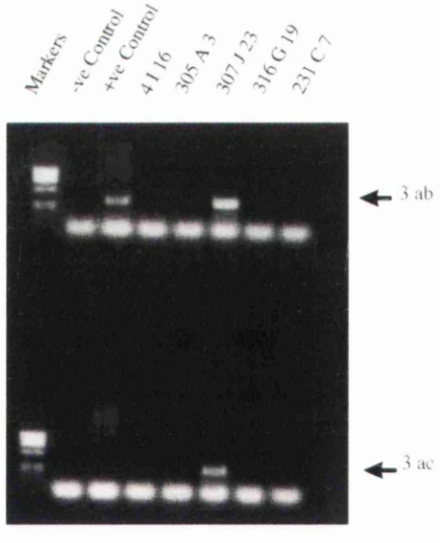
b Possible PAC clones positive for *fak*



c PCR products of different primer pairs



d Identification of clone 307 J 23 to be positive for *fak*



Primer combinations 3ab and 3ac worked well whereas 5ac did not yield any products (Figure 7c). Therefore, 3ab and 3ac were used for future experiments to confirm the *fak* positive clone. PCR reactions confirmed clone **307 J 23** to be positive for *fak* (Figure 7d).

3.3 Localisation of the *fak* probe to the human chromosome 8q24

Having isolated a PAC probe for *fak*, it was important to confirm its genomic localisation. The gene encoding FAK had previously been mapped to mouse chromosome 15 and to human chromosome 8 (Fiedorek and Kay, 1995). Its synteny with mouse chromosome 15, and the previously known linkage of *fak* to the proto-oncogene *c-myc* in the mouse, suggested that human 8q24, where human *c-myc* is located, was the likely site for *fak* localization.

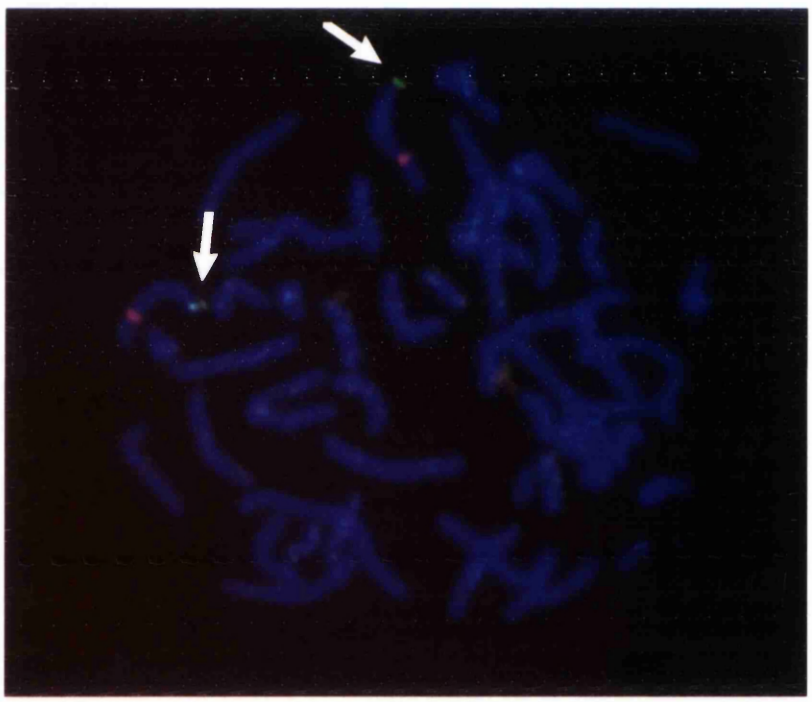
Previously prepared chromosomes from lymphocytes were used. The chromosomes were fixed on a slide as described in Materials and Methods. The PAC probe was biotin labeled using the nick translation kit (Boehringer Mannheim). DIG labelled centromeric probe for chromosome 8 was also used to confirm that *fak* resides on the long arm of chromosome 8. The double hybridization procedure confirmed that the human *fak* gene mapped to the q arm of chromosome 8 and that normal lymphocytes had two copies of the gene (Figure 8).

3.4 *fak* gene copy number gain in cell lines from a variety of human epithelial tumour types

Various studies have shown FAK protein levels to be upregulated in a variety of human epithelial tumour types. To test the generality of *fak* copy number gains, FISH analysis on archived chromosome preparations from cell lines derived from lung, breast, and colon tumours was carried out. Protein analysis for a few selected cell lines with high number of *fak* gene copies from amongst the ones used for FISH analysis, was also carried out to determine if the increase in gene dosage of *fak* was accompanied by high levels of FAK protein expression. A probe with high efficiency is necessary for accurate mapping by FISH and is essential for quantitative analysis

Figure 8 Localisation of *fak* to chromosome 8q: Chromosomes were prepared from lymphocytes that were arrested in metaphase using colcemid and probed with both a DIG-labelled centromeric probe for chromosome 8 (visualised as red) and biotin labelled *fak* probe (visualised in green). *fak* was confirmed to localise to the telomeric end of chromosome 8q.

Localisation of *fak* probe in chromosomes derived from human lymphocytes



of gene copy number (McLeod and Keith, 1996). Since the efficiency of the probe was greater than 95% when hybridised to normal lymphocytes, it was suitable for further anylysis to determine the gene copy number of *fak* in other cell lines. For each cell line, approximately 50 nuclei were counted and the mode of the signals per nuclei was documented.

Out of all the cell lines screened, 8/11 lung cancer cell lines, 5/5 colon cancer cell lines and 7/7 breast cancer lines displayed more than 2 hybridization signals in a high percentage of nuclei (Table 1). LCPH3 (Milroy *et al.*, 1992), one of the lung cancer cell lines, did not show a gain in *fak* gene copy number (Figure 9). The figure shows a number of interphase nuclei with 2 signals each for *fak.*

In addition, the *fak* gene was also amplified in 2 of the cell lines examined, Calu 3 (lung) and HT29 (colon) (Figure 10a, 10b; Table 1). Isochromosomes carrying the q arm of chromosome 8 can also be seen. Figure 10c is the magnified image of a chromosome, carrying the chromosome 8 centromere (labeled in red), showing amplification of the region containing *fak.*

For protein analysis, the cells were lysed in NP40 lysis buffer and 50μg of total protein was separated by gel electrophoresis. Immunoblotting was carried out using anti-FAK antibody. All the epithelial cells examined had readily detectable levels of FAK protein. HT29 and Calu3, both of which have an amplication of *fak* and carry more than 10 copies of the gene, did express readily detectable levels of FAK protein (Fig. 10d). However, LS277, another lung cancer cell line which has an average of 4 signals per nuclus, had a higher level of FAK protein as than Calu3 (Figure 10d; Table 1). Due to unavailability of normal tissue controls for comparison, it was not possible to determine if the difference between the other cell lines was tissue specific. Also, three of the cell lines examined, HT29, Calu3 and BT474, had faster migrating immunoreactive species, which are presumed to be cleavage products of FAK (Figure 10d). The significance of this will be discussed later.

Table 1 **Summary of copy number gains of *fak* locus in lung, colon and breast tumour-derived cell lines**

Cell line	Origin	Signals/nucleus
Calu3	Lung cancer	>10
GLC4	Lung cancer	3
LS274	Lung cancer	3
LS277	Lung cancer	4
LS763	Lung cancer	5
H125	Lung cancer	4
LCPH3	Lung cancer	2
LS111	Lung cancer	2
LS310	Lung cancer	2
LDAN	Lung cancer	5
LS106	Lung cancer	4
HT29	Colon cancer	>10
Colo320	Colon cancer	3
ALT-F	Colon cancer	3
ALT-G	Colon cancer	3
ALT-I	Colon cancer	3
AA/C1/SB10	Colon cancer	3
BE	Colon cancer	3
BT474	Breast cancer	6
MCF-7	Breast cancer	4
MDA436	Breast cancer	4
MDA231	Breast cancer	4
T47D	Breast cancer	4
ZR75	Breast cancer	4

Figure 9 No gain in *fak* copy number in a lung cancer cell line: Interphase nuclei from LCPH3, a lung cancer cell line, were probed with biotin labelled *fak* probe (visualised as green) and were found to have the normal *i.e.* 2 copies of *fak*.

Interphase nuclei derived from LCPH 3, a lung cancer cell line, showing normal number of *fak* gene copy number

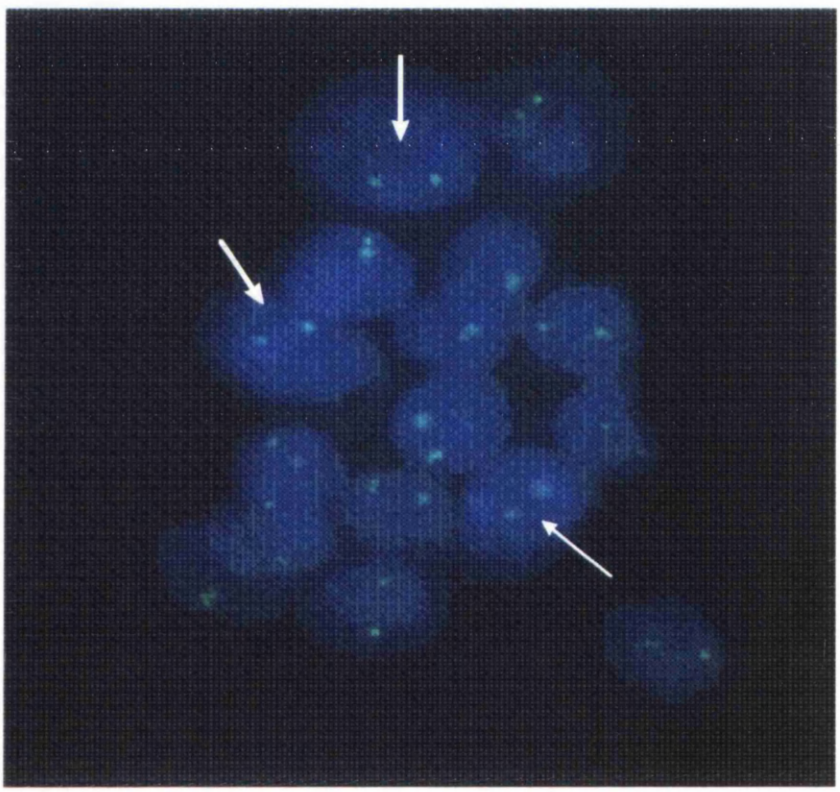
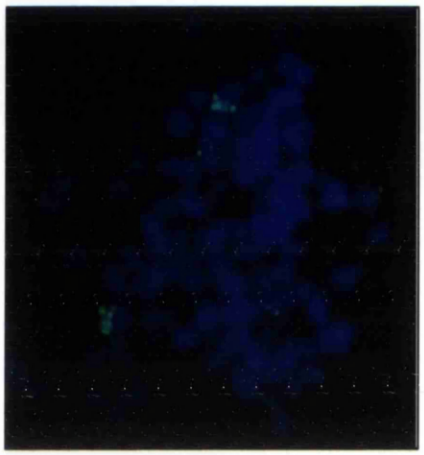
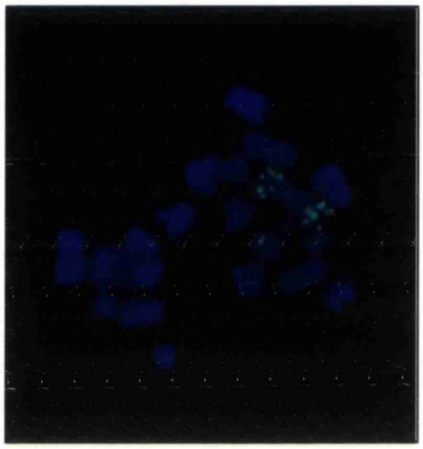


Figure 10 Gain in *fak* gene copy number through amplification in some human cancer cell lines: (a and b) Chromosomes prepared from Calu3 (a lung cancer cell line) and HT29 (a colon cancer cell line) that were arrested in metaphase using colcemid, were probed with biotin labelled *fak* probe (visualised in green) and showed gain in *fak* copy number through amplification. (c) Probing Calu3 chromosomes with both DIG-labelled centromeric probe for chromosome 8 (visualised in red) and biotin labelled *fak* probe showed the presence of an abnormal sized chromosome 8 carrying mainly the amplified region. (d) Protein lysates prepared by lysing the cells from some selected cancer cell lines were separated by SDS-PAGE and probed with anti-FAK antibody to determine the level of FAK protein.

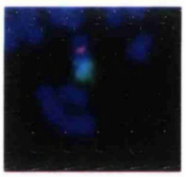
a Gain in *fak* gene copy number: amplification in Calu3, a lung cancer cell line



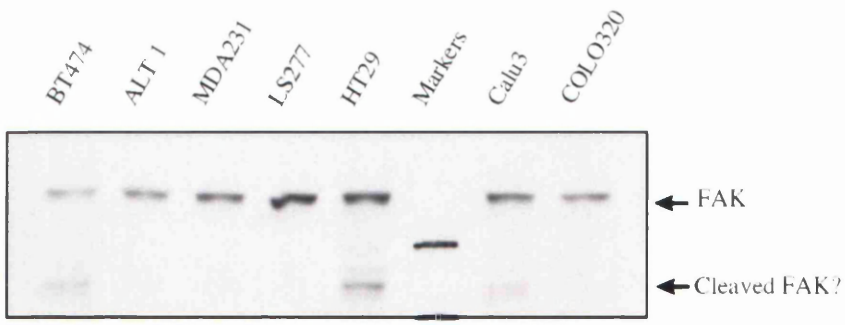
b Gain in *fak* gene copy number: amplification and isochromosome formation in HT29, a colon cancer cell line



c Magnified view of a chromosome carrying *fak* amplification in Calu3 cell line (100X Objective)



d Detection of FAK full length protein and the cleaved form in some cancer cell lines



3.5 Increase in gene dosage of *fak* is accompanied by an increase in protein level in >70% of the squamous cell carcinoma cell lines

Since it was not possible to quantify increase in FAK protein expression due to lack of normal control cell lines for the various colon, lung and breast tumour cell lines screened in the previous section, the BICR series of SCC derived cell lines were selected to study the relationship between gain in gene copy and protein expression. BICR cell lines are malignant keratinocytes derived from squamous cell carcinomas of the head and neck (Edington *et al.*, 1995). Primary human keratinocytes, HNK, were used as the normal control.

FISH analysis of chromosomal preparations from a number of BICR cell lines demonstrated that these generally had more than 2 copies of the *fak* gene in a high percentage of nuclei (Table 2). Two typical examples are shown (Figure 11a and 11b). In the examples shown, BICR6 and BICR31 have 6 and 4 copies of the *fak* gene respectively, and some of these are present at both ends of an isochromosome. All 9 BICR cell lines screened had a high percentage of nuclei with greater than 2 hybridization sites for *fak* (Table 2).

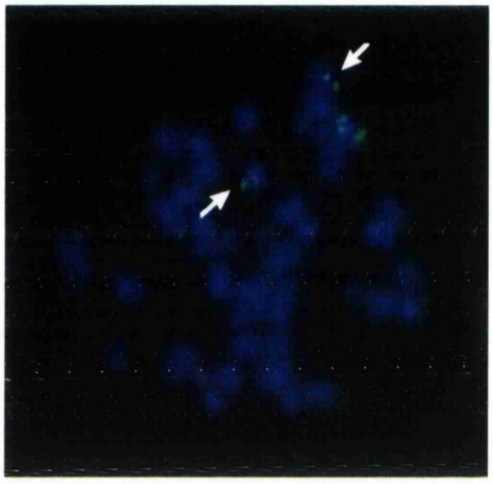
To compare the steady-state levels of FAK protein in the BICR cell lines and the normal keratinocytes, HNK, protein immunoblots were carried out. 25μg of cellular protein were separated by SDS-PAGE, blotted and probed with FAK specific antibody (Figure 12a). The protein levels were then quantified by densitometry and plotted as a histogram (Figure 12b), showing that with the exceptions of BICR16 and BICR63 (2/9 cell lines), the malignant keratinocyes (7/9 cell lines) displayed steady state levels of FAK protein that were elevated above those in normal human keratinocytes (HNK). However, although most cell lines had increased levels of FAK, there was no direct correlation between *fak* gene copy number and protein expression. This is exemplified by BICR6 and BICR16 which each had around 6 copies of *fak* gene, but which express different levels of FAK protein (Figure 12b). As a control for protein loading, p42/ERK2, a protein kinase that is not generally regulated by fluctuations in expression was examined and found not to vary (Figure 12b). Thus, although all the BICR cell lines examined had increased *fak* gene copy

Table 2 Summary of copy number gains of *fak* locus in BICR lines derived from squamous cell carcinomas of the head and neck

Cell line	Site	Signals/nucleus (mode)	Signals/nucleus (maximum)
HNK	(human normal keratinocytes)	2	2
BICR3	Alveolus	4	4
BICR6	Hypopharynx	5	6
BICR10	Buccal mucosa	6	6
BICR16	Tongue	5	6
BICR31	Tongue	4	5
BICR56	Tongue	4	6
BICR63	Tongue	4	6
BICR78	Alveolus	4	6
BICR82	Maxilla	4	5

Figure 11 Increase in *fak* gene dosage in head and neck squamous carcinoma cell lines: (a and b) Metaphase chromosomes were prepared from BICR6 and BICR31 (two of the head and neck carcinoma cell lines tested) were probed with biotin labelled *fak* probe. Arrows point to the copies of *fak* gene. Thick white arrows point to the presence of the gene at both the ends of an isochromosome.

a Gain of *fak* gene copy number in BICR31



b Gain of *fak* gene copy number and presence of isochromosome in BICR6

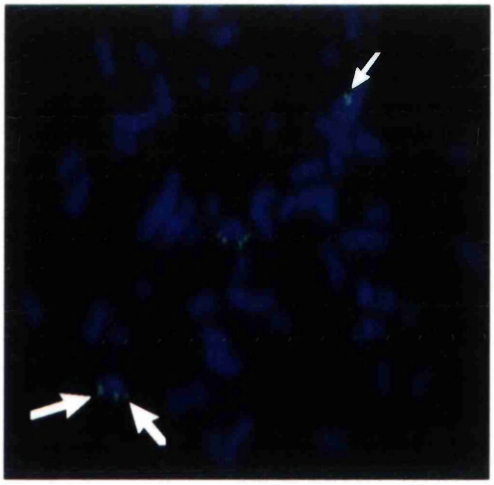
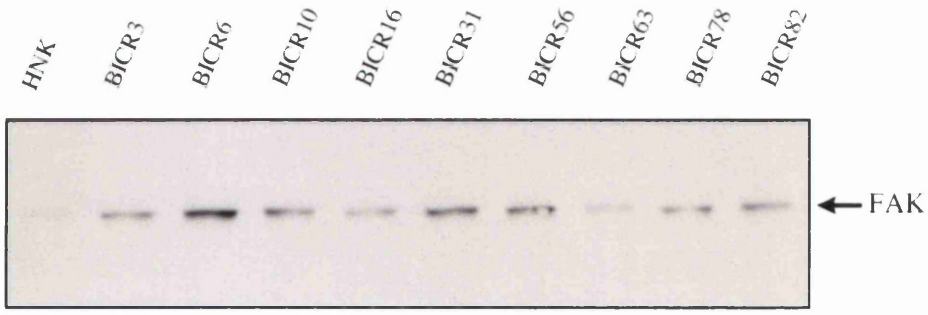
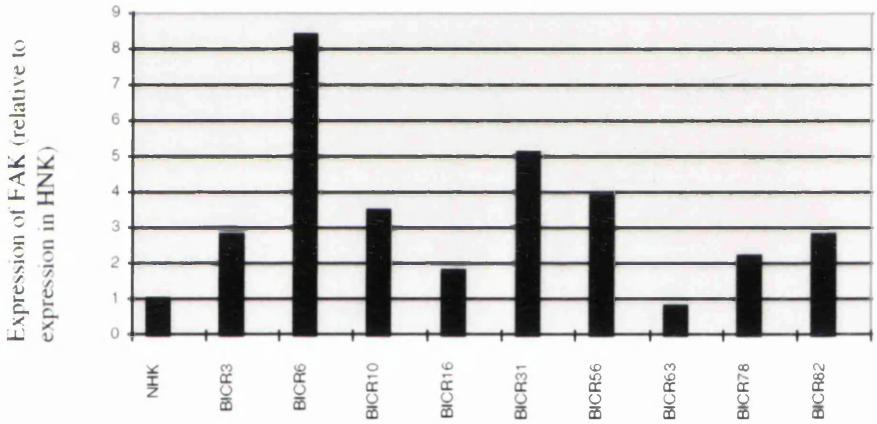


Figure 12 Variations in expression of FAK in BICR cell lines: (a) 25μg of total protein from cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-FAK antibody. (b) Quantitation by scanning laser densitometry of FAK protein in BICR cell lines is shown relative to the expression in HNK control. The immunoblots and quantitation shown are representative of atleast 5 replicate experiments. (c) Lysates separated by SDS-PAGE were also probed with anti-PYK2 (upper panel) or with anti-p42/ERK2 (lower panel).

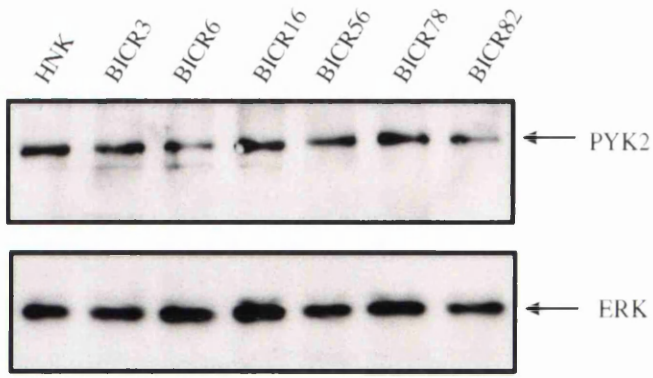
a Expression of FAK protein in BICR cell lines



b Densitometric quantitation of FAK protein expression



c PYK2 and p42/ERK2 do not consistently vary in malignant keratinocytes



number, and this may contribute to elevated protein expressions in some cases, it does not necessarily lead to a related increase in protein in all cases.

The expression of PYK2, a FAK homologue was also examined in the head and neck squamous cell carcinoma cell lines. It is not yet known if PYK2 is involved in cancer, and a recent study has shown that PYK2 is not able to functionally compensate for FAK in *fak* null cells (Sieg *et al.*, 1998). PYK2 protein levels did not vary in the examined cell lines and were the same as in the normal keratinocytes, indicating that in these cells lines, PYK2 is not elevated in expression during tumour development.

3.6 Evidence for increase in *fak* gene dosage also in primary tumour sections

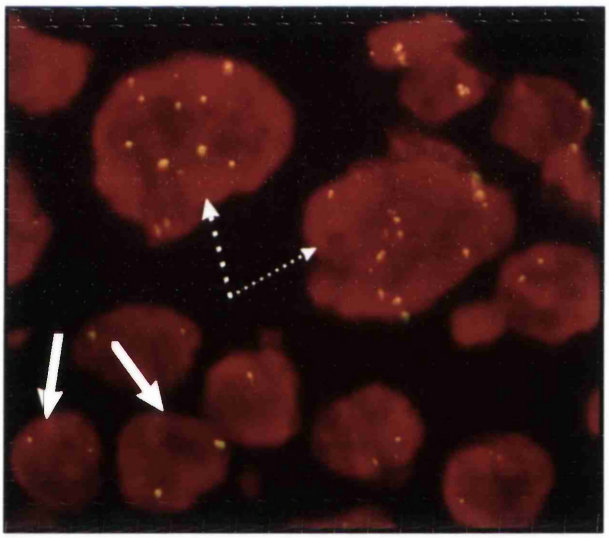
Having shown *fak* to be present in increased copy number in various cancer cell lines tested, it was important to investigate if such was also the case in primary tumour sections, since cultured cells, particularly cell lines, do undergo certain changes after long term growth in tissue culture. Finding *fak* overdose in primary tumours would confirm that increase in *fak* gene copy number in cell lines was not due to extensive passaging in tissue culture, but also occurred *in vivo* during tumour progression. Thus, archived frozen sections of squamous cell carcinomas derived from head and neck were subjected to FISH analysis using *fak* PAC as the probe. A representative of the frozen sections examined is shown (Figure 13). It can be clearly seen that several of the cells within the tumour contain more than two copies of *fak*, whilst some normal cells within the tumour still have the normal 2 copies of the gene, the latter acting as an internal control (Figure 13).

3.7 Increase in *fak* gene dosage accompanies the adenoma to carcinoma progression in an *in vitro* model of colon tumour development

To address the stage during tumour progression that *fak* was increased, an *in-vitro* system that models the conversion from colonic adenoma to carcinoma was used. In this model, a cell line was derived from a large tubular adenoma with mild dysplasia (PC/AA). A clonogenic variant adenoma was established (AA/C1) and a fully

Figure 13 Elevated *fak* copy number in squamous cell carcinoma sections: Frozen squamous cell carcinoma sections were probed with biotin labelled *fak* probe (visualised here as yellow). The nuclei were visualised as red. Cells within the section act as internal controls (arrows point to nuclei with two copies of *fak*, whilst broken arrows point to tumour cell nuclei which contain more than two copies of *fak*).

Elevated *fak* gene copy number in frozen sections of squamous cell carcinoma



tumorigenic and invasive malignant cell line was derived by sequential chemical treatment and tissue culture procedures (AA/C1/SB10; model described in Williams *et al.,* 1990; Brunton *et al.*, 1997). Both the cellular and molecular changes during this *in-vitro* progression are similar to those that occur *in-vivo* (Manning *et al.,* 1991; Williams *et al.,* 1993), making this a relevant model to study colon cancer progression and the changes required for the transition to an invasive phenotype. FAK was found to be phosphorylated accompanying EGF induced cell migration of the carcinoma cells and was thus implied to regulate focal adhesion turnover and tumour cell motility in conjuction with c-Src (Brunton *et al.*, 1997).

FISH analysis on the chromosome preparations derived from the adenoma and the carcinoma cell lines show that while the adenoma cell line (AA/C1), has the normal 2 copies of *fak*, the carcinoma cell line has three copies (Figure 14a). Thus, acquisition of an additional copy of *fak* occurred during malignant conversion and did not occur at an earlier stage of tumour development, at least in this model. This correlates with the earlier findings which suggested that FAK was required for acquiring the malignant phenotype in this model (Brunton *et al.*, 1997). Co-hybridisation with DIG-labelled *c-myc* was carried out at the same time (reasons and results are discussed in section 3.8).

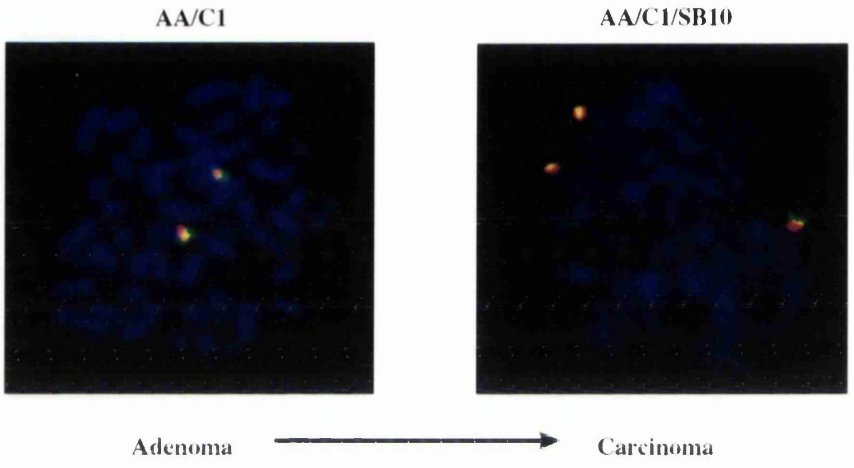
Cell were lysed and cellular protein was separated by SDS-PAGE followed by immunoblotting carried out using anti FAK antibody (Figure 14b). Protein levels were quantified by densitometry (Figure 14c). Quantification results show that the increase in *fak* gene copy number was coincident with up-regulation of FAK protein expression. The several fold increase at protein level cannot, however, be explained by the gain of just one extra copy of *fak*. This suggests that there might be other mechanisms of protein upregulation involved in this case.

3.8 Increase in *fak* gene copy number is accompanied by an increase in gain of *c-myc* gene copy number

As mentioned in section 3.1.1, *c-myc*, a known proto-oncogene, also localises to chromosome 8q24 in the human genome, the region where *fak* has also been

Figure 14 A copy number gain of *fak* gene accompanies increased FAK protein expression during colonic adenoma to carcinoma conversion *in vitro*: (a) Metaphase chromosome preparations of the AA/C1 colon adenoma cell line and its invasive derivative AA/C1/SB10 were probed with biotin labelled *fak* probe (visualised as green), and DIG labelled *c-myc* probe (visualised as red). The chromosomes were stained with DAPI. (b) FAK expression levels in the early colonic adenoma PC/AA, clonogenic adenoma AA/C1 and invasive carcinoma carcinoma AA/C1/SB10 cell lines were determined by immunoblotting using anti FAK specific serum as probe (carried out by V. Brunton, Beatson Institute, Glasgow, UK). (c) Quantitation was carried out by scanning laser densitometry and is expressed as ODXmm^2 (arbitrary units).

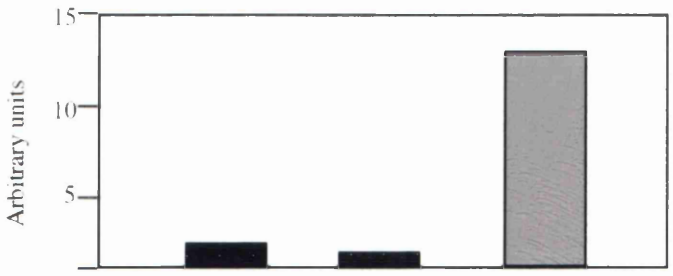
a Progression from adenoma to carcinoma in the *in vito* colon cancer model is accompanied by gain in *fak* gene copy number



b Expression of FAK protein at different stages of *in vitro* colon cancer progression

PC/AA ⟶ AA/C1 ⟶ AA/C1/SB10

c Densitometric quantitation of FAK protein level in the adenoma and carcinoma cell lines



PC/AA ⟶ AA/C1⟶AA/C1/SB10

predicted to lie. There is, thus, some likelihood of *c-myc* exerting the selective pressure for the gain of multiple copies of the region during tumour progression. It was therefore important to determine if there was a co-gain of the *c-myc* locus in the cell lines that showed an increase in *fak* gene copy number.

FISH analysis was carried out on the cell lines that showed an increase in *fak* copy number. *fak* probe was biotin labelled as before while DIG-labelled *c-myc* probe was obtained commercially (Appligene). Different labelling made it possible to visualise both the loci simultaneously. HT29, the colon cancer cell line which had earlier been shown to contain an amplification of *fak* also displayed *c-myc* amplification (Figure 15a). BICR6 along with all the BICR cell lines tested also had the same number *c-myc* gene copies as *fak* (Figure 15b). The adenoma to carcinoma conversion in the *in-vitro* colon cancer progression model was also accompanied by a gain in *c-myc* gene copy (Figure 14c).

3.9 Increase in *c-myc* copy number is not always associated with an increase in c-Myc protein level

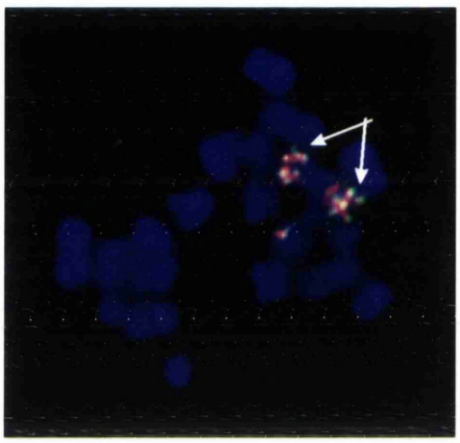
Results from the previous section raised the possibility that perhaps the gain in *fak* gene copy number was coincidental, given the established oncogenic nature of *c-myc*. If this were indeed the case, one prediction would be an associated increase in c-Myc protein levels in the cell lines since that would lead to a selective advantage to the cell during tumour progression.

To investigate if c-Myc was elevated and thus potentially responsible for the acquisition and retention of the whole region (through amplification, isochromosome formation or increased gene dosage) by the cell lines, equal amounts of protein were separated by SDS-PAGE, blotted and quantified (Figure 16a). The experiment was carried out at the same time as immunoblotting for FAK, and therefore the loading control was the same as shown in Figure 12a. The protein levels were quantified and plotted (Figure. 16b). The graph shows the comparison between the FAK and c-Myc protein levels in the BICR cells and the HNK normal keratinocyte. Amongst the BICR cell lines examined, there was considerable variation in c-myc expression.

Figure 15 Gain in *fak* gene copy number in cancer cell lines is accompanied by a gain in *c-myc* copy number: (a and b) Metaphase chromosome preparations of HT29, a colon cancer cell line, and BICR6, a squamous cell carcinoma cell line were probed with biotin labelled *fak* probe (visualised as green) and DIG labelled *c-myc* probe (visualised as red). The chromosomes were stained with DAPI.

a Co-amplification of *c-myc* gene in HT29, a colon cancer cell line, along with *fak*

HT29 (Colon)

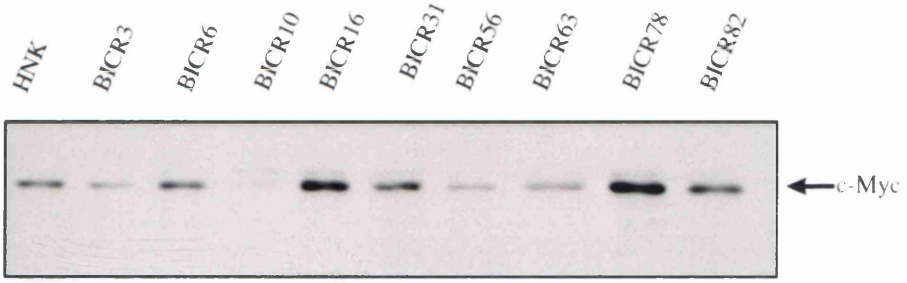


b Co-gain of *c-myc* gene in BICR6, on of the BICR lines tested

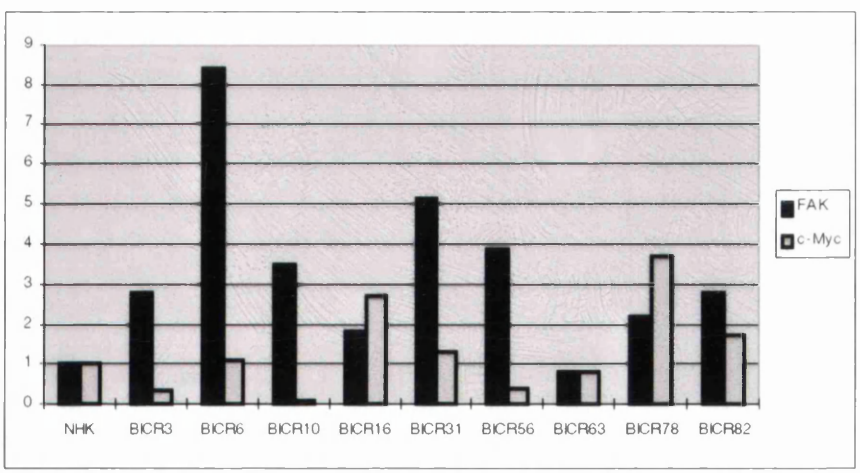
BICR 6 (SCC)

Figure 16 Variation in c-Myc protein levels in BICR cell lines: (a) 25μg of total protein from cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-c-Myc antibody. (b) Quantitation by scanning laser densitometry is shown relative to the expression of FAK in each of the cell lines.

a Expression of c-Myc protein in BICR cell lines and normal keratinocytes (HNK)



b Densitometric quantitation of c-Myc protein expression and comparison with FAK protein levels



Only two cell lines BICR 16 and BICR 78 (2/9) displayed more than two fold elevated levels of c-Myc when compared to normal keratinocytes. Thus, although *fak* and *c-myc* genes are both similarly increased in copy number in the BICR cell lines, c-Myc protein expression levels are not always coordinately increased, at least *in vitro.*

In addition, unlike FAK, no increase in the expression of c-Myc protein was seen as the adenoma cells progressed to become invasive carcinoma cells *in-vitro* (C Paraskeva, unpublished observation).

3.10 Discussion

Linkage of *fak* to *c-myc* in the mouse had suggested that *fak* was most likely to localize to chromosome 8q24, where *c-myc* is located and the region has synteny with the mouse chromosome 15 where *fak* was shown to localize in mouse (Fioderek and Kay, 1995). Results obtained through FISH analysis in this chapter confirm that *fak* does indeed localize to the telomeric end of the long arm of chromosome 8.

Several studies have found FAK protein levels to be elevated in different cancer cell lines and samples from cancerous tissue. FISH results indicate that one of the important mechanisms through which cells are able to overexpress FAK protein is by genetic changes. Two of the cell lines examined, CALU3, and HT29 carried amplification of *fak*. Some of the cell lines had isochromosomes involving the q arm of chromosome 8. In other cell lines with more than two copies of *fak*, the gain in copy number was accounted for by the gain of extra copies of the chromosome or parts of chromosome 8. 100% (5/5 and 7/7) of colon and breast cancer cell lines examined showed an increase in the *fak* gene copy number, while 72% (8/11) of the lung cancer cell lines displayed more than 2 hybridization signals in a high percentage of nuclei. It was, however, not possible to correlate this gain in copy number with an increase at the protein level of FAK due to lack of normal control cell lines for colon, breast or lung. A study carried out by Owens *et al.*, (1995) reported that FAK was found to be overexpressed at the protein level in 17/17

metastatic colonic lesions and 22 out of 25 invasive and metastatic breast tumours compared to normal tissue in patients from whom the tumour samples were taken. This was further confirmed by analysis carried out in the BICR cell lines. FISH analysis showed that all the 9 BICR cell lines examined showed greater than two hybridization sites for *fak*. Comparison of protein levels of FAK showed 7/9 (>70%) cell lines to have a higher steady state levels of FAK than in normal human keratinocytes, HNK. To confirm that genetic alterations involving *fak* were actually involved in the process of carcinogenesis, and not just an artifact of cell culturing *in vitro*, FISH was carried out on primary tumour sections. A vast majority of the cells within the section showed more than two hybridisation signals for *fak*. Some normal cells that were also part of the section had the normal two copies of the gene.

Although gain in *fak* gene copy number through genetic alterations in the cell was generally accompanied by increased levels of FAK protein, there was no direct correlation between the two. For example, both BICR6 and BICR16 have around 6 copies of *fak* but they do not express the same level of FAK protein. In fact, BICR16 was found to express lower levels of FAK as compared to even the normal HNK cells which have the normal 2 copies of *fak*. BICR63 is another cell line that had lower level of FAK expression than the normal keratinocytes. Thus there are cases, though few, where increase in *fak* gene copy number is not accompanied by overexpression of the protein. This might mean that not all the copies gained by the cell are expressed or that they were expressed but the turnover rate for the protein was increased. This finding is contrary to the results of another study that established positive correlation between copy number and protein expression of the *topoisomerase 1* gene in colon and breast cancer lines (McLeod and Keith, 1996).

Given the potential role of FAK in cell growth, migration and apoptosis (see section 1.3), all properties required by a cell to acquire invasive characteristcs, it is not surprising that so many of the cancer cell lines show an upregulation of FAK protein and gain in *fak* gene copy number. Results showing an increase in FAK protein level accompanied by gain in *fak* gene copy number during conversion from adenoma to carcinoma in the *in vitro* model of colon progression, support this. An earlier study

had suggested the potential role for FAK in regulating enhanced cell invasion showed by the carcinoma cells upon EGF stimulation (Brunton *et al.*, 1997).

Comparisons between gene copy number gain and protein quantification for FAK also showed that in some cell lines, the number of copies gained is not able to account for all the elevation at protein level. This is best exemplified in the case of the *in vitro* model of colon tumour development. Here, the progression from the adenoma to the carcinoma was accompanied by a gain of one gene copy of *fak* but the increase at the protein level was about 5 fold. Obviously, one extra copy of *fak* cannot account for a five-fold increase in protein expression. There should, thus, exist other mechanisms by which some cells might upregulate FAK protein level. A mutation in the promoter region or translocation of the gene to a more heavily transcribed region of the genome that enhances the transcription of the gene might play an important role in enhancing the protein levels. Increase in transcription can be quantified using RNA protection assays. These assays involve protection of the 5' end of the mRNA with a radiolabelled probe, digestion of the unprotected part with a nuclease, followed by detection of the radiolabelled probe. The intensity of the band indicates the amount of mRNA present. Increase in mRNA level could also be due increased mRNA stability. The assay to determine mRNA stability would involve inhibition of transcription using actinomycin D followed by a time-course detection of mRNA levels. Increased protein stability can also account for increased protein levels. However, earlier pulse-chase experiments carried out in the lab had indicated FAK to be a relatively stable protein (Val Fincham unpublished data), unless stimulated to undergo proteolysis during apoptosis or focal adhesion turnover (see section 1.3).

The presence of genetic alterations like amplifications and isochromosomes indicate a selective advantage conferred on the cells by them. These structures are never found in normal cells and won't be retained by a cell unless their presence is of importance to the cell. Cancer cells are under constant selective pressure and only those cells that exhibit growth, motility (important in invasion) and survival advantages over other cells are allowed to expand further. Thus, genes like *fak* that localize to these alterations should potentially be able to influence any of the above

mentioned processes. As suggested earlier, in the case of FAK, one possibility is that up-regulated FAK contributes to ECM-dependent tumour growth by mediating signaling from fibronectin to the Ras/MAP kinase pathway that is implicated in proliferation control (Schlaepfer *et al.*, 1994). Another potential role proposed for FAK is in making transformed cells able to grow in an anchorage-independent manner (Frisch *et al.*, 1996b). As well as in anchorage-deprived cells, FAK can also act as a survival factor for adherent cells (Hungerford *et al.*, 1996; Xu *et al.*, 1996). Thus, FAK may contribute to ECM-dependent growth and survival of tumour cells.

As well as tumour cell growth and survival, another likely role for elevated FAK in tumour cells is in the acquisition of an invasive phenotype. Cancer cell invasion is a complex process that requires cells to adhere and migrate through underlying ECM. Since FAK functions as a key regulator of ECM-dependent cell migration (Ilic *et al.*, 1995, 1996; Gilmore and Romer, 1996; Cary *et al.*, 1996), and since endogenous levels of FAK expression can limit the rate of motility in some cell types (Cary *et al.*, 1996), it seems likely that elevated FAK in tumour cells may release normal constraints on the rate of cell motility, thus enhancing invasive potential. Consistent with this possibility it was found in this study that the gain of an additional copy of the *fak* gene, associated with elevated FAK protein level, occurred relatively late in an *in vitro* model of colon carcinoma development and was coincident with acquisition of an invasive phenotype (Brunton *et al.*, 1997).

FISH analysis carried out on the cell lines showed that there was a gain in *c-myc* gene copy number along with *fak*. Indeed, one of the main reasons behind this study was the finding that CALU3, a lung cancer cell line was found to carry an amplicon containing *c-myc* (Hoare *et al.*, 1997). The size of the amplicon suggested that there might be other genes within the region that could be co-amplified along with *c-myc* and might be responsible for exerting the selective advantage. Finding *fak* to be co-amplified with *c-myc*, prompted an investigation into which of these two genes was selected for in the cells. Comparison of levels of expression of the two proteins was carried out in the BICR cell lines. Only 2/9, BICR16 and BICR78, showed an increase in c-Myc protein levels when compared to normal keratinocytes, while as many as 7/9 of the BICR cell lines showed an increase in the level of FAK protein.

Furthermore, although an additional copy of *c-myc* was gained at the same stage as *fak* during colon carcinoma development in the *in vitro* model, there was no concomitant increase in the expression of c-Myc. An earlier study had shown that tumour cells overexpressing *c-myc* had reduced growth factor requirement and a shortened G1-phase of cell cycle (Prins *et al.*, 1993). Thus, overexpression of *c-myc* is required by the cells for it to confer any growth advantage. However, the data implies that *fak*, at least in some cells, is as strong a candidate as *c-myc* as the gene responsible for the maintenance of increased dosage of this region of the genome. However, the possibility of the existence of other gene/s within this region, that might be responsible for the retention of the region in increased dosage, still exists.

Chapter 4: Results 2

Role of focal adhesion kinase in cell survival

4.1 Introduction

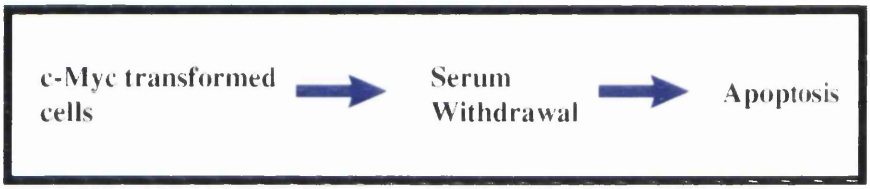
As stated earlier, FAK has been implicated in cell survival by several studies. Earlier work in our lab had raised the possibility of proteolysis of FAK preceding commitment to c-Myc induced apoptosis in CEF after serum withdrawal (Crouch *et al.*, 1996). Results of another study by Frish *et al.,* (1996a), showed that constitutively activated forms of FAK were able to rescue two established epithelial cell lines from anoikis (detachment induced cell death), suggesting apoptosis requires the inactivation of FAK, possibly through proteolysis. One of the objectives of this study was, therefore, to determine if indeed FAK proteolysis played a causal role during apoptosis. Specifically, we tested whether interfering with FAK proteolysis blocked apoptosis. To address this, the strategy was to first determine the enzyme responsible for FAK cleavage during apoptosis with calpain I and caspases being the likely candidates. Having established that, we wished to inhibit FAK cleavage by using enzyme-specific inhibitors, and ask whether this correlated with cell survival. Finally, if inhibition of FAK proteolysis promoted survival, the strategy would be to identify FAK cleavage sites and to generate non-cleavable FAK mutants and study their effect of the overexpression on survival.

4.2 FAK undergoes proteolysis in c-Myc induced apoptosis in CEF

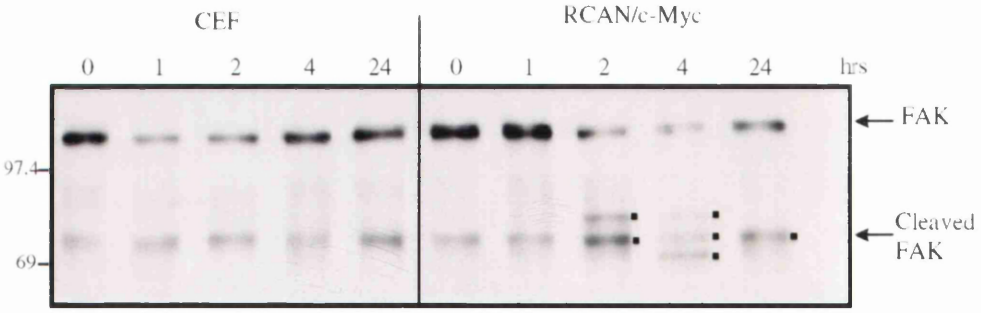
Withdrawal of serum has been firmly established as an inducer of apoptotic cell death in c-Myc transformed fibroblasts (Evan *et al.,* 1992). Transfer of c-Myc transformed CEF to low serum medium induces cell detachment and death in a manner analogous to the effects of these conditions in fibroblasts from other species (Crouch *et al.,* 1996). This latter study showed proteolytic cleavage of FAK during c-Myc-induced apoptosis. The experimental scheme is depicted (Figure 17a). c-Myc transformed CEF were deprived of serum leading to the induction of apoptosis within two hours of serum withdrawal. Levels of FAK were determined by immunoblotting lysates prepared from CEF, or c-Myc transformed CEF at different time intervals after serum withdrawal. In comparison to normal CEF, full length FAK diminished

Figure 17 c-Myc induced apoptosis is accompanied by FAK cleavage: (a) c-Myc transformed CEF were induced to undergo apoptosis by serum-withdrawal. (b) Levels of FAK were determined by immunoblotting lysates prepared from CEF or c-Myc-transformed CEF at various time points after serum-withdrawal (hrs) (carried out by V.Fincham, Beatson Institute, Glasgow, UK).

a Diagramatic representation of induction of apoptosis in c-Myc transformed CEF



b FAK proteolysis during c-Myc induced apoptosis in CEF



in c-Myc expressing CEF as cells were induced to detach and die in c-Myc transformed cultures (Figure 17b). Loss of full length FAK was accompanied by the accumulation of two distinct products of proteolytic cleavage migrating between 60-90 Kd (Figure 17b; described also in Crouch *et al.,* 1996).

4.3 Both calpain and caspases can cleave FAK *in vitro*

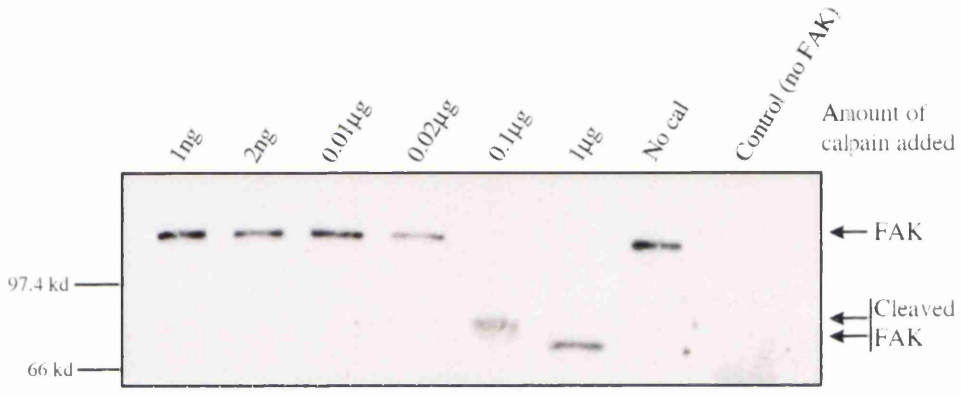
In order to determine if inhibiting FAK cleavage could inhibit apoptosis, it was first important to determine which enzyme/s were responsible for its proteolysis during apoptosis. Calpain was a likely candidate since it is known to localise to the focal adhesions (Beckerle, 1986). Calpain has also been shown to cleave various focal adhesion proteins like talin, α-actinin, Src and even FAK itself (Beckerle *et al.*, 1986; Selliah *et al.*, 1996; Oda *et al.*, 1993; Cooray *et al.*, 1996). Caspases were also likely to play a role in FAK proteolysis during apoptosis as they were already known to cleave various cellular substrates during initiation and execution of apoptosis (reviewed in Harvey and Kumar, 1998).

In vitro cleavage assays with calpain and caspases were carried out using immunoprecipitated FAK from CEF. Cleavage reactions with calpain I were set up using different concentrations of the enzyme to establish the amount required for FAK cleavage. Reactions were carried out at 30^{O}C for 30min, followed by separation of proteins by 7.5% SDS-PAGE. Immunoblotting was carried out using anti-FAK antibody. Results showed that 0.1μg/μl and 1μg/μl of the protease were able to cleave into fragments sized between 60-90 kd (Figure 18a). Earlier studies had also shown FAK to be cleaved into two different sized fragments migrating *in vivo*, between 60-90 kd, and it was suggested that the larger fragment was further cleaved into the smaller fragment (Crouch *et al.*, 1996).

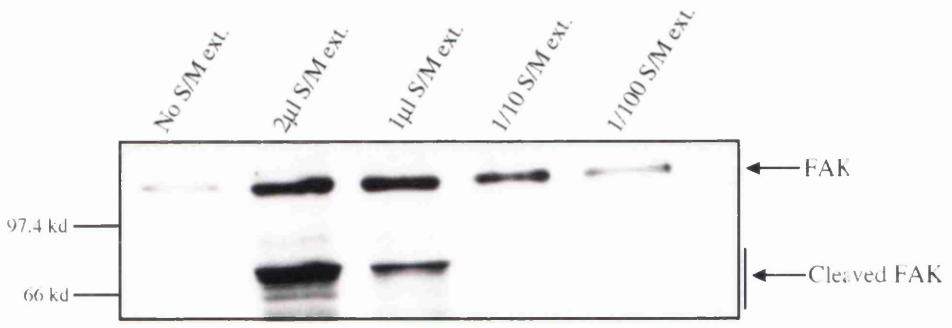
To establish FAK proteolysis by caspase proteases *in vitro*, S/M extracts (a gift from Bill Earnshaw, Edinburgh, UK) were used. S/M extracts are cytoplasmic extracts made from chicken DU249 cells that become committed to apoptosis as a result of an S-phase aphidicolin-induced cell-cycle arrest and are subsequently collected in M-phase (Wood and Earnshaw, 1990; Lazebnik *et al.*, 1993). These extracts are

Figure 18 FAK is cleaved *in vitro* by both calpain I and caspases: (a) To titrate calpain I with respect to FAK cleavage *in vitro*, FAK was immunoprecipitated and cleavage reactions were carried out at $30^{O}C$ for 30min. Proteins separated by SDS-PAGE were probed for full length, and cleaved products of FAK. (b) To examine FAK proteolysis by caspases, *in vitro* reactions were carried out at $37^{O}C$ for 30min. As before, immunoblotting was carried out to detect full length FAK and its cleaved products.

a *In vitro* cleavage of FAK by calpain I



b *In vitro* cleavage of FAK by caspases



enriched for caspases and have been shown to imitate apoptotic events *in vitro* (Lazebnik *et al.*, 1993; Lazebnik *et al.*, 1994). Immunoprecipitated FAK was resuspended in ICE buffer and the cleavage reactions carried out at 37^{o}C for 30min. As in the case of calpain I, different amounts *i.e.*, 2µl, 1µl, 1/10µl, and 1/100µl, of the S/M extracts were used in order to determine the optimal amount required to cleave FAK *in vitro.* Proteins were separated by SDS-PAGE and immunoblotting was carried out using anti-FAK antibody. 1µl, and 2µl of the S/M extracts were found to cleave FAK, generating fragments migrating between 60-90 kd (Figure 18b).

Thus, both calpain I and caspases were able to cleave FAK *in vitro* to generate products of similar lengths as those generated during apoptosis in c-Myc transformed CEF *in vivo*.

4.4 Caspases, not calpain are most likely responsible for FAK proteolysis during apoptosis

In order to determine whether calpain or the caspases were more likely to be responsible for cleavage of FAK during apoptosis *in vivo*, it was important to compare the sizes of the *in vitro* and the *in vivo* products. Thus, experiments were initially carried out to induce apoptosis in c-Myc-transformed CEF after serum withdrawal. These experiments were, however, technically difficult to reproduce with respect to the apoptotic response induced upon serum withdrawal. The reasons for the weak response of the culture observed on many occasions, after serum withdrawal from c-Myc-transformed cells, were not clear, but the inconsistency of the response was noted by a number of members of the lab (V Fincham, M Frame, M Agochiya). One possible explanation for the inconsistency is the culture density, which is thought to have an effect on the sensitisation of the cells to c-Myc-induced apoptosis. Therefore, another mode of inducing apoptosis, which is outlined below, was utilised to study the importance of FAK proteolysis during apoptosis.

The method for inducing reproducible apoptosis was changed to one where apoptosis was induced as a consequence of v-Src inactivation in serum-deprived v-Src

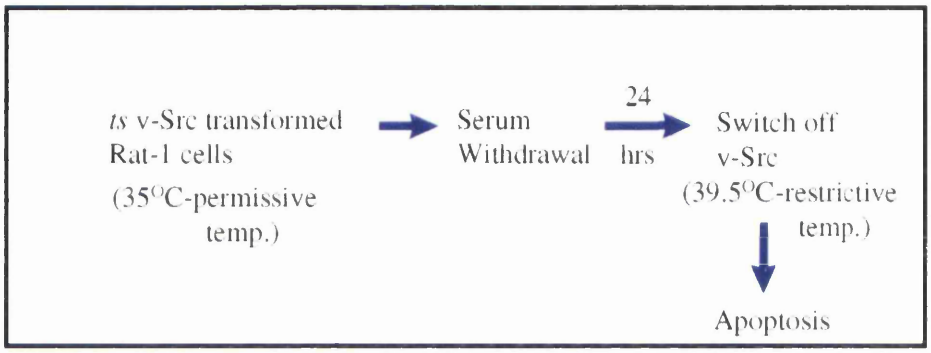
transformed Rat-1 cells. This particular method of inducing apoptosis in *ts LA29 v-src* transformed Rat-1 fibroblasts was developed by D. Johnson (Beatson Institute, Glasgow, UK). Under these conditions, induction of apoptosis in v-Src transformed Rat-1 cells was accompanied by FAK proteolysis producing cleavage products of 60-90 kd. The experimental scheme is outlined (Figure 19a). Characterisation of the induced cell death to be apoptosis is described in the following chapter (section 6.2). This apoptotic response provided a good and reproducible alternative to study apoptosis and the role of FAK in cell survival.

In vitro cleavage assays were carried out to compare cleavage products to the ones generated *in vivo* during apoptosis. For this, FAK cleavage products, generated by caspase, were compared to the *in vivo* products. Calpain I cleavage products were not compared since earlier experiments had shown that both calpain and caspases cleaved FAK to generate similar sized fragments (section 4.3). Immunoprecipitated FAK from v-Src transformed Rat-1 fibroblasts was used in reactions carried out at $37^{O}C$ for 30min using the S/M extracts (described before in section 4.3). Proteins were separated using SDS-PAGE. Lysates from transformed Rat-1 cells undergoing apoptosis (harvested 8 hours after induction of apoptosis), were also separated on the same gel. Immunoblotting was carried out using anti-FAK antibody. *In vitro,* FAK was cleaved into three different fragments that migrated around 60-90 kd (Figure 19b). The smaller of the two lower fragments was the same size as that generated *in vivo* during apoptosis. Also, the upper fragment detected in the apoptotic lysates, was generated during *in vitro* cleavage, although, the intensity of the band was weaker (Figure 19b). This confirmed that both chick FAK and murine FAK were similarly cleavable both *in vivo* and *in vitro* and further indicated the possibility of either calpain I or caspases to be responsible for FAK proteolysis *in vivo*.

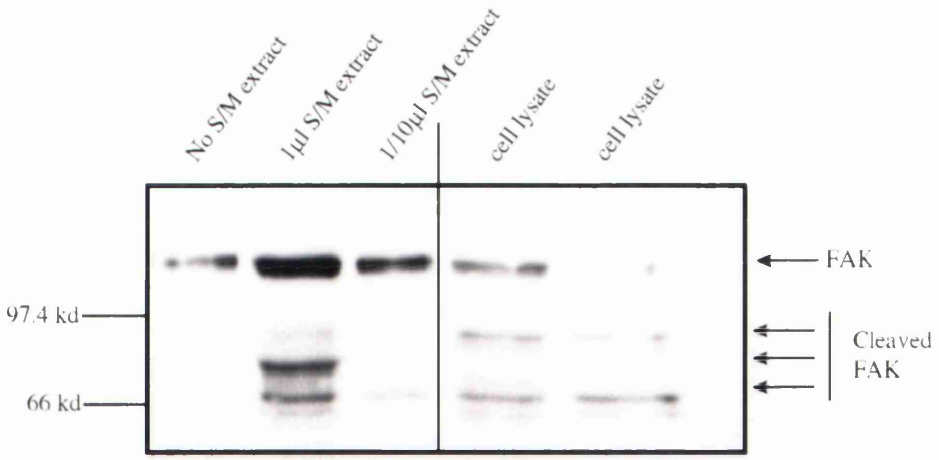
To determine which enzyme/s were responsible for FAK proteolysis *in vivo* during apoptosis, experiments were carried out to study the effect of inhibiting caspase activity on FAK proteolysis during apoptosis. To examine the role played by caspases, a broad spectrum cell permeable caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp(O-methyl)-fluoromethylketone (ZVAD.fmk) was used (McCarthy *et al.*, 1997; Garcia-Calvo, 1998). Also, to investigate if calpain I was responsible for FAK

Figure 19 *In vitro* cleavage of FAK generates similar sized fragments as those detected *in vivo*: (a) To induce an apoptotic response in v-Src transformed Rat-1 cells, they were serum-starved for 24 hours followed by inactivation of v-Src by shifting the cells from 35^{o}C to 39.5^{o}C. Cell lysates from dying cells were obtained at 8 hours after induction of apoptosis and separated by SDS-PAGE. The lysates were probed with anti-FAK antibody. (b) To compare the sizes of the *in vitro* and *in vivo* cleavage products, caspase cleavage products were also separated by SDS-PAGE and immunoblotted at the same time.

a Diagramatic representation of induction of apoptosis in *ts LA29 v-src* transformed Rat-1 fibroblasts



b Comparison of the *in vitro* and *in vivo* cleavage products of FAK



proteolysis during apoptosis *in vivo*, the effects of inhibiting calpain I activity were studied. In order to do this, N-acetyl-leucyl-leucyl-norleucinal (aLLN), a modified peptide which inhibits the activity of calpain I, calpain II by competing for the active site of the enzyme, was used (Wang and Yuen, 1994).

An apoptotic response was induced in transformed Rat-1 fibroblasts as described in Figure 19a. The effects of the inhibitors were studied at two different concentrations of 10μM and 100μM. Both the inhibitors, ZVAD.fmk and aLLN were added an hour before inactivation of v-Src. Cells were harvested at different times after induction of apoptosis. Cell lysates were separated using SDS-PAGE and immunoblotting was carried out.

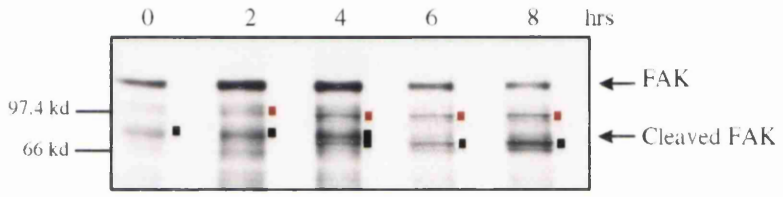
In the control cells, to which no inhibitor was added, FAK was cleaved, as expected, into fragments of sizes between 60-90 kd (Figure 20a). At 2 hours, only the larger of the two lower fragments was present, while the upper band was not, but 4 hours after v-Src inactivation, both of the lower fragments and the upper fragment were visible. At the 6 and 8 hour time points, accumulation of the smaller of the two lower fragments was seen, although there was no change in the upper band. There was also an increase in the products with increase in time, accompanied by a decrease in full length FAK protein.

Addition of 10μM of ZVAD.fmk had no effect on FAK cleavage during apoptosis with the cleavage pattern being similar to that seen in the control cells (Figures 20a and 20b). However, at the concentration of 100μM, ZVAD.fmk was able to block FAK cleavage completely (Figure 20c). There was no change in the amount of full length FAK accompanying progression of apoptosis and no accumulation of the cleavage products was evident.

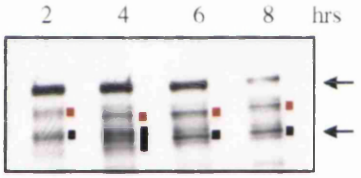
Addition of aLLN, the calpain I and calpain II inhibitor, however, did not have any visible effect on FAK proteolysis (Figures 20d and 20e). However, due to lack of a control to confirm the inhibitor's activity, this could not be established beyond a doubt. Nevertheless, comparison of *in vitro* and *in vivo* cleavage products and

Figure 20 Inhibition of caspases, but not calpain, inhibits FAK proteolysis during apoptosis: (a) v-Src transformed Rat-1 cells were induced to undergo apoptosis in the absence of serum and v-Src activity. Cell lysates obtained at different time points after v-Src inactivation, were separated by SDS-PAGE and immunoblotted with anti-FAK antibody to detect full length FAK and its cleavage products. FAK was cleaved into an upper band (indicated by red symbols), and two lower bands (indicated by black symbols). (b, c, d, e) At the same time, either ZVAD.fmk or aLLN, inhibitors of caspases and calpain respectively, were added to other cells an hour before inactivation of v-Src at 10μM or 100μM. Lysates from these cells were also collected at different time points, saparated by SDS-PAGE and immunoblotted for the detection of FAK cleavage products.

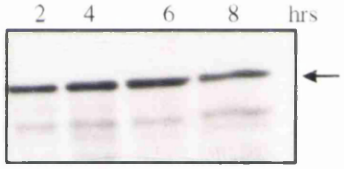
a FAK proteolysis in serum-starved transformed Rat-1 cells undergoing apoptosis



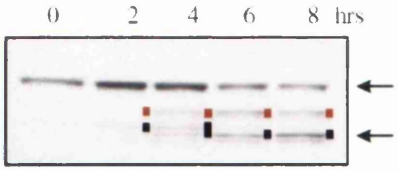
b FAK proteolysis in cells treated with 10μM ZVAD.fmk



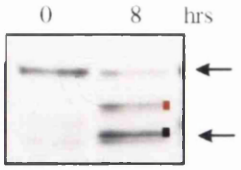
c Inhibition of FAK cleavage in cells treated with 100μM ZVAD.fmk



d FAK proteolysis in cells treated with 10μM aLLN



e FAK proteolysis in cells treated with 100μM aLLN



chemical inhibitor data suggest that caspases were most likely to be responsible for FAK proteolysis during apoptosis in serum-deprived v-Src transformed Rat-1 cells.

4.5 Inhibition of FAK cleavage does not prevent apoptosis in transformed Rat-1 fibroblasts

With results indicating that caspases were the likely proteases responsible for cleaving FAK, it was possible to investigate whether inhibition of FAK proteolysis correlated with suppression of apoptosis. Thus, 100μM ZVAD.fmk was used in order to inhibit FAK cleavage and to study its effects on the induction of apoptosis in serum-deprived v-Src transformed Rat-1 cells. The experiment was carried out as described in the previous section (depicted in figure 19a), and ZVAD.fmk was added one hour before inactivation of v-Src. Cells were harvested at the 0, 2, 4, and 6 hour time points and fixed (as described in the Materials and Methods). Terminal deoxynucleotidyl transferase (TdT)- mediated dUTP-biotin nick end labelling (TUNEL) method was carried out as a measure of cells undergoing apoptosis at the different time points. The percentage of cells undergoing apoptosis in the presence and absence of ZVAD.fmk was determined (Figure 21). The experiment was repeated 3 times and the graph is representative of the results obtained. As is clearly shown, blocking FAK cleavage with ZVAD.fmk did not prevent the cell death. The percentage of cells undergoing apoptosis with ZVAD.fmk was not substantially different from control cells in the absence of ZVAD.fmk (Figure 21). Thus, inhibition of FAK had little or no effect on apoptosis (as measured by TUNEL), indicating that its cleavage was not a critical determinant of the commitment to programmed cell death.

4.6 Discussion

There is a substantial body of evidence implicating FAK in anchorage-independent cell survival. Attenuation of FAK signaling at the integrins induces apoptosis in some cell types (Hungerford *et al.*, 1997; Xu *et al.*, 1996). A study by Cooray *et al.*, 1996, suggested that sequential proteolysis of FAK was perhaps one of the mechanisms used by a cell to attenuate its autokinase acitvity and downstream signalling. Work

Comparison of percentage of serum-deprived transformed Rat-1 cells undergoing apoptosis with and without the caspase inhibitor, ZVAD.fmk, after inactivation of v-Src

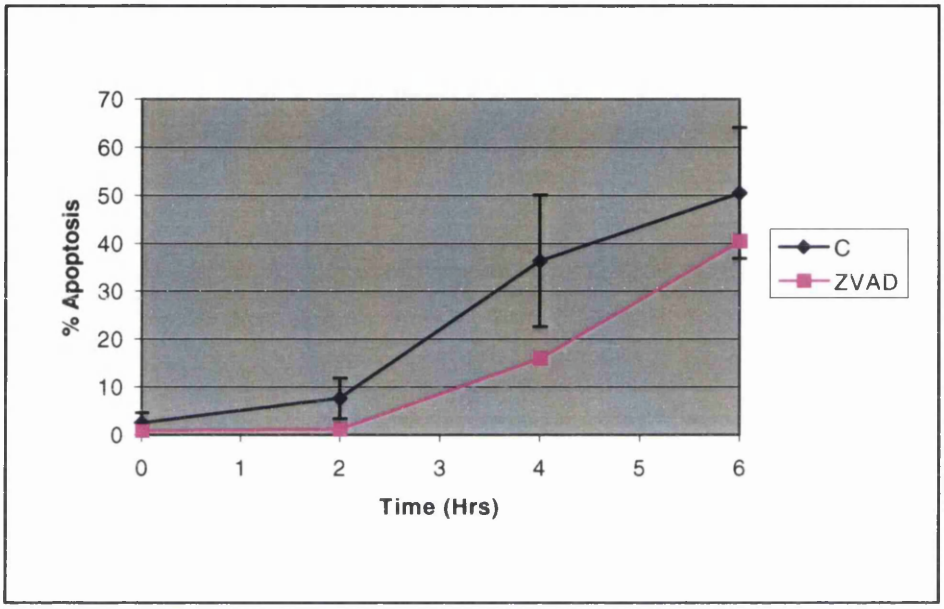


Figure 21 Inhibition of FAK cleavage does not promote cell survival:
v-Src-transformed Rat-1 cells were induced to undergo apoptosis in the absense of serum and after v-Src was switched off. Cells were fixed and TUNEL labelled at different times after inactivation of v-Src. The TUNEL labelled cells represent the percentage of cells undergoing apoptosis. The mean of several experiments was plotted as control, the error bars corresponding to the standard error. Similar experiments with ZVAD.fmk treated cells were also carried out. The plotted line is representative of 3 separate experiments.

done by our group suggested commitment to apoptosis in c-Myc transformed CEF followed proteolysis of FAK (Crouch *et al.*, 1996). Also, suppression of FAK proteolysis by a β1-specific integrin antibody promoted cell survival in these cells. To further investigate the role of FAK proteolysis in the regulation of apoptosis, we first determined which enzymes were responsible for cleaving FAK *in vivo* during induction of apoptosis in c-Myc transformed CEF. Calpain I and caspases were the two proteolytic enzymes investigated. Calpain I localises to focal adhesions like FAK and cleaves it in thrombin stimulated platelets (Beckerle, 1986; Cooray *et al.*, 1996). Caspases were examined because of their well documented role in proteolysis during apoptosis (reviewed in Harvey and Kumar, 1998). Comparison of *in vitro* cleavage products of FAK by the two proteases and subsequent inhibition experiments confirmed that caspases were most likely the proteases involved in FAK cleavage during induction of apoptosis in v-Src transformed Rat-1 cells. A calpain I inhibitor was unable to prevent FAK cleavage, although calpain had been able to cleave FAK *in vitro* to produce products of similar but distinguishable size. This, taken together with the results of an earlier study which showed prevention of FAK cleavage in thrombin stimulated platelets treated with calpain inhibitor-1 (Cooray *et al.*, 1996), suggested that calpain I might be involved in FAK proteolysis, but apparently not during apoptosis. In other words, calpain I cleavage of FAK might have some role in focal adhesion functions like cell migration or growth. FAK proteolysis has been observed during cell transformation induced by v-Src (Fincham *et al.*, 1995). However, instead of being cleaved into distinct 60-90 kd fragments, as is the case during apoptosis in c-Myc transformed CEF and v-Src transformed Rat-1 cells, FAK gets completely degraded during transformation. This degradation of FAK is thought to play a role in focal adhesion turnover and thus in cell migration.

Proteolysis of FAK by caspases during apoptosis has now been confirmed by another study. FAK was sequentially cleaved into two different fragments early in Apo-2L-induced (a member of the tumour necrosis family) apoptosis in suspended Jurkat T cells (Wen *et al.*, 1997). The first cleavage product (85 kd) appeared after 2 hours of addition of Apo-2L, and the second (77 kd) after 4 hours. After 24 hours only the 77 kd fragment remained. This is in agreement with the results presented in this study which also showed that caspase-induced cleavage of FAK generated two fragments

sized between 60-90 kd. The cleavage products were detected after 2 hours of induction of apoptosis in the v-Src transformed Rat-1 cells. Also, there was an increase in the amount of the smaller sized cleavage product with increased time. This suggests that the second fragment is generated through further cleavage of the first fragment. It was suggested by Wen *et al.*, (1997) that cleavage of FAK by caspase 6, which is required for the generation of the second fragment (according to *in vitro* results), is only possible after it has been first cleaved by caspases 3 and 7 to generate the first fragment (Wen *et al.*, 1997).

It has now been confirmed that FAK is cleaved by the capases at two different sites. The cleavage sites in FAK lie within the carboxy terminal half of the protein (Gervais *et al.*, 1998). Cleavage of FAK at either or both of the two sites generates a FRNK like fragment. Thus, proteolysis of FAK is thought to abrogate FAK mediated survival signals by two mechanisms (Gervais *et al.*, 1998). Firstly, it decreases the total amount of full length functioning FAK. Secondly, generation of FRNK like fragments may lead to competitive inhibition of FAK. As stated earlier, FRNK has been shown by other studies before to act as an inhibitor of FAK by competing for common binding proteins (Richardson and Parsons, 1996).

In order to investigate the importance of FAK proteolysis during apoptosis in serum-starved v-Src transformed Rat-1 cells, ZVAD.fmk, a general inhibitor of the caspases was used. Cells were treated with ZVAD.fmk before the induction of apoptosis. Results indicated that although, ZVAD.fmk was able to prevent FAK proteolysis by the caspases during apoptosis, it was unable to prevent apoptosis. Thus, inhibiting FAK cleavage was unable to promote cell survival. This is contrary to the findings of another study where a significant increase in cell survival was observed upon treatment of Jurkat T cells with ZVAD.fmk before induction of apoptosis by Apo-2L treatment (Wen *et al.*, 1997).

Chapter 5: Introduction 2

5.1 Apoptosis: Programmed cell death

Apoptosis is a naturally occuring cell suicide programme intrinsic to metazoan cells (reviewed in Kerr *et al.*, 1972; Wyllie, 1993). It provides an efficient way for the elimination of unwanted cells and is a default pathway which is engaged unless the cells receive sufficient signals to negate it (reviewed in Raff *et al.*, 1994). It is required in the process of development and maintenance of proper shape and function in animals (reviewed in Meikrantz and Schlegel, 1995). Cell death induced in normal and tumour tissues exposed to low or moderate doses of chemotherapeutic agents or ionizing radiation, also has apoptotic characteristics (Wyllie, 1997). The homeostasis of animals is also regulated through apoptosis (Enari *et al.*, 1998). As mentioned before, apoptosis is also induced in some cells on disruption of the interactions between cells and extracellular matrix (Frisch and Francis, 1994). Anoikis is though to prevent tumour cells from anchorage independent growth and may thus protect the animal from malignancy. Tumours require anti-apoptotic mutations, in addition to inappropriate proliferation, in order to survive and propogate (Evan *et al.*, 1995).

5.1.1 Apoptosis vs Necrosis: morphological distinction

Apoptosis was first recognized by its distinct, stereotyped morphology (reviewed in Kerr *et al.*, 1972; Wyllie, 1997). This is an important distinction between apoptosis and necrosis, a form of acute pathological cell death resulting from cell injury. Morphological changes accompanying apoptosis include dramatic shrinkage of cell volume, along with dilation of endoplasmic reticulum and convolution of the plasma membrane (Wyllie, 1993; Wyllie, 1997). The cell gets fragmented into multiple membrane bound bodies, known as apoptotic bodies, which are engulfed by surrounding cells and removed without any inflammation or damage to the surrounding tissue. This is in contrast to necrosis where the injured cell swells up and the plasma membrane breaks up to release pro-inflammatory components (Cotter *et al.*, 1990). Characteristic changes in the organization of the nucleus also distinguishes apoptosis from necrosis. Apoptosis is accompanied by condensation of the chromatin, which segregates into sharply defined bodies within intact nuclear

envelope. The DNA is often digested by endonucleases into distinct sized fragments (Wyllie, 1993; Wyllie, 1997).

5.1.2 Diverse stimuli can activate apoptosis

Apoptosis can be initiated by a variety of stimuli. Cell damaging stress can cause a cell to die by apoptosis. Diverse stress stimuli such as ionizing radiation, osmotic shock, oxidative stress or microtubule disruption can all induce an apoptotic response (Martin and Cotter, 1990; Payne *et al.*, 1995; Jarpe *et al.*, 1998). This ability of cells to undergo apoptosis due to DNA damage induced by ionizing radiation is utilized for cancer treatment by irradiation (Jarpe *et al.*, 1998). Also, the chemicals used as chemotherapeutic agents induce cancer cells to undergo apoptosis (Lutzker and Levine, 1996; Dive *et al.*, 1992; Houghton, 1999; Suzuki *et al.*, 1999). Many of the chemothrapeutic drugs induce apoptosis as a result of DNA damage (Houghton, 1999).

Apart from cellular stress, growth factor deprivation is also known to induce apoptosis in some cell types. For example, deprivation of survival factors like IL-2 in hematopoietic cells can induce apoptosis (Cohen *et al.*, 1992). Anoikis or cell death induced by deprival of survival signals originating from the ECM has been described before in section 1.1.7 of this thesis (Frisch and Francis, 1994). During neuronal development, apoptosis is required to ensure a definite cell number. Neuronal survival is determined by competition for a limited supply of the nerve growth factor (NGF; Levi-Montalcini, 1987; Oppenheim, 1991). This is imitated *in vitro* when withdrawal of NGF induces cell death in neuronal PC12 cells (Mesner *et al.*, 1992).

Some oncoproteins such as c-Myc are also known to induce apoptosis in serum deprived transformed cells (reviewed in Prendergast, 1999). As mentioned before, Evans *et al.*, (1992), discovered that Rat-1 fibroblasts expressing c-Myc constitutively, when deprived of serum, underwent apoptosis instead of growth arrest. Deregulated expression of c-Myc was also found to accelerate apoptotic cell death of interleukin-3 (IL-3) dependent myeloid cells when deprived of the cytokine and was required for activation induced death of T-cell hybridomas (Kagaya *et al.*,

1997). Since c-Myc expression sensitises cells to a wide range of apoptotic stimuli like growth factor withdrawal, p53-dependent response to genotoxic damage *etc.*, it has been suggested that c-Myc does not itself induce apoptosis but rather acts to sensitise cells to other pro-apoptotic insults (Juin *et al.*, 1999). Details of c-Myc sensitisation to CD95/Fas induced apoptosis is described in section 5.1.3.

Certain specific cell surface receptors belonging to the tumour necrosis factor (TNF) receptor family can also induce apoptosis when engaged by their ligands. Two ligand-death receptor pairs, FasL-Fas (also known as CD95L-CD95) and tumour necrosis factor-α (TNF-α)-TNFR1, are well characterised inducers of apoptosis (Pan *et al.*, 1997). Other examples of the receptors belonging to the TNF family include DR3, DR4 and DR5. TRAIL (also called Apo2L) is a cytotoxic protein that binds to DR4 and DR5 (Sherindan *et al.*, 1997). The receptors contain a stretch of 60 to 80 amino acids within their cytoplasmic domain, known as the death domain (Pan *et al.*, 1997). The receptor proximal events have been best characterised for CD95 as it is the most widely expressed and its ligation with its ligand induces apoptosis in every system studied (Jarpe *et al.*, 1998). Stimulation of CD95 results in aggregation of its intracellular death domain, leading to the recruitment of two key signaling proteins, FADD (also known as MORT1) and caspase 8, that together with the receptor form the death inducing signaling complex (DISC) (Scaffidi *et al.*, 1998). TNFR1, on the other hand, binds FADD indirectly through TRADD (Sheridan *et al.*, 1997). Activation of caspase 8 leads to the activation of caspase 3 (Stennicke *et al.*, 1998), and thus provides a link to the apoptosis execution machinery (as discussed later).

5.1.3 Interaction between different stimuli in the regulation of an apoptotic response

Different apoptotic stimuli can sometimes interact functionally to regulate an apoptotic response. C-Myc induced apoptosis requires interaction on the cell surface between CD95-CD95L (Hueber *et al.*, 1997). Experiments were carried out on Swiss 3T3 cells expressing a conditional 4-hydroxytamoxifen (OHT)-dependent c-Myc protein and that die by apoptosis upon OHT-treatment in low serum. Neutralisation of CD95L using monoclonal antibodies reduces and delays c-Myc-induced apoptosis

in a concentration dependent manner (Hueber *et al.*, 1997). Also, when the effects of CD95 ligation were blocked by expression of dominant negative FADD, which was unable to interact with caspase 8, similar effects were noticed. Other experiments suggested that c-Myc acts downstream of the CD95 receptor by sensitizing the cells to the CD95 death signal (Hueber *et al.*, 1997).

5.2 Caspases

Caspases, a family of proteolytic enzymes, were first identified and described in nematode worm *Caenorhabditis elegans* (*C. elegans*) where cell death was found to be dependent on the activation of Ced3, a cyctein protease (Hengartner, 1998; Harvey *et al.*, 1998). In 1993, Yuan *et al.*, discovered that the product of this death gene was similar to human and murine interleukin-1β-converting enzyme (ICE). ICE is an unusual cytoplasmic cysteine protease that was first isolated from cells of monocytic origin (Shibata *et al.*, 1996). These workers also proposed that since Ced3 acts as a cysteine protease involved in apoptosis, cysteine proteases may have a similar function in mammals. As predicted, overexpression of ICE in Rat-1 fibroblasts caused the cells to undergo apoptosis (Miura *et al.*, 1993). Mutations in the active domain of ICE were able to eliminate this effect. In addition, cell death was also prevented by CrmA, a poxvirus protein that inhibits ICE (Miura *et al.*, 1993). Since then, several ICE homologues have been discovered in mammals and are now collectively known as caspases (Alnemri *et al.*, 1996). So far 11 members of this family have been discovered and are named as Caspase-1, through to caspase-11, where the number is assigned based on its date of publication (Table 3 lists the name of all the caspases known and some of the alternate names they are known by) (reviewed in Harvey and Kumar, 1998).

Thus, caspases are a family of sytosolic cysteine proteases that are stored in most cells as zymogens and play an essential role in the execution of apoptosis (Yang *et al.*, 1998). They can be further divided as apical or initiator (Caspases-2, 8, 9, 10) or executioner (caspases-3, 6, 7) depending on the role they play during the process of apoptosis (Yang *et al.*, 1998). These enzymes act by cleaving on the carboxy-

Table 3 Members of the caspase family

Caspase Member	Alternative names
Caspase-1	ICE
Caspase-2	Nedd 2, ICH-1
Caspase-3	CPP32, Yama, apopain
Caspase-4	TX, ICH-2, ICE-rel-II
Caspase-5	TY, ICE-rel-III
Caspase-6	Mch2
Caspase-7	Mch3, ICE-LAP3, CMH-1
Caspase-8	MACH, FLICE, Mch5
Caspase-9	ICE-LAP6, Mch6
Caspase-10	Mch4, FLICE2
Caspase-11	ICH-3

terminal side of aspartate residues within distinct recognition motifs (Harvey *et al.*, 1998).

Each caspase is synthesized as a proenzyme and activated by cleavage at specific internal aspartate residues, potentially by the same or another caspase class (Harvey *et al.*, 1998). The cleavage processes the caspases to form active heterodimeric enzymes. Activation does not require removal of the propeptide but the cleavage between the large and small subunits is thought to be an activating event (Yang *et al.*, 1998). A recent study has shown that caspase-9 can be activated without proteolytic processing (Stennicke *et al.*, 1999). In this study it was found that the mutant forms that disabled one or both of the interdomain processing sites of caspase-9, were still able to activate downstream caspases. The single mutants were only slightly less effective than endogenous caspase-9, whereas the double mutants reconstituted 40% of the wild type activity (Stennicke *et al.*, 1999). However, the activation still required binding to a cytosolic factor, probably Apaf-1, a protein that shares homology with with *ced 4*, the *C. elegans* death gene (Zou *et al.*, 1997; Stennicke *et al.*, 1999).

5.2.1 Function of caspases during apoptosis

Work by several groups has shown that caspases, mainly act by cleaving specific proteins, resulting in the irreversible commitment to cell death (Lazebnik *et al.*, 1994; Janicke *et al.*, 1996; Enari *et al.*, 1998; Widmann *et al.*, 1998). Various different substrates are now known for the caspases whose physiological function helps elucidate the role played by the caspases in apoptosis.

The Retinoblastoma protein (Rb), is one of the proteins cleaved by the capases during apoptosis (Janicke *et al.*, 1996). Rb is an important cell cycle regulator with a known anti-apoptotic function. Phosphorylation of Rb by CDKs leads to the inactivation of its growth suppressive function and Rb-deficient mice are more susceptible to apoptosis than cells with fully functional protein. Janicke *et al.*, (1996), showed that the carboxy-terminal peptide of Rb was specifically cleaved off by the caspases in TNF-and staurosporine-induced apoptosis. Cleaved Rb is unable to bind to the

regulatory protein MDM2, which has been implicated in apoptosis (Janicke *et al.*, 1996).

In contrast to caspase-induced loss of function as with Rb, proteolysis of some proteins by caspases leads to their activation (Brancolini *et al.*, 1995). For example, Gas2, a member of the growth-arrested-specific genes whose expression is strictly coupled to the growth arrest state, was found to be cleaved by caspases in NIH3T3 cells induced to undergo apoptosis after 48 hours of serum-withdrawal (Brancolini *et al.*, 1995). Overexpression of Gas2 deleted in its carboxy-terminal part, similar to the peptide generated by caspase cleavage, induced alterations of the actin cytoskeleton and of the cell shape in different cell types, resembling the changes normally seen during cell death by apoptosis.

As mentioned before, one of the characteristics of apoptotic death is the degradation of the DNA into specific sized fragments. A caspase activated-deoxyribonuclease (CAD) has been identified in the cytoplasmic fraction of mouse lymphoma cells (Enari *et al.*, 1998). CAD is a protein of 343 amino acids which carries a nuclear-localization signal and is produced as a complex with ICAD, its inhibitor, which seems to function as a chaperone for CAD during its synthesis, remaining complexed with CAD to inhibit its DNase activity (Enari *et al.*, 1998). Cleavage of ICAD by caspases allows CAD to enter the nucleus and degrade chromosomal DNA (Enari *et al.*, 1998).

Apart from those mentioned above, several other proteins are also known to undergo proteolysis by caspase action. These include Poly (ADP-ribose) polymerase (PARP), a nuclear enzyme that is recognized as an early event in apoptosis in intact cells (Lazebnik, 1994). Caspase 3 cleaves PARP to a specific 85kd form observed during apoptosis (Tewari *et al.*, 1995b). A 70kd component of the U1 small ribonucleoprotein (U1-70kd) is a proteolytic substrate for the caspases during Fas and TNF induced apoptosis (Tewari *et al.*, 1995a; Casciola-Rosen *et al.*, 1996). DNA dependent protein kinase, sterol-regulatory element binding proteins (SREBPs) are also specifically cleaved during apoptosis (Janicke *et al.*, 1996). In a study carried out by Widmann *et al.*, (1998), Ras, MEKK1 and FAK (as mentioned before) were found

to be cleaved by the caspases in Jurkat cells induced to undergo apoptosis by Fas ligation, exposure to ultraviolet-C or incubation with etoposide. Some of the other proteins cleaved during apoptosis are, Cbl, Cbl-b, Raf-1 and Akt were cleaved later in the apoptotic response (Widmann *et al.*, 1998).

5.3 Role of the Bcl-2 family in cell survival

Bcl-2 was first discovered as an oncoprotein whose deregulated expression through translocation is associated with follicular and B cell lymphomas (Yunis *et al.*, 1987; Tsujimoto *et al.*, 1985; Cleary *et al.*, 1986). Since then several other protein belonging to this family have been discovered (reviewed in Yang and Korsmeyer, 1996; Harrington *et al.*, 1994). These proteins contain a carboxy-terminal transmembrane anchor sequence that allows them to associate with cellular membranes including the outer membrane of mitochondria, endoplasmic reticulum, and the nuclear membrane (Krajewski *et al.*, 1993). Inspite of being homologous at the level of amino acid sequence, some of the family members suppress apoptosis, while the others accelerate it (reviewed in Reed, 1995). For example, while Bcl-2 and its homologue Bcl-X_L inhibit apoptosis in a wide variety of cell systems, Bcl-X_S , Bax, Bak and BAD promote it (reviewed in Reed, 1995). The members of the Bcl-2 family are able to interact with each other to form heterodimers (Sato *et al.*, 1994; Yang *et al.*, 1995). The importance of formation a BAD/Bcl-2 heterodimer in regulating Bcl-2 function is discussed later in section 5.7.3.

The earliest findings implicating Bcl-2 in cell survival showed that overexpression of Bcl-2 increased the viability of certain cytokine dependent cells upon cytokine withdrawal (Nunez *et al.*, 1990; Deng and Podack, 1993). Radiation, chemotherapeutic drugs, and ceramide, a proapoptotic lipid second messenger that is often produced in cells after exposure to γ-radiation, have all been reported to downregulate Bcl-2 expression in leukemia cells, again implying a role in cell survival (reviewed in Reed, 1995). Bcl-2 overexpression also protects the MDCK cells during apoptosis induced by disruption of cell-matrix interactions (Frisch and Francis, 1994). Studies have also implicated a role for Bcl-2 in mediation of suppression of apoptosis in a murine pre-mast cell line by the v-Abl protein tyrosine

kinase (Chen *et al.*, 1997). Overexpression of Bcl-2 inhibits the activation of cytoplasmic caspases after apoptotic stimuli (Krebs *et al.*, 1999). It has also been shown to prevent caspase-independent cell death induced by exogenous nitric oxide in PC12 and HeLa cells (Okuno *et al.*, 1998). Nevertheless, in another study, experiments showed that treatment of Bcl-2 transfected SCC9 cells with anti-Fas antibody did not reduce the number of dead cells significantly (Crowe *et al.*, 1998).

Recent experiments have suggested possible mechanisms by which Bcl-2 and its homologues are able to participate in cell survival. Some studies suggest that Bcl-2 acts by preventing the release of cytochrome C (Yang *et al.*, 1997; Kluck *et al.*, 1997a). Overexpression of Bcl-2 in human acute myeloid leukemia cells prevented the initiation of apoptosis induced by staurosporine and also the efflux of cytochrome C from the mitochondria, suggesting that Bcl-2 acts upstream of cytochrome C release (Yang *et al.*, 1997). On the other hand, in a study carried out by Rosse *et al.*, (1998), overexpression of Bcl-2 prevented Bax-induced apoptosis, but did not stop cytochrome C release, thus showing that Bcl-2 can prevent apoptosis by acting downstream of cytochrome C release. Another more direct approach confirmed that Bcl-2 can act downstream of cytochrome C release. Zhivtovsky *et al.*, (1998) microinjected high concentrations of cytochrome C into the cytosol and found that high levels of Bcl-2 expression were still able to block most of the cell death. A recent study has suggested that Bcl-2 might act in part by blocking the activation of membrane associated caspases (Krebs *et al.*, 1999). However, other studies have shown that overexpression of Bcl-2 prevents CD95 induced apoptosis in SKW6 cells but did not block caspase activities (Scaffidi *et al.*, 1998).

5.4 Role of mitochondrial dysfunction and cytochrome C in apoptosis

Mitochondria have been implicated in the process of apoptosis ever since it was discovered that the members of the Bcl-2 family of anti-apoptotic proteins, are located in the outer mitochondrial membrane (Krajewski *et al.*, 1993). A more direct role for mitochondria in apoptosis was demonstrated by the finding that apoptosis in Xenopus egg extracts *in vitro* requires a dense organelle fraction enriched in mitochondria (Newmeyer *et al.*, 1994). A later study identified cytochrome C as a

necessary component for induction of apoptosis *in vitro* (Liu *et al.*, 1996). Cytochrome C is an essential component of the mitochondrial respiratory chain. It is a soluble protein that is found in the inter membrane space and is loosely attached to the surface of the inner mitochondrial membrane (reviewed in Reed, 1997; Kroemer, 1999). One study used a cell free system based on cytosolic extracts of normally growing cells to reproduce aspects of the apoptotic programme *in vitro* (Liu *et al.*, 1996). Elimination of cytochrome C from the cytosol diminished the ability of the extracts to induce apoptosis; adding back cytochrome C, the apoptotic activity of the extracts was restored. Also, cells undergoing apoptosis *in vivo* showed increased release of cytochrome C to the cytosol, suggesting that mitochondria may function in the apoptotic process by releasing cytochrome C (Yang *et al.*, 1997; Kluck *et al.*, 1997a; Vander Heiden *et al.*, 1997; Bossy-Wetzel, 1998). Another unrelated caspase activating factor (AIF) is also released by the mitochondria from the intermembrane space into the cytosol and is thought to be capable of processing caspase 3 *in vitro* (Susin *et al.*, 1996; reviewed in Green and Reed, 1998). However, the role of cytochrome C in apoptosis has been studied in more detail and is discussed below.

During the apoptotic process, the mitochondrial inner membrane potential is frequently disrupted (Vander Heiden *et al.*, 1997). Opening of the megachannel (a composite ion channel that traverses the inner and outer mitochondrial membrane at sites of contact between them) causes an increase in the matrix volume leading to physical disruption of the outer membrane, facilitating the release of cytochrome C (reviewed in Green and Kroemer, 1998). However, cytochrome C release can also occur independent of mitochondrial transmembrane depolarisation (Bossy-Wetzel *et al.*, 1998). Cell free assays have revealed that cytochrome C is an activator of caspase 3 (Liu *et al.*, 1996). This activation is dependent on dATP. In addition, another cytosolic factor known as Apaf-1 is also required (Zou *et al.*, 1997). Requirement of caspase 9 in cytochrome C mediated activation of caspase 3 was demonstrated when it was found that caspase 9 forms a complex with Apaf-1 in a cytochrome C and dATP-dependent manner (Li *et al.*, 1997) forming an 'apoptosome' (Green and Reed, 1998). Cleavage of caspase 3 is absent in the cytosolic extracts of caspase 9-deficient cells but is restored after addition of *in vitro* translated caspase 9 (Kuida *et al.*, 1998), thus confirming that cytochrome C activation of caspase 3 is mediated by caspase 9.

5.5 Initiation and execution of apoptosis: caspases vs cytochrome C release

Apoptosis, for sometime, has been considered a consequence of a cascade of caspase activation. However, recent studies have shown that the commitment point, as measured as the loss of clonogenic potential or loss of viability does not always correlate with activation of the caspase proteases (reviewed in Green and Kroemer, 1998). Indeed, there are cases in which activation of caspases is observed at a much later time point. For example, during Fas mediated apoptosis in Jurkat and CEM cells, cleavage of caspase 3 and caspase 8 is delayed compared to their activation in SKW6.4 and H9 cells (Scaffidi *et al.*, 1998). Blocking of all mitochondrial apoptogenic activities in these cells by the overexpression of Bcl-2 or Bcl-x_L , not only blocks the process of apoptosis but also blocks the activation of caspases 3 and 8, showing that caspases activation is downstream of mitochondrial dysfunction, at least in these cells (Scaffidi *et al.*, 1998). Other studies have also shown that disruption of mitochondrial function leads to cell death without caspase activation, and caspase-independent pathways of cell death linked to mitochondrial dysfunction also exist (Hirsch *et al.*, 1997).

Inducible expression of Bax, a pro apoptotic Bcl-2 family member, induces apoptosis in Jurkat T cells (Xiang *et al.*, 1996). Inhibition of caspase activity by ZVAD-FMK, a known caspase inhibitor was unable to block Bax-induced cell death. Inhibition of caspase activity was also unable to prevent the fall in mitochondrial membrane potential. Thus Bax-induced alterations in mitochondrial function and subsequent cell death do not require caspase activity (Xiang *et al.*, 1996). Inhibiton of caspases was also unable to prevent cell death induced by the ectopic expression of Bak, another pro-apoptotic homologue of Bcl-2, in Rat-1 cells (McCarthy *et al.*, 1997). In the same study it was found that cell death induced by c-Myc expression in Rat-1 cells was also not prevented by inhibition of caspase activity. However, in the case of both Bak and c-Myc, the cell death resulting after the inhibtion of caspases did not exhibit all of the characteristic biochemical and morphological events associated with apoptosis, including cleavage of cleavage of nuclear lamins and PARP, chromatin condensation and nucleosome laddering (Xiang *et al.*, 1996; McCarthy *et al.*, 1997).

Characteristics associated with the onset of apoptosis, such as surface blebbing were not inhibited. Examples of caspase-independent commitment to cell death can be found in several of the other classic models of apoptosis, including glucocorticoid-induced death of thymocytes and lymphoid lines, death of haematopoietic cell lines induced by cytokine withdrawal and death of target cells of cytotoxic T cells (reviewed in Green and Kroemer, 1998).

Most of the studies carried out on caspase-independent cell death conclude that although the commitment point is independent of caspase activation, caspases are still required for the completion of the process and for the manifestation of certain morphological and biochemical features associated with apoptosis, such as cleavage of PARP and nucleosome laddering. This led Green and Kroemer, (1998), to suggest a mechanism of apoptosis in which caspase activation and disruption of mitochondrial function occur in a circular feedback loop. According to their model, mitochondrial dysfunction would enable the release of caspase activators such as cytochrome C, at the same time as caspases might act on mitochondrial membranes. Such a self amplifying system would have several points of entry (mitochondrial membranes and caspase cascades), allowing for the activation of apoptotic pathway and, at the same time, would be vulnerable to the inhibitory effects of caspase inhibitors and of agents that stabilize mitochondrial membranes (Green and Kroemer, 1998). Figure 22 outlines some of the key regulators of apoptosis and shows the two points where apoptosis can be initiated.

5.6 Pro-apoptotic signal transduction pathways upstream of commitment

Results of several studies are beginning to reveal the complex integration of signal transduction pathways that are involved in the decision of the cell to survive or to undergo apoptosis. Certain kinases have been identified that are able to induce apoptosis when expressed in cells.

MEKK 1, an upstream kinase activator of the ERK and JNK pathways (reviewed in Robinson and Cobb, 1997), has been implicated in the induction of apoptosis (Johnson *et al.*, 1996; Cardone *et al.*, 1997; Widmann *et al.*, 1998). A later study

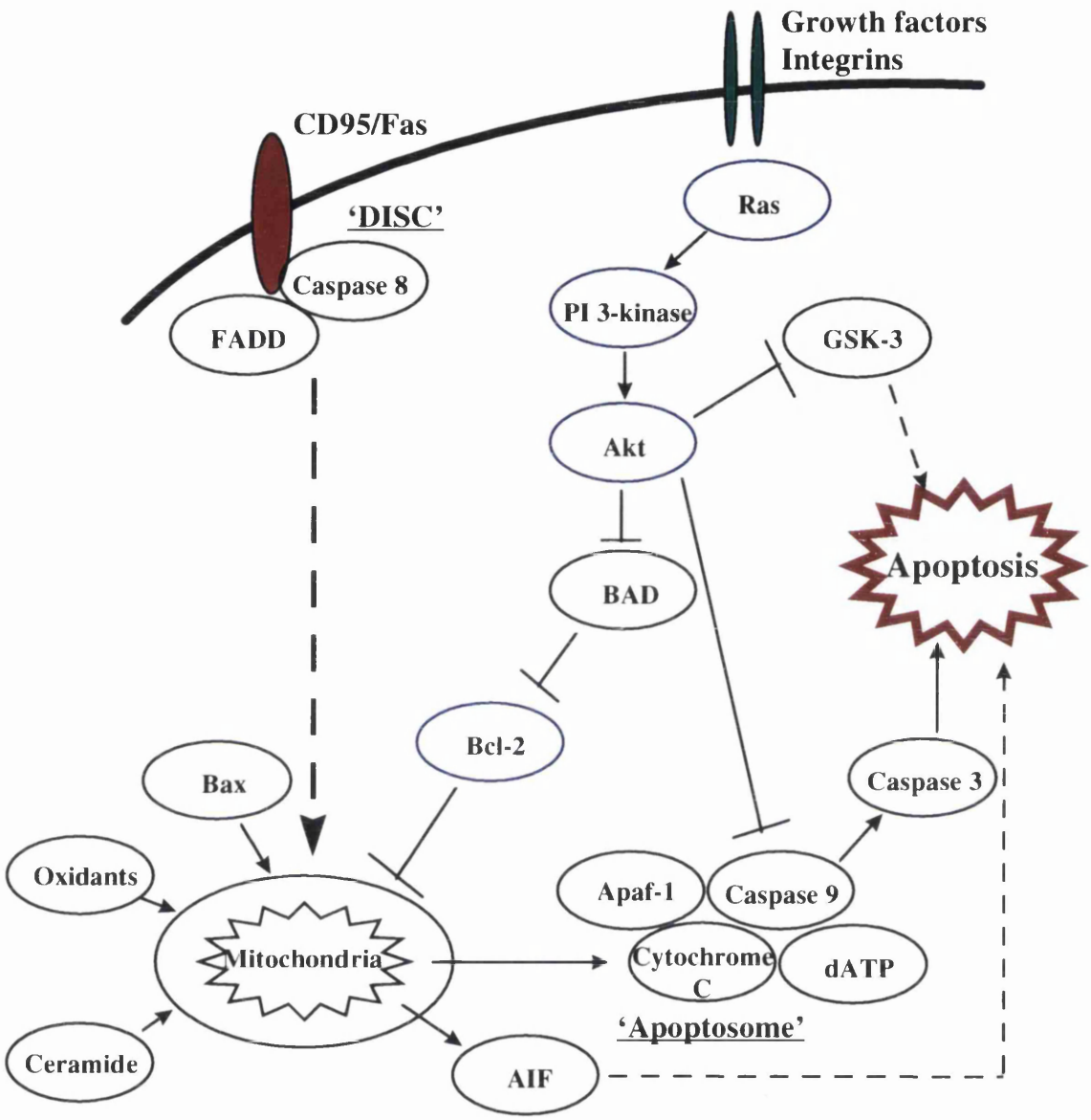


Figure 22 Regulation of apoptosis: (adapted from Prendergast, 1999) The main mediators of the apoptotic process are shown. Regulators of cell survival are encircled in blue, whereas those mediating cell death are encircled in black.

using MDCK cells demonstrated that the activation of MEKK 1 required caspase-mediated proteolysis, generating a fragment that promotes apoptosis (Cardone *et al.*, 1997). Expression of the cleaved product of MEKK 1 was able to induce apoptosis in the cells, while expression of the kinase inactive form protected from cell death (Cardone *et al.*, 1997). Cleavage resistant or kinase inactive mutants of MEKK 1 also prevent complete activation of caspase 7, suggesting that MEKK 1 lies upstream of some caspases. Another study has also reported that the kinase activity of MEKK 1 stimulates caspase 3-activity in cells (Widmann *et al.*, 1998).

Involvement of MEKK 1 in apoptosis focused attention on some of its downstream effectors. C-Jun N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPKs), are likely to be involved in the process of apoptosis. JNK can be activated by various different stimuli such as UV light, γ radiation, protein synthesis inhibtiors, DNA damaging drugs, chemopreventive drugs, TNF-α and interleukin, all implying that JNK may regulate gene expression or other biochemical functions in the cell under stressful conditions (Chen *et al.*, 1996b). A direct correlation between JNK activation and induction of apoptosis by UV-C and γ radiation has been observed (Chen *et al.*, 1996b). Also, overexpression of JNK 1 caused cell death in transfected Jurkat cells, while the expression of a dominant negative mutant prevented UV-C and γ radiation induced cell death (Chen *et al.*, 1996b). JNK activation has also been reported early after the detachment of the epithelial cells from the matrix (Frisch *et al.*, 1996a). Expression of a dominant negative form of JNK reduced JNK activation by fourfold and the transfected MDCK cells were relatively resistant to anoikis, thus showing that the activation of this pathway is linked to anoikis (Frisch *et al.*, 1996a). Inhibition of caspase activity by CrmA attenuated JNK activation. Also, blockage of JNK pathway attenuated the activation of caspases, suggesting a positive feed back loop between the caspases and the JNK pathways (Frisch *et al.*, 1996a). In addition, withdrawal of NGF from PC12 cells also leads to sustained JNK activation (Xia *et al.*, 1995). In SHEP cells, a human neroblastoma cell line, the expression of JNK1 APF (a mutant for form of JNK) inhibited Fas mediated JNK activation and apoptosis, suggesting that JNK is required for Fas mediated cell death (Goillot *et al.*, 1997). Some studies have also investigated the role of JNK substrates to try to determine the pathway in more detail. JNK is

known to phosphorylate transcription factors such as c-Jun, ATF-2 and Elk-1 and strongly augments their transcriptional activity (Gupta *et al.*, 1995; Whitmarsh *et al.*, 1995; Karin, 1995). Expression of a dominant negative form of c-Jun blocks apoptosis induced by NGF withdrawal in PC12 cells (Xia *et al.*, 1995), suggesting that JNK mediated apoptosis might require transcription of other proteins regulated by c-Jun.

Despite the findings mentioned above, there have been reports which question the role played by JNK activation during the process of apoptosis. Expression of an inhibitory mutant of c-Jun did not have any effect on the induction of apoptosis by ionizing radiation in SQ-20-B human cells, and the same mutant was found to protect the T98G glioblastoma cells against cisplatin induced apoptosis (Jarpe *et al.*, 1998). Activation of the JNK pathway also protected HeLa cells from apoptosis following photodynamic therapy (PDT) with hypericin and transfection of cells with dominant inhibitors of the JNK pathway enhanced PDT-induced apoptosis (Assefa *et al.*, 1999). Knockout experiments have also shown similar discrepancy (Nishina *et al.*, 1997). Elimination of an upstream activator of JNK, SEK-1, in ES cells did not make any difference to the normal apoptotic response of the cells to anisomycin induced apoptosis; instead SEK-1 null thymocytes displayed an increased sensitivity to CD3 and Fas mediated apoptosis (Nishina *et al.*, 1997).

p38, another stress-activated member of the MAP kinase superfamily, has also been implicated in the process of apoptosis. It is the physiological MAPKAP-kinase-2 activator recruited in response to Il-1 and stress stimuli (Kyriakis and Avruch, 1996). MAPKAP-kinase-2, in turn, phosphorylates the heat shock protein Hsp25/HSP27. Apoptosis induced by NGF withdrawal in PC12 cells is accompanied by p38 activation (Xia *et al.*, 1995). Furthermore, a constitutively activated form of MKK3, a p38 MAP kinase kinase, was co transfected along with p38 kinase into cells leding to a marked increase in p38 kinase activity and induced a 4.5 fold increase in the number of apoptotic cells in the presence of NGF (Xia *et al.*, 1995). Glutamate induced apoptosis in the Rat cerebellar granule cells is also accompanied by p38 activation and inhibition of p38 activity was able to block glutamate-induced apoptosis (Kawasaki *et al.*, 1997). However, like the JNKs, there have been

contradictory reports concerning the role of p38 kinase in apoptosis. For example, pre-treatment of HeLa cells with a p38 inhibitor enhances PDT-induced apoptosis (Assefa *et al.*, 1999). Thus, the specific role of JNK an p38 stress-activated kinases in supression or promotion of apoptosis may be cell-type specific.

5.7 Anti-apoptotic signal transduction pathways

As mentioned before, both integrin signaling and growth factor signaling have been implicated in cell survival and protection from apoptosis. The signal transduction pathways that act downstream of these signals are just beginning to be described. The roles of Src and FAK in cell survival have already been mentioned. This section will consider other possible signalling proteins downstream of integrins and growth factors that mediate cell survival.

5.7.1 Role of PI 3-kinase/Akt in survival signalling

PI 3-kinase, a known proto-oncogene (Chang *et al.*, 1997; reviewed in Porter and Vaillancourt, 1998), is a heterodimer comprising of an 85kd regulatory subunit and a 110kd catalytic subunit (Fry and Waterfield, 1993). p110 is a dual specificity enzyme that acts not only as a lipid kinase but also as protein serine kinase. It is known to catalyze the phosphorylation of inositol lipids at the D-3 position of the inositol ring, resulting in the formation of phosphatidylinositol 3-phosphate [PIP], phosphatidylinositol 3,4-bisphosphate [PIP_2] and phosphatidylinositol 3,4,5-triphosphate [PIP_3] (Liscovitch and Cantley, 1994). These lipid products act as secondary messengers that act on several different pathways including cell survival, largely through the activation of Akt (also known as protein kinase B or PKB; Delcommenne *et al.*, 1998). Akt, a serine threonine kinase, is also a known proto-oncogene. The lipid products of PI 3-kinase, PIP_2 and PIP_3, are able to bind to the PH domain of Akt (Franke *et al.*, 1997). This binding mediates translocation of the kinase from the cytosol to the plasma membrane (Andjelkovic *et al.*, 1997) which appears to be required in order to present Akt to upstream activating kinases (reviewed in Downward, 1998). Akt becomes phosphorylated at residues Thr308 and

Ser473 by upstream kinases such as protein kinase-1 (PDK1) (Alessi *et al.*, 1996; Alessi *et al.*, 1997).

There is evidence in different cell types demonstrating that cell survival is dependent on the PI 3-kinase/Akt pathway. In particular, PI 3-kinase activity is required for the survival of serum deprived PC12 cells (Yao and Cooper, 1995). In addition, Ras mediated protection of MDCK cells from apoptosis is mediated by PI 3-kinase (Khwaja *et al.*, 1997). A constitutively activated form of PI 3-kinase was able to prevent apoptosis in the cells while blocking PI 3-kinase activity abrogated protection conferred by Ras (Khwaja *et al.*, 1997). Even in non-transformed cells, attachment of cells to the ECM leads to rapid elevation of the levels of the PI 3-kinase lipid products (Khwaja *et al.*, 1997). Overexpression of EGF growth receptor also effectively protects Rat-1 fibroblasts against UV-induced apoptosis (Kulik *et al.*, 1997), an effect that is inhibited by wortmannin, a PI 3-kinase inhibitor. Also, transfection of the cells with constitutively active forms of PI 3-kinase or Akt were sufficient to inhibit UV-induced apoptosis (Kulik *et al.*, 1997). The PI 3-kinase/Akt pathway is also important in CSF-1 regulated macrophage proliferation, differentiation and survival (Hamilton, 1997) and Suppression of c-Myc induced apoptosis by insulin like growth factor (IGF-1) is also mediated by Akt (Rohn *et al.*, 1998). Furthermore, activated Akt suppresses CD95-(Fas) induced apoptosis at a point downstream of FADD but upstream of caspase 8. Thus, there is substantial evidence implicating Akt as a survival factor of importance.

5.7.2 Regulation of PI 3-kinase by PTEN

PTEN, a tumour suppressor protein, modulates cell survival by regulating the PI 3-kinase/Akt pathway (Sun *et al.*, 1999). PTEN is a phosphatase specific for both phospholipids and phosphoproteins. Its substrates include FAK and PIP_3, the lipid product of PI 3-kinase (Li *et al.*, 1998). The role of FAK in cell survival has been discussed before (in section 1.3). Dephosphorylation of PIP_3 negatively regulates the PI 3-kinase/Akt signaling pathway. Inactivation of PTEN in ES cells and in embryonic fibroblasts results in elevated levels of PIP_3, which in turn increases the phosphorylation and activation of Akt and promotes cell survival (Sun *et al.*, 1999).

Another study has reported that *pten* heterozygous mice show impaired Fas mediated apoptosis (Di Cristofano *et al.*, 1999). T lymphocytes from these mice also showed reduced activation-induced cell death. Inhibition of PI 3-kinase activity in these cells was able to abolish Fas responsiveness, thus implicating PTEN and PI 3-kinase/Akt pathway in Fas mediated apoptosis.

5.7.3 Downstream effectors of Akt that direct cell survival and apoptosis

The mechanism by which Akt protects cells from programmed cell death has been the subject of much investigation recently. Akt can phosphorylate the pro-apoptotic Bcl-2 family member, BAD (del Peso *et al.*, 1997; Datta *et al.*, 1997), both *in vitro* and in intact cells. The phosphorylation occurs at Ser136 of BAD (Datta *et al.*, 1997), creating a binding site for 14-3-3 protein, a family of ubiquitous highly expressed adaptor proteins. When BAD is bound to 14-3-3, it is unable to heterodimerize with and inhibit the survival activity of Bcl-2 and Bcl-X_L proteins (Zha *et al.*, 1996). In both haematopoietic precursor cells and fibroblasts, overexpression of BAD induces cell death and this effect is reversed by expression of activated forms of Akt. Death induced by non-phosphorylatable BAD is not suppressed by activated Akt. However, it is not clear if this is the primary way in which the survival signal from Akt is mediated (reviewed in Downward, 1997). This is so because BAD is only expressed in a limited range of tissues and cell lines and most of the studies carried out to investigate its role in Akt mediated survival signal were done using overexpression of the protein.

Glycogen synthase kinase-3 (GSK-3) has recently been identified as an important downstream element of the PI 3-kinase/Akt cell survival pathway (Pap and Cooper, 1998). GSK-3 is a ubiquitously expressed protein serine/threonine kinase whose activity is inhibited by Akt phosphorylation. It is known to phosphorylate a broad range of substrates including translation initiation factor eIF2B and several transcription factors. Overexpression of a catalytically active form of GSK-3 induces apoptosis in Rat-1 and PC12 cells (Pap and Cooper, 1998). Also, a dominant negative form of GSK-3 prevents apoptosis induced by the inhibition of PI 3-kinase activity. GSK-3 is phosphorylated by integrin-linked kinase (ILK), which is also

known to suppress suspension induced apoptosis (Delcommenne *et al.*, 1998). Phosphorylation by ILK also inhibits GSK-3 activity and is believed to be dependent on PI 3-kinase and Akt.

Other substrates of Akt have also been identified which also might mediate cell survival. For example, Akt can phosphorylate recombinant caspase 9 *in vitro* on residue Ser196, and inhibit its protease activity (Cardone *et al.*, 1998). A mutated form of caspase 9 with an Ala substitution at position 196, was resistant to Akt mediated phosphorylation and inhibition *in vitro* and *in vivo*, resulting in Akt resistant induction of apoptosis. Akt can also phosphorylate and regulate the activity of FKHRL1, a member of the Forkhead family of transcription factors, which can induce apoptosis, most likely by inducing the expression of genes that are critical for cell death, such as that encoding Fas ligand (Brunet *et al.*, 1999). Akt phosphorylation of FKHRL 1 in the presence of growth factors leads to its association with 14-3-3 proteins and its retention in the cytoplasm (Brunet *et al.*, 1999). On the other hand, in the absence of survival factors, FKHRL 1 is translocated to the nucleus and targets gene activation (Brunet *et al.*, 1999). There have been reports that Akt itself is translocated to the nucleus following stimulation of cells with growth factors, suggesting that important substrates could also exist in this cellular compartment (Meier *et al.*, 1997).

Another possible downstream target of Akt is p70S6K, a ribosomal protein kinase, (Koh *et al.*, 1999). P70S6K also lies downstream of PI 3-kinase (Chung *et al.*, 1994). It phosphorylates the S6 peptide of the 40S ribosomal subunit (reviewed in Chou and Blenis, 1995). Evidence suggests that p70S6K plays an important role in progression through the G1 phase of the cell cycle. It is possible that Akt mediated survival might involve the activity of p70S6K since regulation of cell cycle progression is linked to cell survival.

5.7.4 Other possible survival pathways

Recent studies have focused attention on another set of proteins known as the signal transducers and activators of transcription or STATs. STATs are latent transcription

factors (reviewed in Bromberg *et al.*, 1999). These are activated by phosphorylation on a single tyrosine residue in response to extracellular ligands. More than 40 different ligands can cause STAT activation, including cytokine receptors which signal through associated Janus tyrosine kinases (Jaks) or growth factors such as EGF, PDGF and CSF 1 (reviewed in Bromberg *et al.*, 1999). STAT dimers are formed by interaction between the SH2 domain of one monomer and the phosphorylated tyrosine residue of the other. These dimers can then translocate to the nucleus and activate specific genes. They are generally thought to play a role in cell growth. However, a recent study showed that cell lines from multiple myelomas that have acquired growth factor independence, require constitutively active STAT3 to protect against apoptosis (Catlett-Falcone et al., 1999). Another study has characterised the role of STAT3 as an oncogene and demonstrated that substitution of two cysteine residues within the carboxy-terminal loop of the SH2 domain, causes cellular transformation in immortalized fibroblasts (Bromberg *et al.*, 1999). STAT1, 3, and 5 have also been implicated in Interleukin-9 (IL-9) signaling. Activation of STATs by IL-9 depends on a single phosphotyrosine at position 367 in IL-9R (Demoulin *et al.*, 1996). IL-9 protects T cells from glucocorticoid-induced apoptosis by a STAT dependent mechanism (Demoulin *et al.*, 1996). Protection from apoptosis was observed by activation of either STAT5 or both STAT1 and STAT3, suggesting redundancy in these cases (Demoulin *et al.*, 1999).

A subgroup of the MAP kinase family, ERKs, have also been implicated in cell survival. The ERKs are activated in response to growth factor stimulation (reviewed in Marshall, 1994). Induction of apoptosis in PC12 cells by NGF withdrawal required suppression of ERK activity (Xia *et al.*, 1995). In this study, expression of constitutively activated forms of MKK1, a protein kinase that activates ERKs, prevented apoptosis induced by NGF withdrawal. Another study demonstrated that activation of the ERK signaling pathway completely suppressed the FTY720-induced apoptosis in T lymphocytes (Matsuda *et al.*, 1999). Furthermore, apoptosis following PDT was accompanied by irreversible inhibition of ERK2 in several cancer cell lines (Assefa et al., 1999). It has been suggested that ERK may also phosphorylate BAD, which is known to play an important role in the induction of apoptosis and its phosphorylation correlates with cell survival (Scheid and Duronio, 1998; section

5.7.3). However, some other studies report that ERK activation does not play any role in cell survival. For example, there was no significant change in ERK2 activity during the early phase of γ or UV induced apoptosis in Jurkat cells (Chen *et al.*, 1996b), although it did gradually decrease only 5-6 hours after radiation. In Rat-1 fibroblasts, the ERK pathway does not have a protective effect on UV induced apoptosis (Kulik *et al.*, 1997).

Chapter 6: Results 3

Characterisation of mediators of apoptosis after removal of v-Src induced survival signal: the role of ZVAD.fmk-sensitive caspases and p38

6.1 Introduction

Results shown in chapter 5 showed that although apoptosis in serum-deprived v-Src transformed Rat-1 cells was accompanied by FAK proteolysis, inhibiting FAK cleavage did not prevent apoptosis. Thus, FAK cleavage was not playing an essential role in the induction of apoptosis, at least in these cells. For this reason, the planned experiments to determine the cleavage sites of FAK followed by generation of cleavage-insensitive FAK mutants were not pursued further. Therefore, further studies concentrated on examining some of the pathways involved in the mediation of apoptosis after attenuation of v-Src induced survival signalling in serum-starved Rat-1 cells.

The following chapter gives a detailed description of the characteristics of cell death in v-Src transformed Rat-1 cells, and the role of both ZVAD.fmk-sensitive caspases and p38 in its induction.

6.2 Characterisation of cell death in v-Src transformed Rat-1 cells

Rat-1 cells expressing a temperature-sensitive mutant (*ts*) of v-Src, *ts LA29*, are transformed at the permissive temperature of 35^{O}C, and morphologically normal at the restrictive temperature of 39.5^{O}C. The mitogenic effects of v-Src have been previously characterised by other studies in the group (Wyke *et al.*, 1993; Wyke *et al.*, 1995; Johnson *et al.*, 1998). In contrast to normal cells, transformed cells placed in 0.2% serum medium (low serum) for 24 hours, continued to cycle at 35^{O}C (Johnson *et al.*, 1998). However, after serum-deprivation for 24 hours, when these cells were shifted to the restrictive temperature to inactivate v-Src, they became loosely adherent or detached and died within a few hours. This was in contrast to cells transformed by other oncoproteins such as c-Myc and v-Jun which died upon withdrawal of serum (Evan *et al.*, 1992; Clark and Gillespie, 1997).

The type of cell death induced in these cells exhibited characteristics of programmed cell death. Specifically, various morphological and biochemical criteria that are known to accompany apoptosis, also accompanied cell death induced after v-Src

inactivation. Experiments were carried out according to the scheme already outlined in section 4.4 (Figure 19a). Cells were harvested at 0, 2, 4, and 6 hours after being shifted to the restrictive temperature for TUNEL analysis to determine the percentage of cells undergoing apoptosis. Figure 23a graphically represents the percentage of cells undergoing apoptosis at each time point.

In order to determine if the dying cells were fragmenting their DNA into nucleosomal ladders, another characteristic feature of apoptosis (reviewed in Kerr *et al.*, 1972; Wyllie, 1997), both detached and adherent cells were harvested separately 6 hours after v-Src inactivation and DNA was isolated. 10μg of isolated DNA from detached and adherent cells was separated into nucleosomal ladders following electrophoresis (Figure 23b). Laddering was more readily detectable in the detached cell DNA (Figure 23b).

Also, when viewed by phase contrast microscopy, the cells exhibited a rounded or blebbing morphology (Figure 23c) and fragmented nuclei (Figure 23d), which are also characteristics associated with cells undergoing apoptosis (reviewed in Kerr *et al.*, 1972; Wyllie, 1997).

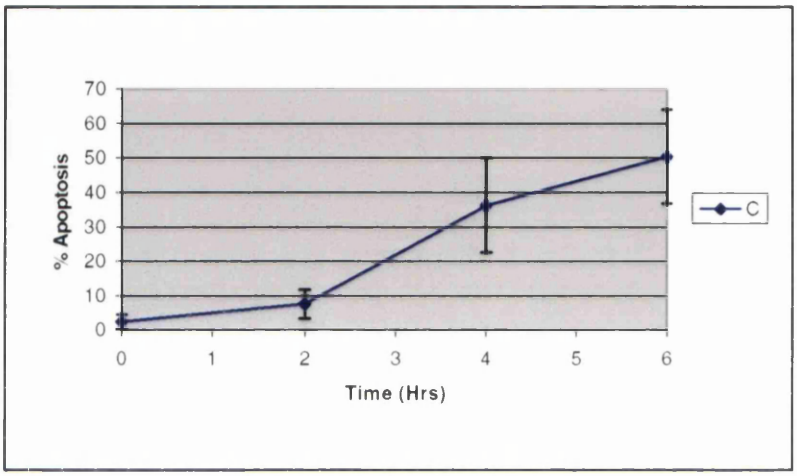
6.3 Biochemical changes associated with apoptosis induced by inactivating v-Src in serum-deprived cells

Apoptosis is frequently associated with the activation of several proteins such as caspases (reviewed in Harvey and Kumar, 1998) and the stress activated kinases, such as p38 (Xia *et al.*, 1995; Kawasaki *et al.*, 1997).

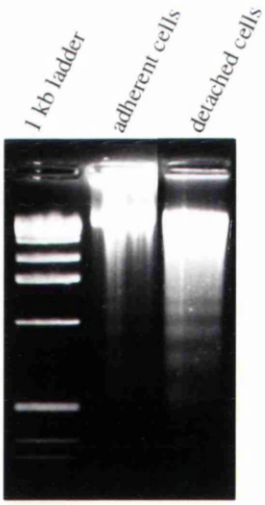
To investigate whether caspases were activated in v-Src transformed Rat-1 cells that were induced to undergo apoptosis after serum-starvation and inactivation of v-Src, cells were harvested at the time of serum withdrawal, 12 hours later, and then, 0, 4, 6 and 8 hours after switching off v-Src. Harvested cells were tested for caspase activity by using a biotinylated peptide that binds to the large sub-units of active caspases. The cell lysates were incubated with the peptide and then separated by SDS-PAGE.

Figure 23 Apoptotic features associated with cell death in transformed Rat-1 cells: (a) To induce an apoptotic response, *ts LA29v-Src* transformed Rat-1 cells were serum starved for 24 hours followed by inactivation of v-Src by shifting the cells from 35^{O}C (permissive temperature) to 39.5^{O}C (restrictive temperature). Cells were fixed and TUNEL labelled to determine the percentage of cells undergoing apoptosis. The mean of several experiments was plotted, with the error bars representing the standard error. (b) DNA isolated from detached and adherent cells 6 hours after the induction of apoptosis, was separated on a 2% tris-borate agarose gel and stained with ethidium bromide to detect nucleosomal ladders. (c) Cells were viewed under the microscope using phase contrast at 6 hours after v-Src inactivation to ascertain the morphology of dying cells (arrow points to blebbing cells). (d) At the same time, the morphology of the nucleus was also examined by staining with DAPI (carried out by D. Riley, Beatson Institute, Glasgow, UK).

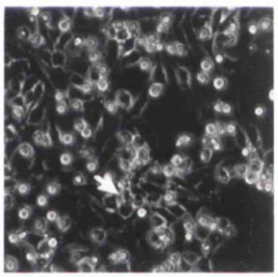
a Percentage of cells undergoing apoptosis in serum-starved transformed Rat-1 cells after inhibtion of v-Src activity



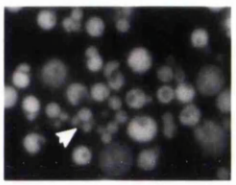
b DNA laddering in detached and adherent cells



c Morphology of cells undergoing apoptosis (20 x objective)



d DAPI stained nuclei at 6 hrs (100 x objective)



Immunoblotting, using streptavidin-coupled antibody, showed the presence of active caspases at 4 hours after v-Src inactivation (Figure 24a). In order to determine more accurately the time at which caspases were activated, caspase activity was tested at more frequent earlier time intervals between 0 to 4 hours. Immunoblot results demonstrated that active caspases can be detected in dying cells around 2.5 hours after switching off v-Src (Figure 24b).

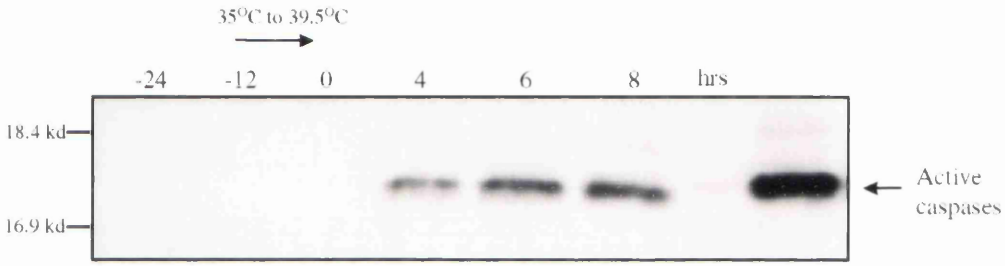
To determine whether the stress activated kinase, p38, was active in dying cells, lysates were obtained at different time intervals, separated by SDS-PAGE and immunoblotted. An antibody, specific for the phosphorylated Thr180/ Tyr182 form of p38, was used to monitor the amount of active p38. Results indicated the presence of active p38 4 hours after the inactivation of v-Src (Figure 25). The same blot was stripped and re-probed for total p38 (both activated and non-activated forms) to confirm that the observed difference was due to enzyme phosphorylation and activation. p38 was present in the cells at all time points in equivalent amounts (Figure 25).

6.4 Effects of interfering with caspase activity

To study the effects of inhibiting caspases, which we showed above to be active at 2.5 hours after v-Src inactivation in serum-starved transformed cells, ZVAD.fmk, a general caspase inhibitor was used (Garcia-Calvo, 1998). The inhibitor was added one hour before inactivating v-Src, at a concentration of 100μM (Figure 26a). Cells were harvested and assayed for TUNEL labelling as before at 0, 2, 4 and 6 hours after switching the serum-starved transformed Rat-1 cells to the restrictive temperature. We observed there was a steady increase in the percentage of labelled cells with time (Figure 26b). The plotted graph represents typical results obtained from a set of 3 experiments. Thus, inhibiting caspase activity was unable to prevent the cells from undergoing apoptosis, at least as measured by TUNEL labelling. However, a comparison of the percentage of cells undergoing apoptosis in the presence and the absence of ZVAD.fmk, showed that there was a small reduction in the number of cells undergoing apoptosis in the presence of the inhibitor (Figure 21). However,

Figure 24 Activation of caspases during apoptosis in transformed Rat-1 cells: (a) Serum starved transformed Rat-1 cells were harvested at different time points before and after v-Src inactivation. Caspase activity in the harvested cells was tested by an assay which involves the binding of biotinylated peptide to the active caspases and its subsequent detection following protein separation by SDS-PAGE and immunoblotting with streptavidin antibody (carried out in collaboration with Kumiko Samejima, Edinburgh, UK). (b) To determine the time of caspase activation more accurately, the experiment was repeated with cells harvested at closer time intervals after induction of apoptosis.

a Caspase activity in serum starved transformed Rat-1 fibroblasts after v-Src inactivation



b Caspase activity in serum-starved transformed Rat-1 fibroblasts after v-Src inactievation at early time points

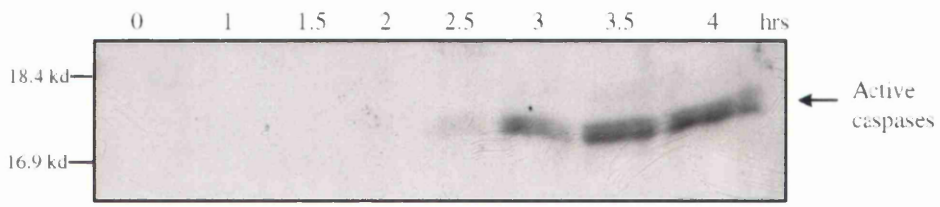


Figure 25 p38 activation during apoptosis in transformed Rat-1 cells: Serum-starved v-Src transformed Rat-1 cells were induced to undergo apoptosis by v-Src inactivation. Cell proteins, obtained at different time intervals after v-Src inactivation were separated by SDS-PAGE and immunoblotted using anti-phospho p38 antibody to detect the presence of phosphorylated/activated p38 (upper panel). The blot was stripped and reprobed for whole p38 to confirm that there was no difference in protein loading (lower panel).

p38 activity during apoptosis in transformed Rat-1 cells in the absence of serum and v-Src activity

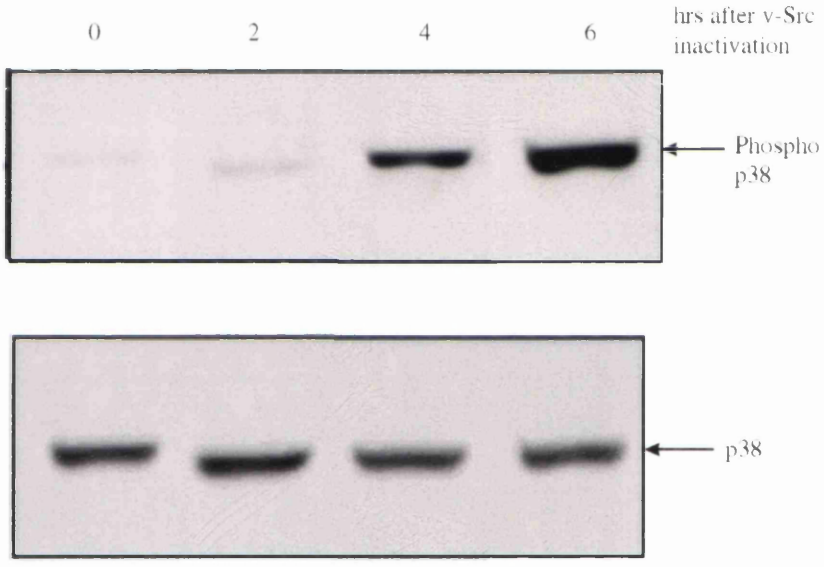
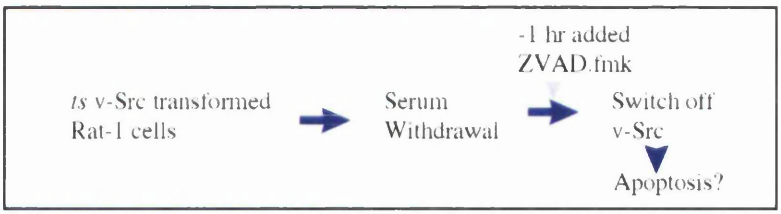
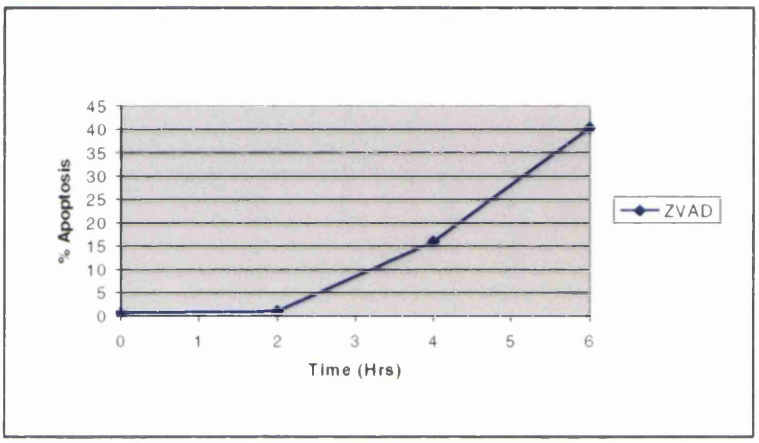


Figure 26 Apoptotic features associated with induction of apoptosis in ZVAD.fmk treated cells: (a) Transformed Rat-1 cells were treated with ZVAD.fmk to inhibit caspase activity, an hour before the induction of apoptosis. (b) Serum-starved cells, induced to die by v-Src inactivation, were harvested at different time intervals and TUNEL labelled to calculate the percentage of cells undergoing apoptosis. The experiment was carried out 3 times and typical results were plotted. (c) DNA isolated from detached or adherent cells 6 hours after induction of the apoptotic response, was separated on a 2% tris-borate agarose gel and stained with ethidium bromide to detect nucleosomal laddering. (d) Cells were viewed using phase contrast microscopy at 6 hours (arrows point to the cells showing membrane blebbing). (e) Cell nuclei were stained with DAPI to detect nuclear fragmentation (carried out by D. Riley, Beatson Institute).

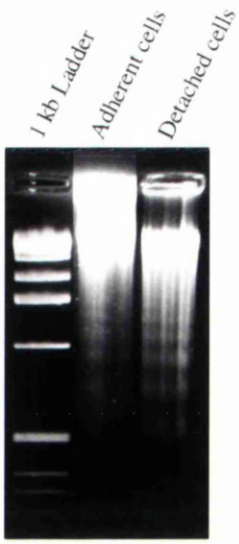
a Diagramatic representation of the experiment carried out with inhibition of caspases



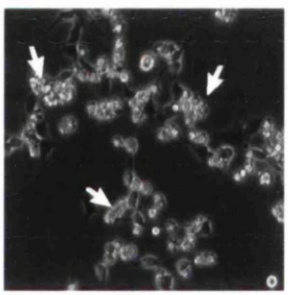
b Percentage of transformed Rat-1 cells undergoing apoptosis in the presence of caspase inhibitor



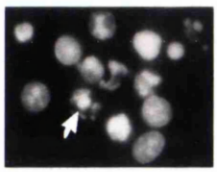
c DNA laddering in detached and adherent cells treated with caspase inhibitor



d Morphology of cells treated with ZVAD.fmk before induction of apoptosis (20 x objective)



e DAPI stained nuclei 6 hrs (100 x objective)



since the percentage of labelled cells lay within the error bars of the controls, it was difficult to ascertain whether this difference was significant.

To determine whether the caspase inhibitor affected DNA laddering, cells were treated with ZVAD.fmk, as before. Detached and adherent cells were harvested separately at 6 hours after switching off v-Src activity. Results showed that, as in the cells without the inhibitor, DNA isolated from both adherent and detached cells separated into approximately 200bp ladders. As before, the laddering was more apparent in the detached cells (Figure 26c and Figure 23b).

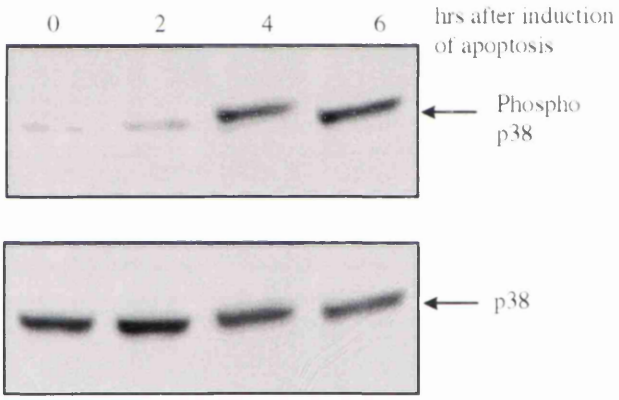
When viewed by phase contrast microscopy, more cells were still in the blebbing state as compared to the control cells (Figure 26d and Figure 22c). Also, the nuclei were fragmented as they were in the cells that had not been treated with ZVAD.fmk (Figure 26e and Figure 22d).

Activation of p38 can be triggered by caspases which, in some cell types, cleave and activate MEKK 1, the upstream kinase (Cardone *et al.*, 1997). Since p38 was active in the cells under study (Figure 25), immunoblots were carried out to determine if inhibiting caspases had any effect on p38 activition. Figure 27 shows that although the amount of p38 was reduced, when compared to the controls (Figure 25), p38 was still activated at about 4 hours after v-Src inactivation (all the samples were run on the same gel and are therefore comparable). Thus, p38 activation was largely independent of caspase activity.

In order to confirm that ZVAD.fmk was indeed inhibiting caspases, its effect on FAK proteolysis, a known caspase substrate (Wen *et al.*, 1997; Chapter 4 of this thesis), was examined. The cleavage of FAK was inhibited by ZVAD.fmk (Figure 20c). Thus, although ZVAD.fmk was inhibiting caspase-mediated proteolysis, it was unable to prevent apoptosis in transformed Rat-1 cells, as measured by TUNEL, DNA laddering or p38 activation.

Figure 27 Biochemical effect of blocking caspase activity: Transformed Rat-1 cells were treated with ZVAD.fmk to inhibit caspase activity, and then induced to undergo apoptosis by v-Src inactivation. Cell lysates obtained at different time points after v-Src inactivation were separated by SDS-PAGE and immunoblotted using anti-phospho p38 antibody to detect p38 activation (upper panel). Blot was stripped and reprobed with antibody against whole p38 (lower panel).

p38 activity in ZVAD.fmk treated cells



6.5 Effects of inhibiting p38 activity

To investigate the role played by p38 in the process of apoptosis, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580), a chemical inhibitor of p38, was used (Saklatvala *et al.*, 1996; Hazzalin *et al.*, 1997).

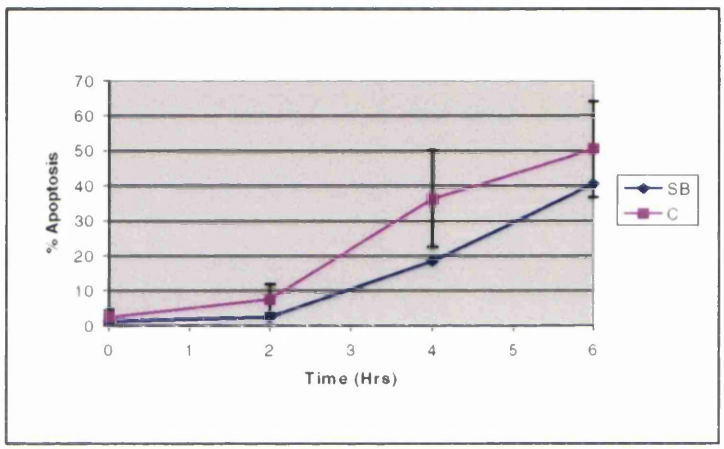
To study the effects of inhibiting p38 activity in serum-starved transformed Rat-1 cells, induced to undergo apoptosis after v-Src inactivation, SB203580 was added one hour before switching the cells to the restrictive temperature (Figure 26a). To determine the percentage of cells undergoing apoptosis, cells, with and without the inhibitor, were harvested at 0, 2, 4, and 6 hours after inactivation of v-Src for TUNEL labelling. A typical result is shown in Figure 28a. There was an increase in the percentage of labelled cells with time. The increase was comparable to that found in control cells without inhibitor (Figure 28a). However, the total percentage of cells undergoing apoptosis in the presence of the inhibitor was apparently slightly less than the control cells, although the significance of this small decrease was not determined.

When viewed at 5 hours, the cells appeared rounded with some clearly in the blebbing stage (Figure 28b), morphological features that were indistinguishable from the control cells (Figure 23c).

In order to verify the state of p38 activity in these cells, cell lysates were separated by SDS-PAGE and immunoblotted using an antibody specific for the phosphorylated Thr 180/ Tyr 182 form of p38. Phosphorylated p38 could be found at 2 hours in cells to which SB203580 was added, as compared to 4 hours in control cells (Figure 29a and Figure 25) and was further increased with time. A possible explanation for why SB203580 did not inhibit p38 activation is that the inhibitor acts downstream of p38. A study by Hazzalin *et al.*, (1997) showed that SB203580 augmented the activity of MKK6, an upstream activator of p38. The increase in MKK6 activity was due to ablation of negative feedback influences which originated from downstream kinases (Hazzalin *et al.*, 1997). SB203580, thus, does not block the activation of p38, which is brought about by the phosphorylation of its Tyr and Thr residues, but inhibits activation of its downstream effectors. Phosphorylated p38 was also detected in cells

Figure 28 Effects of inhibiting p38 activity: (a) Serum-deprived v-Src transformed Rat-1 cells were treated with SB203580, a p38 inhibitor, an hour before being induced to undergo apoptosis as a result of v-Src inactivation. The dying cells were harvested at different time intervals and TUNEL labelled to evaluate apoptosis. The experiment was repeated 3 times and typical results plotted. To compare the number of treated cells undergoing apoptosis to the untreated ones (control), the two were plotted together. (b) Cells were viewed under the microscope using phase contrast to look at the morphology of dying cells.

a Comparison between SB203580 treated and untreated cells undergoing apoptosis



b Morphology of SB203580 treated cells undergoing apoptosis (20 x objective)

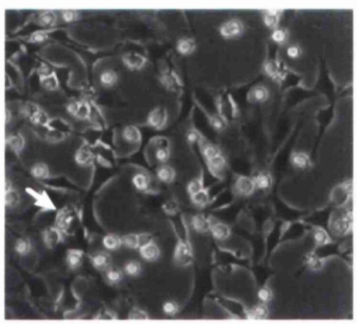
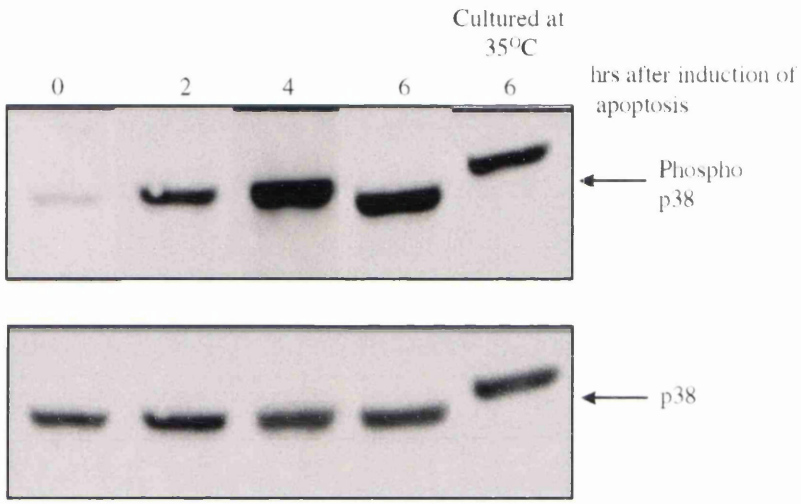
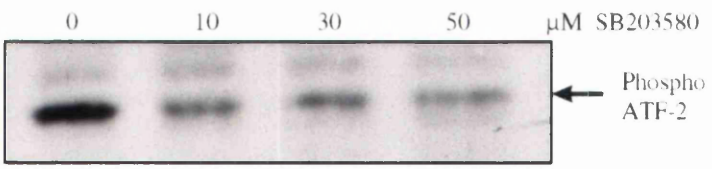


Figure 29 Verification of reduction in p38 activity in the presence of SB203580: (a) In order to determine whether p38 activity was inhibited by SB203580, cell lysates, obtained from cells harvested at different time points after induction of apoptosis in SB203580 treated cells, were separated by SDS-PAGE and immunoblotted for the presence of phospho p38. Lysates prepared from serum-starved transformed Rat-1 cells, which had been treated with the inhibitor but still retained v-Src activity (cultured at $35^{O}C$; harvested at the 6 hour time point), were also probed for phospho p38. (The apparent mobility shift wasn't consistently observed and was a gel artifact). (b) For *in vitro* kinase assay, immunoprecipitated p38, from cells treated with different concentrations of SB203580, was used to phosphorylate ATF-2, a known substrate. Proteins were separated by SDS-PAGE and immunoblotted with anti-phospho ATF-2 antibody to detect amount of p38 activity.

a Detection of phospho p38 in SB203580 treated cells



b *in vitro* kinase assay for p38



harvested at 6 hours, which had been serum-deprived and treated with the inhibitor, but in which v-Src activity had not been switched off (Figure 29a). These data also support the possibility that accumulation of phosphorylated p38 was solely due to the addition of SB203580.

Since the phosphorylation state of p38 could not be used in this case to verify whether p38 activity had been blocked, *in-vitro* kinase assays were carried out. Transformed Rat-1 cells were cultured and serum starved as before. SB203580 was added at 3 different concentrations (of 10μM, 30μM and 50μM), before inactivation of v-Src. Cells were harvested at 6 hours after v-Src inactivation. Cells were lysed and immunoprecipitated p38 was used to determine its ability to phosphorylate ATF-2, a known substrate of p38. Results showed a several fold decrease in p38 activity (Figure 29b).

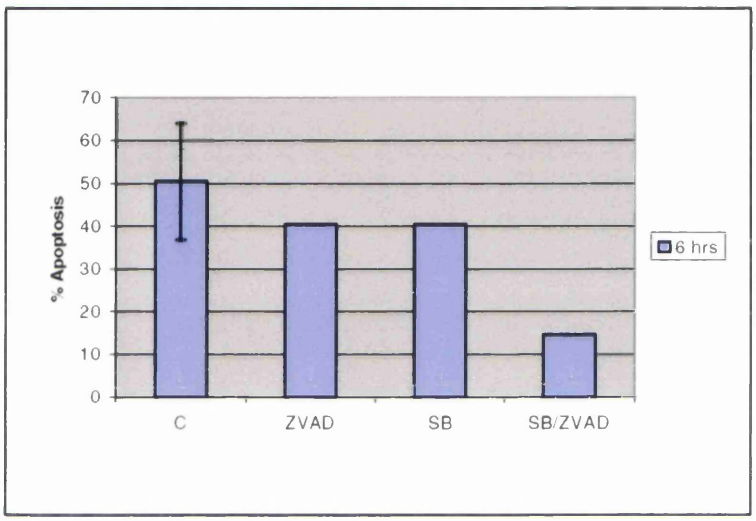
6.6 Inhibiting p38 and caspase activity simultaneously, reduced the percentage of cells undergoing apoptosis

Addition of ZVAD.fmk or SB203580 to serum-starved transformed Rat-1 cells was unable to prevent them from undergoing apoptosis in the absence of v-Src activity. There was, however, an indication that both these inhibitors might have been able to slightly reduce the number of dying cells. Experiments were thus carried out to determine if addition of both ZVAD.fmk and SB203580 together had any additive or synergestic effect on the apoptotic response.

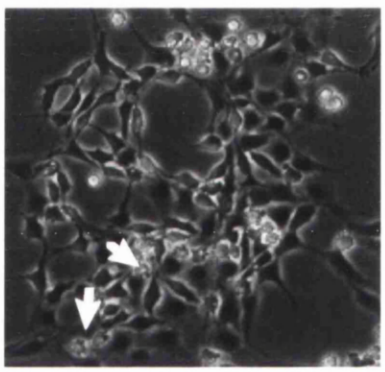
Cells were harvested at 6 hours after switching off v-Src activity to determine the percentage of cells dying by TUNEL labelling. The mean of 4 different values was obtained and was compared to that of the cells undergoing apoptosis at 6 hours in control cells (with no inhibitor added) and to cells which were treated with ZVAD.fmk or SB203580 alone (Figure 30a). The percentage of cells was substantially reduced, (about 70% as compared to the control cells), in the cells treated with both ZVAD.fmk and SB203580 simultaneously.

Figure 30 Inhibition of apoptosis in cells with inhibited caspase and p38 activity: Both ZVAD.fmk (ZVAD) and SB203580 (SB) were added to serum-starved v-Src transformed Rat-1 cells, an hour before v-Src inactivation. (a) Cells were harvested 6 hours after the induction of the apoptotic response and TUNEL labelled to calculate the percentage of cells undergoing apoptosis. The experiment was carried out twice, each time in duplicate, and the mean of the values was compared to the untreated cells (control), and those treated with ZVAD.fmk (ZVAD) and SB203580 (SB). (b) Cells were viewed using phase contrast microscopy. The arrows point to blebbing cells.

a Comparison of percentage of inhibitor-treated and untreated cells undergoing apoptosis



b Morphology of cells treated with ZVAD.fmk and SB203580 (20 x objective)



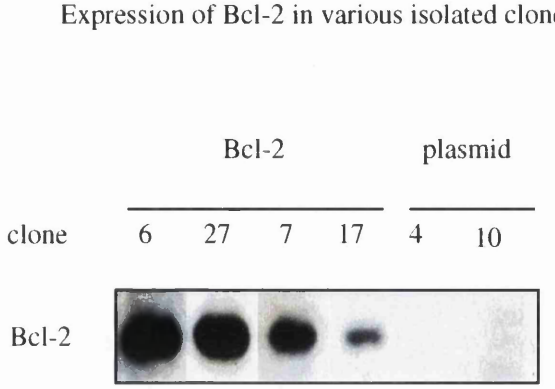
Phase contrast microscopy showed some of the cells in the blebbing state while some were rounded (Figure 30b).

6.7 Bcl-2 overexpression prevented induction of apoptosis

As discussed in section 5.3, overexpression of Bcl-2 can inhibit apoptosis under certain conditions, though not all (Scaffidi *et al.*, 1998). In this study, it was shown that Bcl-2 overexpression inhibited apoptosis only in cells in which caspase activation was delayed, indicating that caspases were not involved in the initiation of the apoptotic response. Results presented in this thesis also show that inhibition of caspases was unable to prevent serum-deprived v-Src transformed Rat-1 cells from dying after switching off v-Src activity, and were thus, possibly not involved in the initiation of the apoptotic response. Therefore, overexpressing Bcl-2 might be able to prevent cell death. To test this, v-Src transformed Rat-1 cells were transfected with pSFFV-Bcl-2 (a mammalian expression vector encoding mouse Bcl-2; all the work presented in this section was carried out by D. Johnson, Beatson Institute, UK). Clones transfected with Bcl-2, or vector alone were isolated after selection for neomycin resistance. Immunoblotting with an anti-Bcl-2 antibody was carried out to confirm Bcl-2 expression in the clones (Figure 31a). Clones transfected with Bcl-2 showed varying expression levels of Bcl-2 protein. On the other hand, those, transfected with plasmid only, did not show any detectable Bcl-2 expression (Figure 31a).

Upon serum-deprivation and v-Src inactivation in the transformed Rat-1 cells, the percentage of dying cells was determined by TUNEL labelling in cells harvested at 6 hours (Figure 31b). Results show that whilst a high percentage of cells transfected with plasmid only (clones 4 and 10) underwent apoptosis, those expressing high levels of Bcl-2 (clones 6 and 27) were prevented from dying (Figure 31b). Also, in clones that did express intermediate levels of Bcl-2 (clones 7 and 17), apoptosis was partially inhibited. Thus, Bcl-2 inhibited death induced by v-Src inactivation in a dose dependent manner.

a



b

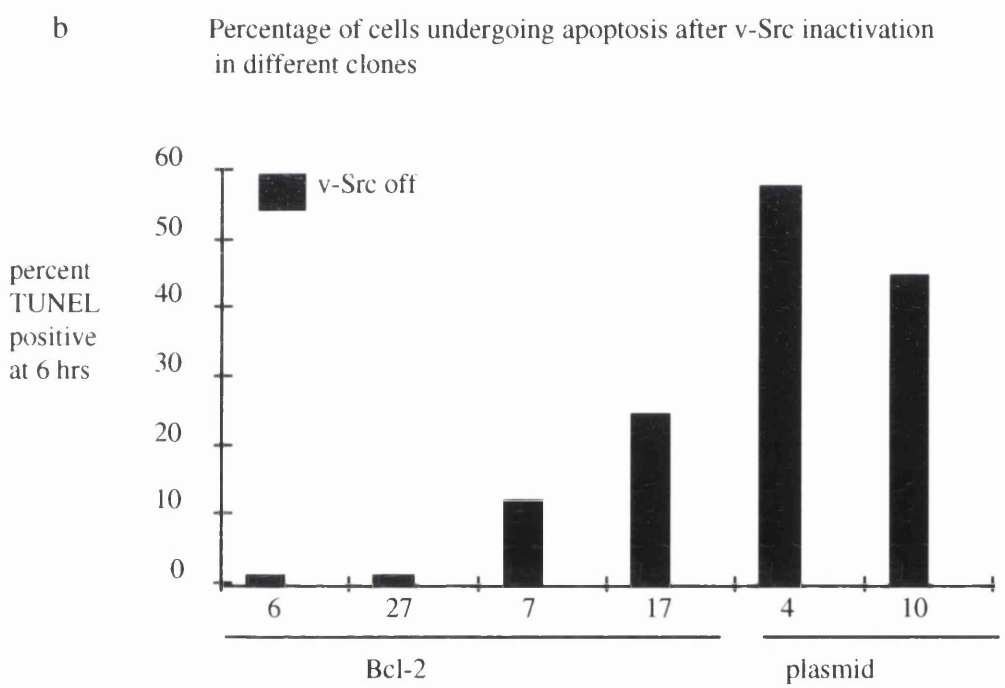


Figure 31 Protection from apoptosis by Bcl-2 overexpression: Transformed Rat-1 cells were transfected with a plasmid construct carrying the gene for mouse Bcl-2. (a) Clones transfected with Bcl-2 and those transfected with vector only, were isolated. Cells from clones resistant to G418 were lysed, the proteins separated by SDS-PAGE and immunoblotted using anti-Bcl-2 antibody to check Bcl-2 expression. (b) Cells derived from the various clones were serum-starved for 24 hours followed by v-Src inactivaion to induce an apoptotic response. Cells were harvested after 6 hours of v-Src inactivation and TUNEL labelled to calculate the percentage of cells undergoing apoptosis. The experiment was carried out several times and typical results were plotted (carried out by D. Riley, Beatson institute, Glasgow, UK).

6.8 Discussion

Apoptosis is a naturally occurring cell suicide pathway. Certain oncoproteins, like c-Myc, are known to induce apoptosis in transformed cells under low serum conditions (Evan *et al.*, 1992). The aim of the study carried out in this chapter, was to characterise the type of cell death induced in serum-deprived Rat-1 fibroblasts that had been transformed with v-Src. However, unlike c-Myc transformed cells, v-Src transformed cells continued to proliferate in low serum and required inactivation of v-Src activity for the induction of cell death in serum-deprived cells. Thus, for the purpose of this study, a temperature sensitive mutant of v-Src was used to transform the Rat-1 fibroblasts. v-Src activity was then abolished by transferring the cells to 39.5°C from the permissive temperature of 35°C, leading to apoptosis.

Cells undergoing apoptosis exhibit certain characteristic features that distinguish them from cells dying through necrosis. The experiments, detailed in this chapter, confirmed that the type of cell death induced in serum-deprived *ts v-src* transformed Rat-1 cells after switching off v-Src activity was apoptotic. The morphology of the dying cells, examined after 5 hours of shifting serum-deprived cells to the restrictive temperature, was similar to the cells undergoing apoptosis, as recorded by previous studies (reviewed in Kerr *et al.*, 1972; Wyllie, 1997). The cells exhibited membrane blebbing, fragmentation of the nuclei and nucleosomal laddering. FACS analysis revealed that there was considerable shrinkage in the cell volume of dying cells. TUNEL labelling was used to quantify apoptosis in serum-starved transformed cells. This assay involves biotin-dUTP labelling of ends of DNA fragments, generated as a result of the processs of apoptosis. The labelling is specific to the process of apoptosis and had been used by some recent studies to quantify the apoptotic response (Okuno *et al.*, 1998; Huang *et al.*, 1999).

Biochemical analysis of cells undergoing apoptosis showed the activation of some enzymes associated with the process of cell death. Caspases were activated about 2.5 hours after switching off v-Src in serum-deprived transformed Rat-1 cells. Caspase activation is known to play an essential role in the execution and, in some cases, the

induction of apoptosis (Yang *et al.*, 1998). Although the assay we used here recognises the binding of a biotinylated substrate to the active site of the caspases that is available only after activation, it cannot be used to distinguish which of the 11 known members of the caspase family are activated. A more detailed analysis to determine which of the caspases was/were activated would provide a better insight into the whether caspases are involved in the execution or induction of apoptosis. Caspase specific antibodies are now becoming available, which will help in such an analysis.

To investigate the role played by the caspases in the commitment to apoptosis and its execution in the transformed cells, a known general inhibitor of the caspases, ZVAD.fmk was used (McCarthy *et al.*, 1997). ZVAD.fmk inhibits caspase activity by competitively binding to the active site. TUNEL labelling results showed that the percentage of ZVAD.fmk treated cells, undergoing apoptosis, was comparable to the untreated ones, indicating that inhibiting caspase activity did not prevent the cells from dying, as measured by TUNEL labelling. Chromosomal DNA was found to be fragmented into nucleosomal ladders and morphologically, the inhibitor treated cells were found to be smaller in size and exhibited membrane blebbing. However, more cells were still in the blebbing stage as compared to the untreated cells. Despite the generally accepted importance of caspase activity in the process of apoptosis, there are studies that report that inhibition of caspases by ZVAD.fmk is unable to prevent apoptosis (Xiang *et al.*, 1996; McCarthy *et al.*, 1997). Membrane blebbing in the ZVAD.fmk treated cells was also reported by McCarthy *et al.*, (1997). They used membrane blebbing as a characteristic of onset of apoptosis. According to their findings, although ZVAD.fmk was unable to block the onset of apoptosis, it inhibited the completion of the process. However, McCarthy *et al.*, (1997), reported that the cells appeared to die by necrosis rather than by apoptosis. Also, unlike the finding of our study, other studies have reported that although unable to prevent cell death in some cases, ZVAD.fmk inhibited DNA laddering (Xiang *et al.*, 1996; McCarthy *et al.*, 1997). Indeed, a caspase activated deoxyribonuclease, CAD, has been identified (Enari *et al.*, 1998). Recent studies showed that the production of nucleosomal laddering required CAD, although chromatin condensation and nuclear fragmentation were independent of CAD activity (Samejima *et al.*, 1998). Instead, AIF, the

mitochondrial apoptosis-inducing factor, is reported to be responsible for chromatin condensation and large-scale fragmentation of DNA (Susin *et al.*, 1999b). However, a previous report had stated that the release and function of AIF was biochemically distinct from the DNAse activity attributed to the mitochondria (Susin *et al.*, 1999a). Thus, there is a possibility that other factors exist that might be responsible for caspase-independent nucleosomal laddering of chromosomal DNA. Other results in our study also support a mitochondrial-dysfunction-dependent mechanism for apoptosis in transformed Rat-1 cells deprived of serum and v-Src activity (discussed below).

The caspase-independent nature of cell death reported through the findings of this chapter, imply that mitochondrial dysfunction might be involved in the initiation of apoptosis. The caspase-independent induction of apoptosis in v-Src transformed Rat-1 cells is also implied by the prevention of apoptosis by overexpression of Bcl-2. Studies concerning the mechanisms by which Bcl-2 prevents apoptosis also show that it involves mitochondrial dysfunction and cytochrome C release (Yang *et al.*, 1997; Rosse *et al.*, 1998; Zhivtovsky *et al.*, 1998). However, it is possible that caspases might still be required for the execution of certain biochemical and morphological changes occurring downstream of DNA laddering and membrane blebbing. If true, this would explain why the cells were positive for TUNEL labelling. To determine if mitochondrial dysfunction is upstream or downstream of caspase activation, and thus, whether the type of apoptosis observed in the transformed Rat-1 cells is caspase-dependent or independent, it would be important to establish if prevention of apoptosis by Bcl-2 is able to prevent caspase activation. Also, comparison of the timing of cytochrome C release with that of caspase activation would throw more light on the sequence of events followed during the apoptotic process.

Apoptosis after switching off v-Src was also accompanied by the activation of p38, a stress activated kinase. p38 has been implicated in the induction of apoptosis in PC12 and Rat cerebellar granule cells (Xia *et al.*, 1995; Kawasaki *et al.*, 1997). MEKK 1, a p38 activator, has also been shown to play an important role in apoptosis (Johnson *et al.*, 1996). However, the role played by p38 in the process of apoptosis is

contentious. A study by Assefa *et al.*, (1999), found that inhibition of p38 enhanced PDT-induced apoptosis. Results presented here showed that, although, p38 activation accompanied the induction of apoptosis in serum-starved transformed Rat-1 cells, inhibition of p38 activity was unable to prevent cell death, as measured by TUNEL labelling. Also, p38 is known to be required for membrane blebbing during apoptosis (Huot *et al.*, 1998), but our results showed that morphological features characteristic of apoptosis, including membrane blebbing and cell shrinkage, were not inhibited. Besides p38, myosin light chain kinase (MLCK) has also been implicated in regulation of membrane blebbing during apoptosis (Mills *et al.*, 1998). Inhibition of MLCK decreases membrane blebbing, while, myosin regulatory light chain (MLC) phosphorylation increases in blebbing cells (Mills *et al.*, 1998). It is thus, possible, that in v-Src transformed Rat-1 cells, MLCK, and not p38, is responsible for membrane blebbing, during apoptosis.

Further investigation into the role played by p38 during apoptosis, could be carried out using a different approach, such as using antisense oligonucleotides. Using this technique, translation of p38 mRNA would be inhibited, ensuring inactivity of p38. Also, the action of some inhibitors can be non-specific. Although SB203580 has been used in several studies to specifically investigate the effects of inhibiting p38 activity, it is possible that it might be acting on other proteins too. The antisense approach would be able to circumvent this problem too.

Results of our study also showed that, unlike the effects of adding p38 and caspase inhibitors separately, adding the two inhibitors together was able to reduce the percentage of cells undergoing apoptosis. This suggests that both p38 and caspases are most likely involved in contributing to cell death independently, but that inhibiting either pathway alone is not sufficient to inhibit cell death. It is possible that these two distinct pathways regulate different aspects of apoptosis. Nevertheless, since the inhibition of cell death upon inhibiting both these pathways was not complete, there is a possibility of the existence of one or more pro-apoptotic pathways in the mediation of apoptosis. One likely candidate is the JNK pathway, another stress activated kinase. As mentioned before in section 5.6, several studies have shown JNK to be involved in the process of apoptosis. Also, work in our lab has

shown that JNK was activated in serum-deprived transformed Rat-1 cells, by 4 hours after v-Src inactivation (D. Riley, unpublished data). JNK activation was partially attenuated in cells treated with ZVAD.fmk, while overexpression of Bcl-2 was able to prevent JNK activation (D. Riley, unpublished data). Further investigations into the role played by JNK in the process of apoptosis could be carried out using JNK specific inhibitors or expression of dominant negative forms. Using antisense for JNK could also provide useful information.

Chapter 7: Results 4

Characterisation of survival signalling induced by v-Src in serum-deprived transformed Rat-1 cells: a possible role for the PI 3-kinase/Akt pathway

7.1 Introduction

It is evident from the results of the previous chapter that inactivation of v-Src induces an apoptotic response in serum-deprived transformed Rat-1 fibroblasts. Since these cells do not die by apoptosis upon withdrawal of serum, this means that v-Src activity induces survival signalling that protects the cells that have otherwise been primed to die, perhaps due to lack of integrin-signalling, when they are serum-starved. It was, thus, of considerable interest to determine the pathway used by v-Src to protect the cells from undergoing apoptosis.

7.2 Role of PI 3-kinase downstream of v-Src

PI 3-kinase is a lipid kinase and a known oncogene (Chang *et al.*, 1997; reviewed in Porter and Vaillancourt, 1998). Studies have shown that v-Src binds to PI 3-kinase through the SH3 domain (Liu *et al.*, 1993; Haefner *et al.*, 1995) and plays a role in v-Src induced cell transformation (Whitman *et al.*, 1985; Fukui and Hanafusa, 1989). PI 3-kinase also mediates Ras-induced survival signalling (Khwaja *et al.*, 1997). It was, therefore, a candidate mediator of the survival signal downstream of v-Src.

In order to determine whether PI 3-kinase is responsible for survival signalling downstream of v-Src, it was necessary to monitor the cellular PI 3-kinase activity, and to investigate whether it was altered after v-Src inactivation. To ascertain the levels of PI 3-kinase activity, Akt phosphorylation in the cells was used as a measure of cellular PI 3-kinase activity. Akt is known to bind to the lipid products of PI 3-kinase and this, in turn, facilitates its translocation to the membrane followed by its phosphorylation and activation by PDK-1 at Thr308 and Ser473 (Franke *et al.*, 1997; Andjelkovic *et al.*, 1997; Alessi *et al.*, 1997).

Cell lysates were prepared from serum-starved v-Src transformed Rat-1 cells before and after inactivation of v-Src. Proteins were separated by SDS-PAGE and immunoblots carried out using an anti-Akt antibody specific for Ser473 in its phosphorylated state. Akt was found to be phosphorylated at all times prior to inactivation of v-Src, followed by complete reduction in phosphorylation of Akt at

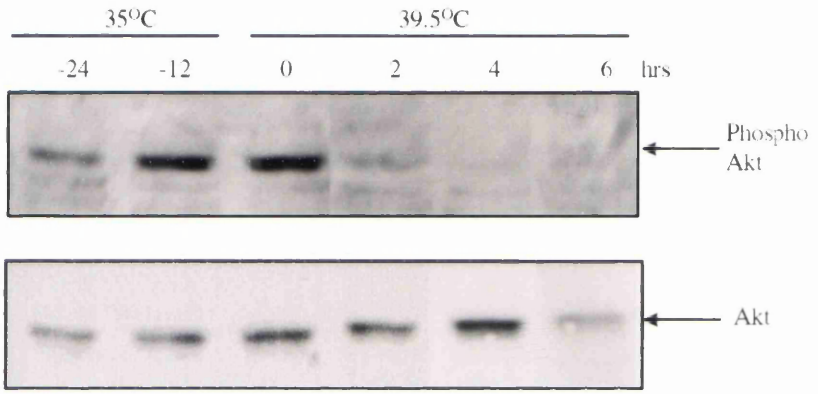
Ser473 at 2, 3 and 6 hours after v-Src inactivation (Figure 32a). The blot was stripped and re-probed with an antibody specific for total Akt (phosphorylated and non-phosphorylated) to ensure that the difference in detection of Akt phosphorylation was not due to unequal protein loading or altered protein expression (Figure 32a). In order to estimate more accurately the time of decrease in Akt phosphorylation, the experiment was repeated with cells being lysed at earlier and more frequent times after v-Src inactivation *i.e.* 0, 15min, 30min, 45min, 1 hour and 1.5 hours after v-Src inactivation. The results showed loss of Akt-Ser473 phosphorylation between 30-45min (Figure 32b).

The decrease in Akt phosphorylation, and its consequent inactivation, could either be a cause or a consequence of activating the apoptotic signal. To investigate whether Akt phosphorylation was decreased in the cells before commitment to apoptosis, experiments were carried out to determine the point at which cells could not be rescued from cell death. Thus, serum was added back to serum-starved transformed cells at different times after v-Src inactivation. Cells were harvested 6 hours after switching off v-Src activity, and TUNEL labelled to determine the percentage of cells undergoing apoptosis. We found that cells could be prevented from undergoing apoptosis if serum was added at 2 hours after v-Src inactivation, but by 4 hours, the cells were already committed to die, and addition of serum after this time was unable to prevent them from death (Figure 33).

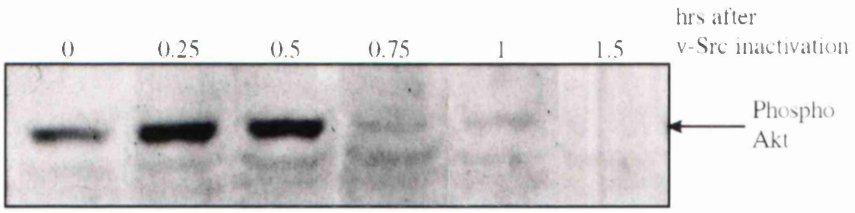
These data indicated that inactivation of Akt, through a decrease in its Ser473 phosphorylation, preceded commitment to cell death. Therefore, activated Akt, probably via PI 3-kinase, was a likely candidate for mediation of the survival signal downstream of v-Src. In order to determine the role played by PI 3-kinase and Akt, a PI 3-kinase specific inhibitor, 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), was used (Yano *et al.*, 1995). To confirm that LY294002 inhibited Akt phosphorylation, an experiment was carried out as outlined (Figure 34a) except that 50μM inhibitor was added one hour before v-Src was inactivated (in cells not treated with LY294002). This was done to allow the inhibitor time to enter the cells. Cell lysates obtained at different times after addition of LY294002 were separated by SDS-PAGE and immunoblotted using anti-Akt antibody specific for phospho-Ser473

Figure 32 Decreased Akt phosphorylation in the absence of v-Src activity: (a) Cell lysates were prepared from serum-starved Rat-1 cells, before and after v-Src inactivation, separated by SDS-PAGE and immunoblotted for the presence of phospho and total Akt. (b) For a more accurate estimate of the time of downregulation of Akt activity, cell lysates were obtained at earlier time points, separated by SDS-PAGE and probed for phospho Akt.

a Decrease in Akt phosphorylation during apoptosis



b Determination of the time at which Akt phosphorylation is decreased



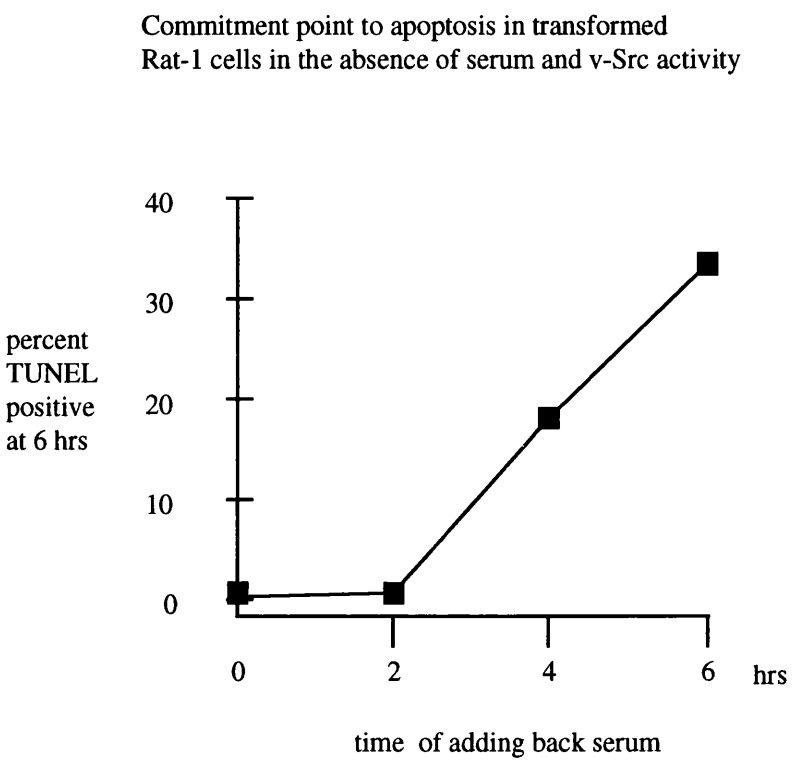
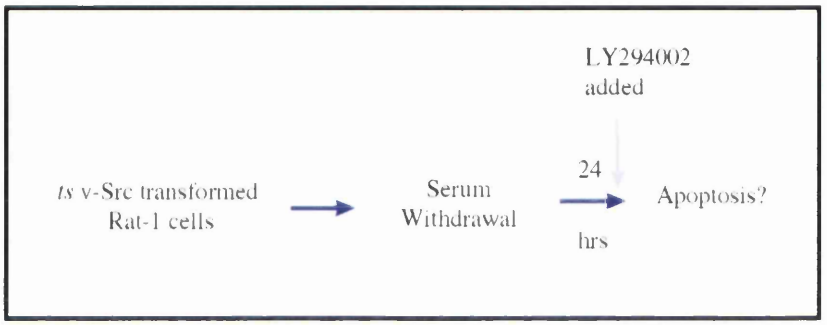


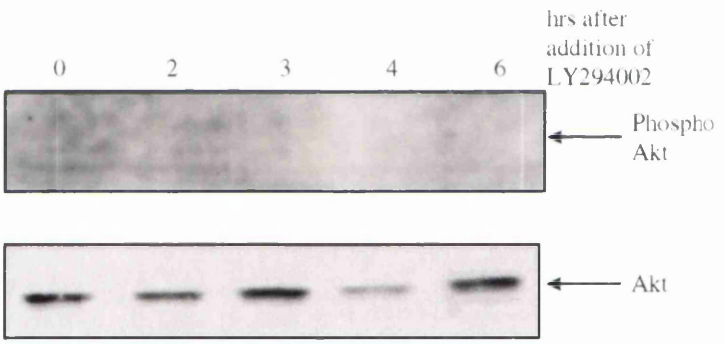
Figure 33 Commitment to undergo apoptosis after 2 hours of v-Src inactivation in serum-starved transformed Rat-1 cells: v-Src transformed Rat-1 cells were serum starved for 24 hours followed by v-Src inactivation. Serum was added back to the cells at different times after v-Src inactivation and the percentage of cells undergoing apoptosis was determined by TUNEL labelling the cells at 6 hours (experiment carried out by D. Riley, Beatson Institute, Glasgow, UK).

Figure 34 Decreased Akt phosphorylation in the presence of PI 3-kinase inhibitor: (a) LY294002, a PI 3-kinase inhibitor, was added to serum-starved transformed Rat-1 cells at a concentration of 50μM. (b and c) Lysates were prepared from LY294002 treated cells at different time intervals, separated by SDS-PAGE and immunoblotting was carried out using anti-phospho Akt antibody or anti-total Akt antibody (lower panel in (b)).

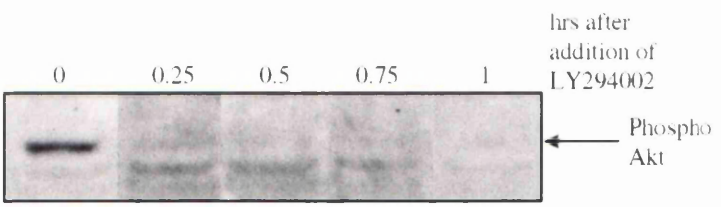
a Diagramatic representation of the experiment carried out using PI 3-kinase inhibitor



b Effect of inhibiting PI 3-kinase on Akt phosphorylation



c Time taken by LY294002 to inhibit Akt phosphorylation



as probe. Akt was found to be dephosphorylated even at 0 hours *i.e.* the one hour pre-treatment with LY294002 had reduced the phosphorylation of Akt (Figure 34b). We next determined the time taken by LY294002 to inhibit Akt phosphorylation. Lysates were prepared as before at the time of adding LY294002 (T=0) and then at 15min, 30min, 45min and 1 hour. We found that LY294002 was able to inhibit Akt phosphorylation within 15min of being added to the cells (Figure 34c). Thus, for all further experiments, LY294002 was added at 0 hours *i.e.* the same time as v-Src was inactivated in control cells.

To determine if the PI 3-kinase inhibitor, LY294002, was able to induce cell death in transformed Rat-1 cells in which v-Src remained active, experiments were carried out as outlined in Figure 34a. Cells were harvested at different times after the addition of LY294002 at 50μM concentration. TUNEL labelling was carried out to determine the percentage of cells undergoing apoptosis when v-Src was active and the PI 3-kinase inhibitor added. The plotted results are representative of a set of three experiments (Figure 35). The percentage of cells undergoing apoptosis increased with time, showing that LY294002 was able to induce apoptosis in serum-starved transformed Rat-1 cells in the presence of v-Src activity. However, compared to the cells induced to undergo apoptosis by v-Src inactivation, the percentage of cells induced to die by addition of LY294002 was lower or the rate of cell death was reduced (Figure 35).

7.3 Effect of LY294002 is specific to v-Src transformed Rat-1 cells

In order to confirm that the induction of apoptosis in serum-deprived v-Src transformed Rat-1 cells induced by LY294002 was a result of specific inhibition of v-Src survival signalling, we compared the effect of drug treatment with that in normal Rat-1 cells.

Thus, LY294002 was added to normal Rat-1 fibroblasts, grown at 35°C (R-35) or 39.5°C (R-39.5), and v-Src transformed Rat-1 cells grown at the restrictive temperature of 39.5°C (*LA*-39.5). The experiment was carried out as depicted (Figure 36a). v-Src activity in the control cells (*LA*-35) was switched off at 0 hours, in the absence of the inhibitor. Cells were harvested for TUNEL labelling at 0 and 6 hours.

Comparison between number of cells undergoing apoptosis in the presence of LY294002, with those in which v-Src has been switched off

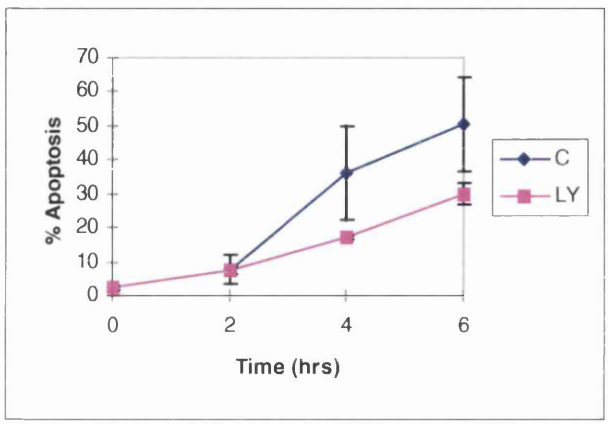
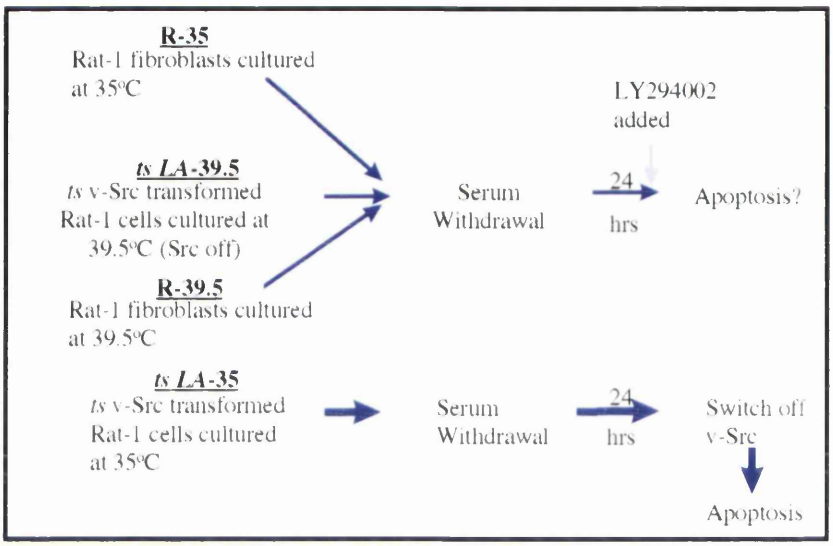


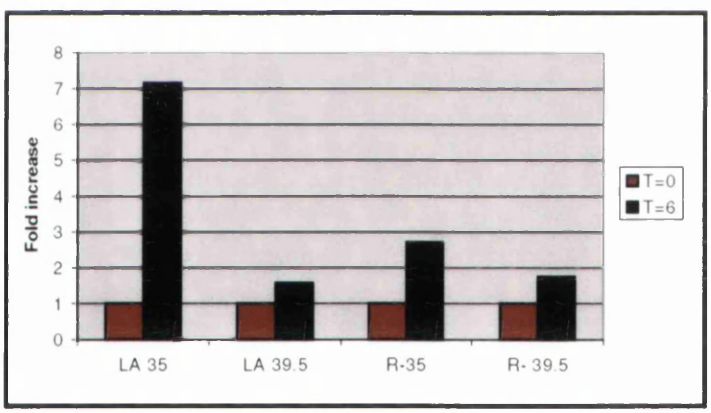
Figure 35 Induction of apoptosis in serum-starved transformed Rat-1 cells after addition of a selective PI 3-kinase inhibitor: Cells were harvested at different times after the addition of LY294002 and TUNEL labelled. The percentage of labelled cells was calculated and typical results plotted. The percentage of cells undergoing apoptosis was compared to the percentage dying in the absence of v-Src activity.

Figure 36 LY294002 did not induce apoptosis in Rat-1 cells displaying normal phenotype: (a) Transformed and non-transformed Rat-1 cells were cultured at either 35^{O}C (permissive temperature for *ts LA29* v-Src) or 39.5^{O}C (restrictive temperature for *ts LA29* v-Src). LY294002 was added to transformed and non-transformed Rat-1 cells cultured at 39.5^{O}C and non-transformed Rat-1 cells cultured at 39.5^{O}C. At the same time, v-Src activity was inhibited in transformed Rat-1 cells cultured at 35^{O}C. (b) Cells were harvested at the time of LY294002 addition or v-Src inactivation and 6 hours after, and percentage of cells undergoing apoptosis was calculated by TUNEL labelling. (c) Also, cell lysates prepared from cells were separated by SDS-PAGE, and screened for the presence of phospho and whole Akt by immunoblotting.

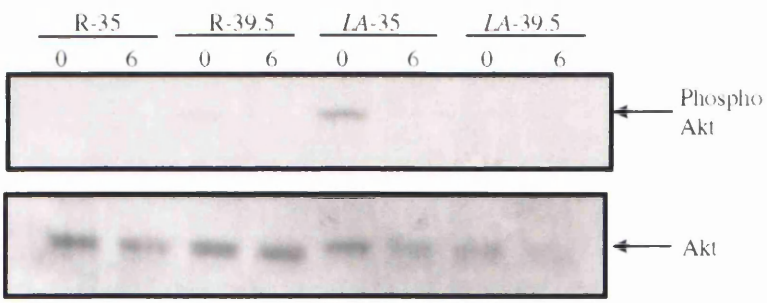
a Diagramatic representation of the experimental protocol to investigate the role of PI 3-kinase in normal and v-Src transformed Rat-1 cells



b Comparison between percentage of cells dying in the presence of LY294002 to the percentage dying after switching off v-Src



c State of Akt phosphorylation



The percentage of labelled cells at 6 hours was plotted as fold increase over the percentage at 0 hours for each cell type (Figure 36b). For control cells, to which no inhibitor was added and v-Src was inactivated, there was a significant increase in the percentage of dying cells at 6 hours compared to those at 0 hours (about 7 fold). In contrast, the difference in the percentage of TUNEL labelled cells at 0 and 6 hours in the drug treated normal Rat-1 cells (including the transformed cells grown at restrictive temperature) was much lower. This indicated that the effect of adding LY294002 was specific to the inhibition of PI 3-kinase activity downstream of v-Src, resulting in death of only the transformed cells.

Also, cell lysates prepared at 6 hours after inactivation of v-Src (in *LA*-35), or addition of LY294002 (in R-35, R-39.5, and *LA*-39.5), were tested for the presence of phospho Akt. The results showed that Akt was in the non-phosphorylated form in all cells except for serum-starved *LA*-35 cells at the time of v-Src inactivation (Figure 36c).

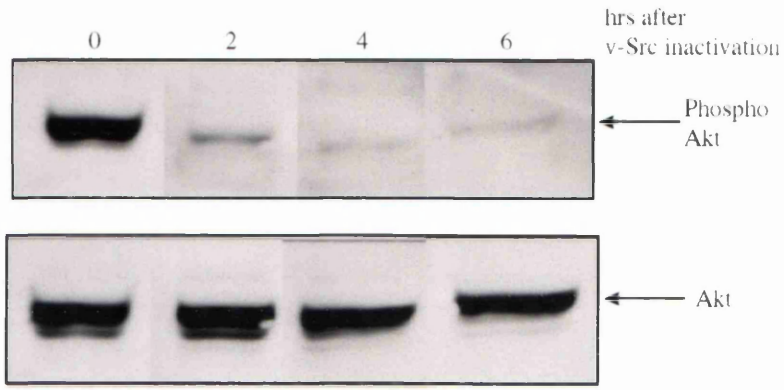
7.4 Decreased Akt phosphorylation in cells treated with SB203580 and ZVAD.fmk and those overexpressing Bcl-2

To determine if the addition of the selective inhibitors of p38 or caspases had any effect on Akt phosphorylation, lysates prepared from transformed cells treated with ZVAD.fmk (caspase inhibitor) or SB203580 (p38 inhibitor), were also screened with the Akt-Ser473 phospho-specific antibody. Akt phosphorylation was decreased in cells treated with ZVAD.fmk (Figure 37a). The decrease was evident within 2 hours of switching off v-Src activity in transformed Rat-1 cells that had been pre-treated with ZVAD.fmk. Also, in cells treated with SB203580, Akt phosphorylation was reduced within 2 hours of v-Src inactivation (Figure 37b).

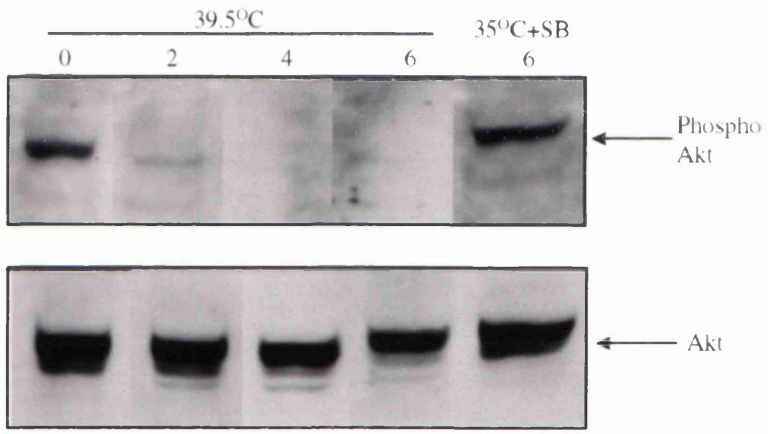
Lysates were prepared from cells transfected with Bcl-2 (cloned into pSFFV) and with the pSFFV vector alone, at different times after v-Src inactivation. Screening with the antibody specific to phospho-Ser473 of Akt, showed that Bcl-2 did not inhibit the decrease in Akt phosphorylation, implying that Bcl-2 blocks cell death downstream of PI 3-kinase/Akt (Figure 37c).

Figure 37 Akt phosphorylation is decreased in cells treated with inhibitors of caspases and p38, as well as those overexpressing Bcl-2: (a and b) Transformed Rat-1 cells were treated with ZVAD.fmk or SB203580 an hour before v-Src inactivation. Cell lysates were prepared at different times after v-Src inactivation, separated by SDS-PAGE and immunoblotting was carried out using anti-phospho Akt or anti-total Akt antibodies. (c) Transformed Rat-1 cells overexpressing Bcl-2 were deprived of serum for 24 hours followed by v-Src inactivation. Cell lysates prepared at different time intervals after v-Src inactivation were screened for phospho and total-Akt (carried out by D.Johnson).

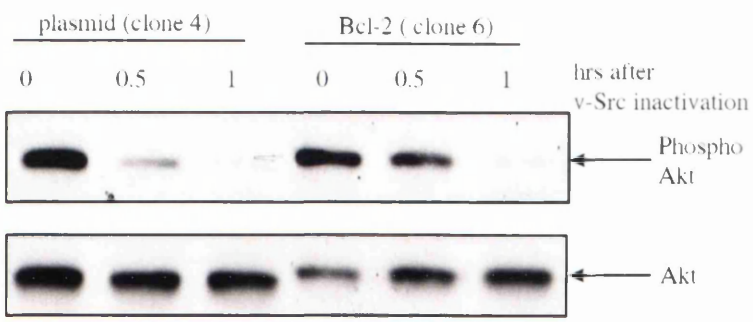
a Akt phosphorylation in cells treated with ZVAD.fmk



b Akt phosphorylation in cells treated with SB203580



c Akt phosphorylation in cells overexpressing Bcl-2



7.5 Decreased MAP kinase activity in cells undergoing apoptosis

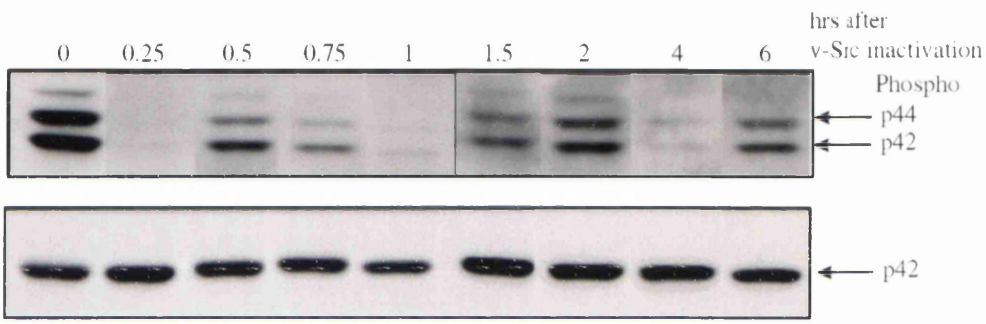
Inhibition of PI 3-kinase activity was able to induce around 50% of the cell death induced after v-Src inactivation, suggesting the possibility that another survival pathway may operate downstream of v-Src. Among the downstream effectors of v-Src transformation is the activation of the MAP kinase (ERK) cascade, which can contribute to survival signalling (Xia *et al.*, 1995; Matsuda *et al.*, 1999). Experiments were therefore carried out to find if any change in ERK activity was associated with apoptosis.

Thus, cell lysates prepared from transformed Rat-1 cells undergoing apoptosis in the absence of serum and v-Src activity, were separated by SDS-PAGE and immunoblotting was carried out using an anti ERK antibody specific for the phosphorylated form of ERK. ERK is activated by phosphorylation on Thr202/ Tyr204 by MEK1 (Kyriakis and Avruch, 1996). A rapid decline in ERK phosphorylation, and therefore presumably its activity, was noticed within 15min after inactivating v-Src (Figure 38a). There was an apparent regain of activity at 30min, which declined again. ERK activity was detected again at 1.30 and 2 hours, which declined at 4 hours and came again at 6 hours. There was, thus, a cyclic gain and loss of ERK activity during apoptosis. At the time points where there was a regain of ERK activity, it was not to the same extent as that found at 0 hours. Slightly different trends were noticed in some other experiments that showed regain of ERK activity at 4 hours. However, in all cells, ERK fluctuated.

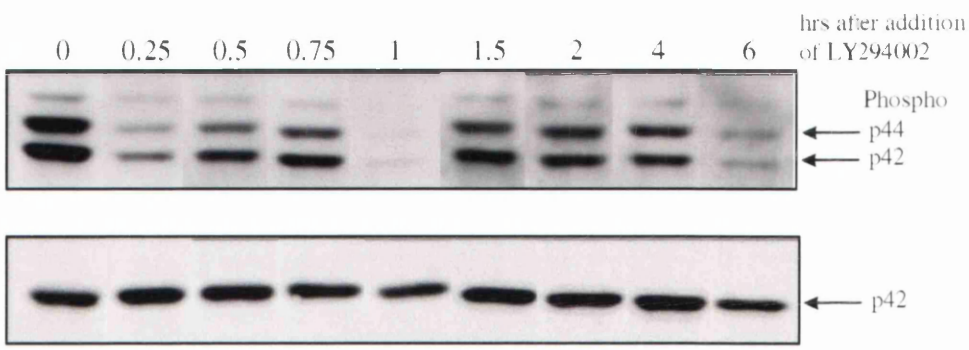
Similar analysis was also carried out in cells treated with LY294002 (Figure 38b). Immunoblot analysis using the anti phospho-ERK antibody showed a decrease in ERK activity at 15min, followed by a regain of activity at 30 and 45min. 1 hour after the addition of LY294002, no ERK activity could be detected. At 1.5, 2 and 4 hours, there was again a gain of ERK activity followed by inactivation again at 6 hours. As in the case of control cells, the gain of ERK activity at the later time points was not to the same extent as that noticed at 0 hours (Figure 38a and Figure 38b). The cyclic trend of activation and inactivation was thus similar to that in the control, although the timing of the fluctuations was variable.

Figure 38 Variation in MAP kinase activity in serum-starved v-Src transformed Rat-1 cells induced to undergo apoptosis in the absence of v-Src activity or in the presence of PI 3-kinase inhibitor: (a and b) Serum-starved v-Src transformed Rat-1 cells were induced to undergo apoptosis either by v-Src inactivation or by inhibition of PI 3-kinase activity. Cell lysates were prepared at different times after induction of apoptosis, separated by SDS-PAGE and probed for the presence of phosphorylated p42/p44 (ERK) or total p42 (ERK2).

a MAP kinase activity in cells undergoing apoptosis after v-Src inactivation



b MAP kinase activity in cells undergoing apoptosis in the presence of LY294002



7.6 ERK does not play a role in the mediation of survival signals downstream of v-Src

To determine whether dephosphorylation of ERK, upon switching off v-Src activity in serum-deprived transformed Rat-1 cells, was responsible for induction of apoptosis, 2-Amino-3-methoxyflavone (PD98059), a specific inhibitor of MEKK 1 (the upstream ERK activating kinase; Alessi *et al.*, 1995; Pang *et al.*, 1995), was used.

Experiments were carried out as described before for LY294002, the selective PI 3-kinase inhibitor. Cells were harvested at different times after addition of 50μM PD98059 for TUNEL analysis. The percentage of labelled cells was plotted as a function of time (Figure 39). There was a minimal increase in the number of cells undergoing apoptosis from about 2% at 0 hours to about 9% at 6 hours. When compared to the number of cells dying after serum-starvation and v-Src inactivation, the percentage of cells dying in the presence of the inhibitor was very small (Figure 39). Thus, inhibiting ERK activation in the presence of v-Src activity was unable to induce apoptosis in serum-deprived transformed Rat-1 cells indicting that ERK was not a key player in inducing survival downstream of v-Src.

In order to determine if addition of PD98059 had any effect on the Akt survival signal induced by v-Src, cell lysates were separated by SDS-PAGE and immunoblotted to detect phosphorylated Akt. There was no decline in the levels of phospho-Akt up to 2 hours (Figure 40a). At 4 and 6 hours after the addition of PD98059, there seemed to be a slight decrease in the amount of Akt activity. After one hour, little phospho-Akt was detected. However, probing the same blot with antibody against total Akt showed that this could be explained by unequal protein loading (Figure 40a).

Effectual working of the inhibitor was confirmed by stripping the blot and reprobing it with anti ERK antibody specific for the phosphorylated form. Results showed a decline in ERK phosphorylation within 15min of adding PD98059 followed by complete dephosphorylation by 30min (Figure 40b).

Comparison between percentage of cells undergoing apoptosis in the absence of v-Src activity and those dying in the presence of PD98059

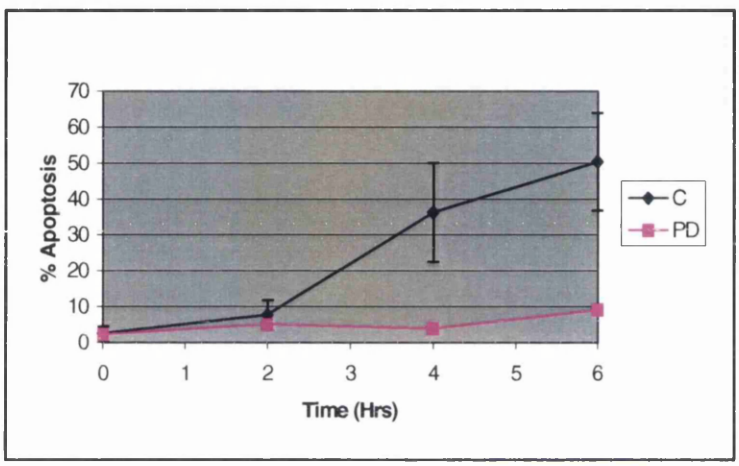
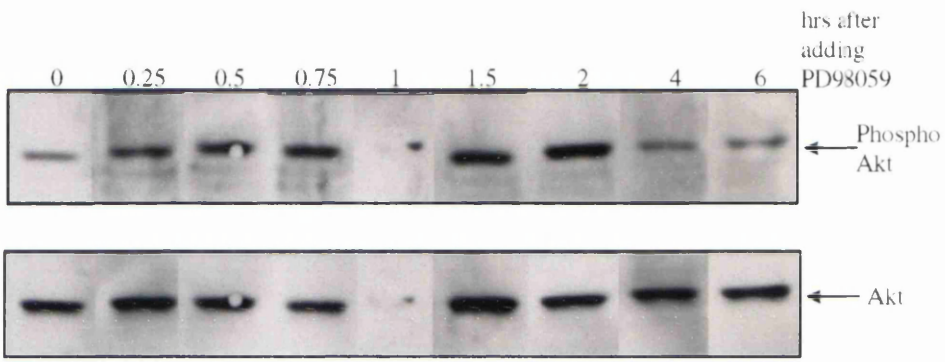


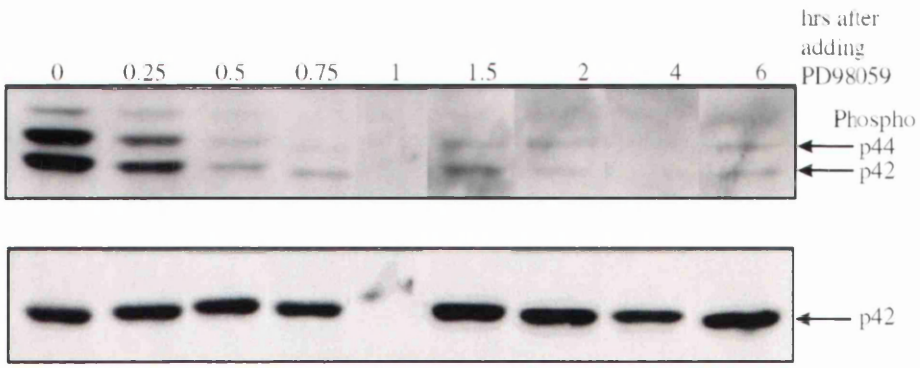
Figure 39 Inhibition of MAP kinase activity does not induce apoptosis in serum-starved transformed Rat-1 cells: PD98059 was added to serum-starved v-Src transformed Rat-1 cells in order to inhibit MAP kinase activity. Cells were harvested at different times to calculate the percentage of cells undergoing apoptosis by TUNEL labelling. The experiment was carried out 3 times and typical results were plotted. The percentage of cells dying in the presence of the inhibitor was compared to those dying in the absence of v-Src activity.

Figure 40 PD98059 was able to inhibit MAP kinase activity but did not affect Akt phosphorylation: PD98059 was added to serum-starved v-Src transformed Rat-1 cells. (a) Lysates were prepared from the treated cells at different time intervals after addition of the inhibitor, separated by SDS-PAGE and screened for phospho and total Akt. (b) cell lysates were also probed for phospho p42/p44 (ERK) and total p42 (ERK).

a Akt phosphorylation in cells treated with MAP kinase inhibitor



b Inhibition of MAP kinase activity in cells treated with PD98059



7.7 Inhibiting PI 3-kinase and ERK activity simultaneously did not increase the effect of blocking PI 3-kinase alone

Results from the previous section confirmed that inhibition of ERK activity was unable to induce apoptosis in serum-deprived v-Src transformed cells. It was, however, possible that inhibiting ERK activity at the same time as PI 3-kinase could enhance the effect of blocking PI 3-kinase activity on its own.

Cells treated with both inhibitors, PD98059 and LY294002, were harvested at different times for TUNEL labelling. The percentage of cells undergoing apoptosis was plotted as a graph and compared to the control cells (with no inhibitor and v-Src activity switched off), and cells treated with LY294002 or PD98059 separately (Figure 41). The percentage of labelled cells was similar to that after addition of LY294002 alone. Therefore, there was no enhanced effect of adding both the inhibitors simultaneously.

7.9 Discussion

Results of the previous chapter showed that in the absence of v-Src signalling, serum-starved v-Src transformed Rat-1 cells undergo apoptosis. This indicated that v-Src was able to provide the serum-starved transformed Rat-1 cells with a survival signal. When v-Src activity was switched off, cells were committed to undergo apoptosis, possibly because of absence of normal integrin-mediated signalling that had been lost as a result of transformation by *ts LA29 v-src.* In this regard, it is known that transformation by v-Src results in reduced production of fibronectin matrix and redistribution of actin and α-actinin (Stoker *et al.*, 1986). The possible deleterious effect of v-Src on cell viability had been implied by an earlier study as well which showed that v-Src transformed cells either died or kept multiplying in low serum (Wyke, 1971). This was not surprising since other mitogenic oncoproteins, such as c-Myc, E2F-1 and v-Jun, have also been shown to induce apoptosis under low serum conditions (Shan and Lee, 1994; Evan, 1992; Clark ad Gillespie, 1997). The difference between v-Src and the other oncoproteins, capable of inducing apoptosis is, that v-Src, while priming the cells to die, also provides them with an

Comparison between percentage of cells undergoing apoptosis after the addition of different inhibitors

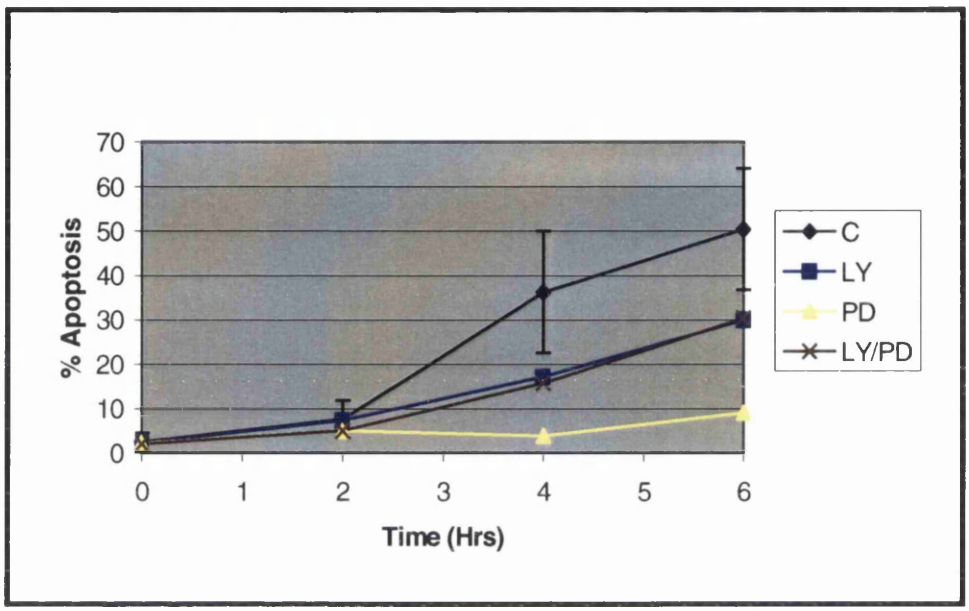


Figure 41 Inhibition of MAP kinase activity at the same time as inhibition of PI 3-kinase activity does not increase the percentage of cells undergoing apoptosis: Both LY294002, the PI 3-kinase inhibitor and PD98059, the MEK kinase inhibitor were added together at the concentration of 50μM each to serum-deprived transformed Rat-1 cells. Cells were harvested at different times and TUNEL labelled to calculate the percentage undergoing apoptosis. The number of cells dying after being treated with both inhibitors was compared to the number of dying cells treated with either inhibitor and to the number undergoing apoptosis after withdrawal of serum and v-Src activity.

overriding survival signal, while other mitogenic oncoproteins require co-operation from other anti-apoptotic oncogenes (Bissonnette, 1992). Although, our work did not focus on investigating further into the mechanisms by which v-Src can possibly prime the transformed cells to undergo apoptosis, other studies offer some possibilities. For example, v-Src upregulates c-Myc, which has been shown by several studies to induce apoptosis in serum-starved cells. It is thus possible that v-Src utilises the apoptotic pathway stimulated by c-Myc to induce cell death.

The experiments detailed in this chapter were carried out to determine some of the mediators of the survival signal induced by v-Src. Initial experiments showed phosphorylation of Akt was reduced within 30-45min of switching off v-Src activity in serum-starved v-Src transformed Rat-1 cells. Since commitment to apoptosis was shown to occur only after 2 hours of switching off v-Src activity, the decrease in Akt phosphorylation preceded commitment to cell death. Further investigation into the role of PI 3-kinase and Akt was carried out using LY294002, a selective inhibitor of PI 3-kinase (Vlahos *et al.*, 1994). Inhibition of PI 3-kinase activity by LY294002 induced the serum-starved transformed Rat-1 cells to undergo apoptosis even in the presence of v-Src activity, as measured by TUNEL labelling. This was accompanied by a decrease in Akt phosphorylation within 15min of adding the inhibitor. The percentage of cells undergoing death induced after drug treatment of transformed Rat-1 cells was about 50% of that induced by switching off v-Src activity in serum-starved transformed Rat-1 cells.

To verify whether the cell death induced by LY294002 was indeed the result of inhibition of the PI 3-kinase/Akt pathway or due to some toxic effects of the drug itself, control experiments using serum-starved normal Rat-1 cells were carried out. v-Src transformed Rat-1 cells, cultured at the restrictive temperature, and thus morphologically normal, were also used. Upon treatment with LY294002, the normal Rat-1 cells showed a very small increase in the percentage of cells undergoing apoptosis at 6 hours after the addition of the inhibitor, as compared to those at 0 hours. It is possible that the small percentage of cell death, induced in drug treated normal Rat-1 cells, might be the result of drug toxicity and not of inhibiting the PI 3-kinase/ Akt pathway.

Some further analysis of LY294002 induced cell death in transformed cells (carried out by D.Riley, Beatson Institute, Glasgow, UK) showed that cell death is accompanied by activation of both p38 and JNK stress kinases. These biochemical events were similar to those induced by switching off v-Src in the transformed cells, thus supporting a role for PI 3-kinase in mediating v-Src survival signalling. Activation of caspases in the drug treated transformed cells has not yet been examined. Also, observation of DNA laddering would provide further support to the idea that the cell death induced by LY294002 in transformed Rat-1 cells is indeed due to apoptosis.

To confirm the roles of PI 3-kinase and Akt downstream of v-Src, experiments were carried out using a constitutively active form of p110, the enzymatic subunit of PI 3-kinase. Results showed that transformed Rat-1 cells, transfected with the constitutively active form of p110, did not show any resistance to apoptosis in the absence of serum and v-Src activity. However, since overexpression of p110 could not be shown by immunoblot analysis, it was not possible to draw any conclusions from this experiment. The experiment, thus, needs to be repeated in order to confirm the role of PI 3-kinase and Akt in mediating the v-Src induced survival signal. Also, experiments using constitutively active forms of Akt could be carried out for the same purpose. Furthermore, the role of downstream effectors of Akt could also be investigated.

Results of our study indicate that although Akt phosphorylation decreased within 30-45min of inactivating v-Src, caspase activity was not detected until much later *i.e.* about two and a half hours after v-Src inactivation. Inhibition of caspases also did not inhibit dephosphorylation of Akt, indicating that activation of caspases lies downstream of Akt inactivation due to its dephosphorylation. Similar observations were also made regarding inactivation of Akt and activation of p38, suggesting that p38 also acts downstream of Akt inactivation upon induction of apoptsis. There are, therefore, missing links that exist between the time of switching off the survival signal and the time of execution of apoptosis, which need to be established. Results of this study also showed that Bcl-2 acts downstream of Akt. Overexpression of Bcl-

2 in v-Src transformed Rat-1 cells was unable to prevent Akt dephosphorylation, but did inhibit the cells from undergoing apoptosis in the absence of serum and v-Src activity.

Since inhibition of PI 3-kinase was able to induce only about 50% apoptosis in serum-starved v-Src transformed Rat-1 cells (not taking into account possible cell death resulting from toxic effects of the inhibitor itself), compared to the percentage of cells dying in the absence of v-Src activity, the role of MAP kinase in the mediation of v-Src induced survival signal was also investigated. Several studies (discussed in section 5.7.4) have reported the involvement of the MAP kinase (ERK) pathway in cell survival. ERK phosphorylation was found to be reduced in serum-starved Rat-1 cell undergoing apoptosis within 15min after v-Src inactivation or addition of LY294002. This raised the possibility of induction of apoptosis being related to decrease in ERK activity. However, inhibition of MAP kinase activity, using PD98059, in the presence of v-Src, was unable to induce cell death in serum-starved transformed Rat-1 cells, although, immunoblot results showed complete inhibition of ERK phosphorylation. There was also no change in Akt phosphorylation in PD98059 treated cells. It is thus possible that the cyclic loss and gain of ERK activity, detected after the induction of the apoptotic stimuli, was more a consequence, rather than a cause of apoptosis or was unrelated. The fact that ERK is not involved in cell survival was also demonstrated when inhibition of ERK activity was unable to further enhance the percentage of cells undergoing apoptosis after treatment with LY294002, the PI 3-kinase inhibitor. In support of results of this study, other groups have also shown cell survival can be independent of ERK activity (Chen *et al.*, 1996b; Kulik *et al.*, 1997).

STAT3 is another likely mediator of the survival signal induced by v-Src (see section 5.7.4). It is required for v-Src transformation of cells (Yu *et al.*, 1995; Bromberg *et al.*, 1998; Turkson *et al.*, 1998). Both c-Src and v-Src have been shown to phosphorylate STAT3 on Tyr705, which is required for effective dimer formation and transcriptional activity of the STAT molecule (Cao *et al.*, 1996; Cirri *et al.*, 1997; Schaefer *et al.*, 1999). v-Src also associates with STAT3 both *in vivo* and *in vitro* (Cao *et al.*, 1996). It is thus possible that STAT3 might be collaborating with

the PI 3-kinase/ Akt pathway to provide the v-Src transformed cells with a survival signal. The role played by STAT3 can be evaluated by examining the effects of inhibiting STAT3 activity, possibly through the use of dominant negative mutants of STAT3. The role of other STAT molecules, *i.e.* STAT1, in survival signalling can also be investigated by carrying out similar experiments.

Chapter 8: Final discussion, Summary and Future Perspectives

8.1 FAK upregulation and oncogenesis

Several studies have reported an upregulation of FAK at the protein level in various different tumour cell lines (see section 1.3.7). However, the mechanism behind this upregulation has not been investigated. Our study revealed that gain of *fak* gene copy number through genetic alterations is one way by which many cancer cell lines may upregulate FAK at the protein level. Furthermore, genetic alterations leading to FAK protein upregulation have been found to accompany acquisition of invasive potential in an *in vitro* colon cancer model. Work by several other groups has also shown that FAK might play a role in the mediation of cell processes such as cell motility, growth and survival (see section 1.3), all of which are likely to be of some importance during tumour progression. These findings, although strongly suggesting the importance of FAK's role in tumorigenesis, do not confirm the oncogenic potential of FAK. Further investigations are thus needed to determine exactly what aspects, if any, of tumour development are influenced by FAK. This can be investigated by combining *in vivo* and *in vitro* studies. For example, studies involving intervention in the functions of FAK, both in cultured cells and in animals, be carried out. In cultured cancer cells, overexpression of effective dominant negatives could help determine the effects on behavioural properties that are associated with malignancy, like cell proliferation, motility and survival.

In vivo studies would require knocking out FAK in animals. Since FAK knockouts are embryonic lethal, the strategy would involve conditionally knocking out FAK in a tissue specific manner after induction of tumour formation in that tissue. In our group, this study is currently underway using transgenic mice carrying *'loxP'* flanked *fak* and the gene coding for a fusion protein between a mutated ligand binding domain of the human estrogen receptor (ER) and the Cre recombinase (Indra *et al.*, 1999). The fusion protein can be activated by 4-hydroxy-tamoxifen (OHT), but not natural ER ligands (Indra *et al.*, 1999). Upon activation, Cre recombinase will facilitate excision of DNA between the two *loxP* sites. These mice could be induced to develop papillomas by chemical treatment and the effects of knocking out FAK at different stages of tumour development could be monitored by stimulating Cre recombinase using OHT. If the results showed an alteration in tumour development,

then FAK could be considered a potential therapeutic target. In this respect, several drug companies are already showing a keen interest in FAK as a potential target for anti-cancer therapy (M.Frame, personal communicaton).

FAK as a potential anti-cancer target

Being a tyrosine kinase, the enzymatic activity of FAK might be crucial in its biological functions. However, not much is known about the importance or regulation of FAK's kinase activity. In this regard, overexpression of the kinase dead mutant of FAK does not appear to act in a dominant negative manner (V.Fincham, unpublished data), perhaps implying that the kinase activity of FAK may not be crucial in its function. However, this needs to be further tested. Targetting the kinase activity through develpoment of specific small molecular weight inhibitors is a possible to approach to test this.

Another possibility is to examine the potential of interfering with FAK's role as an adaptor protein. FAK is known to bind to proteins like Src, PI 3-kinase, CAS and Grb-2 (see section 1.3.3). It is thus possible that the binding activity plays an important role in FAK's mediation of intracellular signalling leading to cell survival, focal adhesion turnover during cell motility and/or cell proliferation. One way to approach this problem could be to observe the effects of mutating the putative binding sites and overexpressing these mutants in cells. Analysis of the amino acid sequences of FAK and its binding partners that are involved in the binding interaction, will help design specific competing peptides, introduction of which might interfere with the adaptor function of FAK. Inhibition of the adaptor function of FAK through these approaches might provide a useful insight into the role of specific binding interactions in various aspects of FAK biological activities.

FAK is phosphorylated on at least six tyrosine residues. Phosphorylation of these residues is thought to be important both in activation of FAK and its binding to other proteins. Mutation of these sites to phenylalanine residues (work currently being carried out in our group) and overexpression of these mutants will also provide valuable information regarding which tyrosine phospho-acceptor sites mediate the

biological responses and might reveal novel ways of intervening in FAK-mediated cell survival, motility or proliferation.

8.2 FAK proteolysis is not critical for apoptosis

We examined the role of FAK proteolysis in the apoptotic response induced in v-Src transformed Rat-1 cells. FAK is a downstream substrate of Src and is phosphorylated by it. If FAK was critical in mediating the survival signalling downstream of Src, then inhibiting the attenuation of its activity, induced by its cleavage (Cooray *et al.*, 1996), should have prevented the transformed Rat-1 cells from undergoing apoptosis in the absence of serum and v-Src activity. FAK proteolysis has also been shown to accompany c-Myc induced apoptosis in serum-starved transformed fibroblasts (Crouch *et al.*, 1996). Our results indicated that although FAK was cleaved upon induction of apoptosis in v-Src transformed cells, most likely by caspases, inhibiting this proteolysis did not protect the transformed cells against apoptosis. This indicates that if at all, FAK retention is not sufficient in mediating the survival signal downstream of v-Src. Whether it is necessary or not for survival, can be determined by further experiments involving inhibition of FAK activity through the use of dominant negative or antisense approaches.

8.3 Switching off v-Src in serum-deprived transformed Rat-1 cells induces apoptosis

A major finding of the work presented here was that switching off v-Src in transformed Rat-1 cells induced an apoptotic response. The inhibitor studies carried out suggested that blocking either caspases or p38 independently did not prevent the serum-starved v-Src transformed Rat-1 cells from undergoing apoptosis after inactivation of v-Src. However, when caspases and p38 were inhibited simultaneously, there was a significant, though not complete, reduction in the percentage of dying cells. This implied that the two pathways were redundant and independent of each other. These results, although providing significant information about the signalling pathways involved in the induction of apoptosis in v-Src transformed Rat-1 cells, give rise to many questions that remain to be answered. For

example, the decrease in the percentage of cells undergoing apoptosis, associated with the inhibition of both caspases and p38, was incomplete. This argues that another independent pathway is involved in the mediation of the apoptotic signal. JNK, another known stress activated kinase that mediates apoptosis, is a possible candidate. Studies carried out by our group have shown JNK to be active in the cells undergoing apoptosis at about the same time as the activation of p38 (D.Riley, BICR, Glasgow). The role of p53, a known tumour suppressor, in the induction of apoptosis could also be examined (reviewed in Wang, 1999). Whether the type of apoptosis is caspase (those insensitive to ZVAD.fmk inhibition) dependent or independent, also needs to be established. Preliminary data from our experiments has suggested that caspases are not involved in the induction of apoptosis. They also do not appear to be required for DNA laddering, a hallmark of apoptosis. Overexpression of Bcl-2 protected the cells from undergoing apoptosis. This indicates that mitochondrial dysfunction might play an important part in the induction of cell death. To test this, the time of release of cytochrome C in cells dying in the absence of serum and v-Src activity should be ascertained. This would give some indication as to whether the activation of caspases or the release of cytochrome C, is a primary event in the decision of cells to commit to apoptosis after switching off v-Src.

The importance of Fas ligands in the induction of apoptosis is well documented. To test whether the v-Src induced survival signal supresses the pro-apoptotic pathway initiated by CD95 stimulation, it is first important to determine if an intact CD95 apoptotic signalling pathway is present in the v-Src transformed fibroblasts. Cell surface CD95 expression in fibroblast cells can be determined by flow cytometry analysis using the method described in Hueber *et al.*, 1997. If the cells were found to express detectable amounts of cell surface CD95 receptors, then these could be incubated with CD95 antibody (to stimulate activity)and apoptosis in the presence of v-Src activity monitored. Similar experiments could be carried out to test the effect of CD95 stimulation in v-Src transformed cells in the presence of serum and v-Src activity. If an intact CD95 apoptotic signalling pathway was found to be present in the v-Src transformed cells, then its involvement in the apoptotic pathway initiated in these cells after switching off v-Src could also be examined. To do this, serum-deprived cells, in which v-Src activity had been switched off, would be incubated

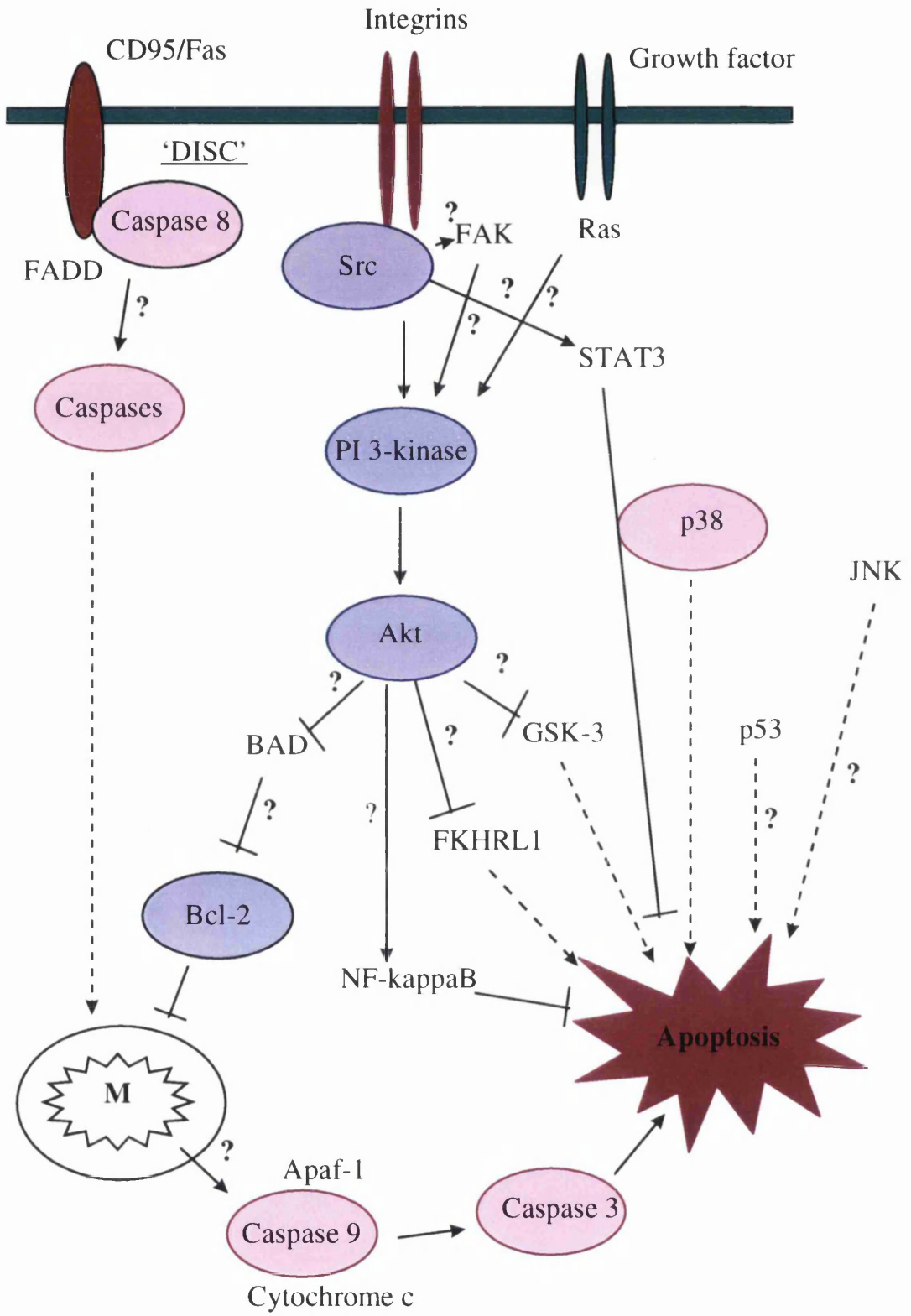
with a monoclonal antibody to CD95L that neutralises CD95 (Hueber *et al.*, 1997). Using serum-deprived cells with inhibited v-Src activity and cells with added PI 3-kinase inhibitor as controls would enable us to determine whether or not, in the absence of v-Src activity, the serum deprived cells underwent apoptosis through the Fas L/R initiated pathway. If that was the case, further experiments might reveal how v-Src induced survival signals impinge on this apoptotic pathway.

8.4 v-Src mediates survival *via* PI 3-kinase

Another major conclusion from the work presented in this thesis was that serum-deprived v-Src transformed cells are primed to die and are kept alive solely by a survival signal induced by v-Src. We also studied some of the possible pathways that might lie downstream of v-Src in the mediation of cell survival. These are outlined in Figure 42. The data obtained implicated the PI 3-kinase/Akt pathway to lie downstream of v-Src in survival signalling. The requirement of FAK for the Src/PI 3-kinase mediated survival pathway in the transformed Rat-1 cells has not yet been examined. This could be studied by introducing dominant negative mutants of FAK, capable of inhibiting the association of FAK with PI 3-kinase, and then observing the effect on cell survival that these might have. The *in vitro* model of apoptosis that was used by us has several advantages. It is a tractable model for investigating Src survival signalling and is thus, very good for examining the molecular mechanisms lying downstream of v-Src. However, since the study was carried out in an *in vitro* model using v-Src, the relevance of the finding with regards to the function of c-Src in cell survival is not very clear. In particular, it does not tell us whether the endogenous Src family kinases play a role in mediating cell survival *in vivo.* Translating the relevance of our *in vitro* results to tumour development *in vivo* is a goal for future work. Nevertheless, in advanced cancer cells, the activity of Src family kinases, specifically Src itself, is often increased. The role that Src might play in the development of malignancy is not well understood but there have been some studies that have independently shown c-Src to play an important role in the process of cell survival. A recent study has shown c-Src to be required during VEGF-induced angiogenesis (Eliceiri *et al.*, 1999). Specifically, c-Src was required for the survival of endothelial cells during VEGF mediated angiogenesis. Introduction of a dominant

Figure 42 Pathways involved in mediation of v-Src induced survival signalling and the apoptotic pathway it impinges on, in transformed Rat-1 cells: Mediators of v-Src induced survival signalling in transformed Rat-1 cells are shown in blue, whereas those mediating the apoptotic response in the absence of serum and v-Src activity are shown in pink. Various other candidate pathways that might mediate either the survival signal or the apoptotic pathway are also shown.

Some of the known and possible regulators of apoptosis in serum-deprived v-Src transformed Rat-1 cells upon inactivation of v-Src



negative form of c-Src into cells treated with VEGF for the stimulation of angiogenesis, prompted extensive apoptotic response.

The PI 3-kinase/Akt pathway has also been shown by other studies to mediate cell survival (see section 5.7.1). Although, the inhibitor experiments, carried out in the work presented here, indicated a role for PI 3-kinase and Akt in the mediation of v-Src induced survival signal in serum-starved v-Src transformed Rat-1 cells, molecular studies using a constitutively active form of PI 3-kinase were inconclusive. Further molecular experiments thus need to be carried out to confirm the role of PI 3-kinase/Akt in survival signalling. For this purpose, dominant negative mutants of both PI 3-kinase or Akt could be used. Another approach could be to study the effect of overexpressing PTEN, a known PI 3-kinase antagonist (see section 5.7.2). PTEN dephosphorylates PIP3, a product of PI 3-kinase, thus enabling the transfer of Akt to the membrane and its subsequent activation.

Resistance to apoptosis or increased cell survival might play an important role in the process of metastasis. Recent work in our lab showed that levels of c-Src protein were elevated in metastatic cells, as compared to the non-metastatic cells from which they were derived (R.Jones, unpublished data). The non-metastatic cell line was derived from a primary human colorectal carcinoma (KM12 C), while the metastatic cell lines were obtained from metastases resulting after injection of the primary cells into the spleen of nude mice (KM12 SM and KM12 L4A; Giavazzi *et al.*, 1986 a and b). Levels of phospho-Akt were also elevated in the metastatic cell lines (R.Jones, unpublished data). More work needs to be carried out to establish whether there is a link between the upregulation of c-Src and phospho-Akt levels. However, it is possible that the elevated levels of Src and Akt phosphorylation are indeed linked and might be contributing to enhanced cell survival during the metastatic process. To address this, Rob Jones and Anne Wyke in our group are expressing a dominant negative Src protein in metastatic variants and constitutively activated Src in the non-metastatic variant. The metastatic cell lines are also being transfected with dominant negative Akt, whilst the non-metastatic KM12 C cells are being transfected with active Akt. Once stable transfectants expressing the exogenous proteins are obtained, these will then be tested for their ability to survive detachment and growth factor

deprivation. They will also be tested for their ability to form tumours in nude mice and to metastasize to the liver after tail vein injection. These experiments will be able to establish whether the survival pathway identified by work described in this thesis has any role in tumour cell behaviour, particularly cell survival and metastasis.

Therapeutic potential

It is possible that Src-mediated cell survival signals might be playing a part in conferring drug resistance to tumour cells by enhancing their survival potential. For example, cancer cells treated with cytotoxic agents, such as apoptosis-inducing anti-cancer drugs, might be more resistant to cell killing if upstream survival signalling is activated. Src inhibitor studies could thus be carried out in conjunction with drug treatment to determine if inhibition of Src activity makes the cell more sensitive to drug treatment. This possibility could be investigated in the metastatic cell lines derived from the model described above and the sensitivity of KM12 C colon cancer transfectants currently being generated, to cytotoxic drugs like 5FU or cis-platin examined. Such information would indicate that drugs that specifically target components of the survival pathways, such as, Src or PI 3-kinase, might be of therapeutic benefit when given in combination with conventional anti-cancer agents.

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Oncogene (1999) 18, 5646-5653

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Increased dosage and amplification of the focal adhesion kinase gene in human cancer cells

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Focal adhesion kinase (pp125^{FAK}) is present at sites of cell/extracellular matrix adhesion and has been implicated in the control of cell behaviour. In particular, as a key component of integrin-stimulated signal transduction pathways, pp125^{FAK} is involved in cellular processes such as spreading, motility, growth and survival. In addition, a number of reports have indicated that pp125^{FAK} may be up-regulated in human tumour cells of diverse origin, and consequently, a role has been proposed for pp125^{FAK} in the development of invasive cancers. However, to date the mechanisms that lead to elevated pp125^{FAK} expression in tumour cells have not been determined. Here we used *in situ* hybridization to confirm chromosome 8q as the genomic location of the human *fak* gene and report that elevation of pp125^{FAK} protein in cell lines derived from invasive squamous cell carcinomas is accompanied by gains in copy number of the *fak* gene in all cases examined. In addition, we observed increased *fak* copy number in frozen sections of squamous cell carcinomas. Furthermore, increased dosage of the *fak* gene was also observed in many cell lines derived from human tumours of lung, breast and colon, including two cell lines Calu3 and HT29, in which *fak* was amplified. In addition, in an *in vitro* model for human colon cancer progression there was a copy number gain of the *fak* gene during conversion from adenoma to carcinoma, which was associated with increased pp125^{FAK} protein expression. Thus, we show for the first time that many cell lines derived from invasive epithelial tumours have increased dosage of the *fak* gene, which may contribute to the elevated protein expression commonly observed. Although other genes near the *fak* locus are co-amplified or increased in copy number, including the proto-oncogene c-*myc*, the biological properties of pp125^{FAK} in controlling the growth, survival and invasiveness of tumour cells, suggest that it may contribute to the selection pressure for maintaining increased dosage of the region of chromosome 8q that encodes these genes.

Keywords: focal adhesion kinase; tyrosine kinase; protein expression; cancer; FAK

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Received 29 December 1998; revised 10 May 1999; accepted 10 May 1999

Introduction

Focal adhesion kinase (pp125^{FAK}) is a tyrosine kinase that localizes to cellular focal adhesions and associates with a number of other proteins such as integrin adhesion receptors (reviewed in Richardson and Parsons, 1995), focal adhesion components, such as paxillin and talin (Turner and Miller 1994; Schaller and Parsons, 1994; Tachibana *et al.*, 1995; Hildebrand *et al.*, 1995; Chen *et al.*, 1995), and signalling proteins including protein tyrosine kinases of the Src family (Cobb *et al.*, 1994; Xing *et al.*, 1994; Cary *et al.*, 1996; Fincham and Frame, 1998), phosphatidyl inositol (PI) 3-kinase (Chen *et al.*, 1994), the Rho GTPase activating protein Graf (Hildebrand *et al.*, 1996) and adaptor molecules, including GRB2 (Schlaepfer *et al.*, 1994; Kharbanda *et al.*, 1995) and p130^{CAS} (Harte *et al.*, 1996; Polte and Hanks, 1995). Although the precise regulation and biological function of pp125^{FAK} remain to be determined, its diverse range of binding partners places it at a crossroads between adhesion and growth-regulatory signal transduction.

Studies addressing the role of pp125^{FAK} in cell adhesion suggest that it can contribute to both focal adhesion assembly (Richardson and Parsons, 1996; Richardson *et al.*, 1997) and focal adhesion turnover (Fincham *et al.*, 1995; Ilic *et al.*, 1995; Fincham and Frame, 1998). Pp125^{FAK} is extensively regulated by tyrosine phosphorylation, which might, in turn, control the processes of focal adhesion assembly and disassembly that contribute to cell motility. In keeping with this, there is strong evidence that pp125^{FAK} is a key regulator of cell migration. Specifically, cells derived from pp125^{FAK} $-/-$ mouse embryos exhibit reduced migration as a result of impaired adhesion turnover (Ilic *et al.*, 1995, 1996). Moreover, displacement of pp125^{FAK} from focal adhesions reduces cell migration (Gilmore and Romer, 1996), whilst overexpression of pp125^{FAK} in CHO cells stimulates migration (Cary *et al.*, 1996), suggesting that the level of pp125^{FAK} protein may limit cell movement in some cell types.

Since the expression level of pp125^{FAK} may be a determinant of the rate of cell movement, and since elevated pp125^{FAK} may also enhance tumour cell survival (Frisch *et al.*, 1996; Xu *et al.*, 1996), it is perhaps not surprising that a number of groups have found that pp125^{FAK} is up-regulated in cancer cells. In particular, pp125^{FAK} is elevated in cell lines from human melanomas, with pp125^{FAK} levels correlating with the rate of cell migration on fibronectin (Akasaka *et al.*, 1995), in cervical carcinoma cell lines

(McCormack *et al*., 1997), in prostatic carcinoma tumours and cell lines (Tremblay *et al*., 1996) and in colon and breast tumours and cell lines (Owens *et al*., 1995), the latter study inferring a link between

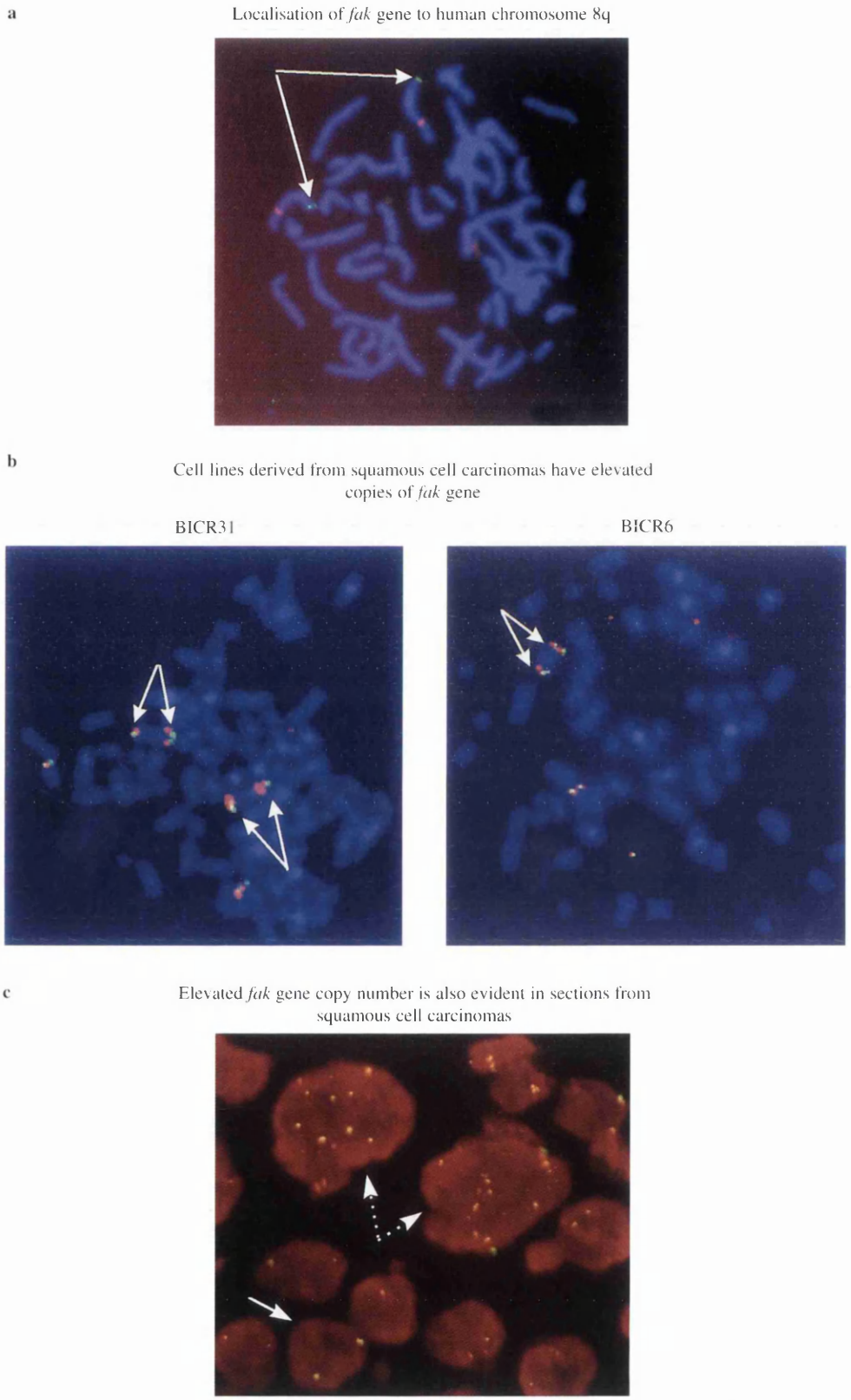

Figure 1 Localization of *fak* to chromosome 8q in proximity to the c-*myc* locus and its copy number gains in BICR31 and BICR6. (**a**) Chromosomes were prepared from lymphocytes that were arrested in metaphase using colcimid and probed with both a DIG-labelled centromeric probe for chromosome 8 (visualized as red) and biotin-labelled *fak* probe (visualized as green). (**b**) Metaphase chromosome preparations from BICR31 and BICR6 were probed with both DIG-labelled c-*myc* and biotin-labelled *fak*. White arrows show *fak* and c-*myc* genes at the ends of isochromosomes. The chromosomes were stained with DAPI. (**c**) 5 μ frozen squamous cell carcinoma tumour sections were probed with FITC-labelled *fak* (signals visualized as yellow). The nuclei were visualized as red. Cells within the sections act as internal controls (arrow points to nuclei with two copies of *fak*, whilst broken arrows point to tumour cell nuclei which contain more than two copies of *fak*

pp125^{FAK} expression and tumour invasiveness. However, despite the mounting evidence that the expression of pp125^{FAK} is elevated in tumour-derived cell lines, the mechanism of up-regulation has not been addressed. Here we report that in malignant keratinocytes derived from squamous cell carcinomas (SCC), *fak* is up-regulated at the genetic level and that this is often associated with increased expression of pp125^{FAK} protein. Furthermore, we observed increased *fak* gene dosage in a variety of cell lines derived from human lung, breast and colon tumours, including two cell lines in which *fak* was amplified. In addition, the progression of colonic adenoma cells to carcinoma cells *in vitro* was accompanied by increased pp125^{FAK} expression and a copy number gain of the *fak* gene. Thus, we conclude that in many cells derived from human epithelial tumours, there is increased *fak* gene dosage which is often associated with increased pp125^{FAK} protein expression. Although the c-*myc* proto-oncogene is almost always co-amplified or increased in copy number along with *fak*, increased expression of c-Myc protein was less frequently observed in the invasive cell lines we examined. These data, together with the important roles now defined for pp125^{FAK} in cell motility, invasion, growth and tumour cell survival, suggest that pp125^{FAK} could contribute to the selection pressure that results in the frequent increase in dosage or amplification of the region of chromosome 8q that contains the *fak* and c-*myc* genes.

Results and discussion

Copy number gains of the fak *locus in the BICR series of SCC-derived cell lines*

The gene encoding pp125^{FAK} has previously been mapped to mouse chromosome 15 and to human chromosome 8 (Fioderek and Kay, 1995). Linkage of *fak* to the protooncogene c-*myc* in the mouse suggested that human 8q24, where c-*myc* is located, is the likely site of the *fak* gene in the human genome (Fioderek and Kay, 1995). To examine the *fak* gene in human tumour-derived cell lines by fluorescent *in situ* hybridization (FISH), we isolated a PAC clone containing *fak* sequences as described in the methods. We confirmed that the *fak* gene mapped to the q arm of chromosome 8 and that normal lymphocytes had two copies of the gene (Figure 1a). Furthermore, FISH analysis of chromosome preparations from a number of malignant keratinocyte cell lines derived from SCCs of the head and neck (the BICR cell lines, Edington *et al*., 1995) demonstrated that these had more than two copies of the *fak* gene in a high percentage of nuclei and that it co-localized with c-*myc*, confirming its position at human chromosome 8q24 (shown for BICR6 and BICR31 in Figure 1b). In the examples shown, BICR6 and BICR31 have five and four copies of both *fak* and c-*myc* respectively, and some of these are present at both ends of an isochromosome (shown by arrows in Figure 1b). We found that all nine BICR cell lines examined had greater than two copies of both *fak* and c-*myc* (shown in Table 1). In addition, we observed elevated dosage of the *fak* gene in frozen sections of squamous cell carcinomas derived from head and neck (Figure 1c), indicating that increased *fak* was not a consequence of tumour cell growth in culture and occurred *in vivo* during tumour progression.

Increased fak *gene dosage is associated with increased protein expression in most cell lines*

To compare the levels of pp125^{FAK} and c-Myc protein in the BICR cell lines, we carried out protein immunoblots. Protein concentrations were carefully determined and equal amounts of total cell lysate were separated by SDS–PAGE, blotted and probed with pp125^{FAK}- or c-Myc-specific antibodies. With the exceptions of BICR16 and BICR63 (2/9 cell lines), the malignant keratinocytes examined (7/9 cell lines) displayed steady state levels of pp125^{FAK} protein that were elevated more than twofold above those in human normal keratinocytes (HNK). Although most cell lines had increased levels of pp125^{FAK}, there was no precise correlation between *fak* gene copy number and protein expression. This is exemplified by BICR6 and BICR16 which each have five copies of the *fak* gene, but which express substantially different levels of pp125^{FAK} protein (Figure 2a and b). Thus, although all of the BICR cell lines examined have increased *fak* gene copy number, and this may contribute to elevated protein expression in some cell lines, it does not necessarily lead to a related increase in protein in all cases. These observations imply that additional controls operate, in at least some malignant cell lines, to control the level of pp125^{FAK} protein.

Amongst the BICR cell lines examined, there was considerable variation in c-Myc expression (Figure 2a and b). Only two cell lines BICR16 and BICR78 (2/9) displayed more than twofold elevated levels of c-Myc when compared to normal keratinocytes (Figure 2a and b). Thus, although the *fak* and c-*myc* genes are both similarly increased in copy number in the BICR cell lines, we found that pp125^{FAK} was elevated in 6/9 cell lines without an associated increase in c-Myc, indicating that the protein expression levels are not always co-ordinately regulated. These data further imply that c-Myc function is unlikely to provide the selection pressure for maintaining increased dosage of the region of 8q24 which contains *fak* and c-*myc* in

Table 1 Summary of copy number gains of *fak* locus in BICR cell lines derived from squamous cell carcinomas of the head and neck

Cell line	*Site*	*Signals/nucleus (mode)*	*Signals/nucleus (maximum)*
HNK	(human normal keratinocytes)	2	2
BICR3	Alveolus	4	4
BICR6	Hypopharynx	5	6
BICR10	Buccal mucosa	6	6
BICR16	Tongue	5	6
BICR31	Tongue	4	5
BICR56	Tongue	4	6
BICR63	Tongue	4	6
BICR78	Alveolus	4	6
BICR82	Maxilla	4	5

The BICR series of cell lines were all derived from invasive carcinomas. Those tested (BICR3, BICR6, BICR10, BICR16, BICR31 and BICR56) are all invasive *in vitro*, as determined by an assay that monitors upward movement into the reconstituted basement membrane Matrigel *in vitro*, an assay that discriminates between benign and malignant cells derived from head and neck tumours (EK Parkinson, unpublished)

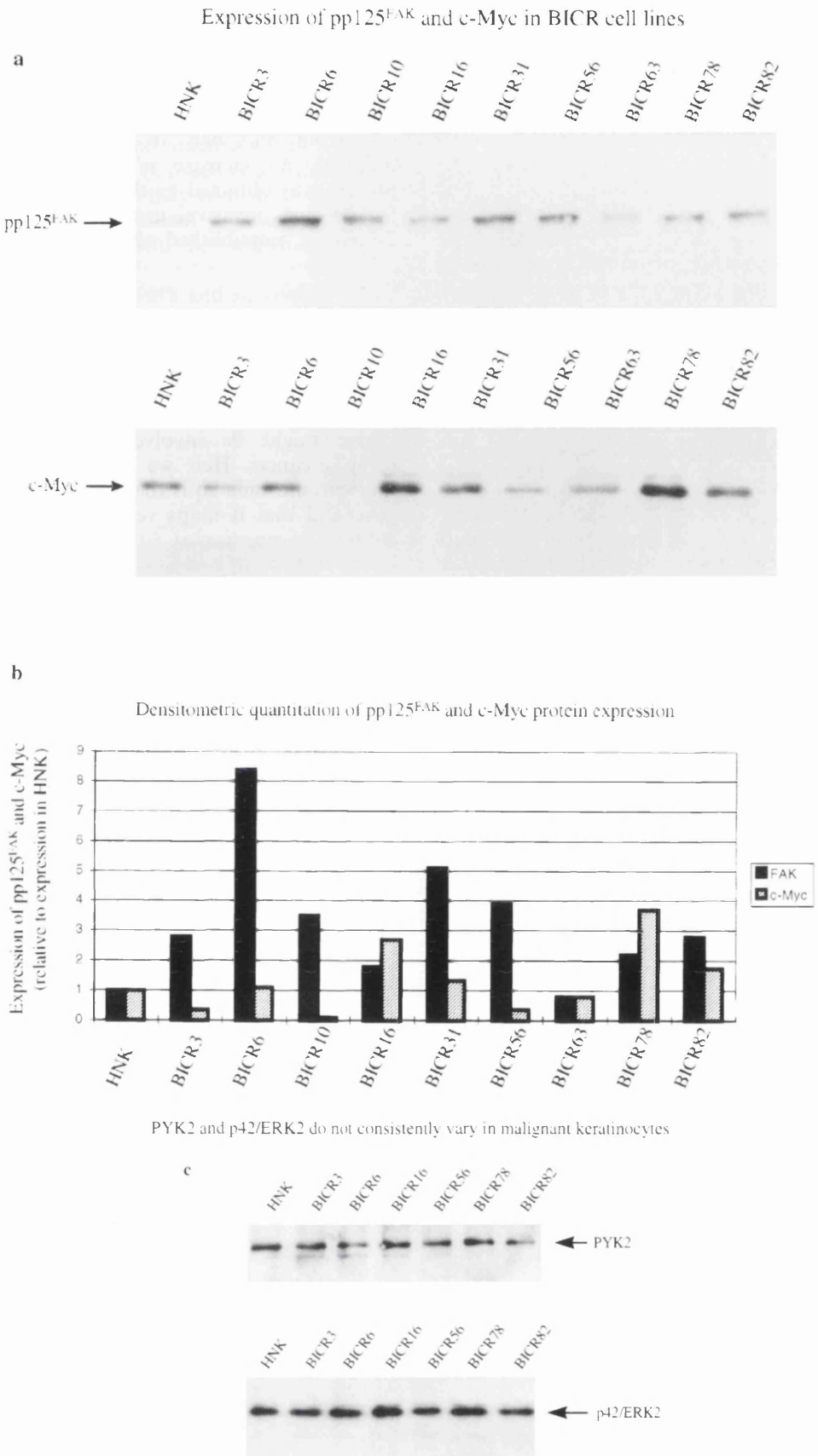

Figure 2 Variations in expression of pp125^{FAK} and c-Myc proteins in BICR cell lines and human normal keratinocytes (HNK). (**a**) Total cell lysates (25 μg) were separated by 7.5% SDS–PAGE (upper panel) and 10% SDS–PAGE (lower panel), transferred to nitrocellulose and probed with anti-pp125^{FAK} (upper panel) or anti-c-Myc (lower panel). (**b**) Quantitation by scanning laser densitometry of pp125^{FAK} (solid bars) and c-Myc (shaded bars) protein in BICR cell lines is shown relative to the expression of each in HNK controls. The immunoblots and quantitations shown are representative of several replicate experiments. (**c**) Lysates separated by 7.5% or 10% SDS–PAGE respectively, were probed with anti-PYK2 (upper panel) or anti-p42/ERK2 (lower panel)

many of the BICR cell lines examined, and suggests that pp125^{FAK} could be a potential driving force for maintaining these genetic changes in some tumour cells, at least *in vitro*. However, our data do not rule out roles for other genes at this locus in the frequent selection of copy number gains in malignant cells.

As a control for protein loading and to determine whether other proteins similarly fluctuated in malignant keratinocytes, we examined the expression of the pp125^{FAK} homologue, PYK2 (Lev *et al.*, 1995; Avraham *et al.*, 1995; Sasaki *et al.*, 1995), which can substitute for some aspects of pp125^{FAK} signalling (Sieg *et al.*, 1998), and p42/ERK2, a protein kinase that is not generally regulated by fluctuations in expression. We found no consistently altered expression of either protein in the BICR cell lines tested when compared to normal keratinocytes (Figure 2c).

fak *gene copy number gains in cell lines from a variety of human epithelial tumour types*

To test the generality of *fak* copy number gains, we carried out FISH analysis on archived chromosome preparations from cell lines derived from lung, breast and colon tumours. We found that 8/11 lung cancer cell lines, 6/6 colon cancer cell lines and 6/6 breast cancer cell lines had greater than two copies of *fak* per nucleus (Table 2). In addition, the *fak* gene was amplified in two cell lines examined, Calu3 (lung) and HT29 (colon) tumour cells (Figure 3, Table 2). Although we were unable to compare the pp125^{FAK} protein levels in these cell lines with normal lung or colon cell controls, both cell lines expressed abundant pp125^{FAK} (not shown). Thus, many tumour-derived cell lines from a variety of epithelial tumour types have up-regulated *fak* at the genetic level.

fak *copy number gain accompanies the adenoma to carcinoma progression in an* in vitro *model of colon tumour development*

To address the stage during tumour progression that *fak* was increased, we utilized an *in vitro* system that models the conversion from colonic adenoma to carcinoma. In this model, a cell line was derived from a large tubular adenoma with mild dysplasia (PC/AA). A clonogenic variant adenoma was established (AA/C1) and a fully tumorigenic and invasive malignant cell line was derived by sequential chemical treatment and tissue culture procedures (AA/C1/SB10; model described in Williams *et al.*, 1990; Brunton *et al.*, 1997). Both the cellular and molecular changes during this *in vitro* progression are similar to those that occur *in vivo* (Manning *et al.*, 1991; Williams *et al.*, 1993), indicating that this is a relevant model to study colon cancer progression and the changes required for the transition to an invasive phenotype. Using FISH analysis, we showed that the clonogenic, non-invasive adenoma cell line AA/C1 had two copies of the *fak* gene (Figure 4a); in contrast, the invasive carcinoma derivative AA/C1/SB10 had three copies. Thus, acquisition of an additional copy of *fak* occurred during malignant conversion and did not occur at an earlier stage of tumour development, at least in this model. The timing of the increase in *fak* gene copy number was coincident with up-regulation of pp125^{FAK} protein expression (Figure 4b) and the acquisition of an invasive phenotype (Brunton *et al.*, 1997). These data are consistent with the up-regulation of pp125^{FAK} not being required for tumour initiation, or other early stages of tumour development, but support its proposed role in the later acquisition of an invasive phenotype. In addition to *fak*, we again found that the carcinoma cells had an additional copy of c-*myc*, although no increase in the expression of c-Myc protein was obtained as the adenoma cells progressed to become invasive carcinoma cells *in vitro* (C Paraskeva, unpublished observation).

General discussion

The finding that pp125^{FAK} is often overexpressed in human tumours and tumour-derived cell lines, strongly suggests that this cell/ECM adhesion-linked tyrosine kinase might be involved in the development of invasive cancer. Here we have used FISH to confirm that chromosome 8q is the genomic location of the *fak* gene and that it maps very close to the c-*myc* gene, defining its position at 8q24 where c-*myc* is known to reside. We further demonstrate that genetic mechanisms including copy number gains, amplification and isochromosome formation involving the *fak* locus, are often associated with elevated pp125^{FAK} protein in human epithelial tumour cell lines and in tumours *in vivo*. This, together with the fact that pp125^{FAK} protein has been shown to be elevated in tumour tissues (Owens *et al.*, 1995), suggests that increased pp125^{FAK} expression may have biological consequences for tumour development by providing the malignant cells with a selective advantage. In this regard, the properties of pp125^{FAK}, and the nature of its documented binding partners, suggest that it may have several possible functions during tumour progression.

One possibility is that up-regulated pp125^{FAK} contributes to ECM-dependent tumour growth by mediating signalling from fibronectin to the Ras/

Table 2 Summary of copy number gains of *fak* locus in lung, colon and breast tumour-derived cell lines

Cell line	*Origin*	*Signals/nucleus*
Calu3	Lung cancer	>10
GLC4	Lung cancer	3
LS274	Lung cancer	3
LS277	Lung cancer	4
LS763	Lung cancer	5
H125	Lung cancer	4
LCPH3	Lung cancer	2
LS111	Lung cancer	2
LS310	Lung cancer	2
LDAN	Lung cancer	5
LS106	Lung cancer	4
HT29	Colon cancer	>10
Colo320	Colon cancer	3
ALT-F	Colon cancer	3
ALT-G	Colon cancer	3
AA/C1/SB10	Colon cancer	3
BE	Colon cancer	3
BT474	Breast cancer	6
MCF-7	Breast cancer	4
MDA436	Breast cancer	4
MDA231	Breast cancer	4
T47D	Breast cancer	4
ZR75	Breast cancer	4

MAP kinase cascade that is implicated in proliferation control (Schlaepfer *et al*., 1994). Another potential role proposed for pp125^{FAK} is in making transformed cells able to grow in an anchorage-independent manner (Frisch *et al*., 1996). As well as in anchorage-deprived cells, pp125^{FAK} can also act as a survival factor for adherent cells (Hungerford *et al*., 1996; Xu *et al*., 1996) and is specifically cleaved and inactivated by caspases during the apoptotic process (Crouch *et al*., 1996; Wen *et al*., 1997). Thus, pp125^{FAK} may contribute to ECM-dependent growth and survival of tumour cells under conditions *in vivo* that would be unfavourable for normal cells.

As well as tumour cell growth and survival, another likely role for elevated pp125^{FAK} in tumour cells is in the acquisition of an invasive phenotype. Cancer cell invasion is a complex process that requires cells to adhere and migrate through the underlying ECM. Since pp125^{FAK} functions as a key regulator of ECM-dependent cell migration (Ilic *et al*., 1995, 1996; Gilmore and Romer, 1996; Cary *et al*., 1996), and since endogenous levels of FAK expression can limit the rate of cell motility in some cell types (Cary *et al*., 1996), it seems likely that elevated pp125^{FAK} in tumour cells may release normal constraints on the rate of cell motility, thus enhancing invasive potential. Consistent with this possibility, we found that the gain of an additional copy of the *fak* gene, which was associated with elevated pp125^{FAK} protein, occurred relatively late in an *in vitro* model of colon carcinoma development and was coincident with acquisition of an invasive phenotype (Brunton *et al*., 1997).

The results presented here do not allow us to conclude whether neighbouring genes, including that encoding c-Myc, that are also increased in dosage or co-amplified along with *fak*, contribute to malignant development. Our results with the BICR series of cell lines, and their normal keratinocyte counterparts, show that pp125^{FAK} protein is up-regulated to a greater or lesser extent in many tumour-derived cell lines. However, although c-*myc* is also up-regulated at the genetic level, increased expression of c-Myc protein was less frequently observed. Furthermore, although an

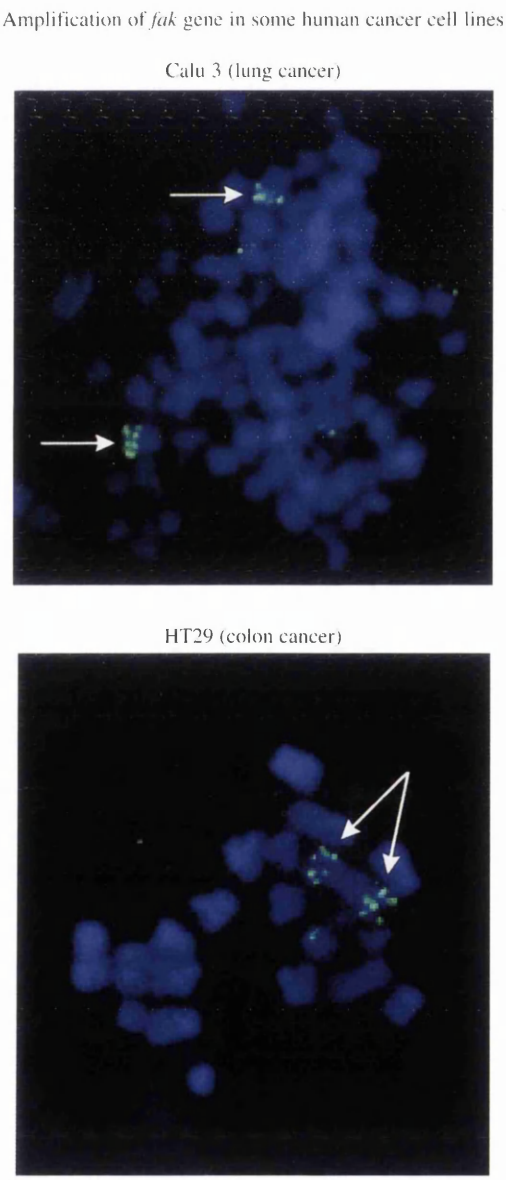

Figure 3 Amplification of *fak* gene in Calu3 and HT29 cancer cells. FISH was carried out on archived metaphase chromosome preparations from Calu3 lung cancer cells (upper panel) and HT29 colon cancer cells (lower panel) using biotin-labelled *fak* probe. Regions of amplified *fak* are shown by arrows. The chromosomes were stained with DAPI

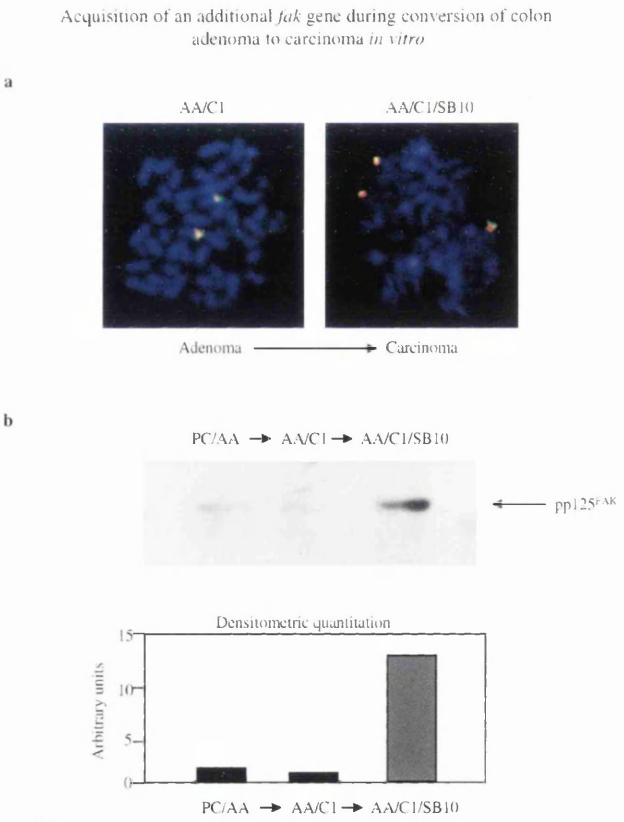

Figure 4 A copy number gain of the *fak* gene accompanies increased pp125^{FAK} protein expression during colonic adenoma to carcinoma conversion *in vitro*. (**a**) Metaphase chromosome preparations of the AA/C1 colon adenoma cell line and its invasive derivative AA/C1/SB10 were probed with both DIG-c-*myc* and biotin-labelled *fak*. The chromosomes were stained with DAPI. (**b**) pp125^{FAK} expression levels in the early colonic adenoma PC/AA, clonogenic adenoma AA/C1 and invasive carcinoma AA/C1/SB10 cell lines were determined by immunoblotting using anti-pp125^{FAK}-specific serum as probe. Quantitation was by scanning laser densitometry and is expressed as OD × mm^2 (arbitrary units)

additional copy of the c-*myc* gene was gained at the same stage as *fak* during colon carcinoma development in the *in vitro* model, there was no concomitant increase in the expression of c-Myc. Together, these data imply that *fak*, at least in some cells, is a stronger candidate than c-*myc* as the gene responsible for maintenance of increased dosage of this region of the genome. The frequent genetic up-regulation of *fak* that we observed in cell lines derived from lung, breast and colon cancers suggests that it could be an important event in the development of invasive epithelial tumours. This, together with the role of pp125^{FAK} as a regulator of cellular adhesion, motility, invasion, survival and cell growth, processes that are perturbed in cancer, implies that pp125^{FAK} may be intimately involved in tumorigenesis.

Materials and methods

Cell culture

Normal human keratinocytes (HNK) were prepared from human foreskin essentially as described in Parkinson *et al.* (1986), except that type I-S trypsin inhibitor (Sigma Chemical Co., UK) was used instead of FCS-containing medium to neutralize trypsin. These normal cells and the BICR cell lines, which were derived from squamous cell carcinomas by a method modified from that described in Rheinwald and Beckett (1981), were grown on lethally irradiated NIH3T3 feeder cells in DMEM supplemented with 10% foetal bovine serum (FBS) and hydrocortisone (0.4 μg/ml). The AA/C1 colon adenoma and AA/C1/SB10 colon carcinoma cells were cultured in 3T3 conditioned medium–DMEM with 10% FBS, insulin (0.2 units/ml) and hydrocortisone (1 μg/ml) – as described in Brunton *et al.* (1997).

Protein immunoblots

Cells were lysed in 10 mM Tris (pH 7.5), 1% Triton X-100, 0.5% NP40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaPPi, 2 mM PMSF, 100 μM Na_3VO_4, 0.5 mM NaF and 0.1% aprotinin (Sigma). Extracts were assayed for protein content using the Micro BCA Protein Assay Kit (Pierce, USA) after clarification by high speed centrifugation at 4°C. Lysates were boiled in high SDS (2.5%) sample buffer and 25 μg total protein separated by discontinuous SDS–PAGE (7.5% gel for pp125^{FAK} and 10% gel for c-Myc) under reducing conditions prior to transfer to nitrocellulose. Proteins were detected by probing with 1:500 dilution of anti-pp125^{FAK} (Transduction Laboratories, USA) or 1:100 dilution of anti-c-Myc serum (a gift from David Gillespie, Beatson Institute, Glasgow, UK) diluted in 5% non-fat milk in PBS/0.2% (v/v) Tween 20. Detection of bound antibody was by reaction with horseradish peroxidase-conjugated secondary antibody (Amersham, UK) and visualization was by enhanced chemiluminescence (Amersham, UK). Quantitation was by scanning laser densitometry using appropriate ECL exposures. p42/ERK2 and PYK2 were detected similarly after probing with 1:2000 anti-p42 (anti-C-terminal peptide rabbit polyclonal serum) or anti-PYK2 (Affiniti, Cambridge, UK).

FISH analysis

Probe isolation cDNA encoding pp125^{FAK} (a gift from Alan Richardson and Tom Parsons) was radiolabelled with ^{32}-phosphate dCTP* and used to screen a human PAC library. Filters containing the human genomic library were obtained from the MRC Human Genome Mapping Project Resource. Filters were hybridized overnight at 65°C in hybridization buffer (5 × SSPE, 5 × Denhardt's, 0.5% SDS, 1 mg/ml yeast RNA type IV) containing 200 ng radiolabelled pp125^{FAK} cDNA. Positive clones were screened to isolate a PAC covering the region of the genome encoding pp125^{FAK}.

Chromosome preparation Chromosome preparations, probe labelling, *in situ* hybridization and visualization were as described previously (Murphy *et al.*, 1995) using the Hybaid Omnislide system. The chromosome preparations from the lung, breast and colon cell lines that were analysed by FISH (results in Table 2) were from archived material. The c-*myc* and the chromosome 8 centromeric probes were obtained commercially (Appligene). Fluorescent images were captured using a digital camera on a Zeiss Axioscope. The number of signals was determined in more than 50 nuclei for each cell line and the mode and maximum number of copies of the *fak* gene were recorded. To examine tumour tissue, 5 μ frozen sections of squamous cell carcinoma derived from head and neck were probed as described above. Nuclei were stained with DAPI.

Acknowledgements
We thank Adam Hurlstone for his help with PAC isolation, members of Nicol Keith's lab for help with FISH analysis, David Gillespie for the anti-c-Myc antibody, Alan Richardson and Tom Parsons for pp125^{FAK} cDNA and John Wyke for reading the manuscript. This work was supported by the Cancer Research Campaign (UK). V Brunton and D Owens were supported by the Medical Research Council (UK).

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Regulation of both apoptosis and cell survival by the v-Src oncoprotein.

Running Title
Regulation of apoptosis and survival by v-Src

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Abstract

The v-Src oncoprotein, unlike other well studied oncoproteins including Myc, v-Jun and E2F-1, can induce cycling under low serum conditions without inducing cell death. Here we demonstrate that growth of transformed cells in low serum is dependent on v-Src induced survival signals. We used Rat-1 cells transformed by a temperature sensitive v-Src to show that when we switched off v-Src under low serum conditions, the cells rapidly exited the cell cycle and entered a programme of cell death. Death was accompanied by activation of caspases and the stress-activated kinases, JNK (Jun N-terminal kinase) and p38 MAP (mitogen activated protein) kinase, and was inhibited by overexpression of the anti-apoptotic protein Bcl-2 or by combined treatment with a caspase inhibitor, Z-VAD-FMK and a p38 inhibitor, SB203580. This suggests that under low serum conditions v-Src transformed cells were primed to enter apoptosis, a process involving both p38 and caspases, but this was prevented by v-Src or serum induced survival signals. Amongst other effects, v-Src activates two downstream kinases, PI3-K (phosphatidylinositol 3-kinase) and ERK1/2 (extracellular signal-regulated kinase), which have been shown to mediate growth and survival signals and switching off v-Src led to down regulation of the activity of both kinases. Using specific inhibitors of PI3-K (LY294002) and the ERK activating kinase MEK1 (MAP kinase kinase) (PD98059) we showed that, unlike normal Rat-1 cells, v-Src transformed Rat-1 cells grown in low serum could be induced to die by the PI3-K inhibitor. We conclude that, under low serum conditions, v-Src transformed cells are primed to die by apoptosis but are prevented from doing so by v-Src induced survival signals mediated by PI3-K.

Keywords: v-Src; transformation; apoptosis

Abbreviations: JNK, Jun N-terminal kinase; Z-, benzyloxycarbonyl; FMK, flouromethylketone; PI3-K, phosphatidylinositol 3-kinase; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, MAP kinase kinase/ERK kinase; PAK, p21-activated kinase; MEKK, MEK kinase; Z-EK(bio)D-aomk, N-(N^{α}-benzyloxycarbonylglutamyl-N^{ε}-biotinyllysyl)aspartic acid[(2,6-dimethylbenzoyl)oxy]methyl ketone; DAPI, (4',6-Diamidino-2-pheylindole); BrdU 5-Bromo-2'-deoxyuridine; BAD, Bcl-XL/Bcl-2 associated death factor; PBS, phosphate buffered saline; EDTA, ethylenediaminetetraacetic acid; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; ECL, enhanced chemiluminescence; TUNEL, TdT-mediated dUTP nick end labelling; TdT, terminal deoxynucleotidyl transferase; RSV, Rous sarcoma virus; *ts*, temperature sensitive; DMSO, dimethylsulphoxide; GSK-3, glycogen synthase kinase-3.

Introduction

A number of cell lines and primary cells of mesenchymal origin become quiescent when deprived of growth factors or matrix interactions. Even in the absence of both conditions such quiescent cells remain viable for some time [1-3]. In contrast, the same cells transformed by either polyomavirus or RSV (Rous sarcoma virus) do not enter viable quiescence when denied both serum growth factors and matrix; they either multiply in suspension or become non-viable, a phenomenon that permitted the selective isolation of revertants of polyomavirus transformed cells and of *ts* (temperature sensitive) transformation mutants of RSV [2,3]. We recently characterised how v-Src, the oncoprotein of RSV, prevented withdrawal from the cell cycle in the absence of growth factors [4] and we now wish to understand how v-Src influences viability in these non-quiescent cells.

Several oncoproteins can induce the paradoxical outcomes of cell multiplication and senescence or apoptosis, depending on the cellular context [5]. For example c-Myc, E2F-1 and v-Jun induce both cell proliferation and apoptosis [6-8] whilst Ras, operating through the Raf pathway has similar effects [9]. However, Ras stimulation of PI3-K protects against apoptosis [9] and this is thought to be a reason why Ras and other anti-apoptotic oncogenes such as Bcl-2 demonstrate oncogenic cooperation with c-Myc [10-12], each oncogene countering the other's antiproliferative properties.

The way in which oncoproteins such as Myc and E2F-1 induce apoptosis is beginning to be characterised [13,14]. v-Src transformation stimulates c-Myc expression [15,16] suggesting a likely pro-apoptotic effect. However v-Src can also activate several kinases which can inhibit cell death including ERK [17,18] and PI3-K [19] which induces cell survival in epithelial cells following detachment via the downstream serine/threonine kinase, Akt [20]. This suggests that v-Src may promote cell death or protect from cell death under different conditions.

We made use of Rat-1 cells transformed by a temperature sensitive v-Src (*ts LA 29*) which we used previously to characterise the mitogenic effects of v-Src [4,18,21]. Here we use the reversibility of *ts* v-Src to examine the effect of inactivating v-Src in transformed cells grown under low serum conditions. We found that the cells exited the cell cycle and were committed to a programme of cell death. We characterised the death as apoptosis by morphological changes including membrane blebbing, DNA and nuclear fragmentation and biochemical changes including the activation of caspases [22] and the activation of the stress induced kinases, JNK and p38 [23-25]. Over expression of the anti-apoptotic protein Bcl-2 [26] prevented cell death and treatment with the caspase inhibitor Z-VAD-FMK [27] and the p38 inhibitor SB203580 [28] simultaneously but not independently significantly reduced cell death. This suggested that the apoptotic pathway required either caspases or p38 and interacted functionally with Bcl-2.

Since v-Src transformed cells did not undergo apoptosis when v-Src was active we assessed whether apoptosis seen when v-Src was switched off was due to loss of v-Src survival signals. We investigated the effect of switching off v-Src on PI3-K and ERK activity and found that ERK and Akt (which is activated downstream of PI3-K) were rapidly inactivated. We tested whether inactivation of either kinase could induce cell

death in v-Src transformed cell using LY294002 to inhibit PI3-K [29] and PD98059 to inhibit ERK activating kinase (MEK1) [30]. We found that LY294002 was sufficient to induce apoptosis when added to v-Src transformed cells in low serum but did not induce apoptosis when added to normal Rat-1 cells grown under low serum conditions. This suggests that, in the absence of serum survival signals, v-Src promotes apoptosis but the transformed cells are protected from cell death by survival signals from v-Src mediated by PI3-K which could explain why v-Src does not require a co-operating oncoprotein. We suggest that the anti-apoptotic effects of v-Src could be as important to the transforming ability of the v-Src oncoprotein as the effects on mitogenesis.

Results

v-Src activity prevents transformed cells undergoing apoptosis in low serum

Rat-1 cells expressing the *ts LA 29* v-Src mutant of RSV (*ts LA 29* Rat-1) are transformed at the permissive temperature for v-Src activity (35°C) and morphologically normal at restrictive temperature (39.5°C). When such cells were placed in 0.2 % serum medium (low serum) for 24 h they continue to cycle at 35°C (Figure 1A, 0 h and 4). On inactivating v-Src by shift to 39.5°C we used flow cytometry to show that there was the expected rapid reduction in the number of cells in S-phase (as judged by the number of cells which incorporated BrdU during a 1 h pulse, Figure 1A, 4 h and 8 h). The same phenomenon is seen initially when v-Src is inactivated in the presence of serum growth factors 4. In contrast to the cells' behaviour in high serum, however, v-Src inactivation in low serum led to many cells becoming loosely adherent (detached) within a few hours, with rounded or blebbing morphology (Figure 1B) and fragmented nuclei (Figure 1C) which could be labelled using the TUNEL (TdT-mediated dUTP nick end labelling) technique 31 (Figure 1D). Almost all cells had detached in this way by 24 h after v-Src inactivation.

When detached and still adherent cells were harvested separately 8 h after inactivating v-Src in low serum, flow cytometry showed the detached cells to have a peak DNA content less than the G_0/G_1 peak (Figure 1E, left) and a smaller cell size (Figure 1E, right) The still adherent cells were smaller than cells in which v-Src was active (Figure 1E) but not as small as the detached cells. DNA isolated from detached and adherent cells separated into 200 base pair ladders, again the adherent cells had an intermediate phenotype with the laddering being more apparent in the detached cell DNA (Figure 1F). Thus inactivation of v-Src in transformed cells which lack serum survival factors led to the rapid onset of changes characteristic of apoptosis; cell shrinkage, blebbing and detachment, nuclear fragmentation, TUNEL labelling and DNA laddering. These changes could be prevented by replacing serum up to 2 h after inactivating v-Src but from 4 h after v-Src inactivation, the cells were progressively committed to a programme of cell death (Figure 1G) that eventually involved the whole population. Our results indicate that v-Src protected from cell death under low serum conditions but cells became committed to death when v-Src was switched off. Thus under low serum conditions survival signals from v-Src prevent commitment to death in v-Src transformed cells.

Biochemical changes associated with apoptosis induced by switching off v-Src

Apoptosis is frequently associated with activation of specific proteases, caspases and with the activation of the stress activated kinases p38 and JNK. Using a biotinylated peptide, which binds to the large subunits of all active caspases tested 32, we detected active caspases in extracts from cells harvested 2.5 h after v-Src inactivation (Figure 2A). All members of the MAP kinase family including JNK and p38 are activated by dual tyrosine, serine/threonine phosphorylation in the catalytic domain by specific MAP kinase kinases 33. We used antibodies specific for the dual Thr183/Tyr185 form

of JNK or Thr180/Tyr182 form of p38 to determine the amount of active p38 and JNK. We detected phosphorylated p38 and JNK 4 h after v-Src inactivation (Figure 2B). Several different isoforms of JNK have been reported around 54 and 46 kD which are produced from three JNK genes by alternative spicing [34]. We found an isoform of JNK in apoptotic cells whose appearance correlated with a decrease in the amount of p54 JNK (Figure 2B), suggesting that it might be derived from the p54 isoform. We could also detect two JNK isoforms around 45 kD. Thus activation of caspases, JNK and p38 and the appearance of morphological changes characteristic of apoptosis (Figure 1) were all initiated between 2 and 4 h after inactivating v-Src.

Activation of JNK and p38 can be triggered by caspases which, in some cell types, cleave and activate two upstream kinases, MEKK1 and PAK-2 (p21-activated kinase-2) [35,36]. Since caspases, JNK and p38 were activated simultaneously (Figure 2A and B), we investigated whether caspase activity was required for the activation of JNK or p38. We added a general caspase inhibitor Z-VAD-FMK [27,37] 1 h before switching off v-Src and harvested the adherent and detached cells separately after 6 h. Treatment with Z-VAD-FMK prevented the cleavage of two known caspase substrates FAK (data not shown) and p27 (Figure 3C) [38], indicating that caspase activity was inhibited. We found that Z-VAD-FMK slightly reduced the amount of active JNK compared to total JNK in detached cells but increased the amount of active JNK in the adherent cells (Figure 3A). More markedly, Z-VAD-FMK reduced active p38 in adherent cells and almost abolished it in detached cells (Figure 3B). This suggests that during apoptosis initiated by switching off v-Src under low serum conditions, the activation of p38 was largely dependent on the activation of caspases, while activation of JNK was not.

The new form of JNK (Fig 3A, arrows) was present in extracts from control detached cells but not in extracts from Z-VAD-FMK treated detached cells, which also retained the p54 isoform of JNK. This suggests that the new isoform could have been formed by caspase cleavage of p54 JNK. The p54 isoform has a longer C-terminal tail [34] not present in p46 JNK which could be the location of the cleavage site. The general caspase inhibitor Boc-D-FMK also inhibited the formation of the new JNK isoform whereas other caspase inhibitors including Z-DEVD-FMK, Z-YVAD-FMK, Z-IETD-FMK, which are designed to inhibit particular subsets of caspases, had no effect (data not shown). These results suggest that several caspases are activated in the apoptotic cells and that these are responsible for the appearance of the new JNK isoform and loss of the p54 isoform.

The role of caspases and p38 in apoptosis

Since we found that activation of caspases 2.5 h after switching off v-Src (Figure 2A), correlated with commitment to cell death (Figure1G), we investigated whether inhibiting caspases would prevent cell death. We found that the caspase inhibitor Z-VAD-FMK, added at the same time as inactivating v-Src, reduced cell shrinkage in the detached cells (compare Figure 1E and Figure 4A) but did not prevent cell detachment or membrane blebbing (Figure 4B), nuclear fragmentation (Figure 4C), DNA laddering (Figure 4D) or TUNEL labelling (Figure 4E). Between 4 and 6 h the control cells stopped blebbing and became rounded whereas the cells treated with Z-VAD-FMK remained in a blebbing state (compare the number of rounded cells in

Figure 1B and Figure 4B). This suggests that caspases were not required for cell death but are required for cell shrinkage and the cessation of cell blebbing.

Previous work has shown that cell blebbing in the presence of Z-VAD-FMK requires the activity of p38 [24]. Cells treated with Z-VAD-FMK retained some p38 activity (Figure 3) raising the possibility that signalling through p38 was sufficient to induce blebbing and cell death. We added a selective inhibitor of p38, SB203580 [28] at the same time as inactivating v-Src and investigated the effect on cell blebbing and TUNEL labelling. We found that SB203580 had no effect on the number of cells undergoing cell blebbing (data not shown) and no significant effect on the number of TUNEL positive cells (Figure 4E). However, when we added both Z-VAD-FMK and SB203580 we observed a 65 % drop in the number of TUNEL positive cells (Figure 4E) and a similar fall in the number of cells undergoing cell blebbing (data not shown). Thus, in order to prevent cell death it was necessary to block both caspases and p38 suggesting that either was sufficient to induce cell death under these conditions.

v-Src mediated protection against apoptosis

Cell death following v-Src inactivation in low serum may be caused by down regulation of v-Src survival signals. Among the many down stream effects of v-Src transformation is the activation of two kinases PI3-K and ERK (MAP kinase) both of which have been shown to mediate survival signals [17,20]. Therefore we investigated the effect of switching off v-Src on the activities of PI3-K and ERK using antibodies specific for phospho Ser473 Akt and phospho p44/42 ERK. Akt phosphorylation is a good indicator of PI3-K activity since it is recruited to the membrane by the phospholipid products of PI3-K where it is activated by phosphorylation at Thr308 and Ser473 [39]. ERK is activated by phosphorylation on Thr202/Tyr204 by MEK1 [33]. We found a rapid decline in the activated Akt and ERK after switching off v-Src (Figure 5A, upper). The upper band detected with the phospho-ERK antibody at 0 h did not appear in all experiments and its identity is not known. ERK phosphorylation returned at 4 h, simultaneous with the phosphorylation of p38 and JNK (Figure 2C) and the onset of apoptosis, whereas Akt protein was down regulated from 4 h consistent with cleavage by caspases as reported by Widmann *et al.*, (1998) [40]. Since both the ERK and PI3-K were rapidly inactivated when v-Src was switched off, this showed that both were controlled by v-Src activity in *ts LA 29* Rat-1 cells and are candidate mediators of v-Src induced survival signals.

To test whether either pathway relayed v-Src induced survival signals we used PD98059, a specific inhibitor of MEK1, [30] to inactivate ERK or LY294002, a specific inhibitor of PI3-K [29]. We added these two inhibitors to v-Src transformed cells cultured for 24 h in low serum medium and harvested them at intervals for immunoblotting or TUNEL. Figure 5B shows that adding PD98059 reduced ERK phosphorylation while Figure 5c shows that adding LY294002 reduced Akt phosphorylation. Figure 5D shows that LY294002 induced about half the number of TUNEL positive cells as switching off v-Src whereas PD98059 had no effect either alone or when added with LY294002. Importantly, the effect of LY294002 on cell survival is specific to v-Src transformed cells since when we added LY294002 to serum deprived normal Rat-1 we did not observe any increase in apoptosis after 6 h

(Figure 5E). Furthermore, the susceptibility of v-Src transformed cells to inhibitor induced cell death is lost after they are cultured for several days at 39.5°C (Figure 5E). This suggests v-Src transformed cells under low serum conditions were dependent on survival signals mediated by v-Src induced PI3-K activity.

If PI3-K is responsible for mediating v-Src survival signals we would expect that death induced by inhibiting PI3-K would be mediated by the same pathway as death induced by switching off v-Src. To investigate this we harvested adherent and detached cells treated with LY294002 to examine the appearance of the new isoform of JNK, which we showed was dependent on caspase activity (Figure 3A) and the activation of p38 and JNK. Cells treated with PD98059 (most of which remained attached) served as a control. Treatment of serum deprived v-Src transformed cells with LY294002 induced p38 and JNK phosphorylation and the new isoform of JNK in the detached cells whereas PD98059 had no effect (Figure 5F). In contrast to results shown in Figs 2B and 3B we did not see any active p38 in the adherent cells in Figure 5F suggesting that cells with active p38 were weakly attached and could be harvested with detached cells. These results indicate that apoptosis induced by inhibiting PI3-K under low serum conditions is accompanied by similar biochemical events to that induced by switching off v-Src, supporting the role for PI3-K in mediating v-Src survival signals.

Cell death is inhibited by the anti-apoptotic protein Bcl-2

Over expression of Bcl-2 or Bcl-X_L can inhibit some but not all types of apoptosis [41,42]. Therefore we tested whether over expression of Bcl-2 would inhibit cell death induced by switching off v-Src. We transfected *ts LA 29* Rat-1 cells with a mammalian expression vector for mouse Bcl-2 and isolated clones which over express Bcl-2 to differing extents (Figure 6A). We found that Bcl-2 inhibited cell death induced by v-Src inactivation or by LY294002 in a dose dependent manner (TUNEL labelling shown in Figure 6B). However, Bcl-2 did not block the fall in Akt or ERK phosphorylation after v-Src was switched off (Figure 6C). These results show that Bcl-2 blocks cell death due to switching off v-Src or inhibiting PI3-K without blocking inactivation of Akt.

The Bcl-2 family of anti-apoptotic proteins inhibit apoptosis by preventing activation of caspases, JNK and p38 [26,41,43-45]. Our results suggest that Bcl-2 blocked cell death downstream of Akt, thus to define more precisely where Bcl-2 acted we determined if it could inhibit phosphorylation of JNK and p38 or the caspase dependent mobility shift in JNK. JNK and p38 did not become phosphorylated in clones expressing high levels of Bcl-2 whereas they were phosphorylated in clones containing the plasmid alone (Figure 6D). Similarly, the new JNK isoform (indicated by arrows) was not seen in clones with high Bcl-2 levels (Figure 6D). These results suggest that Bcl-2 blocks cell death upstream of the activation of caspases, JNK and p38 and downstream of PI3-K and Akt.

Discussion

The apoptotic activities resulting from the activation of mitogenic oncoproteins may protect normal cells from unscheduled proliferation and this is one postulated reason for co-operation between mitogenic and anti-apoptotic oncogenes [10-12]. Unlike other oncoproteins, the deregulated tyrosine kinase v-Src does not require a co-operating partner to induce full neoplastic transformation, however, as a potent mitogen, v-Src might also be expected to promote apoptosis. If so, then v-Src itself must induce a countervailing anti-apoptotic process if it is to succeed in inducing neoplasia on its own. We show here that v-Src does indeed induce both pro-apoptotic and anti-apoptotic effects in Rat-1 cells cultured with insufficient serum survival factors.

The potentially deleterious effect of v-Src on cell viability was implied in very early studies, which showed that, in low serum, v-Src expressing cells either multiplied or died, but could not become quiescent [3]. We demonstrate here that v-Src transformed cells in low serum are primed to die by apoptosis when v-Src is switched off (Figure 1) or PI3-K is inhibited (Figure 5D). Apoptosis was specific to v-Src transformed cells in low serum, since inhibition of PI3-K in low serum did not lead to apoptosis in normal Rat-1 cells or *ts LA* 29 Rat-1 cells in which v-Src had been inactive for several days (Figure 5E). Since *ts LA* 29 Rat-1 cells display this apoptosis upon switching off v-Src in low serum, either the oncoprotein or serum must provide an overriding survival signal (Figure 1G). The speed and magnitude of the apoptotic response in this conditional system may make it useful for studying details of apoptotic commitment and execution. We, however, have concentrated on the dual roles of v-Src in this phenomenon and their consequences for Src biology.

Possible mechanisms of priming apoptotis by v-Src under low serum conditions

A proapoptotic effect of v-Src in low serum has not been previously described and, although we do not know how it is mediated, the properties of v-Src offer several interrelated possibilities.

Firstly, v-Src upregulates c-Myc, which has been shown both to promote cell proliferation and to prime Rat-1 cells for apoptosis under low serum conditions (comprehensively reviewed recently in [46]). Secondly, there is evidence that activated Ras promotes apoptosis through Raf signalling [9] and v-Src also activates this pathway [18]. Thirdly, v-Src disrupts cell cycle controls [4,18,21] and the ability to induce apoptosis under low serum conditions, common to mitotic oncoproteins such as E2F-1 [6,47], v-Jun [8] and c-Myc [7], could be a consequence of deregulated cell cycle progression. Variations of the dual signal model for Myc function postulate that the oncogene coordinately stimulates distinct pathways for apoptosis and proliferation [46]. v-Src may also show this functional duality since addition of the MEK1 inhibitor, PD98059, throughout the 24 h incubation in low serum arrested v-Src transformed Rat-1 in G_0/G_1 but did not prevent apoptosis when v-Src was switched off (data not shown).

Finally, v-Src phosphorylation leads to disruption and turnover of the components of cellular adhesions and linking cytoskeletal elements [48], a process that became more pronounced during the 24 h incubation in low serum employed in our experiments (data not shown). The consequent attenuation of integrin signalling may be a signal

for apoptosis, analogous to detachment induced cell death ("anoikis") in epithelial cells [49]. Serum-deprived mesenchymal cells can survive in the absence of integrin signalling [1-3] and *ts LA29* Rat-1 cells at the restrictive temperature and normal Rat-1, which both have a normal morphology, resist apoptosis following PI3-K inhibition (Figure 5E). This implies that, in mesenchymal cells, either integrin mediated or other signals are sufficient to induce survival. These signals can substitute for one another since we showed previously that surrogate integrin signalling can protect serum-deprived fibroblasts from Myc-induced apoptosis [50]. In serum-deprived *ts LA*29 Rat-1 cells at the permissive temperature inactivation of v-Src or inhibition of PI3-K may remove another survival signal from cells whose integrin survival signalling has been disrupted by morphological transformation, resulting in the onset of cell death.

v-Src induced survival signals

We showed that the v-Src survival signal is mediated, at least in part, by PI3-K, since the PI3-K inhibitor, LY294002, induced significant apoptosis in v-Src transformed cells grown under low serum conditions (Figure 5D). v-Src can bind and activate PI3-K [19] and, when we switched off v-Src, we observed inactivation of PI3-K, as evidenced by reduced Akt phosphorylation (Figure 5A). Furthermore, LY294002 inhibited Akt phosphorylation, showing that this inhibitor did, indeed, act on the PI3-K pathway (Figure 5C). Taken together, these results show that v-Src survival signals mediated by PI3-K protect cells from v-Src-primed apoptosis. Previous reports showed that v-Src survival signals mediated by PI3-K protected epithelial cells from anoikis [51] and Rat-1 cells from UV induced apoptosis [52], suggesting that this is a general mechanism whereby v-Src can provide a survival signal to protect against different apoptotic stimuli.

The effectors of v-Src primed apoptosis

Survival signals mediated by Akt have been shown to impinge on the apoptotic machinery in a number of ways including phosphorylation of Bad (Bcl-XL/Bcl-2 associated death factor) [53] and pro-caspase 9 [54]. Phosphorylation prevents pro-caspase 9 activation while phosphorylated Bad is held within the cytosol in a complex with 14-3-3 protein [53,55]. When Akt is inactivated Bad is de-phosphorylated and moves to the mitochondrial membranes where it may titrate out the anti-apoptotic Bcl-2 family members. This may allow pro-apoptotic Bcl-2 family members to homodimerize and interact with pores in the mitochondrial membranes resulting in release AIF [56] and cytochrome c [26] which in turn activates caspase 9. Thus dephosphorylation of Bad and/or caspase 9 provides a possible mechanism of both caspase activation and its inhibition by Bcl-2 when v-Src or PI3-K was switched off. Consistent with this we have found that *ts LA29* Rat-1 cells express Bad protein (D. Johnson and C. Dive unpublished).

Additional mechanisms are required to explain why apoptosis induced by switching off v-Src or inhibiting PI3-K was simultaneous with the activation of both caspases and stress activated kinases, JNK and p38 (Figure 2). Either caspases or p38 were sufficient to induce cell death, although not necessarily all the stigmata of apoptosis, but inhibiting both enhanced cell survival (Figure 4). In addition the activation of p38 was partly caspase dependent (Figure 3B) which may be due to the cleavage and

activation of an upstream kinase analogous to cleavage of PAK 2 [36] or MEKK1 [35]. Therefore switching off v-Src or inhibiting PI3-K engaged at least two independent pathways. These are similar to those reported downstream of Fas [45,57] since ligation of Fas or other death receptors activated the p38 and JNK pathways [58-61] and independently stimulated the activation of caspase 8 [57]. Ras induced PI3-K activity down regulated Fas expression [62]. If v-Src induced PI3-K activity has a similar effect then switching off v-Src or inhibiting PI3-K would increase Fas expression which may activate the Fas pathway. Bcl-2 can inhibit Fas induced cell death in some cells and can inhibit activation of JNK [45] and caspase 8 [41] at the cell membrane. We found that Bcl-2 inhibited the activation of JNK, p38 and caspases when v-Src or PI3-K were switched off in low serum. If inhibiting PI3-K in *ts LA* 29 Rat-1 cells in low serum induced Fas expression then Bcl-2 may prevent cell death by blocking JNK, p38 and caspase activation downstream of Fas. Possible pro-apoptotic signalling downstream of switching off v-Src is summarised in Figure 7.

Conclusions

v-Src can both prime for apoptosis and protect against it. Apoptosis priming is not unique to *ts LA*29 Rat-1, since we observed it also in v-Src transformed chick embryo fibroblasts but not in transformed NIH3T3 cells (data not shown). Pro- and anti-apoptotic functions are also shown by activated Ras [9] v-Src and Ras share, at least in part, the same anti-apoptotic pathway. However, Ras, unlike v-Src, requires cooperating oncogenes for primary cell transformation, in which its pro-apoptotic function prevails over the anti-apoptotic effect. With v-Src, the priming for apoptosis is only apparent when serum survival factors are limiting, conditions under which v-Src transformed cells are finely balanced between proliferation and death. These phenomena may, nonetheless, have implications for a role for Src in neoplasia. Many tumours, particularly those of the colon, have high levels of c-Src expression [63,64] and a few metastatic colon tumours have mutated c-Src which could be important in tumour progression [65]. We showed previously how v-Src induces transformation via effects on cell growth and behaviour [4,18,21,66]. Here we suggest that the ability to induce cell survival could be an equally important transforming activity of v-Src and could be another mechanism whereby deregulated c-Src contributes to tumour formation *in vivo*. Manipulating pathways involved in the alternative death priming effects of v-Src could have therapeutic potential, therefore it would be interesting to characterise these pathways in v-Src transformed Rat-1 cells.

Materials and Methods

Cell culture and induction of cell death

Rat-1 cells and *ts LA* 29 Rat-1 cells were maintained as in 4. BrdU incorporation and labelling was as in 4. For the induction of cell death cells were seeded at 1.5 x 10^6 per 90 mm plate, the following day the medium was replaced with medium containing 0.2% serum. After 24 h they were either shifted to restrictive temperature (39.5°C) to inactivate v-Src or various inhibitors were added.

To harvest adherent and detached cells we seeded 6 x 10^6 cells on a 150 mm plate. The following day the medium was replaced with medium containing 0.2% serum. After 24 h they were shifted to 39.5°C for 6 to 8 h. The detached cells were harvested by washing carefully and the adherent cells were either harvested by trypsinization or lysed on the plate.

BrdU immunostaining, Propidium iodide staining and flow cytometry

As in 4.

Generation of cell lines which over express Bcl-2

pSFFV-Bcl-2 expression plasmid was from Stanley J Korsmeyer, Washington University, St Louis, Missouri, USA. *ts LA 29* Rat-1 cells were transfected with pSFFV-Bcl-2 or pSFFV using DOTAP (Boehringer Mannheim). Single G418 resistant colonies were isolated, grown up and tested for expression of Bcl-2 by immunoblotting. All colonies isolated from cells transfected with pSFFV-Bcl-2 expressed Bcl-2 but we could not detect Bcl-2 in colonies from cells transfected with pSFFV.

Western blot analysis

As in 4 except phospho-specific antibodies were used as recommended by the manufacturers.

Antibodies

Phospho-specific antibodies to Akt, JNK and p38 were from New England Biolabs (#9271S, #9251L, #9211S), phospho-ERK was from Promega (#V8031). Total JNK antibody, raised against full length recombinant JNK was a gift from Billy Clark, Beatson Institute, Glasgow and the p42 ERK antibody, raised against a peptide from the C-terminus of p42 ERK (EETARFQPGYRS), 67 was a gift from Anne Wyke, Beatson Institue, Glasgow. Bcl-2 antibody was from Pharmingen (#15021A), p27 andtibody was kind gift of Steve Coats, Amgen, CA, USA.

Inhibitors

Inhibitors were purchased from CalBiochem. Stocks were made in DMSO (dimethylsulphoxide) as follows: Z-VAD-FMK 10 mM stock, used at 100 μM, SB203580 66mM stock, used at 50μM, LY294002 80 mM stock, used at 50 μM, PD98059 10 mM stock used at 50 μM.

DAPI (4',6-Diamidino-2-pheylindole) staining

Cells were grown in chamber slides, 1 x 10^4 cells per chamber in 0.4 ml culture medium. They were washed twice in PBS (phosphate buffered saline) then fixed in 3.7% formaldehyde in PBS for 10 mins. They were washed in PBS and permeabilized in 0.5% Triton-X-100, 1% BSA in PBS for 30 mins. They were washed once in PBS then mounted in Vectasheild (Vector Labs) containing 0.1 μg/ml DAPI.

DNA Laddering

Cells were harvested by trypsinization, washed in PBS then suspended in TNE (10 mM Tris-Cl pH 8.2, 400 mM NaCl, 2 mM EDTA (ethylenediaminetetraacetic acid)) at a concentration of 5 x 10^6 per ml. Cells were lysed by addition of 0.5% SDS and proteins digested by adding 0.25 mg/ml proteinase K and incubating at 37°C overnight. The lysates were extracted with water saturated phenol then chloroform and precipitated with 1.5 volumes of 100% ethanol and spun for 5 mins in a microfuge. After a 70% ethanol wash the pellet was dissolved in 100 μl 10 mM Tris pH 8.0, 1 mM EDTA and 0.1 mg/ml DNase free RNase A was added. Tubes were incubated at 37°C for 30 mins then 5 to 10 μg was run on a 2 % agarose gel.

TUNEL

31. Cells were harvested and washed once in PBS. They were fixed for 15 mins on ice in 1% formaldehyde in PBS then centrifuged at 1000 rpm for 5 mins. They were resuspended in 100 μl PBS and 1 ml ice cold 70% ethanol was added. They were stored overnight at 4°C and the next day they were rehydrated in PBS on ice for 30 mins. Aliquots of 10^6 cells were spun and resuspended in 0.2 M potassium cacodylate, 2.5 mM Tris-HCl pH 6.6, 2.5 mM $CoCl_2$, 0.25 mg/ml, BSA, 5 units of TdT (terminal deoxynucleotidyl transferase) and 0.5 nmoles of biotinylated dUTP per tube. They were incubated at 37°C for 30 mins, washed in 1 ml of PBS then spun and resuspended in 100 μl 4 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M NaCitrate), 0.1% Triton X-100, 5% low fat dried milk and 5 μg/ml avidin-FITC (fluorescein isothiocyanate). They were incubated for 30 mins at room temperature in the dark then washed by addition of 1 ml PBS containing 0.1% Triton X-100, spun and resuspended in 500 μl of PBS containing 10 μg/ml propidium iodide. They were analysed by flow cytometry and the percentage TUNEL positive cells determined by FITC fluorescence.

Detection of active caspases

32 Cells were lysed by freezing on dry ice and thawing at 37°C three times then incubated with Z-EK(bio)D-amok (N-(N^{α}-benzyloxycarbonylglutamyl-N^{ε}-biotinyllysyl)aspartic acid[(2,6-dimethylbenzoyl)oxy]methyl ketone) (1μM added from a 25μM stock in DMSO) at 37°C for 15 mins. They were diluted with 2 x SDS

sample buffer, boiled for 3 minutes and sonicated briefly. They were subject to SDS-PAGE (polyacrylamide gel electrophoresis) on 16% gels, transferred to nitrocellulose, probed with peroxidase-labeled streptavidin and visualised using ECL (enhanced chemiluminescence).

Acknowledgements

We are grateful to Stanley Korsmeyer, Washington University, MO, USA for the Bcl-2 expression plasmid, C. Dive, Manchester University, UK for help with Bad westerns, Steve Coats, Amgen, CA for p27 antibodies and Dave Gillespie for many helpful discussions. This work was supported by The Cancer Research Campaign, project grant number SP 2281/0101. Association for International Cancer Research project grant number, Sylvia Aitkin trust, and CRC studentship to M. Agochiya.

Figure Legends

Figure 1. Inactivation of v-Src under low serum conditions causes cell cycle exit and apoptosis. (A) At intervals after inactivation of v-Src cells were allowed to incorporate BrdU for 1 h. They were then fixed and stained with FITC labelled antibodies against BrdU and the DNA stain, propidium iodide and analysed by flow cytometry. The dot plots show propidium iodide staining on the X-axis and FITC labelling (BrdU) on the Y-axis. Cells which incorporated BrdU and were in S-phase are indicated. (B and C) *ts-LA 29* Rat-1 cells 6 h after inactivation of v-Src. (B) photographed under phase contrast and (C) fixed and stained with DAPI. (D) Cells were harvested at intervals after v-Src inactivation, fixed and labelled by the TUNEL technique. Graph shows the means and standard errors of 7 experiments. (E and F) Flow cytometric and DNA analysis of v-Src transformed cells under low serum conditions (0h) and following inactivation of v-Src for 8 h when loosely adherent (det.) and adherent cells (adh.) were harvested separately. (E) Cells were fixed, stained with propidium iodide and analysed by flow cytometry. Left panel shows cell number against propidium iodide staining while right panel shows cell number against cell size (forward scatter). (F) DNA extracted from the cells was analysed by 2 % agarose gel electrophoresis and stained with ethidium bromide (M - markers). (G) At intervals after inactivation of v-Src we added 5% serum. Cells were harvested 6 h after v-Src inactivation, fixed and labelled by the TUNEL technique. A representative experiment is shown.

Figure 2. Activation of caspases, JNK and p38 during apoptosis. (A) We extracted proteins from cells harvested at intervals after v-Src inactivation and incubated with a biotinylated peptide (Z-EK(bio)D-amok) which binds to the active site of caspases. The proteins were separated by SDS PAGE and immunoblotted with streptavidin linked to horseradish peroxidase. Active caspases were detected by ECL. (B) We extracted proteins from cells harvested at intervals after v-Src inactivation and immunoblotted using antibodies against Thr183/Tyr185 JNK or Thr180/Tyr182 p38 (upper panels) or total JNK or p38 (lower panels). Arrows indicate an isoform of JNK which appeared at 4 h.

Figure 3. The effect of the caspase inhibitor Z-VAD-FMK on (A) p27 (B) JNK activation and (C) p38 activation. (A) Proteins extracted from control or Z-VAD-FMK treated cells at 0 h and adherent and detached cells 6 h after v-Src inactivation were analysed by immunoblotting antibodies against p27. (B and C) Proteins extracted from control or Z-VAD-FMK treated cells at 0 h and adherent and detached cells 6 h after v-Src inactivation were analysed by immunoblotting antibodies against Thr183/Tyr185 JNK or Thr180/Tyr182 p38 (upper panels) or total JNK or p38 (lower panels) (Arrows indicate the new isoform of JNK in detached cells)..

Figure 4. The effect of Z-VAD-FMK and SB203580 on cell death. (A) Z-VAD-FMK treated adherent or detached cells were harvested 6 h after inactivating v-Src. They were fixed and stained with propidium iodide then analysed by flow cytometry. Left panel shows cell number against propidium iodide staining while right panel shows cell number against cell size (forward scatter). (B) 6 h after inactivating v-Src cells treated with Z-VAD-FMK were photographed under phase contrast or (C) fixed and stained with DAPI. (D) We extracted DNA from control [C] or Z-VAD-FMK treated

[Z] adherent or detached cells harvested 6 h after inactivating v-Src and analysed it by 2% agarose gel electrophoresis and staining with ethidium bromide. (E) 6 h after inactivating v-Src cells treated with Z-VAD-FMK or SB203580 were harvested, fixed and labelled by the TUNEL technique. Results shown are the average and standard errors of 4 replicates from 2 different experiments.

Figure 5. The role of ERK and PI3-K pathways in cell death. (A) We extracted proteins from the cells at intervals after v-Src inactivation and analysed them by immunoblotting using antibodies against phosphoThr202/Tyr204 p42/44 ERK or phospho-Ser473 Akt (upper panels) or p42 ERK or total Akt (lower panels). (B and C) v-Src transformed cells were incubated in low serum at the permissive temperature for 24h. (B) Cells were harvested at intervals after the addition of PD980959 and proteins extracted from the cells were analysed by immunoblotting using antibodies against phosphoThr202/Tyr204 p42/44 ERK (upper) or p42 ERK (lower). (C) Cells were harvested at intervals after the addition of LY294002 and proteins extracted from the cells were analysed by immunoblotting using antibodies against phospho-Ser473 Akt (upper) or total Akt (lower). (D) v-Src transformed cells incubated in low serum for 24h were harvested at intervals after the addition of PD980959 (dashed line), LY294002 (fine line), both inhibitors (dotted line) or after switching off v-Src (heavy line). Cells were fixed and labelled using the TUNEL technique. The graph shows a representative experiment of cells treated with inhibitors and means and standard errors of 7 experiments when v-Src was switched off. (E) Rat-1 cells or *ts LA 29* Rat-1 cells were grown at the permissive (35°C) or restrictive (39.5°C) temperature for the v-Src oncoprotein. Cells were transferred to low serum for 24 h then treated with LY294002 for a further 6 h, harvested, fixed and labelled by the TUNEL technique. (F) v-Src transformed cells incubated in low serum for 24h were harvested 6 h after treatment with PD98059 or LY294002 or switching off v-Src. Proteins extracted from the adherent or detached cells were analysed by immunoblotting using antibodies against Thr183/Tyr185 JNK or Thr180/Tyr182 p38 (upper panels) or total JNK or p38 (lower panels). Arrows show the new isoform of JNK seen in detached cells.

Figure 6. Inhibition of cell death by Bcl-2. (A) We extracted proteins from cell lines engineered to over express Bcl-2 or containing plasmid DNA and analysed the level of Bcl-2 expression by immunoblotting with antibodies specific for Bcl-2. (B) v-Src transformed Bcl-2 expressing or control cells were incubated in low serum for 24 h. LY294002 was added or v-Src switched off and the cells were harvested 6 h later, fixed and stained using the TUNEL assay. The results shown are representative of several different experiments. (C) v-Src transformed Bcl-2 expressing and control cells were incubated in low serum for 24 h then harvested cells at intervals after switching off v-Src. Proteins were extracted and analysed by immunoblotting using antibodies against phospho-Ser473 Akt or phosphoThr202/Tyr204 p42/44 ERK (upper panels) or Akt or p42 ERK(lower panels). (D) The v-Src transformed Bcl-2 expressing and control cells were incubated in low serum for 24 h then harvested 6 h after switching off v-Src (-Src) or adding LY294002 (LY). Proteins were extracted and analysed by immunoblotting using antibodies against Thr183/Tyr185 JNK or Thr180/Tyr182 p38 (upper panels) or total JNK and p38 (lower panels). Arrows indicate the new isoform of JNK.

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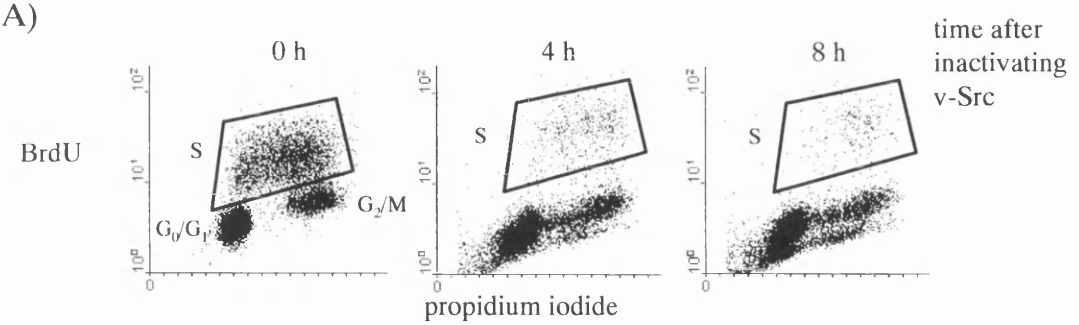

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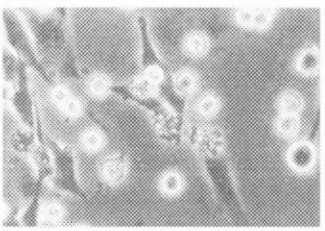

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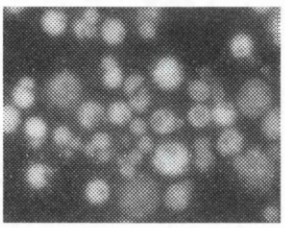

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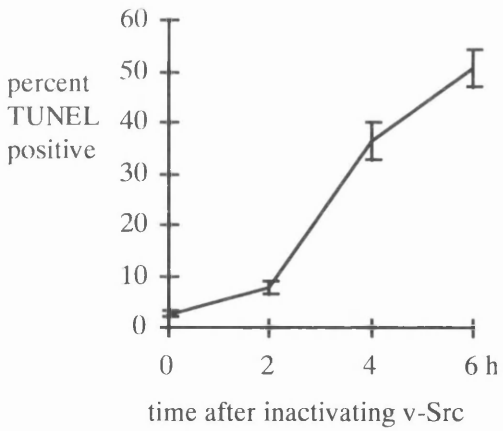

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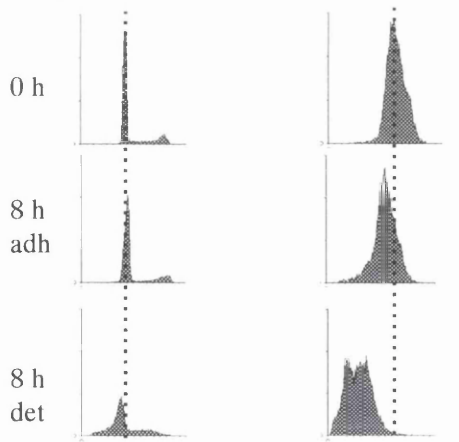

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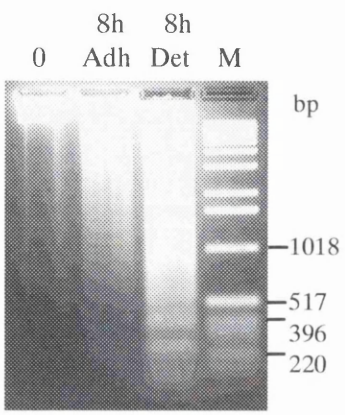

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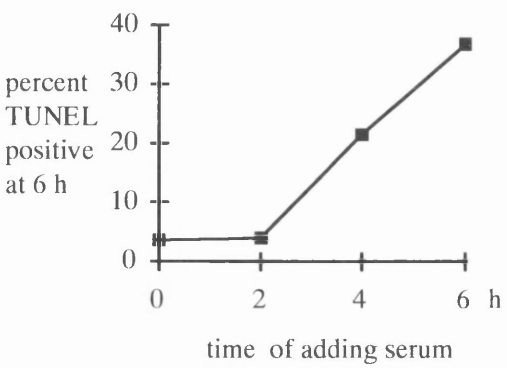

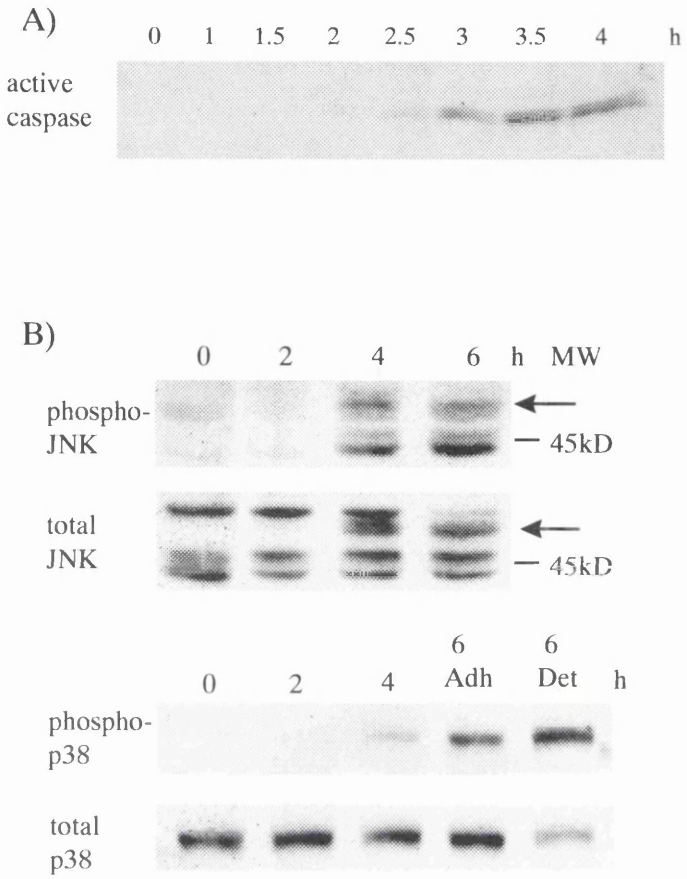
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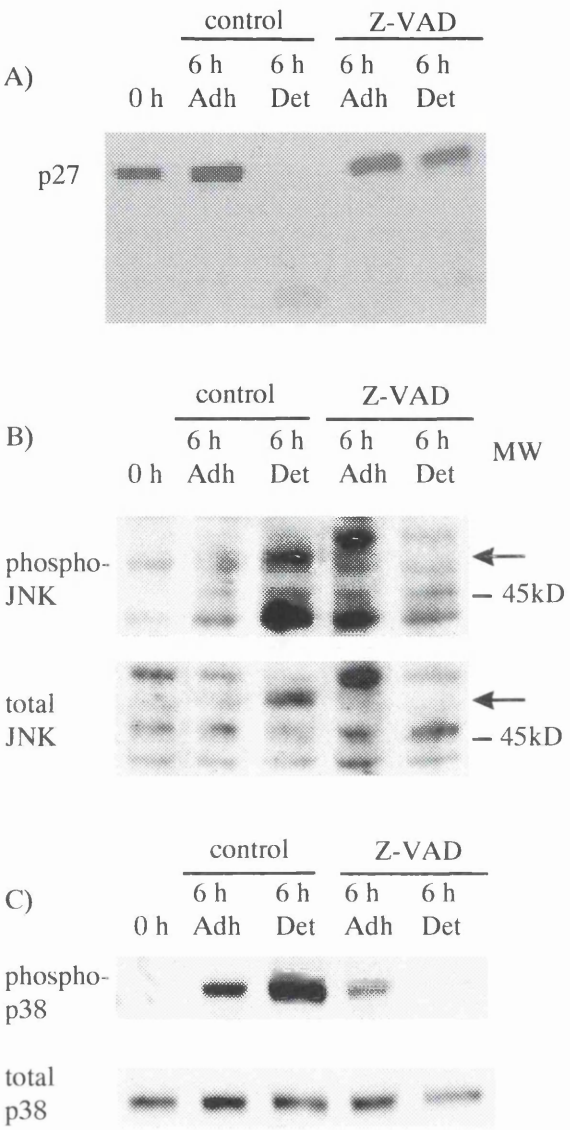
phospho-p38
total p38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6 h Adh
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6 h Adh
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p27
B)
control
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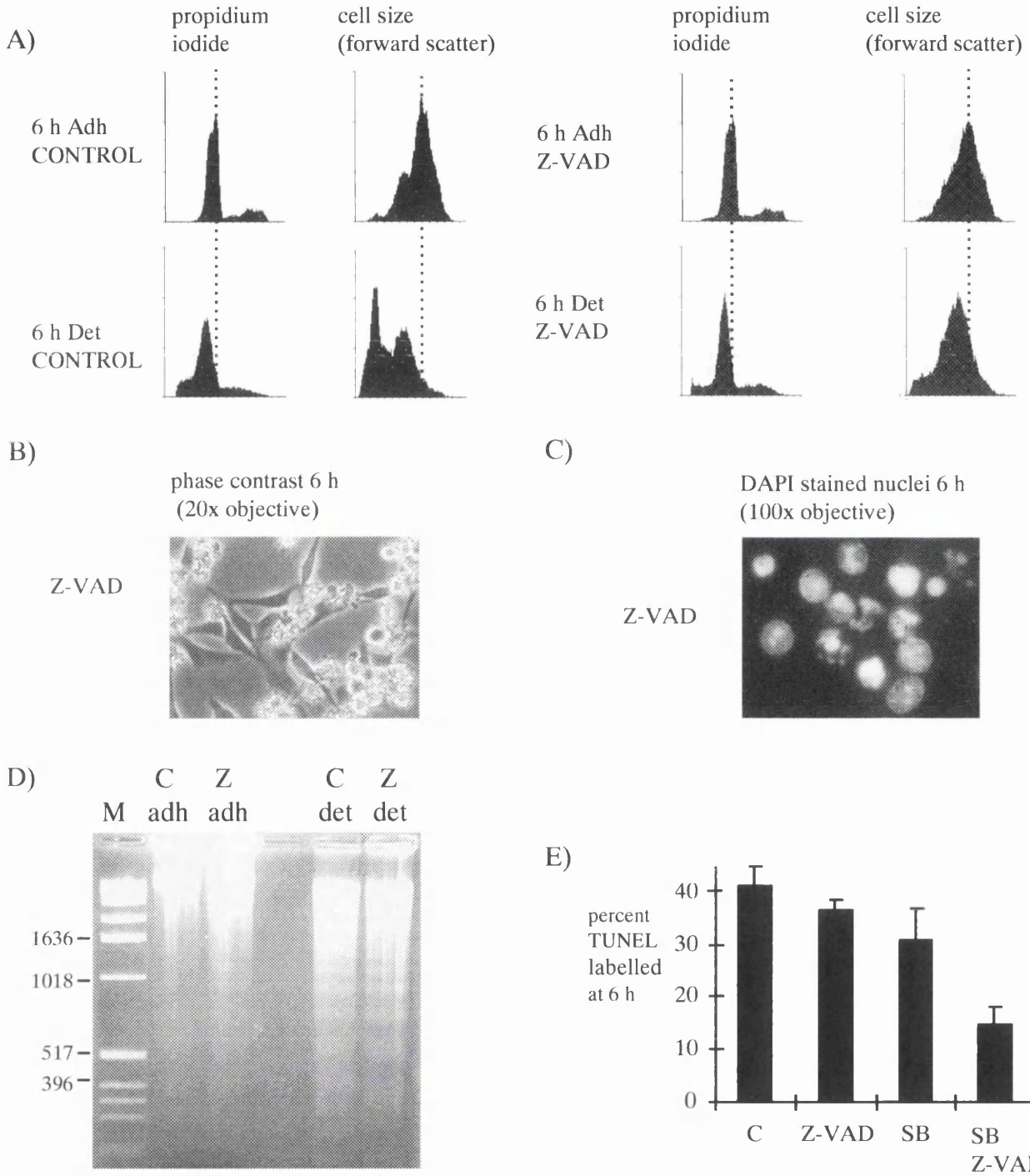
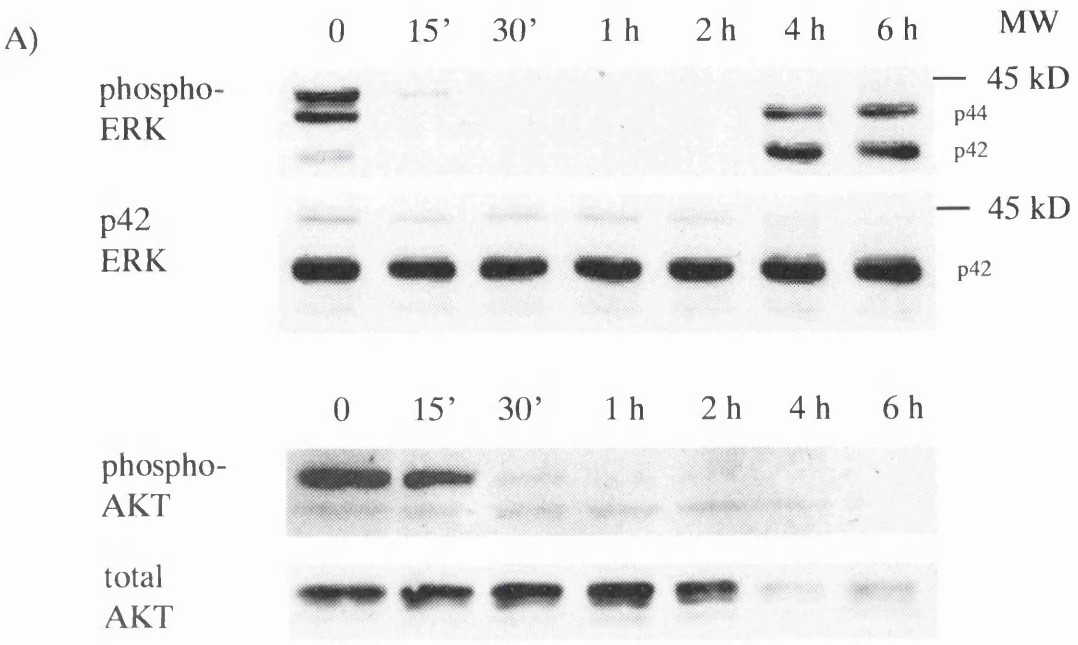
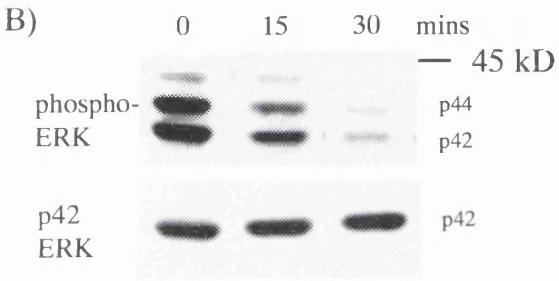
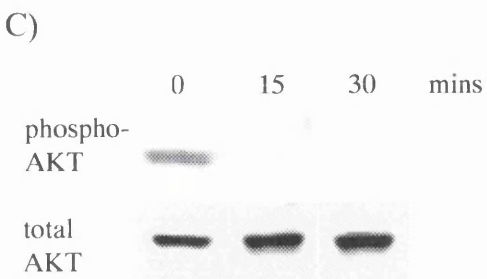
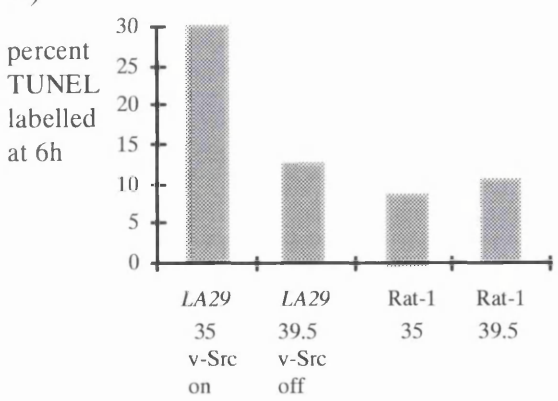
6 h Det
6 h Adh
6 h Det
MW
phospho-JNK
45kD
total JNK
45kD
C)
control
Z-VAD
0 h
6 h Adh
6 h Det
6 h Adh
6 h Det
phospho-p38
total p38

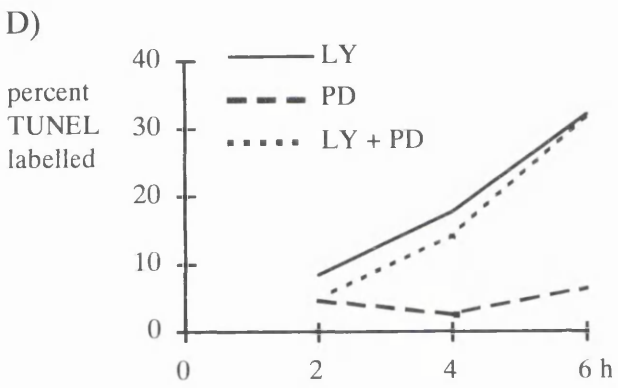

A)
propidium iodide
cell size (forward scatter)
6 h Adh CONTROL
6 h Det CONTROL
6 h Adh Z-VAD
6 h Det Z-VAD
B)
phase contrast 6 h (20x objective)
Z-VAD
C)
DAPI stained nuclei 6 h (100x objective)
Z-VAD
D)
M
C adh
Z adh
C det
Z det
1636
1018
517
396
E)
percent TUNEL labelled at 6 h
40
30
20
10
0
C
Z-VAD
SB
SB Z-VAD

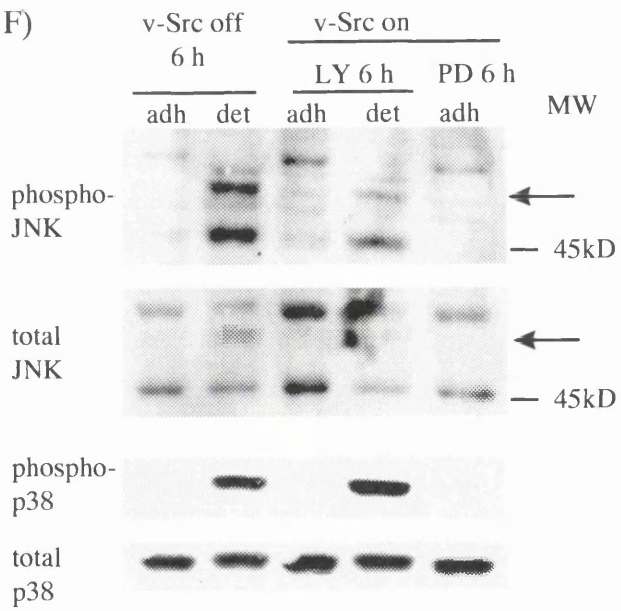

A)
0
15'
30'
1 h
2 h
4 h
6 h
MW
phospho-
ERK
45 kD
p44
p42
p42
ERK
45 kD
p42
phospho-
AKT
total
AKT

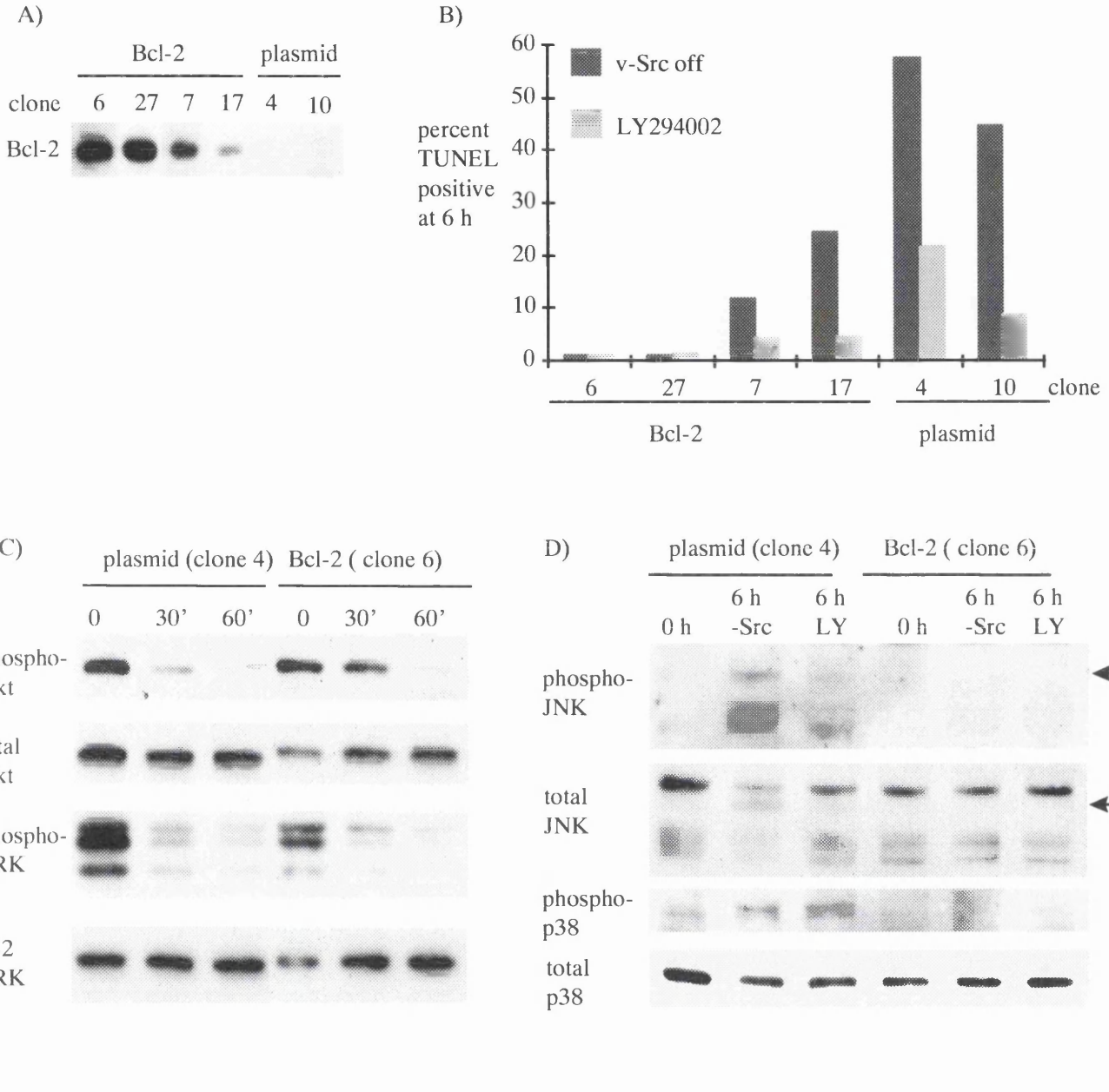

B)
0
15
30
mins
45 kD
phospho-
ERK
p44
p42
p42
ERK
p42

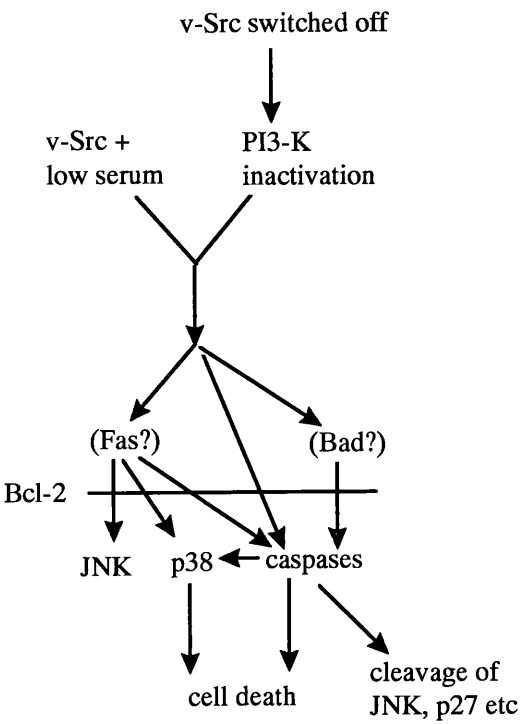

C)
0
15
30
mins
phospho-
AKT
total
AKT


E)
percent
TUNEL
labelled
at 6h
30
25
20
15
10
5
0
LA29
35
v-Src
on
LA29
39.5
v-Src
off
Rat-1
35
Rat-1
39.5


D)
percent
TUNEL
labelled
40
30
20
10
0
LY
PD
LY + PD
0
2
4
6 h


F)
v-Src off
6 h
v-Src on
LY 6 h
PD 6 h
adh
det
adh
det
adh
MW
phospho-
JNK
45kD
total
JNK
45kD
phospho-
p38
total
p38


A)
Bcl-2
plasmid
clone 6 27 7 17 4 10
Bcl-2
B)
v-Src off
LY294002
percent TUNEL positive at 6 h
60
50
40
30
20
10
0
6 27 7 17 4 10 clone
Bcl-2
plasmid
C)
plasmid (clone 4)
Bcl-2 (clone 6)
0 30' 60' 0 30' 60'
phospho-Akt
total Akt
phospho-ERK
p42 ERK
D)
plasmid (clone 4)
Bcl-2 (clone 6)
0 h
6 h -Src
6 h LY
0 h
6 h -Src
6 h LY
phospho-JNK
total JNK
phospho-p38
total p38


v-Src switched off
v-Src +
low serum
PI3-K
inactivation
(Fas?)
(Bad?)
Bcl-2
JNK
p38
caspases
cell death
cleavage of
JNK, p27 etc