Focal adhesion kinase signalling in endothelial cells

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PhD Thesis

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Focal adhesion kinase in endothelial cell signalling.

Abstract

The angiogenic factor vascular endothelial growth factor (VEGF) induces migration and cytoskeletal changes in endothelial cells. The mechanism by which VEGF signals was not known but it was possible an adhesion associated protein such as focal adhesion kinase (FAK) was involved. Further investigation revealed VEGF caused a marked activation of tyrosine phosphorylation of FAK in HUVE cells, which was time and concentration dependent. The PI3K inhibitor wortmannin partially inhibited VEGF stimulated FAK phosphorylation and it inhibited phosphorylation in response to lysophosphatidic acid and VEGF induced membrane ruffling. The tyrosine kinases Src and Fyn were associated with FAK upon VEGF stimulation of HUVE cells. VEGF stimulated recruitment to FAK would allow Src family kinases to stimulate tyrosine phosphorylation of FAK thereby mediating the effects of VEGF on FAK tyrosine phosphorylation. Analysis of a functional consequence of VEGF stimulated FAK tyrosine phosphorylation was assessed by displacement of endogenous FAK by microinjection of a truncated C-terminal FAK. A reduction in the distance migrated by VEGF stimulated endothelial cells was demonstrated, indicating VEGF signals via FAK to induce migration. To investigate the role of FAK in other endothelial cell functions, epitope tagged FAK mutants were generated and characterised. Co-expression of the FAK mutants and in addition, a Fyn kinase inactive protein, gave some insight into the mechanism of FAK tyrosine phosphorylation. Expression of the mutant FAK proteins revealed neither the autophosphorylation site or the kinase ability of FAK are required to provide survival signals from FAK in adherent endothelial cells however the truncated C-terminal FAK did induce apoptosis.

CONTENTS

List of Figures

1. Introduction

2. Materials and methods

3. Modulation of FAK phosphotyrosine in response to VEGF and adhesion.

List of tables

Abbreviations

AEBSF	4-(2-aminoethyl) benzene-sulphonyl fluoride
ATCC	American Type Culture Collection
BAE cells	Bovine endothelial cells
CE cells	Chicken embryo cells
CHO cells	Chinese hamster ovary cells
Csk	C-terminal Src kinase
DABCO	1,4-diazobicyclo-(2,2,2)-octane
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dsDNA	Double stranded DNA
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
EGF	Epidermal growth factor
ERK	Extracellular signal-related kinase
FAK	p125 Focal adhesion kinase
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
Graf	GTPase regulator associated with focal adhesion kinase
Grb2	Growth factor receptor bound protein 2
GST	Glutathione S-transferase
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
HUVE cells	Human umbilical vein endothelial cells
Ig	Immunoglobulin
IPTG	Isopropyl ß-D-thiogalactoside
JNK	c-Jun amino-terminal kinase

Kb	Kilobase
LPA	Lysophosphatidic acid
MAP	Mitogen activated protein
MDCK cells	Madin-Darby Canine Kidney cells
MW	Molecular weight
NTP	Nucleotide triphosphate
P130Cas	p130 Crk-associated–substrate
PAE cells	Porcine aortic endothelial cells
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
pFF397F	Flag tagged FAK with tyrosine residue 397 mutated to phenylalanine
pFF454R	Flag tagged FAK with lysine residue 454 mutated to an arginine.
pFF792	Flag tagged C-terminal FAK containing residues 792-1052
pFF925F	Flag tagged FAK with tyrosine residue 925 mutated to phenylalanine
pFFfl	Full length flag tagged FAK
PI3K	Phosphatidylinositol 3-kinase
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PKB	Protein kinase B
PRR-1	Proline rich region-1
PRR-2	Proline rich region-2
PTB domains	Phosphotyrosine binding domains
SDS	Sodium dodecyl sulphate
SDW	Sterile distilled water
SH2	Src-homology-2
SH3	Src-homology-3
TEMED	N,N,N',N'-tetramethylethylenediamine
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor receptor-1
VEGFR-2	Vascular endothelial growth factor receptor-2
(v/v)	Volume by volume ratio
(w/v)	Weight by volume ratio

CHAPTER ONE

Introduction

1. Introduction

1.1 Angiogenesis.

Angiogenesis is the formation of new capillaries from pre-existing small blood vessels. Angiogenesis differs from the similar process of vasculogenesis in which blood vessels develop from mesodermal precursors called angioblasts (Risau *et al.* 1988, Folkman and Shing 1992). Angiogenesis is required for a number of normal physiological processes, such as embryonic development, endometrial proliferation and wound healing (Blood and Zetter 1990, Fidler and Ellis 1994, Paku and Paweletz 1991). However, uncontrolled angiogenesis is also known to be associated with various pathological conditions such as arthritis, diabetic retinopathy, tumour growth and tumour metastasis (Blood and Zetter 1990, Fidler and Ellis 1994, Paku and Paweletz 1991). An understanding of the process of angiogenesis, at the molecular level, would be beneficial in providing treatment for these pathological conditions.

Capillary blood vessels consists of endothelial cells and pericytes which when stimulated can form new capillary networks. The formation of new capillaries involves a multistep process; initiation is caused by angiogenic molecules binding to their respective receptors which leads to secretion of proteolytic enzymes and degradation of the basement membrane. This is followed by endothelial migration, capillary tube formation and endothelial proliferation. Ausprunk and Folkman (1977) reported endothelial cells migrate towards an angiogenic stimulus with mitosis occurring at a more distal aspect of the new capillary

Many angiogenic factors have been identified, including fibroblast growth factor (Esch *et al.* 1985, Lobb *et al.* 1985, Shing *et al.* 1985), angiogenin (Fett *et al.* 1985), tumour necrosis factor-α (Leibovich *et al.* 1987, Frater-Schroder *et al.* 1987), transforming growth factor-α and ß (Roberts *et al.* 1986, Schreiber *et al.* 1986) and platelet derived endothelial cell growth factor (Ishikawa *et al.* 1989). In addition vascular endothelial growth factor is also angiogenic (Connolly *et al.* 1989, Ferrara and Henzel 1989, Levy *et al.* 1989) and has generated interest due to its potential therapeutic effects.

1.1.1 Evidence of VEGFs role in angiogenesis.

VEGF is a secreted growth factor (Leung *et al.* 1989) and is also mitogenic for endothelial cells and therefore a potential angiogenic stimulus (Ferrara and Henzel 1989, Plouet *et al.* 1989, Conn *et al.* 1990). VEGF is produced by vascular smooth muscle cells (Ferrara *et al.* 1991) which would enable the VEGF to act in a paracrine fashion on the neighbouring endothelial cells. Strong evidence that VEGF is an important factor in blood vessel growth is that it stimulates angiogenesis in vivo as shown by experiments performed on rat and rabbit cornea, (Levy *et al.* 1989, Connolly *et al.* 1989), chick choroallantoic membrane (Leung *et al.* 1989, Plouet *et al.* 1989) and the rabbit bone graft model (Connolly *et al.* 1989). VEGF mRNA expression is temporally and spatially related to blood vessel proliferation in developing tissue, ovary and in the healing wound suggesting that VEGF is involved in physiological angiogenesis. VEGF is also involved in tumour angiogenesis as monoclonal antibodies to VEGF were shown to suppress vascularisation and inhibit tumour growth in nude mice (Kim *et al.* 1993).

1.1.2 Therapeutic potential of VEGF.

The therapeutic angiogenic potential of VEGF was investigated by administering VEGF to rabbit hind limbs in which ischemia had been induced by removal or ligation of the femoral artery. VEGF produced statistically significant augmentation of collateral vessel development. This demonstrated VEGF is sufficiently potent to achieve therapeutic benefits (Takeshita *et al.* 1994a and b). In addition, administration of VEGF resulted in accelerated re-endothelialisation following balloon angioplasty-induced endothelial denudation of rabbit carotid arteries suggesting it may also be a useful therapeutic agent in large vessels.

1.2 The discovery of VEGF.

Senger *et al.* (1983) partially purified a factor from the conditioned medium of guinea pig tumour cells and human histiocytic lymphoma U-937 cells which enhanced the permeability of blood vessels. The enhanced permeability was measured by the extravasation of Evans blue dye after intradermal injection in guinea pigs. This protein was named vascular permeability factor (VPF).

In 1989 several groups independently identified an endothelial cell specific mitogen which was purified from conditioned medium of pituitary folliculostellate cells (Ferrara and Henzel 1989) and AtT-20 pituitary cells (Plouet *et al.* 1989). The groups named the molecule vascular endothelial growth factor and vasculotropin respectively. Subsequently VEGF and VPF were cloned and were discovered to be the same molecule (Leung *et al.* 1989, Keck *et al.* 1989).

Additional growth factors belonging to the VEGF family have since been discovered, namely placental growth factor-1 (PlGF1), (Maglione *et al.* 1991), PlGF2 (Maglione *et al.* 1993), VEGF-B (Olofsson *et al.* 1996), VEGF-C (Joukov *et al.* 1996, Lee *et al.* 1996), VEGF D (Orlandini *et al.* 1996) and more recently VEGF-E (Ogawa *et al.* 1998).

1.2.1 VEGF structural and genetic properties.

The human VEGF gene is located on chromosome 6p21.3 (Vincenti *et al.* 1996) and is organised into eight exons separated by seven introns (Tischer *et al.* 1991). VEGF is a basic 45 KDa heparin-binding glycoprotein consisting of two identical disulphide linked subunits (Ferrara and Henzel 1989). VEGF exists in five isoforms of 121, 145, 165, 189 and 206 amino acid residues, which are the products of alternative splicing from one gene (Houck *et al.* 1991, Tischer *et al.* 1991). $VEGF_{121}$ and $VEGF_{165}$ are diffusible proteins that are secreted into the medium, however $VEGF_{165}$ remains bound to the cell surface or ECM while $VEGF_{121}$ is soluble. $VEGF_{189}$ and $VEGF_{206}$ have a high affinity for heparin and are mostly bound to heparin containing proteoglycans in the ECM (Houck *et al.* 1992, Park *et al.* 1993). $VEGF_{145}$ is a secreted isoform which binds to the extracellular matrix (Poltorak *et al.* 1997). $VEGF_{165}$ is the predominate isoform (Houck *et al.* 1992) and is referred to as VEGF in this thesis.

The amino acid sequence of VEGF exhibits a limited but significant homology to the A chain and B chain of PDGF. Both growth factors are dimers and all eight cysteine residues involved in the intra and inter chain disulphides in PDGF B are conserved in VEGF which indicates the folding of the two proteins could be similar. In addition, clusters of basic amino acids in the COOH terminal halves of human VEGF and PDGF B commonly occur (Keck *et al.* 1989, Tisher *et al.* 1989).

1.2.2 VEGF gene knockouts.

Inactivation of the encoding VEGF A gene in mice resulted in embryonic lethality in heterozygous embryos, between day 11 and 12. Embryos lacking a single VEGF allele displayed impaired blood vessel development demonstrating that even reduced concentrations of VEGF lead to abnormal development. The defects observed demonstrated most steps of early vascular development were impaired including angiogenesis, lumen formation, formation of large vessels and establishment of interconnections (Carmeliet *et al.* 1996, Ferrara *et al.* 1996).

1.2.3 Regulation of VEGF gene expression.

VEGF gene expression is upregulated by a number of factors including hypoxia, growth factors, cytokines, extracellular molecules and inactivation of tumour suppressor genes. During hypoxia VEGF transcription is induced by hypoxia-inducible factor-1 (HIF-1) binding to a HIF-1 binding site located in the VEGF promoter region (Levy *et al.* 1995, Liu *et al.* 1995). In addition hypoxia also stabilises VEGF mRNA (Stein *et al.* 1995). Factors which increase VEGF production include FGF-4 (Deroanne *et al.* 1997), PDGF (Finkenzeller *et al.* 1997), TNF ß (Pertovaara *et al.* 1994), IGF-1 (Goad *et al.* 1996), interleukin-1 ß, (Li *et al.* 1995), IL-6 (Cohen *et al.* 1996) and nitric oxide (Tuder *et al.* 1995, Chin *et al.* 1997, Murohara *et al.* 1998). Inactivation of the tumour suppressor gene von Hippel-Landau induces VEGF expression (Siemeister *et al.* 1996, Stratmann *et al.* 1997) and mutant p53 also had the same effect (Kieser *et al.* 1994).

1.2.4 VEGF receptors

Two VEGF receptor tyrosine kinases have been identified, the Flt-1 (fms-like tyrosine kinase) (de Vries *et al.* 1992), and KDR (kinase insert domain containing receptor) (Terman *et al.* 1992). The murine homologue of KDR was named Flk-1, (fetal liver kinase-1) and it has 85% amino acid sequence identity with KDR (Matthews *et al.* 1991, Quinn *et al.* 1993). Flt-1 has the highest affinity for VEGF with a dissociation constant (K_d) of approximately 9pM whereas the KDR has a lower affinity for VEGF with a K_d of 770pM demonstrated in HUVE cells (Waltenberger *et al.* 1994).

The KDR was originally cloned from a human umbilical vein endothelial cell cDNA library (Terman *et al.* 1991), Flt-1 from a human placenta cDNA library (Shibuya *et al.* 1990) and Flk-1 from a fetal liver stem cell cDNA library (Matthews *et al.* 1991). Each were identified using polymerase chain reaction (PCR) and degenerate oligonucleotide primers complimentary to conserved tyrosine kinase domains of known receptor tyrosine kinases. Between the KDR and Flt1 receptors there is a 33% identical match in amino acid sequence in the extracellular domains, 80% match in the kinase domain, 43% match in the kinase insert domain and a 22% match in the cytosolic tail.

The VEGF receptors are more recently referred to as VEGFR-1 (Flt-1) and VEGFR-2 (KDR/flk-1). Another receptor, VEGF-3 (Flt-4) has also been cloned, which appears to fit into the family of VEGFR-1 and 2 genes (Pajusola *et al.* 1992) although this receptor is found on lymphatic endothelial cells and is not found in vascular endothelial cells (Kaipainen *et al.* 1993 and 1995). In addition, VEGFR-3 does not bind VEGF but the more recently identified VEGF-related peptide (VEGF-C) (Joukov *et al.* 1996, Lee *et al.* 1996). VEGFR-1, VEGFR-2 and VEGFR-3 all contain seven extracellular immunoglobulin-domains, a single membrane spanning domain and a tyrosine kinase sequence which is interrupted by a kinase-insert domain (Shibuya *et al.* 1990, Terman *et al.* 1991, Pajusola *et al.* 1992).

A soluble truncated form of VEGFR-1 has been identified which is missing the seventh immunoglobulin-like domain, the transmembrane spanning region and the intracellular domain. The soluble receptor binds VEGF with high affinity and has been demonstrated to be produced naturally by HUVE cells. The addition of soluble VEGFR-1 inhibited VEGF induced mitogenic activity and has been shown to form a VEGF-stabilised complex with the extracellular domain of VEGFR-2 *in vitro*. This would suggested the soluble receptor could act as a suppressor of VEGF action (Kendall and Thomas 1993, Kendall *et al.* 1996).

More recently an additional receptor which binds $VEGF_{165}$ has been identified as neuropilin-1 (Soker *et al.* 1998). This receptor was previously identified for binding semaphorins, which act as repellents of nerve growth cones (He and Tessier-Lavigne 1997, Kolodkin *et al.* 1997). The neuropilins have a short intracellular domain and are therefore unlikely to act as independent receptors. Interestingly, neuropilin-1 appears to act as a co-

receptor enhancing the binding of VEGF to VEGFR-2 and VEGF chemotactic, migratory and mitogenic response (Soker *et al.* 1998).

1.2.5 VEGF receptor gene knockouts.

There is impaired vessel formation in VEGFR-1 and 2 deficient embryos and mice with either receptor mutated die in utero between day 8.5 and 9.5. In embryos deficient for VEGFR-1, the endothelial cells failed to organise into normal vascular channels (Fong *et al.* 1995). Embryos with an inactivated VEGFR-2 lacked vasculogenesis and also failed to develop blood islands (Shalaby *et al.* 1995). Gene knockout experiments have demonstrated that both VEGFR-1 and 2 are essential for development of the vasculature in mice. Interestingly the tyrosine kinase activity of VEGFR-1 does not appear necessary for murine embryo development or angiogenesis as normal development was observed in VEGFR-1 tyrosine kinase deficient homozygous mice (Hiratsuka *et al.* 1998).

1.2.6 Signal transduction

VEGF binds to the second immunoglobulin-like domain of VEGFR-1 and 2 (Davis-Smyth *et al.* 1996, Barleon *et al.* 1997). On binding the VEGF ligand, the VEGFR-1 and VEGFR-2 show an increase in the level of phosphorylation of their tyrosine residues. This autophosphorylation was shown in NIH 3T3 cells stably expressing VEGF-1 (Seethram *et al.* 1995) and also in porcine aortic endothelial cells stably expressing both receptors although the VEGF-2 was autophosphorylated much more efficiently (Waltenberger *et al.* 1994). Indeed, Yamane *et al.* (1994) and De Vries *et al.* (1992), both reported almost undetectable levels of autophosphorylation of VEGFR-1 after VEGF stimulation in sinusoidal endothelial cells from rat liver and xenopus oocytes transiently expressing Flt-1 receptors. Recently, both the VEGFR-1 and 2 have been demonstrated to become tyrosine phosphorylated in response to VEGF activation of HUVE cells (Kanno *et al.* 2000).

VEGF stimulation of human endothelial cells has been shown to lead to an increase in intracellular Ca^{2+} concentration and inositol triphosphate (IP_3) formation. (Brock *et al.* 1991). Many agonists which increase $[Ca^{2+}]_i$ do so through receptors coupled to phospholipase C (PLC) (Berridge *et al.* 1993.). Activated PLC generates the intracellular messengers inositol 1,4,5- triphosphate (IP_3) and 1,2-diacylglycerol (DAG) by hydrolysis

of phosphatidylinositol 4,5-bisphosphate. IP_3 increases the $[Ca^{2+}]_i$ by binding to the IP_3 receptor on intracellular Ca^{2+} compartments. The increase in IP_3 observed in human endothelial cells (Brock *et al.* 1991) supports the hypothesis that PLC is involved in the increase of $[Ca^{2+}]_i$ induced by VEGF stimulation.

1.2.7 Tyrosine phosphorylation of cellular substrates.

Guo *et al.* (1995), reported that VEGF induced tyrosine phosphorylation of numerous proteins in cultured bovine aortic endothelial cells (BAEC). The proteins identified which contained SH2 domains were phospholipase Cγ (PLCγ), Ras GTPase activating protein (GAP), the oncogenic adaptor protein Nck and phosphatidylinositol 3-kinase (PI3K) although he did not identify which VEGF receptor was involved.

VEGFR-1 and 2 appear to activate different signal transduction cascades. Waltenberger *et al.* (1994), stably expressed VEGFR-1 in porcine aortic endothelial cells and demonstrated that members of the Src family such as Fyn and Yes showed increased levels of tyrosine phosphorylation on VEGF stimulation. Cunningham *et al.* (1995), demonstrated using the yeast two-hybrid system that the p85 subunit of P1-3 kinase interacts with a VEGFR-1 tyrosine autophosphorylation site and Seetharam *et al.* (1995) have observed that VEGF induced phosphorylation on tyrosine residues of PLCγ and GAP in VEGFR-1 transfected NIH3T3 fibroblasts.

Waltenberger *et al.* (1994) however unlike Guo *et al.* (1995), Seetharam *et al.* (1995) and Cunningham *et al.* (1995), observed no effects on tyrosine phosphorylation of P1-3 kinase and PLCγ and only a slight increase in phosphorylation of GTPase upon VEGF binding to VEGFR-1. An explanation for this difference is that the PAE cells used by Waltenberger may lack a component of the signalling pathway required in certain signal transduction events. Seetharam *et al.* (1995) demonstrated a strong activation of MAP kinase only in endothelial cells and not VEGFR-1 transfected NIH3T3 fibroblasts. In addition, unlike many receptor tyrosine kinases which phosphorylate the adaptor protein Shc, Seetharam observed very weak tyrosine phosphorylation of Shc in VEGFR-1 expressing NIH3T3 fibroblasts, suggesting VEGFR-1 may utilise a unique signal transduction system.

Stimulation of VEGFR-2 with VEGF results in phosphorylation of GAP, PLCγ, all three Shc isoforms as well as strong activation of p42 MAP kinase (Waltenberger *et al.* 1994, Takahashi and Shibuya 1997, Kroll and Waltenberger 1997). Upon ligand binding VEGFR-2 associates with Grb2, Nck, shc and GAP. In addition, the tyrosine phosphatases SHP-1 and SHP-2 physically associate with VEGFR-2 in PAE cells overexpressing this receptor and therefore may be involved in the regulation of VEGF signalling (Kroll and Waltenberger 1997). See figure 1.1 for schematic representation of VEGF signalling.

1.2.8 Biological consequences of VEGF binding.

Several groups have demonstrated VEGF induces migration in endothelial cells (Yoshida *et al.* 1996, Senger *et al.* 1996, Abedi and Zachary 1997). VEGF has been shown to affect the cytoskeleton, inducing the formation of actin stress fibres, membrane ruffling and cell shape changes (Waltenburger *et al.* 1994, Abedi and Zachary 1997). Rousseau *et al.* (1997) suggested that VEGF mediated the actin reorganisation and cell migration by activating p38 (stress activated protein kinase-2). See figure 1.1 for schematic representation of VEGF induced signalling.

The role played by each individual VEGF receptor has also been studied. Binding of VEGF to VEGFR-2 stably expressed on PAE cells resulted in changes in gross morphology of the cell, actin reorganisation, membrane ruffling, chemotaxis and mitogenicity, however VEGFR-1 expressing PAE cells showed none of these responses. (Waltenburger *et al.* 1994, Seetharam *et al.* 1995). However PAE cells are reported not to express VEGF receptors and if this is the case they may lack important components of the intracellular signalling pathway required for VEGFR-1.

Initially no function could be assigned to VEGFR-1 however Clauss *et al.* (1996) demonstrated that monocytes endogenously express VEGFR-1 but not VEGFR-2. Monocytes exposed to VEGF mediated chemotactic activity and tissue factor production and therefore these responses to VEGF must be mediated via VEGFR-1. Having demonstrated VEGF induced tissue factor production in HUVE cells Clauss *et al.* (1996) used neutralising antibodies to VEGFR-2 and still observed some tissue factor production and concluded some, although not all, tissue factor production was induced via VEGFR-1.

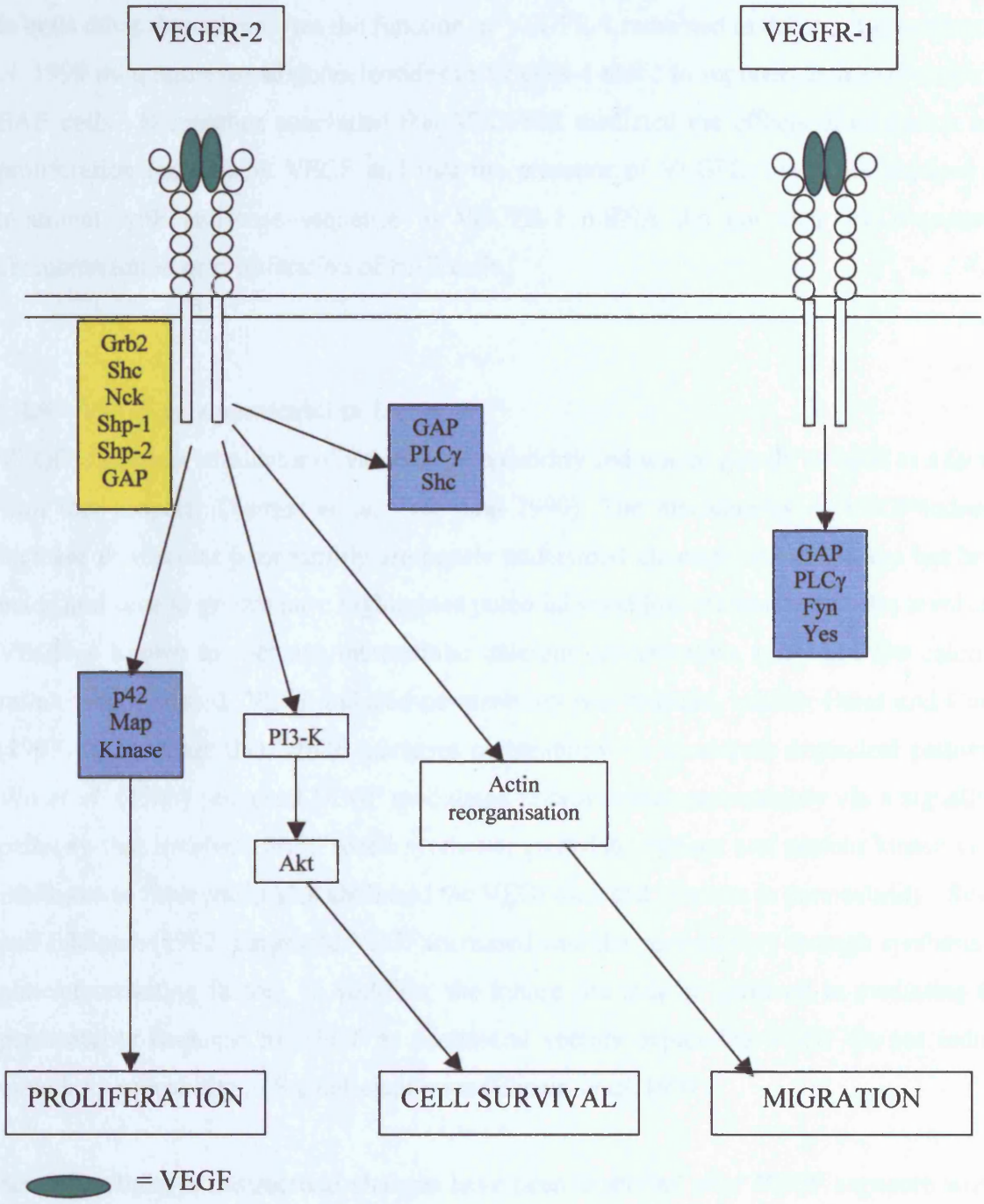

Figure 1.1. Simplified scheme of some of the signalling events initiated upon VEGF activation. Signalling of VEGFR-1 and 2 known at the time of study. Blue boxes represent proteins which become tyrosine phosphorylated upon VEGF receptor activation. Yellow box represents proteins which become associated with the VEGF receptor upon its activation.

In cells other than monocytes the function of VEGFR-1 remained in debate. Bernatchez *et al.* 1999 used antisense oligonucleotides to VEGFR-1 and 2 to suppress their expression in BAE cells. Bernatchez concluded that VEGFR-2 mediated the effects of migration and proliferation induced by VEGF and that the presence of VEGFR-1 was not required as treatment with antisense sequence to VEGFR-1 mRNA did not alter VEGF-induced chemoattraction or proliferation of BAE cells.

1.2.9 VEGF as a permeability factor.

VEGF is a potent stimulator of vascular permeability and was originally isolated as a factor with this property (Senger *et al.* 1983 and 1990). The mechanisms of VEGF-induced increase in vascular permeability are poorly understood although some progress has been made and several groups have highlighted potential signalling/effector molecules involved. VEGF is known to increase intracellular calcium concentration and when the calcium influx was inhibited, VEGF-induced permeability was reduced, leading Bates and Curry (1997) to conclude that VEGF increases permeability via a calcium dependent pathway. Wu *et al.* (1996) proposed VEGF modulated microvascular permeability via a signalling pathway that involved nitric oxide synthesis, guanylate cyclase and protein kinase G as inhibitors to these molecules abolished the VEGF-induced increase in permeability. Sirois and Edelman (1997) suggested VEGF increased vascular permeability through synthesis of platelet-activating factor. In addition, the kinase Src may be involved in mediating the permeability response to VEGF as adenoviral vectors expressing VEGF do not induce vascular permeability in Src deficient mice (Eliceiri *et al.* 1999).

Several cellular ultrastructural changes have been described after VEGF exposure which contribute to the increase in permeability. These changes include transcellular gaps (Neal and Michel 1997), intercellular gaps (Kevil *et al.* 1998, Roberts and Palade, 1995), vesiculo-vacuolar organelle formation (Feng *et al.* 1997, Kohn *et al.* 1992) and the appearance of fenestrations (Roberts and Palade, 1995 and 1997).

1.3 Integrins

The process of angiogenesis involves cell adhesion, migration and proliferation and these processes are mediated in part by cell adhesion molecules such as integrins. A critical role for integrins in angiogenesis has now been established (Brooks 1996).

Integrins are transmembrane proteins and act to link the extracellular matrix components (such as fibronectin, vitronectin, laminin and collagen) with the cytoskeleton and focal adhesions inside the cell. (Hynes 1992). Integrins are comprised of α and ß subunits. There are several different forms of the α and ß subunits which combine to form more than 20 receptors with distinct cellular and adhesive properties (Hynes 1992). Each integrin heterodimer can recognise several ECM proteins and an ECM ligand may also be recognised by more than one integrin (Hynes 1992, Damsky and Werb 1992).

Integrins are important in many cellular processes. For example many cells such as fibroblasts and endotheial cells can only replicate if anchored to a solid substratum (Stoker *et al.* 1968, Folkman and Moscona 1978). Activation of integrins can also inhibit apoptosis usually seen in cells in suspension (Meredith *et al.* 1993, Frisch and Francis 1994), and antibodies which block certain integrins can induce apoptosis (Brooks *et al.* 1994 a, b). This suggests integrins are essential in cell survival and in many cells, for replication. Since integrins have no intrinsic enzymatic activity they must depend on their association with other cellular proteins to initiate their signals. Integrin receptor engagement and clustering leads to formation of focal adhesions (Guan *et al.* 1991).

1.4 Focal adhesions

Focal adhesions develop at the plasma membrane at sites where the cell contacts the extracellular matrix. Focal adhesions are specialised structures which comprise of a number of interacting proteins which link extracellular matrix (ECM) proteins to cytoskeletal proteins inside the cell. Focal adhesions were first identified in fibroblasts by electron microscopy and reflection contrasts microscopy (Abercrombie *et al.* 1971). Similar structures with similar functions are observed *in vivo* and include dense plaques in smooth muscle cells, myotendinosis junctions (cell-ECM interactions) in skeletal muscle, the basal surface of epithelial cells and some cell-cell junctions and cell-ECM junctions (Geiger *et al.* 1984, Burridge *et al.* 1988).

Many proteins are localised at focal adhesions, these include vinculin, talin, focal adhesion kinase and VASP. Structurally, focal adhesions attach the cell membrane to the ECM and anchor and stabilise the actin cytoskeleton (Burridge *et al.* 1988). It is also thought focal adhesions play a role in signal transduction (Damsky and Werb 1992, Juliano and Haskill 1993, Lo and Chen 1994, Clark and Brugge 1995). Signals received from integrins via focal adhesions can effect migration, proliferation, apoptosis and differentiation. One protein thought to be involved in integrin mediated signalling is focal adhesion kinase (FAK) as it is phosphorylated on its tyrosine residues upon integrin activation (Guan *et al.* 1991).

1.5 Focal adhesion kinase (FAK).

Focal adhesion kinase (FAK) is a cytoplasmic protein tyrosine kinase and was originally identified by its high level of tyrosine phosphorylation in v-Src transformed cells (Kanner *et al.* 1990). At a similar time Guan *et al.* (1991) described a 120kDa protein that localised to focal adhesions but was distinct from any other known focal adhesion protein. This 120 kDa protein was tyrosine phosphorylated in cells that had spread on fibronectin but became dephosphorylated when the cells were detached by treatment with trypsin. Integrins were suggested to be involved in regulating FAK tyrosine phosphorylation as cross linking the integrins to artificially cluster them resulted in FAK phosphorylation whereas plating cells on polylysine which does not activate integrins did not.

FAK is broadly expressed in most cell lines and tissues (Hanks *et al.* 1992, Andre and Becker-Andre 1993, Guan and Shalloway 1992, Kornberg *et al.* 1992, Burridge *et al.* 1992, Schaller *et al.* 1992). FAK is highly conserved structurally and the amino acid sequence from chicken, (Schaller *et al.* 1992), murine (Hanks *et al.* 1992), human (Andre and Becker-Andre 1993, Whitney *et al.* 1993, Choi *et al.* 1993) and *Xenopus* (Hens and DeSimone 1995) sources of FAK are over 90% identical. In addition a Drosophila FAK homolog, Dfak56 has been identified that is 33% identical at the amino acid level to vertebrate FAK, with the highest similarity seen in the kinase and focal adhesion targeting domains and also in several protein-protein interaction motifs (Fox *et al.* 1999). Several FAK isoforms are produced from internal promoters within the gene and through alternative splicing and are discussed in section 1.5.2.

1.5.1 Structure of FAK.

FAK differs from many nonreceptor tyrosine kinases as it does not possess any SH2 or SH3 domains which mediate protein interactions. As shown in figure 1.2, FAK has a central kinase domain flanked by large NH_2 and C-terminal domains (Schaller *et al.* 1992). FAK contains several phosphoacceptor tyrosines (discussed in section 1.5.4) and two proline rich regions in the C-terminal half (Hildebrand *et al.* 1995 and 1993, Tachibana *et al.* 1995). Mutation studies were used by Hildebrand *et al.* (1993), to investigate the domain structure of FAK and to identify the regions that determined subcellular localisation. Using a series of FAK deletion variants Hildebrand identified a sequence in the C-terminal region necessary for the localisation of FAK to focal adhesions. Mutants which did not contain this 159 amino acid sequence were not localised to focal adhesions. This sequence was termed the focal adhesion targeting (FAT) sequence. In addition, when the last 199 residues from the FAK C-terminus, which contains the FAT sequence, were fused in frame with a cytosolic protein it became localised to focal adhesions (Hildebrand *et al.* 1993). This suggests the FAT sequence is sufficient for focal adhesion targeting.

It was observed that some cells autonomously expressed the C-terminal domain of FAK as a 41/43 KDa protein named FAK related non kinase (FRNK). FRNK, like FAK, was localised to focal adhesions providing further evidence FAK is directed to focal adhesions by sequences in its C-terminal domain (Schaller *et al.* 1993).

FAK is probably located to focal adhesions by interacting with other proteins in the focal adhesion. Although the FAT sequence is known, it has yet to be elucidated which protein(s) in the focal adhesion 'tether' FAK. Hildebrand *et al.* 1995 showed using fusion proteins and immunoprecipitation assays that the C-terminal domain of FAK binds with paxillin, another focal adhesion protein. The paxillin binding site on FAK has been localised to the C-terminal domain of FAK spanning residues 919-1042 (Tachibana *et al.* 1995, Hildebrand *et al.* 1995). Although the paxillin binding site overlaps the FAT sequence it is unlikely to be solely responsible for recruiting FAK to focal adhesions as a single mutation in FAK which prevents the binding of paxillin does not prevent FAK targeting to focal adhesions (Hildebrand *et al.* 1995). In addition, FAK has been shown to be localised to focal adhesions before paxillin (Miyamoto *et al.* 1995).

Chen *et al.* (1995) identified residues 965-1012 in the carboxyl-terminal domain of FAK necessary for the binding of another cytoskeletal protein, talin. This association also occurred with kinase-deficient FAK indicating talin association to FAK may be independent of FAK phosphorylation and activation. Talin, for the same reasons as paxillin, has been ruled out as the protein which targets FAK to focal adhesions (Hildebrand *et al.* 1995, Miyamoto *et al.* 1995). Talin can associate with ß integrin cytoplasmic tails which may link FAK to integrin activation (Chen *et al.* 1995).

FAK also posses two conserved proline-rich regions in its C-terminal half. Using a two-hybrid screen and co-immunoprecipitation it was demonstrated that Cas binds via its SH3 domains to FAK at the proline rich sequence of residues 711-717 (Polte and Hanks 1995, Harte *et al.* 1996). Cas also binds to a second proline-rich region (PR-2) in FAK encompassing residues 872-878 although the proline rich region 1 binds the majority of the p130Cas (Polte and Hanks 1997). The p85 subunit of PI3K has also been shown to interact via its SH3 domain with a proline rich sequence in FAK which leads to significant activation of PI3K (Guinebault *et al.* 1995).

Another protein which binds to FAK is Graf (GTPase regulator associated with focal adhesion kinase) (Hildebrand *et al.* 1996). Graf is a GTPase activating protein (GAP) which rapidly converts active GTP bound G protein to the inactive GDP bound state. Graf binds via its SH3 domain to the second proline rich region in the C-terminal domain of FAK (Hildebrand *et al.* 1996). In Swiss 3T3 cells Graf was demonstrated to be a GAP for Rho (Taylor *et al.* 1999) as microinjection of Graf blocked Rho mediated stress fibre formation. Graf may function to integrate signalling between tyrosine kinases and the Rho family GTPases. Recently a novel protein CAP has also been shown to bind to FAK via its SH3 domains (Ribon *et al.* 1998).

Using an *in vitro* assay Schaller *et al.* (1995), demonstrated FAK binds directly to a membrane-proximal site in the cytoplasmic tail of $ß_1$ integrin subunit. Further analysis using purified fusion proteins of FAK containing residues 31-376 revealed this N-terminal sequence to be involved in the direct binding of FAK to the $ß_1$ peptide. FAK was also found to bind to synthetic peptides of the cytoplasmic domain of $ß_2$ and $ß_3$ integrin subunits (Schaller *et al.* 1995). The direct interaction of FAK with ß integrins however have not been demonstrated *in vivo*.

1.5.2 Isoforms of FAK.

Several isoforms of FAK are formed by alternative splicing and alternative promoters. Chicken fibroblasts express a C-terminal form of FAK which is generated from a promoter located within an intron lying downstream of the catalytic domain (Schaller *et al.*1993). This form of FAK is termed FRNK (FAK-related nonkinase) and can act as a negative regulator of full-length FAK (Richardson and Parsons 1996).

Another variant of FAK, FAKB, has been identified in lymphocytes. FAKB, differs in the C-terminus and is activated upon T and B cell receptor activation (Kanner *et al.* 1994). However the existence of FAKB has not been confirmed by DNA sequencing.

Rat brain contains a FAK variant which contains an insertion of 3 amino acid within the C-terminal domain. This FAK variant is termed FAK + (Burgaya and Girault 1996). Burgaya *et al.* (1997) discovered FAK mRNA from rat brain contained several additional short exons coding for peptides of 6, 7 and 28 residues. These alternatively spliced forms of FAK were termed FAK box 6, box 7 and box 28 and yield proteins with amino acid insertions surrounding the FAK autophosphorylation site at tyrosine 397. The presence of box 6 or 7 leads to increased autophosphorylation activity. Many of these FAK isoforms are specifically expressed in brain tissue suggesting neuronal FAK may be regulated and activated uniquely from non neuronal FAK.

1.5.3 FAK related protein tyrosine kinase. Proline-rich tyrosine kinase 2 (Pyk2).

Another member in the FAK family of non receptor protein tyrosine kinase is proline-rich tyrosine kinase 2 (Pyk2) (Lev *et al.* 1995). Pyk2 is also known as related adhesion focal tyrosine kinase (RAFTK) (Avraham *et al.* 1995), cell-adhesion kinase-beta (CAKß) (Sasaki *et al.* 1995), and calcium-dependent protein-tyrosine kinase (CADTK) (Yu *et al.* 1996).

Pyk2 is highly expressed in cells of the central nervous system and is also found in cells of hematopoietic lineage. Pyk 2 is slightly smaller than FAK with a molecular mass of 112 kDa. FAK and Pyk2 have an amino acid sequence similarity of 45% and also share an overall structural similarity, both containing a central catalytic domain and two proline-rich domains in the C-terminal domain. Pyk2 is activated by extracellular signals that lead to

calcium influx or calcium release from internal stores (Lev *et al.* 1995). Immunofluorescence staining has showed that endogenous and recombinant Pyk2 only weakly associate with focal contacts in adherent cells and the protein is found diffusely distributed throughout the cell (Schaller and Sasaki 1997, Zheng *et al.* 1998).

1.5.4 FAK phosphorylation.

1.5.4.1 Serine phosphorylation

FAK is phosphorylated on multiple serine residues in adherent fibroblasts (Calalb *et al.* 1995). Schlaepfer and Hunter (1996) reported FAK became dephosphorylated on serine residues when fibroblasts cells were held in suspension and strong serine phosphorylation of FAK was observed in cells replated on poly-lysine. The biological significance of this has not been determined.

1.5.4.2 Tyrosine phosphorylation

FAK becomes tyrosine phosphorylated in response to adhesion of cells to ECM proteins and transformation by v-Src and this phosphorylation correlates with increased FAK tyrosine kinase activity (Guan and Shalloway 1992, Burridge *et al.* 1992, Kornberg *et al.* 1992). Detachment of cells by trypsinisation reduces the phosphotyrosine content of FAK (Hanks *et al.* 1992). FAK also becomes tyrosine phosphorylated in response to a number of other stimuli including PDGF (Rankin and Rozengurt 1994), neuropeptides bombesin and endothelin (Zachary *et al.* 1992, Sinnett-Smith *et al.* 1993), and lysophosphatidic acid (LPA) (Seufferlein and Rozengurt 1994). PDGF activates a transmembrane receptor whereas LPA, bombesin and endothelin stimulate G protein linked receptors (Rozengurt 1991, Durieux and Lynch 1993). Interestingly these non-integrin stimulated tyrosine phosphorylation of FAK are all inhibited by cytochalasin D which prevents actin polymerisation.

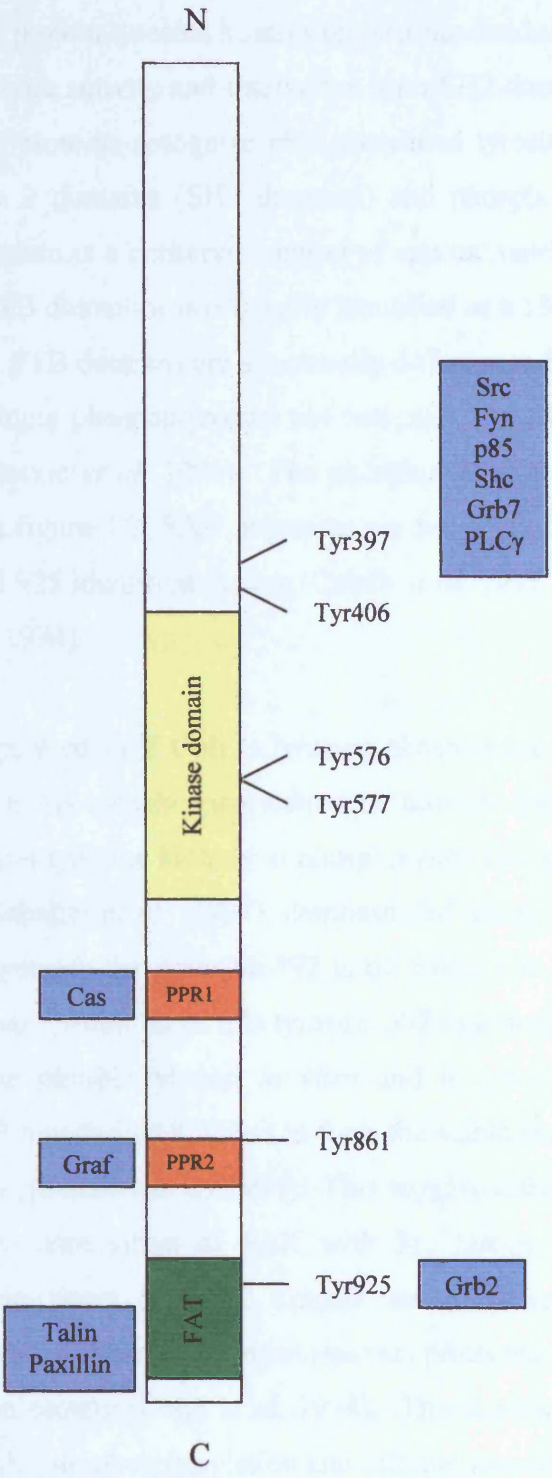

Figure 1.2. FAK structure, sites of tyrosine phosphorylation and protein interactions. A variety of proteins have been shown to associate with FAK as indicated by the blue boxes (see text in section 1.5.4.2 for details). Proline rich region-1 (PPR1) and proline rich region-2 (PPR2) are both indicated by the orange boxes. The focal adhesion targeting domain (FAT) is indicated by a green box and the kinase domain is indicated by the yellow box boxes (see text in section 1.5.1 for details).

The phosphorylation of protein tyrosine kinases on tyrosine residues is commonly involved in regulating their catalytic activity and interaction with SH2 domain containing proteins. Cytoplasmic signalling proteins recognise phosphorylated tyrosine residues and interact through Src homology 2 domains (SH2 domains) and phosphotyrosine binding (PTB) domains. The SH2 domain is a conserved region of approximately 100 amino acids long (Pawson 1988). The PTB domain was originally identified as a 186-residue segment of the signalling protein Shc. PTB domains are structurally different to SH2 domains and bind to sequence motifs containing phosphotyrosine not recognised by SH2 domains (Kavanaugh and Williams 1994, Blaikie *et al.* 1994). The phosphoacceptor sites of FAK have been mapped. As shown in figure 1.2, FAK possesses six tyrosine phosphorylation sites 397, 407, 576, 577, 861 and 925 identified to date (Calalb *et al.* 1995 and 1996, Schaller *et al.* 1994, Schlaepfer *et al.* 1994).

Recombinant FAK expressed in E Coli is tyrosine phosphorylated at residue 397. This suggests tyrosine 397 is an autophosphorylation or transphosphorylation site as E Coli lacks endogenous protein tyrosine kinases to phosphorylate this site (Schaller *et al.* 1994, Calalb *et al.* 1995). Schaller *et al.* (1994), demonstrated using phosphopeptide mapping and site-directed mutagenesis that tyrosine 397 is the major site of FAK phosphorylation both *in vitro* and *in vivo*. Mutation of this tyrosine 397 to a nonphosphorylatable residue impaired FAK tyrosine phosphorylation *in vitro* and *in vivo*. Chicken embryo cells expressing Src and 397 mutated FAK failed to form the stable complexes of FAK and Src seen with wildtype FAK (Schaller *et al.* 1994). This suggested that autophosphorylation of FAK may regulate the association of FAK with Src family kinases. The Src/FAK association required an intact Src SH2 domain and dephosphorylation of *in vitro*-autophosphorylated FAK by tyrosine phosphatases was protected by incubation with GST-Src SH2 domain fusion protein (Cobb *et al.* 1994). This demonstrated Src bound via its SH2 domain to the FAK autophosphorylation site. Stable association of Fyn, another Src family kinase, with tyrosine phosphorylated FAK in normal chicken embryo cells has been demonstrated (Cobb *et al.* 1994) and the tyrosine 397 residue was required for this association (Polte and Hanks 1995). Several other proteins have been reported to bind to the autophosphorylation site of FAK *in vivo* via their SH2 domain. These include the p85 subunit of PI3K (Chen and Guan 1994a, Chen *et al.* 1996), the adapter protein Shc (Schlaepfer *et al.* 1998), the signalling protein Grb7 (Han and Guan 1999) and the γ1 isoform of phospholipase C (Zhang *et al.* 1999) (see figure 1.2).

FAK which was mutated at 397 from a tyrosine to a phenylalanine still possessed kinase activity even though it was not significantly tyrosine phosphorylated. Tyrosine 397 mutated FAK displayed approximately 50% of the wild type level of enzymatic activity during *in vitro* phosphorylation of an exogenous peptide substrate (Schaller *et al.* 1994). However it exhibited reduced autophosphorylating activity.

Calalb *et al.* (1995), used tryptic phosphopeptide mapping to identify tyrosine sites which become phosphorylated in response to adhesion and Src transformation. Four tyrosine sites were identified 397, 407, 576 and 577 in mouse BALB/3T3 fibroblast cells in response to adhesion. Tyrosines 576 and 577 lie within the catalytic domain of FAK and maximal kinase activity is seen when these tyrosines are phosphorylated. Tyrosines 407, 576 and 577 were shown to be phosphorylated by Src *in vitro* and v-Src *in vivo*. Calalb *et al.* (1996) also identified tyrosine 861 as a phosphoacceptor sites which was efficiently phosphorylated by Src *in vitro*.

Schlaepfer *et al.* (1994) demonstrated adhesion of murine NIH3T3 cells to fibronectin induced association of Src and the adapter protein Grb2 to FAK whereas plating on poly-L-lysine did not. The Grb2 association was via its SH2 domain and mutation of tyrosine residue 925 to a phenylalanine blocked Grb2 SH2 domain binding to FAK *in vitro*. Schlaepfer and Hunter (1996) discovered that mutation of FAK tyrosine 397, the Src binding site, disrupted not only Src binding but also the association of Grb2 with FAK *in vivo*. They proposed Src family protein tyrosine kinases are recruited to FAK following integrin induced tyrosine phosphorylation and bind via tyrosine 397. Src then phosphorylates FAK at tyrosine 925 creating a binding site for Grb2 via its SH2 domain.

In order to investigate binding of Grb2 to FAK several mutants were expressed in human 293 cells. These cells were either serum starved or serum starved and replated onto fibronectin. Interestingly in serum starved cells the F925 FAK mutant and an N-terminally truncated form of FAK which was missing the first 100 residues (Δ1-100) both contained elevated levels of phosphotyrosine and both were associated with Src. The constitutive association of Src with these two mutants may be due to altered conformation of FAK which exposes tyrosine 397 which enables Src to bind. This may also explain the increase in tyrosine phosphorylation of FAK seen in serum starved cells with these mutants. Grb2 was only associated with Δ1-100 FAK in serum starved cells. Wild type FAK was tyrosine

phosphorylated in serum starved cells but was not associated with Src or Grb2 (Schaelpfer and Hunter 1996).

Replating of the serum starved 293 cells lead to an increase in phosphorylation of other FAK mutant including F397, F861, F407 and R454 the kinase inactive FAK. All of the mutants demonstrated association with Src except for F397. The kinase inactive FAK R454 was also associated with Src and may have resulted from either endogenous FAK or some other endogenous kinase phosphorylating tyrosine 397 of R454 enabling Src to bind (Schlaepfer and Hunter 1996).

1.5.5 Substrates of FAK

Paxillin has been suggested as a potential substrate for the tyrosine kinase activity of FAK as FAK contains two paxillin binding sites (Tachibana *et al.* 1995, Hildebrand *et al.* 1995) and both FAK and paxillin demonstrate similar tyrosine phosphorylation responses to integrin activation (Burridge *et al.* 1992). Although paxillin is phosphorylated equally well in cells from FAK deficient mice (Ilic *et al.* 1995, Hamasaki *et al.* 1996), tyrosine phosphorylation of paxillin has also been shown to be FAK dependent (Schaller and Parsons 1995). FAK must be autophosphorylated and localised to focal adhesions in order to induce this tyrosine phosphorylation of paxillin however two catalytically inactive FAK mutants retained the ability of FAK to tyrosine phosphorylate paxillin (Shen and Schaller 1999). Given that paxillin is a good substrate of activated Src (Thomas *et al.* 1995) and phosphorylation of FAK tyrosine 397, the Src binding site, is required for paxillin phosphorylation, it is possible that Src associated to FAK is responsible for paxillin phosphorylation. However, as Src, Fyn or FAK deficient cells were not compromised in paxillin phosphorylation it suggests there is redundancy amongst these kinases (Hamasaki *et al.* 1996).

The signalling/adaptor protein Shc also binds to FAK at tyrosine 397. (Schlaepfer and Hunter 1996, Schlaepfer *et al.* 1998). FAK binds to and directly phosphorylates Shc at tyrosine 317 which promotes Grb2 binding to the adaptor and signalling to ERK2 (Schlaepfer *et al.* 1998).

P130Cas is another potential substrate of FAK. Crk associated substrates (Cas) form a protein family of at least three distinct proteins, p130Cas, Cas-L or HEF1 and Efs or Sin. P130Cas was first identified as a 120-130kDa protein which was highly tyrosine phosphorylated in v-Src and v-Crk transformed fibroblasts (Matsuda *et al.* 1990, Birge *et al.* 1992, Sakai *et al.*1994). Cas-L (lymphocyte type Cas) or HEF1 (human enhancer of filamentation 1) is a 105kDa protein which is tyrosine phosphorylated by the engagement of β_1 integrins in T lymphocytes (Minegishi *et al.* 1996) and interacts with p130Cas (Law *et al.* 1996). Efs (Embryonal Fyn-associated substrate) or Sin (Src interacting or signal integrating protein) is a 83kDa protein reported to bind to Src family SH3 domains (Ishino *et al.* 1995, Alexandropoulos and Baltimore 1996).

Cloning of p130Cas revealed it contains an SH3 domain in its N-terminal domain and many putative SH2 domains (Sakai *et al.* 1994). P130Cas has been considered a substrate for FAK for several reasons. P130Cas is localised to focal adhesions (Harte *et al.* 1996). Integrin mediated cell adhesion promotes tyrosine phosphorylation of p130Cas in rat and human fibroblast cell lines and the kinetic profile of p130Cas phosphorylation was very similar to that of FAK (Nojima *et al.* 1995, Vuori *et al.* 1995). p130Cas and FAK phosphorylation are both inhibited by treating cells with cytochalasin D, an agent which disrupts polymerisation of actin stress fibres (Nojima *et al.* 1995, Vuori *et al.* 1995).

Expression of a constitutively activated FAK in fibroblasts resulted in constitutive tyrosine phosphorylation of p130Cas with the autophosphorylation site of FAK being necessary for this phosphorylation (Vouri *et al.* 1996). However, in mutant cell lines lacking FAK, c-Src or c-Fyn, only cells deficient for Src failed to phosphorylate Cas during integrin mediated adhesion (Vouri *et al.* 1996, Hamasaki *et al.* 1996). In addition, cells lacking csk, which negatively regulates Src, demonstrated enhanced Cas phosphorylation indicating Src kinase plays a major role in Cas phosphorylation (Vouri *et al.* 1996). It would appear the role of FAK may be to recruit Src via tyrosine 397 which enables Src to phosphorylate the Cas bound to FAK. Src may also phosphorylate Cas via other routes which would explain phosphorylation of p130Cas in FAK deficient cells.

More recently others have found FAK tyrosine 397 not necessary for Cas tyrosine phosphorylation and have demonstrated FAK mutated at its autophosphorylation site phosphorylates Cas at tyrosine 762 or 764. Phosphorylation of these residues allowed Fyn

and Src to bind to p130Cas (Tachibana *et al.* 1997). This led Tachibana *et al.* (1997) to suggest a model in which FAK becomes activated upon integrin engagement and phosphorylates p130Cas at tyrosine residues 762 and/or 764. Src family kinases are then recruited to the phosphorylation sites and further phosphorylates p130Cas at tyrosine residues. Crk can bind to p130Cas via its SH2 domains (Nakamoto *et al.* 1996) and the Src family phosphorylation may create the phosphotyrosine sites for Crk to bind. However in Src deficient cells p130Cas is not detectably phosphorylated, even though FAK is activated, so FAK alone may not be able to phosphorylate p130Cas (Vuori *et al.* 1996, Schlaepfer *et al.* 1997). This suggests a complex of Src family kinases and FAK leads to the phosphorylation of p130Cas. Supporting this theory, Schlaepfer *et al.* (1997) demonstrated that expressing a Src mutant which contained the SH2 and SH3 domains of Src but not its kinase domain still resulted in Cas phosphorylation. As FAK could tyrosine phosphorylate Cas *in vitro*, Schlaepfer *et al.* (1997) suggested that FAK mediated the phosphorylation of Cas in the absence of Src kinase domain and that Src acted as an adapter protein linking FAK and p130Cas. Nakamoto *et al.* (1997) demonstrated that the SH3 domain of p130Cas and the association of p130Cas with Src played a role in the localisation of p130Cas to focal adhesions. Src could localise p130Cas to focal adhesions for FAK to phosphorylate. In Src deficient cells p130Cas would not be localised to focal adhesions and therefore not become phosphorylated.

1.6 The role of FAK in apoptosis

Apoptosis is gene directed, energy dependent cell death as opposed to accidental cell death often termed necrosis (Cotter *et al.* 1990). Anoikis is a term which describes apoptosis in adherent cells which occurs due to loss of attachment from the ECM (Frisch and Francis, 1994) and is thought to occur to prevent detached cells reattaching and growing at inappropriate locations.

The morphological changes which occur during apoptosis have been well documented. The dying cell first severs attachments with other cells and the extracellular matrix and starts to round up. The membrane starts what is termed 'blebbing', during which the plasma membrane protrudes and retracts. The chromatin starts to condense and once the nucleus is fully condensed it disassembles into nuclear fragments. The cell contents are compartmentalised into membrane bound vesicles termed apoptotic bodies. These

apoptotic bodies are taken up by phagocytes and neighbouring cells (Kerr *et al.* 1972). This avoids the release of intracellular contents and therefore avoids inflammation. Apoptosis is considered an injury limiting mode of cell disposal.

The role of FAK in apoptosis has been highlighted by a number of studies. In particular the role of FAK in providing an anti-apoptotic signal during integrin engagement. Inhibition of FAK by microinjection of antibodies to an epitope near the FAT sequence resulted in apoptosis in fibroblasts (Hungerford *et al.* 1996). Expression of a constitutively activated form of FAK prevents epithelial cells from undergoing apoptosis upon cell detachment (Chan *et al.* 1994). Some cancer cells demonstrate high levels of FAK expression (Weiner *et al.* 1993 and 1994, Owens *et al.* 1995 and 1996). Expression of FAK antisense oligonucleotides in tumour cell lines which expressed high levels of FAK caused the treated cells to become detached and succumb to apoptosis (Xu *et al.* 1996).

The mechanism by which FAK signalling suppresses anoikis/apoptosis is still under investigation but some progress has been made. Frisch *et al.* (1996) not only demonstrated that a constitutively active form of FAK expressed in MDCK cells prevented the cells from undergoing anoikis but both the Tyr397 autophosphorylation site and the kinase activity of FAK were required. Akt/PKB is a survival-promoting serine-threonine protein kinase regulated by PIP_3 (Kulik *et al.* 1998). Constitutively activated PI3K or PKB/Akt blocked anoikis in MDCK cells (Khwaja *et al.* 1997) and it is possible FAK signals via this route due to the potential binding of PI3K to Tyrosine 397. Recently the tumour suppressor protein, PTEN has been implicated in this pathway. PTEN is a phosphatase and induces apoptosis in response to cell detachment via FAK (Tamura *et al.* 1999). PTEN is suggested to negatively regulate the PI3K/Akt cell survival pathway by dephosphorylation of FAK. This dephosphorylation prevents the binding of PI3K to FAK and therefore reduces production of the PI3K products which activate Akt (Tamura *et al.* 1998 and 1999).

Ilic *et al.* (1998) demonstrated when serum is absent fibronectin survival signals transduced by FAK, suppress a p53-regulated apoptosis in primary fibroblasts and endothelial cells. Fibroblasts and endothelial cells devoid of FAK could survive if the p53 tumour suppressor gene was inactivated. This suggests survival signals from the ECM are transduced via FAK and are monitored by p53.

Once apoptosis has been initiated FAK is cleaved into two fragments of 90 and 35kDa in human endothelial cells. FAK is cleaved sequentially by caspases at two sites. Caspase-3 preferentially cleaves FAK at $DQTD^{772}$ while the site $VSWD^{704}$ is preferentially cleaved by caspase-6 (Wen *et al.* 1997, Gervais *et al.* 1998). Both these sites are in the carboxyl-terminal half of FAK separating the kinase domain from the FAT domain. Once cleaved the C-terminal portion of FAK is proposed to act like FRNK, the natural variant of FAK, and compete with full length FAK for localisation to focal adhesions. Engineered C-terminal FAK fragments identical to the ones produced during apoptosis were expressed in HeLa cells. The tyrosine phosphorylation levels of full length FAK were reduced by expression of either of the FAK C-terminal apoptosis fragment (Gervais *et al.* 1998). Interestingly although human, chicken, rat and mouse FAK all posses the cleavage site $VSWD^{704}$, rat and mouse FAK do not posses the second cleavage site $DQTD^{772}$. The caspase cleavage sites lie either side of the proline rich region 2 however the significance of this is not clear at present (Gervais *et al.* 1998). Ilic *et al.* (1998) expressed the FAT domain of FAK and FRNK and demonstrated that the FAT domain induced a much higher level of apoptosis compared to FRNK. During growth factor deprived-induced apoptosis in HUVE cells, p130Cas but not paxillin, was still able to bind to the N-terminal 90kDa fragment of FAK whereas paxillin, but not p130Cas, was still able to bind to the 35kDa C-terminal fragment of FAK (Levkau *et al.* 1998).

It has been reported dephosphorylation of FAK preceded caspase cleavage (van de Water *et al.* 1999). Whether FAK cleavage occurs before or after loss of focal adhesions is not clear. Levkau *et al.* (1998) reported during growth factor deprivation-induced apoptosis in HUVE cells that cleavage of FAK preceded cell detachment and coincides with the loss of FAK from focal adhesions. In renal epithelial cells during chemically induced apoptosis FAK cleavage by caspases occurred after the cells had lost focal adhesions (van de Water *et al.* 1999).

1.7 The role of FAK in migration

FAK is localised to focal adhesions upon integrin engagement and therefore a potential role for FAK could be modulation of cell adhesion during migration. Indeed there is much evidence linking FAK to a role in cell migration. Ilic *et al.* (1995) generated FAK deficient cells and noted they exhibited reduced mobility compared to wild type cells. Overexpression of FAK in CHO cells increased their migration on fibronectin (Cary *et al.* 1996). Microinjection of the C-terminal portion of FAK to displace endogenous FAK from focal adhesions, caused the microinjected cells to display decreased migration (Gilmore and Romer 1996). Evidence of a role during *in vivo* migration comes from Dfak 56, the *drosophila* homolog of FAK and its expression in cells just before and during their migration during embryogenesis (Fox *et al.* 1999).

The recruitment of Src to FAK is also likely to play a role in FAK mediated migration. Overexpression of FRNK reduces FAK tyrosine phosphorylation and cell spreading (Richardson *et al.* 1996 and 1997) however overexpression of Src rescues this (Richardson *et al.* 1997). For FAK overexpression to increase migration, an intact tyrosine 397 site on FAK is necessary (Cary *et al.* 1996) and this is a site to which Src binds. Further evidence is obtained from Src deficient fibroblasts which exhibit slower spreading (Kaplan *et al.* 1995).

Grb2 and p130Cas also bind to FAK which led Cary *et al.* (1998) to investigate their role in FAK mediated migration. FAK mutants which did not bind Grb2 promoted cell migration as efficiently as FAK suggesting Grb2 does not mediate FAK induced migration. However a FAK mutant with an impaired ability to bind p130Cas demonstrated migration similar to control levels. Furthermore, expression of p130Cas with FAK further increased migration on fibronectin whereas expression of the SH3 domain of p130Cas with FAK decreased cell migration. All these data strongly suggest a role for p130Cas in FAK mediated cell migration. Autophosphorylation and activation loop sites are required for maximum adhesion induced FAK enhanced cell spreading and migration responses (Owen *et al.* 1999). Decreased phosphorylation of p130Cas was observed during expression of the FAK autophosphorylation mutant however expression of FAKs activation loop sites mutant did not effect p130Cas phosphorylation. This might suggest Src binding to FAKs autophosphorylation site is responsible for phosphorylation of p130Cas. The pathway appears to include Crk. Klemke *et al.* (1998) demonstrated Crk bound via its SH2 domain

to tyrosine phosphorylated p130Cas to promote migration and preventing their interaction blocked the increase. Furthermore, this migration was Rac dependent.

It has recently been proposed that PI3K is involved in FAK induced migration as the PI3K inhibitors, wortmannin and LY294002, inhibited FAK promoted cell migration (Reiske *et al.* 1999). PI3K and Src both bind to tyrosine 397 on FAK as discussed earlier. In contrast Reiske *et al.* (1999) used a mutant FAK capable of binding Src but not PI3K which when overexpressed did not induce cell migration even though Src was bound. Grb7 binding to FAK has also been suggested to play a role in cell migration with Grb7 being downstream of FAK (Han and Guan 1999).

Focal adhesion turnover is required in cell migration to allow the cells to move (Sankar *et al.* 1995). As FAK deficient fibroblasts demonstrate an elevated number of focal adhesions and a rounded morphology, it is possible that FAK tyrosine phosphorylation levels are involved in their turnover to allow cell spreading and migration (Ilic *et al.* 1995). Indeed, cells lacking the tyrosine phosphatase Shp-2 demonstrated reduced FAK dephosphorylation and were impaired in their ability to spread and migrate. Interestingly, fibroblast cells deficient for a functional Shp-2 displayed similar properties to cells lacking FAK (Yu *et al.* 1998). In contrast, overexpression of the phosphatase PTEN, has been reported to reduce the tyrosine phosphorylation levels of FAK as well as reducing migration (Tamura *et al.* 1998). Migration is a dynamic process. It is possible that spatially separate pools of FAK undergo cycles of tyrosine phosphorylation and dephosphorylation which results in focal adhesion turnover, however the role of FAK in focal adhesion turnover it is still not clear and further investigations are required.

1.8 Aims of the project

The angiogenic factor vascular endothelial growth factor stimulates enhanced vascular permeability, migration and cytoskeletal changes in endothelial cells. The intracellular mechanism by which VEGF regulates endothelial cell function are not known. Cell migration and cytoskeletal changes involve modulation of cell:substrate adhesions and permeability is dependent on cell:cell adhesions. Focal adhesion kinase (FAK) is an intracellular tyrosine kinase that has been implicated in regulating cell adhesion and

migration and localises to focal adhesions. FAK is therefore a potential candidate for involvement in VEGF modulation of endothelial migration and other adhesion related phenomenon. The aim of this study was to determine whether FAK is involved in the VEGF signalling cascade and to define its role in mediating VEGF action. This will be achieved by (i) determining the effects of VEGF in FAK tyrosine phosphorylation. (ii) Determining the effects of VEGF on recruitment of signalling intermediates by FAK. (iii) Investigating the tyrosine residues and domains of FAK involved in VEGF activation by generating mutant FAK constructs. (iv) Determining the role of FAK in VEGF activated cell survival and migration.

CHAPTER TWO

Materials and Methods

2. Materials and Methods

2.1 General Reagents.

All chemicals were of analytical grade unless otherwise stated. Most were obtained from Sigma Aldridge, (Poole, Dorset, UK).

2.2 Antibodies.

The series of cell antibodies is given in appendix one.

2.3 Human umbilical vein endothelial (HUVE) cells.

2.3.1 Isolation and culture.

Human umbilical vein endothelial (HUVE) cells were obtained by G. Williams, Department of Surgery, University of Leicester. Tissue culture flasks were coated with 1% gelatin in phosphate buffered saline (PBS; 140mM sodium chloride, 10 mM sodium phosphate, pH 7.4) and HUVE cells cultured in medium 199 (Gibco, Paisley, Glasgow. Scotland), containing 100u/ml penicillin, 100μg/ml streptomycin, 20% foetal calf serum (FCS), 5u/ml heparin and 100μg/ml endothelial growth supplement and incubated at 37°C in 5% CO_2. Cells were passaged usually in a ratio of 1:3 when they reached confluency. For passage cells were washed twice with PBS and incubated with 2ml trypsin-EDTA solution (0.05% trypsin and 0.02% EDTA diluted in PBS), for approximately two minutes, until cells lifted off the flask with gentle agitation. Complete medium was then added to inactivate the trypsin which was then removed by centrifuging the cells (400g for 6 minutes) and resuspending them in an appropriate volume of complete medium before plating them out. HUVE cells were used for experiments between passages two - five. All tissue culture plastic was obtained from Nunc products.

2.3.2 Agonist treatment of HUVE cells.

All agonists were obtained from Sigma Aldridge, (Poole, Dorset, UK). Prior to stimulation with agonists, the media was removed and the cells washed twice with Optimem 1 (Gibco), incubated for 16 hours in Optimem with one medium change two hours before the start of the experiment. For VEGF stimulation cells were stimulated with 2.5 nM Vascular endothelial growth factor (VEGF) for 10 minutes unless otherwise stated. VEGF was dissolved in filtered sterile distilled water to a concentration of 100μg/ml and stored at -20° C.

For challenge with lysophosphosphatidic acid (LPA) cells were treated with 2 μM LPA, (Sigma-Aldridge) for 5 minutes. LPA was dissolved in sterile water at 1mg/ml just prior to use (then stored at -20°C).

Fibroblast growth factor (FGF) challenge was by addition of 5μg/ml FGF for 20 minutes. FGF stored at -20°C.

All control cells were treated with the relevant volume of sterile water or other appropriate solvent as described for each individual experiment.

2.3.3 Treatment of serum starved cells with inhibitors.

Cells were treated with 100nM Wortmannin (Sigma-Aldridge, Poole, Dorset, UK). Wortmannin was dissolved in ethanol to produce a 1 mM stock solution which was stored at 4°C. All control cells treated with relevant amount of ethanol as described.

2.4 Bovine aortic endothelial (BAE) cells.

2.4.1 Source of cells and culture conditions.

BAE cells were a kind gift from Dr. M Bower, Department of Cell Physiology and Pharmacology, University of Leicester. BAE cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) containing 10% FCS, 1% penicillin and streptomycin and 1% L-Glutamine. Cells were grown to confluency before passaging and plating out at split

ratios of 1:10 or 1:20. BAE cells were passaged using the same method as used for HUVE cells except BAE cell culture medium (described in section 2.4.1) was used. All experiments were performed with BAE cells between passages 6 and 14.

2.4.2 Freezing and storage of BAE cells.

80cm^2 flasks of confluent cells were trypsinised as described in 2.4.1. cells were resuspended in 500µl DMEM containing 10% FCS, 1% penicillin and streptomycin and 1% L-Glutamine before the addition of 400µl FCS. The cell suspension was placed in a sterile freezing vial with 100µl of DMSO and vortexed. The cell suspension was placed at 4°C for 1 hour, -20°C for 1 hour, -80°C for 1 hour (or overnight) and finally stored in liquid nitrogen. For retrieval of cells from liquid nitrogen storage vials were thawed at 37°C and the contents mixed with 10ml of DMEM containing 10% FCS, 1% penicillin and streptomycin and 1% L-Glutamine. The cells were centrifuged at 400g for 6 minutes to remove DMSO resuspended in DMEM containing 10% FCS, 1% penicillin and streptomycin and 1% L-Glutamine and plated in 80cm^2 tissue culture flask. Within 18 hours the cells had grown to confluency and were then passaged and used for experiments.

2.4.3 Agonist treatment of BAE cells.

Prior to challenge with agonists BAE cells were washed twice in PBS then incubated overnight in DMEM containing 1% penicillin and streptomycin and 1% L-Glutamine (serum free).

2.5 Preparation of whole cell lysates.

HUVE or BAE cells were usually grown in a 6 well plate (Nunc). Cells were washed twice in PBS and lysed by the addition of 50 µl of reducing sample buffer (50mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue, 5mM EDTA) containing 100mM dithiothrietol (DTT). The contents of the well was scraped using a cell scraper and placed into microfuge tubes. Samples were boiled at 95°C for 5 minutes, sonicated for 10 seconds and finally centrifuged for 5 minutes at 10000g. Samples were either stored at -20°C or used directly for SDS-PAGE.

2.6 Immunoprecipitation.

2.6.1 Immunoprecipitation of FAK.

HUVE or BAE cells for immunoprecipitation were grown in 90mm tissue culture dishes (Nunc). Following challenge with agonists flasks were placed on a tray of ice and cells washed once with PBS containing 100μM sodium orthovanadate. PBS was removed before the addition of 0.5ml modified RIPA buffer, pH7.4 (50mM Tris, 1% (v/v) Triton X-100, 0.25% deoxycholate, 150mM NaCl, 1mM EGTA, 1mM sodium orthovanadate, 1mM sodium fluoride, 1mM AEBSF, 1μg/ml aprotinin, 1μg/ml leupeptin and 1μg/ml pepstatin. Flasks were rocked at 4°C for 15-45 minutes. Cell lysates and debris were scraped using a cooled cell scraper and placed in microfuge tubes. The lysate was centrifuged at 13,000g for 10 minutes at 4°C to pellet the insoluble material. 0.6ml of the supernatant was transferred to a new microfuge tube. Anti-FAK antibody conjugated to agarose beads were washed three times with modified RIPA and 20 μl of 1:1 (v/v) beads in modified RIPA was added to each tube and the mixture rotated at 4°C overnight. The agarose beads were collected by centrifugation, 8000g for 2 seconds, and washed three times with ice cold RIPA. The beads were resuspended in 30μl reducing sample buffer (50mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue, 5mM EDTA) containing 200mM DTT. The samples were immediately boiled at 95°C for 5 minutes to dissociate the immunocomplex from the beads. Samples were centrifuged at 10000g for 5 minutes and proteins in the supernatant were resolved by SDS-PAGE.

2.6.2 Co-immunoprecipitation analysis.

In early experiments it was observed that proteins previously reported to immunoprecipitate with FAK failed to be recovered using the modified RIPA lysis buffer. In order to minimise disruption of these associations a gentle lysis buffer was used for co-immunoprecipitation analysis. The lysis buffer described by Backer *et al.* (1992) was used. This lysis buffer was designated as Backer buffer and comprised of 20mM Tris-HCL, pH 8.0, 1% NP-40, 137mM NaCl, 10% glycerol. Prior to use 1mM Na_3VO_4, 1mM NaF and 0.1mM AEBSF was added. All other steps during the immunoprecipitation remained unchanged.

2.6.3 Immunoprecipitation of phosphorylated proteins.

To immunoprecipitate tyrosine phosphorylated proteins monoclonal anti-phosphotyrosine clone PT-66 (Sigma-Aldridge) was used. PT-66 directly conjugated to agarose beads was washed three times in Backer buffer and 50 µl 1:1 (v/v) beads in Backer buffer was added to each 0.6ml of lysis prepared as described in section 2.6.2. All other steps in the immunoprecipitation remained as for FAK immunoprecipitation.

2.6.4 Immunoprecipitation of epitope tagged constructs.

Immunoprecipitation of proteins incorporating a flag epitope tag from transfected cell lysates were performed as follows. Transfected cells from a 90mm tissue culture dish were placed on ice and washed once with PBS containing 100µM sodium orthovanadate and the cells lysed by the addition of 0.5ml Backer buffer as described in section 2.6.2. Cell lysates were prepared as follows. Flasks were rocked at 4°C for 15-45 minutes. Cell lysates and debris were scraped using a cooled cell scraper and placed in microfuge tubes. The lysate was centrifuged at 13,000g for 10 minutes at 4°C to pellet the insoluble material. 0.6ml of the supernatant was transferred to a new microfuge tube. Anti-Flag M2 antibody covalently attached to agarose (Sigma-Aldridge) was used for the immunoprecipitation. 50µl of the 50% slurry was spun down and washed three times with Backer buffer, resuspended to a final volume of 45µl in Backer buffer before addition to lysate. The beads were left rotating with the lysates at 4°C for 4 hours. The agarose beads were collected by centrifugation at 6000g for 2 seconds and washed three times with ice cold Backer buffer. The beads were resuspended in 30µl reducing sample buffer (50mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue, 5mM EDTA) containing 200mM DTT. The samples were immediately boiled at 95°C for 5 minutes to dissociate the immunocomplex from the beads and centrifuged at 13000g for 5 minutes before the proteins from the supernatant were separated using SDS-PAGE.

2.6.5 Immunoprecipitation of Fyn.

Fyn was immunoprecipitated from transfected cell lysates as follows. Transfected cells from a 90mm tissue culture dish were placed on ice and washed once with PBS containing 100µM sodium orthovanadate and lysed by the addition of 0.5ml Backer buffer as

described in section 2.6.2. Cell lysates were prepared as described in section 2.6.4. 2.4μg of anti-Fyn monoclonal antibody was added to the cell lysate and rotated for 2 hours at 4°C. Approximately 7μg of Protein G:sepharose was added and incubated for a further 2 hours. The sepharose beads were collected and proteins prepared for SDS-PAGE analysis as described in section 2.6.4.

2.7 Separation of proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

All protein PAGE was conducted using a Biorad microgel. Acrylamide gels were prepared as in Table 2.1.

% acrylamide gel	7.5%	10%	Stacking gel (6%)
30%Acrylamide / 0.8%Bisacrylamide	3.8	5	1.7
2M Tris pH 8.8	2.8	2.8	1.3 *1M Tris pH6.8
DdH_2O	8.2	7.2	6.9
10% SDS	0.15	0.15	0.1
10% Ammonium persulphate	0.1	0.1	0.1
TEMED	0.01	0.01	0.01

All volumes shown in ml

Table 2.1 SDS-polyacrylamide gel components.

The gels were prepared from stock 30% acrylamide/ 0.8% bisacrylamide and filtered using a 0.45μm acrodisc to remove particles. Ammonium persulphate and TEMED were added after filtering. Samples were boiled at 95°C for 5 minutes in reducing sample buffer (50mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue, 5mM EDTA) containing 100mM DTT (unless otherwise stated) and spun at 10000g for 5 minutes prior to loading onto gel. Routinely pre-stained MW markers were also run to identify the size of the proteins. Gels were run in running buffer, 2.5mM Tris, 19.2 mM glycine and 0.01% SDS at 200V for approximately 45 minutes.

If visualisation of proteins with Coomassie Brilliant Blue was required, gels were stained with 1% Coomassie Brilliant Blue in 10% acetic acid, 10% methanol, 80% H_2O and the excess stain removed by washing in the above staining solution without the Coomassie Blue (Laemmli, 1970).

2.8 Western blotting.

Separated proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane, (Hybond ECL, Amersham) as described by Towbin *et al.* 1979. Proteins were transferred for 14-16 hours at 0.1 amps or in cold room with pre-cooled transfer buffer, 192mM glycine, 25mM Tris base, 20% methanol for 1 hour 30 minutes at 0.75amps. The nitrocellulose membrane was then probed as described below.

2.9 Immunoblotting to detect proteins on nitrocellulose membrane.

2.9.1 Detection of Tyrosine phosphorylated proteins.

The nitrocellulose membrane was removed from western blotting tank and blocked in TBS containing 0.1 % TX-100 and 5% BSA for 45 min at 37°C shaking. The membrane was then probed by incubation with anti-phosphotyrosine antibody directly conjugated to horseradish peroxidase (HRP) diluted 1 in 2500 in blocking buffer for 30 minutes at 37°C with shaking. The membrane was thoroughly rinsed with TBS containing 0.1% TX-100 with at least 4 changes of wash buffer over 1 hour. Bound antibody was visualised by enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) and emitted light detected by exposing membrane to Kodak imaging film (Sigma-Aldridge, Poole, Dorset, UK).

In some experiments unconjugated monoclonal anti-phosphotyrosine Clone PT-66, (Sigma) was used. This antibody was used to probe membrane at a dilution of 1:1000 in blocking buffer for 45 minutes at room temperature. After rinsing and two washes of 10 minutes with TBS containing 0.1% TX-100, the membrane was incubated with the anti-mouse HRP conjugated antibody diluted 1:2500 in blocking buffer for 45 minutes at room temperature. A final wash was performed with at least five changes of wash buffer in 45

minutes before developing with the ECL or the enhanced chemiluminescent protocol described in section 2.10.

2.9.2 Detection of FAK on western blots

Re-probing of the nitrocellulose membrane to detect the relative amount of FAK protein present in each lane was performed. To do this the membrane was stripped as described in section 2.11 and then blocked in TBS containing 4% milk and 0.1% TX-100 for 1 hour at room temperature, washed briefly in TBS containing 0.1% TX-100 before being incubated for 1 hour with polyclonal DRC FAK antibody diluted 1:20000 in TBS containing 0.1% TX-100 and 1% BSA. After rinsing the membrane and two further washes of 10 minutes with TBS containing 0.1% TX-100, the membrane was incubated with the goat anti-rabbit HRP conjugated antibody (Sigma-Aldridge, Poole, Dorset, UK), diluted 1:3500 in blocking buffer for 45 minutes at room temperature. A final wash was performed with at least five changes of wash buffer in 45 minutes before developing with the ECL or enhanced chemiluminescent protocol (section 2.10).

2.9.3 Detection of flt-1 (VEGFR-1) and flk (VEGFR-2) on western blots.

To detect flt-1 (VEGFR-1) or flk (VEGFR-2) the nitrocellulose membrane was blocked in TBS containing 0.1 % TX-100, 1% milk powder and 1% BSA for 1 hour at room temperature followed by incubation for 1 hour at room temperature with the flt-1 (VEGFR-1) or flk (VEGFR-2) antibody which was diluted 1:500 in blocking buffer. After rinsing and two washes of 10 minutes with TBS containing 0.1% TX-100, the membrane was incubated with the anti-rabbit HRP conjugated antibody diluted 1:2500 in blocking buffer for 45 minutes at room temperature. A final wash was performed with at least five changes of wash buffer in 45 minutes before developing with the ECL protocol.

2.9.4 Detection of Src, Fyn Grb2 and p85 subunit of PI 3-Kinase on western blots

The detection of the above proteins was performed by blocking the nitrocellulose membrane in TBS containing 1% milk, 1%BSA and 0.05% Tween-20 for 45 minutes at room temperature. The rabbit polyclonal anti-Src antibody was diluted 1:500 in blocking buffer, (final concentration of 0.2μg/ml) and incubated with the membrane for 45 minutes

at room temperature. After rinsing and two washes of 7 minutes with TBS containing 0.05% Tween-20, the membrane was incubated with the anti-rabbit HRP conjugated antibody diluted 1:2500 in blocking buffer for 45 minutes at room temperature. A final wash was performed with at least five changes of wash buffer in 45 minutes before developing with the ECL or enhanced chemiluminescent protocol.

To detect Fyn the same method was used as that for the detection of Src except the Fyn monoclonal was diluted 1:250 in blocking buffer before use and an anti-mouse HRP conjugated antibody was used as a secondary antibody diluted 1:2500 in blocking buffer.

To detect Grb2, a rabbit polyclonal was used at a final concentration of 2μg/ml (diluted 1:500). The same method was followed as that used for the detection of Src.

To detect the p85 subunit of PI 3-Kinase a rabbit polyclonal antibody was used at a final concentration of 2μg/ml. The same method was followed as that used for the detection of Src.

2.9.5 Detection of Flag constructs on nitrocellulose membrane.

To detect the flag constructs the nitrocellulose membrane was blocked with 5% non-fat dry milk in TBS for 30-60 minutes at 37°C. After three changes of TBS in five minutes the membrane was incubated with anti-FLAG M2 antibody diluted to 10μg/ml in TBS for 30 minutes at room temperature. The membrane was washed with TBS for 20 minutes with five changes of wash buffer before incubation with anti-mouse HRP diluted 1:2500 in TBS at room temperature for 45 minutes. The membrane was washed for 45 minutes with five changes of TBS before detection using the enhanced chemiluminescence protocol (section 2.10).

2.10 Enhanced chemiluminescence protocol.

HRP conjugated antibodies bound to the nitrocellulose membrane were visualised by enhanced chemiluminescence. This was done using the ECL system (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) or the protocol described by Matthews *et al.* (1985). For this latter protocol 22μl of 90mM p-Coumaric acid in DMSO,

50µl of 250 mM Luminol, 5-amino-2,3-dihydro-1,4-pthalazinedione in DMSO and 3µl H_2O_2 was added to 10ml of 0.1M Tris-HCl, pH 8.5. The nitrocellulose membrane was immediately incubated with solution for two minutes before wrapping in Saranwrap and exposing to biomax light film (Sigma-Aldridge, Poole, Dorset, UK).

2.11 Stripping nitrocellulose membranes.

In order to reprobe the nitrocellulose membrane with other antibodies it was submerged in stripping buffer (62.5nM Tris-HCl, 2% SDS and just before use 100 mM 2-Mercaptoethanol was added), for 30 minutes at 60°C with gentle agitation. The nitrocellulose membrane was washed in TBS containing 0.1% TX-100 to remove the stripping buffer before being blocked with an appropriate blocking solution and probed.

2.12 Far Western blots.

This method, from Grasser *et al.* 1993, was used to detect the potential binding of SH2 domain containing proteins to FAK immobilised on nitrocellulose membranes. The nitrocellulose membrane from a western blot was incubated for 1 hour in hepes binding buffer, HBB (20mM Hepes, pH 7.5, 5mM $MgCl_2$, 1mM KCl) containing 5% dried milk powder at room temperature. The membrane was then incubated for 18 hours at 4°C with HBB containing 5% milk, 5mmM DTT and the GST fusion protein of interest at a concentration of 2µg/ml. Subsequent incubation steps were carried out in PBS and 5% dried milk powder and washing steps with PBS containing 0.1% TX-100. After incubation with the GST fusion protein the membrane was washed for 45 minutes with four changes of wash buffer before incubation with an anti-GST antibody diluted 1:2000 for 1 hour at room temperature. The membrane was washed as before, followed by incubation with an anti-rabbit conjugated HRP antibody for 1 hour at room temperature. The membrane was washed as before and developed as usual using the chemiluminescence protocol described in section 2.10.

2.13 Adhesion experiments

Tissue culture plates were coated with fibronectin (Sigma-Aldridge, Poole, Dorset, UK) diluted with PBS to a concentration of 25μg/ml and incubated overnight at 4°C. Before use the plates were warmed to 37°C, excess fibronectin removed and plates rinsed briefly with PBS. An $80cm^2$ flask of cells was trypsinised as previously described and cells resuspended in media. Half of the cells were plated onto the fibronectin coated plate and the other half was kept in suspension. Cells were incubated for 60-90 minutes at 37°C. The plated cells were washed with PBS to remove any cells which had not adhered and the cells in suspension were collected by centrifuging at 400g for 5 minutes and rinsed with PBS. Cells were lysed by addition of 0.5ml of modified RIPA to the adherent cells or resuspending the pellet following centrifugation in 0.5ml of modified RIPA. Lysates were analysed by immunoprecipitation (section 2.6).

2.14 Immunofluorescence.

Cells were grown on sterile autoclaved glass coverslips until reaching desired confluency. Cells were fixed for 10-15 minutes at room temperature in 4% paraformaldehyde in cytoskeletal buffer, 10mM MES pH 6.1, 138mM KCl, 3mM MgCl, 2mM EGTA and 0.32M sucrose with the sucrose added just prior to use. The cells were rinsed in TBS before permeabilizing in TBS containing 0.5% TX-100 for 10 min. Following permeabilisation the cells were rinsed three times in TBS-0.1% TX-100 for 3 minutes each, before blocking for 10 minutes at room temperature in abdil (2% BSA in TBS containing 0.1% TX-100, made fresh on day of use). The cells were then treated as described below according to the cytoskeletal structures or tagged proteins being examined.

2.14.1 Detection of filamentous actin

Detection of filamentous actin was performed with fluorescein (FITC) conjugated phalloidin. Cells were fixed and permeabilised as described in 2.14. After blocking in abdil the cells were incubated for 30 min at 37°C in a humidified chamber with FITC-phalloidin diluted in abdil to a final concentration of 2μg/ml. Coverslips were washed in

TBS containing 0.1% TX-100 for 30 minutes with three changes of wash buffer and dipped in distilled water to remove the salt. Excess water was removed by dabbing the edge of the coverslip on a tissue before mounting in 220mM diazobicylclo-octane (DABCO), dissolved in 90% glycerol, 10% PBS, pH8.6. Cover slips were fixed in place using clear varnish and viewed under a fluorescence microscope. Photos taken using 400 ASA film on an Olympus OM4 camera.

2.14.2 Immunostaining cells for vinculin.

The focal adhesion protein vinculin is detected in permeabilised cells by immunofluorescence staining. Cells were fixed and permeabilised as described in 2.14 and then blocked in TBS containing 0.1% TX-100 and 10% BSA at room temperature for 15 minutes followed by incubation with monoclonal anti-vinculin VIN-11-5 diluted 1:100 in blocking buffer for 1 hour at 37°C. The coverslips were washed in TBS containing 0.1% TX-100 for 30 minutes with three changes of wash buffer before incubation with Cy3 labelled anti-mouse antibody diluted 1:200 in blocking buffer for 45 minutes at 37°C. The coverslips were washed for five minutes in blocking buffer followed by washing with TBS containing 0.1% TX-100 for 30 minutes with 4 changes of wash buffer. The coverslips were dipped in distilled water and mounted as described for actin staining.

2.14.3 Immunostaining cells for epitope tagged proteins.

The anti-flag BioM2 antibody was used to detect the presence and location of proteins containing the FLAG epitope tag. This antibody recognises the FLAG epitope by sequence and is directly conjugated to biotin. Twenty-four hours post transfection, cells were fixed and permeabilised as described in section 2.14. Following blocking in abdil the cells were incubated for 1 hour in a humidified chamber with anti-flag BioM2 antibody diluted in abdil to a concentration of 10μg/ml. Coverslips were washed in TBS containing 0.1% TX-100 for 15 minutes with three changes of wash buffer before incubation with ExtrAvidin-FITC conjugate diluted 1:200 in abdil for 1 hour at 37°C in a humidified chamber. The coverslips were washed for 30 minutes with three changes of wash buffer and then dipped in distilled water. The coverslips were then mounted in DABCO and viewed as described previously for actin staining. When dual staining with phalloidin which is conjugated to FITC, an anti-flag M2 antibody which was not conjugated to biotin

was used at 10 μg/ml and an alternative fluorescence marker, Cy3 labelled anti-mouse antibody was used diluted 1:250 in place of the ExtrAvidin-FITC conjugate. The FITC-phalloidin incubation was performed last. All other steps remained unchanged.

2.14.4 Immunostaining cells for GST.

Cells which had been micro-injected with GST or a GST fusion protein were immunostained for GST to detect their location. To stain for GST the cells were fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature. The coverslips were rinsed in TBS before permeabilizing in TBS containing 0.5% TX-100 for 10 minutes Following permeabilisation the coverslips were rinsed three times in 10 minutes in TBS containing 0.1% TX-100, before blocking for 10 minutes at room temperature in TBS containing 1% BSA and 0.1% TX-100. The coverslips were then incubated with anti-GST diluted 1:2000 in TBS containing 0.1% TX-100 for 1 hour at 37°C. The coverslips were washed in TBS containing 0.1% TX-100 for 15 minutes with three changes of wash buffer before incubation with FITC labelled anti-rabbit antibody diluted 1 in 200 in TBS containing TX-100 at 37°C for 1 hour. The coverslips were washed for 20 minutes with four changes of wash buffer, dipped in distilled water mounted as described previously (section 2.14.1).

2.14.5 Double Immunofluorescence staining.

For staining cells with two different antibodies or an antibody and phalloidin, coverslips were fixed, permeabilised, blocked and probed as described with the appropriate antibody. After the final wash cells were probed with phalloidin or the appropriate second antibody using the relevant protocol detailed above. When cells were probed with two different antibodies they were raised in different species and the secondary used in the detection steps were species specific and conjugated to different fluorophors. Coverslips were mounted as above and the cells visualised by fluorescence microscopy using the appropriate filters.

2.15 Microbiological media recipes.

L Broth (LB) was prepared using 10g tryptone, 5g yeast extract, 10g NaCl and distilled water to 1 litre, pH 7.0. Autoclaved for 20 min at 120°C. Ampicillin was added before use to a final concentration of 100μg/ml. Once ampicillin added, broth stored at 4°C.

L Agar plates were prepared using 10g tryptone, 5g yeast extract, 10g NaCl, 15g agar and made up to 1 litre with distilled water, pH 7.0. Autoclaved for 20 min at 120°C. Once solution cooled to 50°C ampicillin was added to a final concentration of 100μg/ml. Agar (25ml) poured into 100mm petri dishes.

SOB was prepared as follows. 2% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl, autoclaved for 20 minutes at 120°C before the addition of 10mM $MgCl_2$ and 10mM $MgSO_4$, pH 6.7-7.0.

SOC was prepared as follows. To 100ml of SOB, 1ml of 2M filter-sterilized glucose solution was added prior to use. Filter sterilised before use.

NZY was prepared as follows. Prepared with 10g of NZ amine (casein hydrolysate), 5g of yeast extract, 5g of NaCl, autoclaved for 20 minutes at 120°C. Prior to use 12.5mL of 1M $MgCl_2$, 12.5mL of 1M $MgSO_4$ and 10ml of 2M filter sterilised glucose solution was added per litre of NZY. Solution filter sterilised before use.

Transformation buffer (TB) for preparation of competent cells.

10mM Pipes, 55mM $MnCl_2$, 15mM $CaCl_2$, 250mM KCl, pH 6.7. The solution was filter sterilised. This solution was prepared by reducing the pH to 6.7 to dissolve the Pipes before the addition of a solution of the other components.

2.16 Preparation of competent XL1-Blue cells.

Epicurian Coli XL1-Blue supercompetent cells were purchased from Stratagene and the manufacturers instructions followed for use. Alternatively cells were prepared as described by Inoue *et al.* (1990). In brief, a 1 litre flask containing 125ml of SOB was inoculated with 1ml of a 10 ml LB culture grown up over night from a single colony. The cells were grown at 18°C with gentle rotation until an OD_{600} of 0.6 was reached (~16 hours). The cultures were placed on ice for 10 minutes before pelleting the cells by centrifuging at 3500g for 10 min at 4°C. The cells were resuspended gently in 40 ml of ice cold transformation buffer (TB) and left on ice for 10 minutes before centrifuging at 3500g for 10 minutes at 4°C. The cells were then resuspended gently in 10ml of ice cold TB and

DMSO added to a final concentration of 7%. Finally the cells were placed on ice for 10 minutes before aliquoting into 0.5ml volumes in sterile 1.5ml microfuge tubes and snap freezing in liquid nitrogen. Aliquots were stored at -80°C.

2.17 Storage of bacterial cultures.

E. Coli containing plasmid was stored at -80°C as described by Sambrook *et al.* 1989. Bacterial cultures (0.85ml) were placed in sterile plastic freezing vial with sterile glycerol (0.15ml) and vortexed to mix thoroughly before snap freezing in liquid nitrogen.

2.18 Preparation of DNA.

2.18.1 Hybaid midi prep.

For large amounts (50-200μg) of transfection quality DNA, a Hybaid midi DNA preparation kit was used. 50-100ml of LB containing 100μg/ml ampicillin was inoculated with a single colony of desired plasmid and grown for 16-18 hours at 37°C with shaking at 125rpm. Bacterial cells were collected by centrifuging at 3500g for 10 min and manufacturers instructions followed. DNA was dissolved in 100μl sterile distilled water (SDW) and DNA concentration determined by measuring the absorbance of the sample at OD_{260} and purity assessed by agarose gel electrophoresis (described in 2.20) and ethidium bromide staining.

2.18.2 Qiagen mini prep.

For smaller amounts (15-40μg) of transfection quality DNA, a Qiagen mini prep was performed. 10mL of LB containing 100μg/ml ampicillin was inoculated with a single colony and grown for 16-18 hours at 37°C with shaking at 125rpm. Bacterial cells were collected by centrifuging at 3500g for 10 min and manufacturers instructions followed. DNA was dissolved in 20μl SDW and DNA concentration determined by measuring the absorbance of the sample at OD_{260} and purity assessed by agarose gel electrophoresis (described in 2.20) and ethidium bromide staining.

2.18.3 Wizard Plus SV Minipreps DNA purification system.

For small amounts (15-40μg), of non-transfection quality DNA, a Wizard Plus SV Miniprep DNA purification kit was used. 10ml of LB containing 100μg/ml ampicillin was inoculated with a single colony of desired plasmid and grown for 16-18 hours at 37°C with shaking at 125rpm. Bacterial cells were pelleted by centrifuging at 3500g for 10 min and manufacturers instructions followed. DNA was dissolved in 30μl sterile distilled water and the DNA concentration determined by measuring the absorbance of the sample at OD_{260} and purity assessed by agarose gel electrophoresis (described in 2.20) and ethidium bromide staining.

2.18.4 Quick plasmid mini preps.

A rapid method of DNA preparation was used for recovering plasmids when screening many colonies for correct inserts. 3ml LB cultures containing 100μg/ml ampicillin were inoculated with a single colony and grown for 16 hours with shaking at 225rpm. 1.5ml of culture was poured into an microfuge tube and centrifuged at 13000g in a bench top microfuge to collect cells. Excess liquid was removed and the pellet resuspended in 50μl of rapid lysis buffer (10mM Tris, pH 8.0, 100mM NaCl, 1mM EDTA), followed by the addition of 50μl of phenol chloroform. Samples were vortexed and then centrifuged for 5 minutes at 13000 rpm. The top aqueous layer was removed (~50μl) and placed in clean microfuge tubes. The DNA was precipitated by the addition of twice the volume of ethanol (100μl) and left at -20°C for 15 minutes, spun for 2 minutes to pellet DNA and air dried before resuspending in 10μl sterile distilled water. 3μl of this was used in restriction endonuclease digests and 250ng of RNAse was included.

2.19 Spectrophotometric analysis of DNA.

Spectrophotometric determination of DNA concentration was performed by diluting an aliquot of DNA containing solution into 0.5ml of distilled water. DNA concentration was estimated by measuring absorbance at 260nm and calculated assuming 50μg/ml of DNA measured in a cuvette with a 1 cm path length will have an absorbance of 1. Pure DNA has a A_{260}/A_{280} ratio of > 2.0 (Sambrook *et al.* 1989).

2.20 Agarose gel electrophoresis.

DNA was fractionated on 0.8 to 1% agarose gels essentially according to Sambrook *et al.* (1989). Agarose was heated in TAE buffer (40mM Tris-acetate, 1mM EDTA), using a microwave until all the agarose had dissolved. Once the solution had cooled to hand hot (~50°C), ethidium bromide was added (final concentration 1μg/ml) and the solution poured into a taped tray and a comb inserted to form wells. The gel was allowed to cool to room temp before DNA samples, mixed 6:1 with 6X type 4 DNA loading buffer (0.25% bromophenol blue, 40% (w/v) sucrose in water), were loaded into wells. Gels were run at 100 volts in TAE buffer until the samples had run a appropriate distance to ensure separation. DNA fragments were visualised on a transilluminator and fragments sized by comparison with 1Kb ladder DNA molecular markers (GIBCO Life Technologies Ltd, Paisley, UK), also run on each gel. Gels visualised by transillumination with ultraviolet light were photographed using a Polaroid land camera or images were captured on a Multimage light cabinet (Flowgen, Shenstone, UK).

2.21 Restriction digests.

Restriction endonucleases were purchased from Roche. Typically 1μg of DNA was used for a digest in the manufacturers recommended buffer, with 10 units of each restriction endonuclease, in a final volume of 20μl. The reaction was left to proceed for 1 hour for a single digest or 2 hours for a double digest at the appropriate temperature for the restriction enzyme (usually 37°C).

2.22 Gel extraction of bands for ligation.

Following cleavage with restriction enzymes the whole digest reaction was resolved by agarose gel electrophoresis and the gel visualised on a transilluminator. The DNA fragment was excised from the agarose gel using a clean sharp scalpel blade and placed in a clean microfuge tube. A QIAquick gel extraction kit (Qiagen) was used to extract and purify the DNA from the gel. The manufacturers instructions were followed, briefly the gel containing the DNA was dissolved and applied to a column containing silca-membrane to which the DNA adsorbs in the presence of high salt while contaminants pass through the

column. Impurities were washed away and the DNA eluted with 30µl of sterile distilled water.

2.23 Ligation.

Ligations were usually performed using a vector:insert molar ratio of 1:3 and 50-200ng of vector DNA. The following equation was used to convert molar ratios to mass ratios:

(ng of vector x kb size of insert/kb size of vector) x (molar ratio of insert/vector).

In the ligation reaction, 3 units of T4 DNA Ligase was used in a final volume of 10µl and the ligation mixture was incubated at 14°C overnight. Following the ligation reaction, the ligated DNA was transformed into competent cells.

2.24 Transformations

Supercompetent XL1-Blue cells purchased from Stratagene were used in accordance with manufacturers instructions, typically 50-100µl of cells and 50-100ng of DNA was used in each transformation reaction. XL1-Blue cells made as described in section 2.16 were used as follows, XL1-Blue cells were thawed on ice and 100µl aliquoted into prechilled Falcon 2059 polypropylene tubes on ice. Typically 50ng of DNA was added to each aliquot of cells, swirled gently and incubated on ice for 30 minutes. The tubes were heat pulsed at 42°C for 45 seconds, incubated on ice for two minutes and 0.9ml of preheated (42°C) SOC added. The tubes were incubated at 37°C for 1 hour with shaking at 225 rpm. 50µl and 150µl of the transformation mixture were spread onto agar plates containing the appropriate antibiotic. The plates were then incubated overnight at 37°C.

2.25 Site directed mutagenesis.

Site directed mutagenesis was performed using Stratagene QuikChange site directed mutagenesis kit. In this method two synthetic oligonucleotide primers, each complimentary to opposite strands of the vector and containing the desired mutation, annealed to the plasmid during temperature cycling. The mutagenic primers were

incorporated and extended with high fidelity proof reading DNA polymerase (Pfu), resulting in nicked circular strands. The products were then treated with *Dpn*1 to digest the methylated parental DNA (DNA isolated from almost all *Escherichia coli* is *dam* methylated). The circular nicked DNA was transformed into XL1-Blue competent cells which then repaired the nicks in the mutated plasmid.

Primers were designed following manufacturers instructions and obtained from Genosys Biotechnologies Ltd., (Cambridge, UK). In brief, primers were between 25 and 45 base pairs in length and the melting temperature was ~10°C above the extension temperature of 68°C. The mutation was in the middle of the primer with 10-15 bases of correct sequence either side. Primers had a minimum GC content of 40% and terminated in one or more C or G bases. The primers were purified by polyacrylamide gel electrophoresis (PAGE).

Manufacturers instructions were followed and typically 10ng and 50ng of dsDNA template was used. The reaction was set up in thin walled reaction tubes as follows:

10 x reaction buffer	5µl
DNA template (10ng/µl) 10ng and 50ng	1/5µl
Primer 1 125ng	1µl
Primer 2 125ng	1µl
10mM dNTP mix (2.5mM each NTP)	1µl
SDW	40/36µl
Pfu DNA polymerase (2.5units/µl)	1µl

Reaction was overlaid with one drop of mineral oil. Using a Perkin-Elmer heat cycler, the reaction was subjected to 95°C for 30 seconds followed by 16 cycles of 95°C for 30 seconds, 55°C for 1 minute and 68°C for 2 minutes per kilobase of plasmid length.

After the reaction had cooled, 1µl of *Dpn* 1 restriction enzyme (10u/µl) was added below the mineral oil layer and mixed by pipetting up and down gently. After a brief spin the reaction tubes were incubated at 37°C for 1 hour to digest the parental (the nonmutated) supercoiled dsDNA.

2μl and 4μl of this reaction was used to transform Epicurian Coli XL1-Blue competent cells as described earlier. After transformation the XL1-Blue competent cells repair the nicks in the mutated plasmid.

2.26 Endothelial cell transfections.

BAE cells were trypsinised and plated at approximately 20% confluence the day before transfection into a 90mm plate. Cells were 60-80% confluent when used for transfection. Transfection was performed using Qiagen Superfect™ transfection reagent. Typically 7.5μg of DNA was diluted in DMEM containing no serum or antibiotics to a final volume of 225μl. 38μl of Superfect was added to the DNA and thoroughly mixed by pipetting up and down six times. Samples were incubated for 10 minutes at room temperature to allow complex formation. Cells were washed with 4 ml of PBS and 2 ml of fresh complete DMEM added, followed by the DNA solution and thoroughly mixed. The cells were incubated with the DNA complexes for 2.5 hours before washing twice with PBS and fresh complete medium added. Cells were incubated for 20-24 hours to allow expression of construct.

If whole cell lysates were being analysed for initial expression of constructs, all procedures were as for 90mm plates except cells were seeded into 6 well plates and typically 2μg of DNA was used in the transfection. The DNA was diluted with DMEM containing no serum or antibiotics to a final volume of 150μl and 10μl of Superfect™ was added to the DNA.

2.27 Vectors

The pCR3 vector was obtained from Invitrogen (CH Groningen, The Netherlands). PFLAG-CMV2 was obtained from Sigma-Aldridge (Poole, Dorset, UK).

The pGEX-C terminal FAK and pGEX were a kind gift from Prof. D Critchley, Dept. of Biochemistry, University of Leicester, UK.

The pGreen Lantern-1 expression vector was obtained from Gibco Life Technologies Ltd. (Paisley, UK).

2.28 Cloning of focal adhesion kinase into pFLAG-CMV2 expression vector.

cDNA corresponding to mouse FAK was obtained from the American Tissue Culture Company in the PT7-7 vector (ATCC clone code 63207). The FAK cDNA was subcloned from the PT7-7 vector into pFLAG-CMV2 expression vector (Sigma-Aldridge, Poole, UK). To do this a Not1 site was introduced into the 5' end of the full length FAK insert of PT7-7-FAK using site directed mutagenesis as described in section 2.25. Primers were designed to alter nucleotide 115 from an A to a G and nucleotide 118 from a T to a C therefore introducing a Not1 restriction endonuclease site.

FAKNF	5'- GC AAA AGA ATG GC**G** GC**C** GCT TAT CTT GAC CC-3'
FAK cDNA	102-GC AAA AGA ATG GCA GCT GCT TAT CTT GAC CC-132

The nucleotide mismatches are shown in bold and the newly introduced Not1 site is underlined.

Following site directed mutagenesis, FAK cDNA was subcloned into pFlag-CMV2 by restriction digests of both plasmids with Not1 and Blg2 (see appendix 2 for pFLAG-CMV2 multiple cloning site and vector map). The FAK insert was digested by Not1 restriction endonuclease at nucleotide 114 and Blg2 restriction endonuclease at nucleotide 3306. This FAK fragment was gel purified and ligated into pFlag-CMV2 which had also been digested with Not1 and Blg2. Competent XL1-Blue cells were transformed with the ligation product DNA as described in section 2.24. Ampicillin resistant transformants were screened for the presence of FAK cDNA inserts by quick plasmid mini preparations as described in section 2.18.4 followed by restriction digests with Not1 and Bgl2. The entire FAK sequence excluding the start methionine was subcloned into the pFlag-CMV2 construct resulting in N-terminally flag tagged FAK which was assigned the name pFFfl and corresponding protein, FFfl (see figure 2.1 for schematic representation of FFfl).

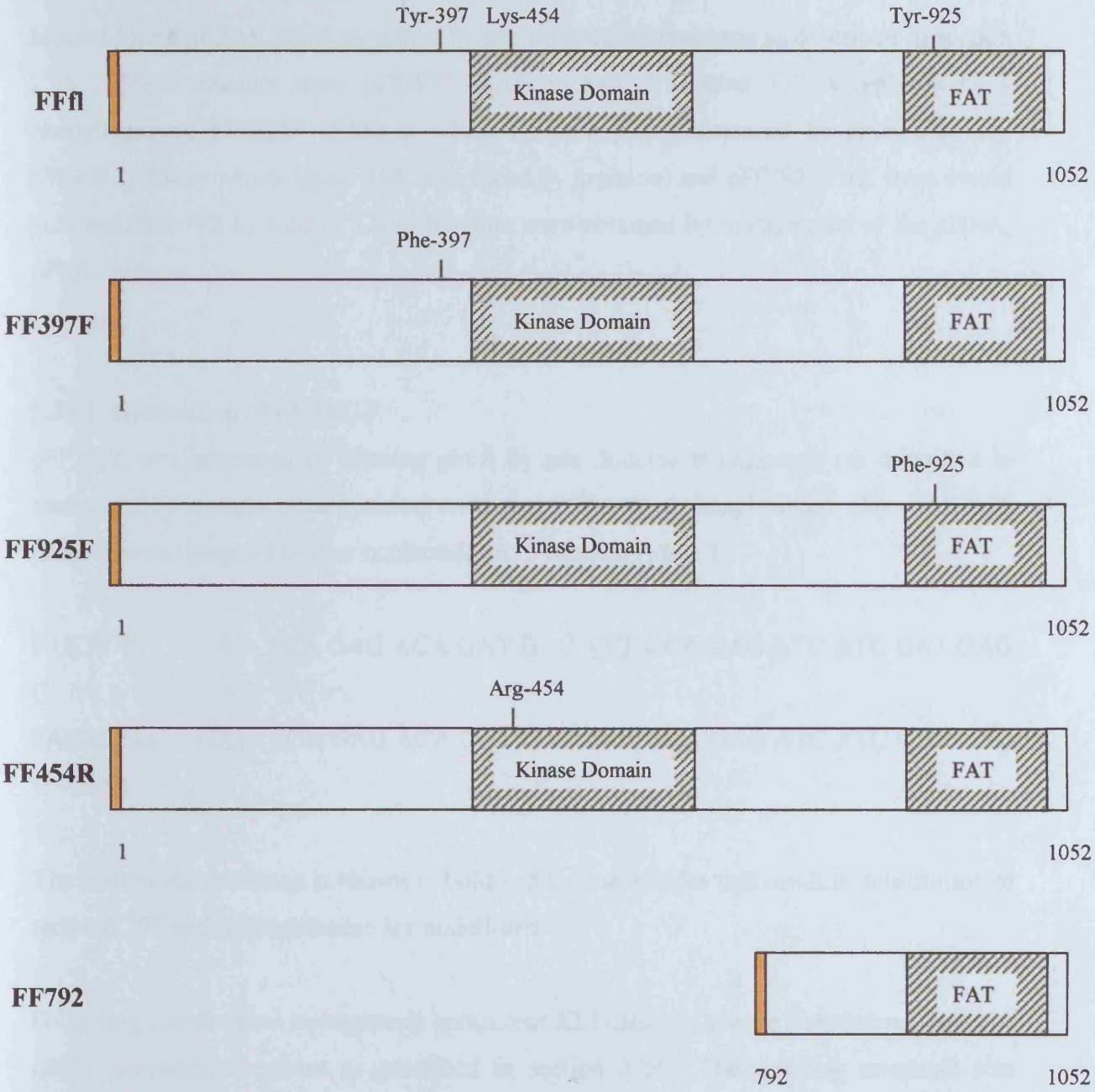

Figure 2.1. Schematic representation of the mutant FAK proteins generated.

The flag tag is represented by the orange rectangle. Indicated are the kinase domain and the FAT domain (focal adhesion targeting domain) of FAK. The amino acids encoded by each construct and the mutation introduced into each FAK mutant are indicated. See sections 2.29.1 - 4.

2.29 Generation of FAK mutant constructs

Mutant forms of FAK were generated by site directed mutagenesis as described in section 2.25. These mutants were pFF397F (FAK in which tyrosine 397 is replaced by a phenylalanine), pFF925F (FAK in which tyrosine 925 is replaced by phenylalanine), pFF454 (FAK in which lysine 454 is replaced by arginine) and pFF792 (FAK from amino acid residues 792 to 1052). These mutants were obtained by mutagenesis of the cDNA, pFFfl.

2.29.1 Generation of pFF397F

pFF397F was generated by altering pFFfl by site directed mutagenesis (as described in section 2.25) to replace the tyrosine residue at 397 with a phenylalanine. The FAK397F primers were designed to alter nucleotide 1299 from an A to a T.

FAK397F 5' - TCA GAG ACA GAT GAC **TTT** GCA GAG ATC ATC GAT GAG G –3'

FAK cDNA 1283 - TCA GAG ACA GAT GAC TAT GCA GAG ATC ATC GAT GAG G - 1319

The nucleotide mismatch is shown in bold and the nucleotides that result in substitution of tyrosine 397 with phenylalanine are underlined.

Following site directed mutagenesis competent XL1-Blue cells were transformed with the newly generated construct as described in section 2.24. The resulting construct was assigned the name pFF397F and the resulting protein FF397F (see figure 2.1 for schematic representation of FF397F).

2.29.2 Generation of pFF925F

pFF925F was generated by altering pFFfl by site directed mutagenesis to replace the tyrosine residue at 925 with a phenylalanine. The FF925F primers were designed to alter nucleotide 2883 from an A to a T.

FF925F 5' – G TCC AAT GAC AAG GTA **TTT** GAG AAT GTG ACA GGC C – 3'

FAK cDNA 2866 - G TCC AAT GAC AAG GTA TAT GAG AAT GTG ACA GGC C - 2900

The nucleotide mismatch is shown in bold and the nucleotides that result in substitution of tyrosine 925 with phenylalanine are underlined.

Following site directed mutagenesis competent XL1-Blue cells were transformed with the newly generated construct as described in section 2.24. The resulting construct was assigned the name pFF925F and the resulting protein FF925F (see figure 2.1 for schematic representation of FF925F).

2.29.3 Generation of pFF454R

pFF454R was generated by altering pFFfl by site directed mutagenesis to replace lysine 454 with an arginine. The FF454F primers were designed to alter nucleotide 1470 from an A to a G.

FAK454F 5' – CCA GCT TTG GCT GTT GCA ATC **AGA** ACA TGT AAA AAC TGT AC – 3'

FAK cDNA 1448 - CCA GCT TTG GCT GTT GCA ATC AAA ACA TGT AAA AAC TGT AC - 1488

The nucleotide mismatch is shown in bold and the nucleotides that result in substitution of lysine 454 with arginine are underlined.

Following site directed mutagenesis competent XL1-Blue cells were transformed with the newly generated construct as described in section 2.24. The resulting construct was assigned the name pFF454R and the resulting protein FF454R (see figure 2.1 for schematic representation of FF925F).

2.29.4 Generation of pFF792

To generate truncated C-terminal FAK, pFF792 (which comprised of amino acids 792-1052), a Not1 restriction site was introduced at nucleotide position 2581 by site directed mutagenesis as described in section 2.25. Primers were designed to alter nucleotide 2580 from a T to a G and nucleotide 2585 from an A to a C therefore introducing a Not1 restriction endonuclease site.

FAK792F 5'- AGT GTG GAG GAT **G**CG GC**C** GCT TTG GAC CTT CG -3'
FAK cDNA 2568- AGT GTG GAG GAT TCG GCA GCT TTG GAC CTT CG- 2599

The nucleotide mismatches are shown in bold and the newly introduced Not1 site is underlined.

Following the introduction of a Not1 site by directed mutagenesis the newly generated construct was digested with the restriction endonuclease Not1 which resulted in the production of two fragments, one consisting of the first two thirds of FAK (residues 1-791) and the second comprising of the C-terminal third of FAK (residues 792-1052) and pFlagCMV2 in a linearised form. The fragments were separated by agarose gel electrophoresis and the appropriate band excised and gel purified. A simple religation of the linearised fragment containing pFlag and the C-terminal third of FAK was performed. Competent XL1-Blue cells were transformed with the ligation product DNA as described in section 2.24. Ampicillin resistant transformants were screened for the presence of FAK cDNA inserts by quick plasmid mini preparations as described in section 2.18.4 followed by restriction digests with Not1 and Bgl2. This construct was assigned the name pFF792 and the corresponding protein FF792 (see figure 2.1 for schematic representation of FF792).

2.30 Production of pCR3-FAK

To enable expression of FAK which was not epitope tagged, full length FAK was subcloned from pFFfl into the pCR3 expression vector (see appendix 2 for pCR3 multiple cloning site and vector map). Full length FAK cDNA insert was excised from pFFfl by restriction digest with the endonuclease Not1 and Xba1. The pCR3 vector was also digested with the same restriction endonucleases. The excised FAK fragment and

linearised pCR3 vector were gel purified and ligated. Competent XL1-Blue cells were transformed with the ligation product DNA as described in section 2.24.

2.31 Preparation of GST-C-terminal FAK fusion protein.

The pGEX-C-terminal FAK construct was a kind gift from Prof. D Critchley, Dept. of Biochemistry, University of Leicester, UK. E. Coli JM101 cells (or E. Coli BL21 cells) were transformed with the pGEX-C-terminal FAK plasmid were induced to express the GST-C-terminal FAK fusion protein. To do this approximately 500ml of 2YT media was inoculated with 5ml of overnight GST fusion protein culture and grown at 37°C with shaking at 200rpm until an $O.D._{600}$ of approximately 1.0 was reached. IPTG was added to a concentration of 0.1mM to induce expression of the fusion protein. After a further 1-2 hours of growth at 37°C at 200rpm the bacterial cells were pelleted by centrifuging at 3500g for 10 minutes at 4°C. The pellet was resuspended in 25ml of ice-cold PBS containing a cocktail of protease inhibitors (complete mini tablets, Roche Diagnostics, Lewes, UK) and divided into five separate 5ml samples and lysed by sonication. The tubes were kept on ice during sonication of 10 second periods which were repeated four times. Triton X-100 was added to the pooled sonicate to a final concentration of 1% and this was incubated at 4°C for 30 minutes. The insoluble material was pelleted by centrifuging at 20000g for 15 minutes at 4°C. The supernatant was incubated with 700µl of 50% glutathione-agarose slurry on a rotary mixer for one hour at 4°C. The G-agarose beads were pelleted by centrifuging at 500g at 4°C for 1 minutes and the supernatant discarded. The beads were washed three times with PBS containing protease inhibitors. The GST fusion protein was eluted from the agarose beads by incubation with one bead volume of 10mM reduced glutathione in 50mM Tris-HCl pH 8.0 for 1 hour at room temperature.

The yield of the fusion protein was calculated using GeneQuant and the solution diluted to a concentration of 2mg/ml in microinjection buffer (75mM KCL, 10mM Potassium phosphate). To analyse the purity of the expressed GST-C terminal FAK fusion protein a 10 µl sample was boiled for 5 minutes with 40µl of reducing sample buffer (containing 200mM DTT), centrifuged at 10000g for 5 minutes before loading onto a 10 % SDS-PAGE gel. After electrophoresing the samples for approximately 45 minutes the gel was stained with 0.05% Coomassie brilliant blue as described in section 2.7.

E. Coli JM101 cells transformed with the control pGEX plasmid (kind gift from Prof. D Critchley, Dept. of Biochemistry, University of Leicester, UK) were induced to express GST protein following the same procedure as for pGEX-C-terminal FAK as described above. The GST was diluted to concentration of 2mg/ml in micro-injection buffer (75mM KCL, 10mM Potassium phosphate).

2.32 Micro-injection of HUVE cells

HUVE cells for micro-injection were grown on gridded coverslips to confluence before serum starving overnight. One-half of the cells were removed from the coverslip using a cell scraper. After 30 minutes incubation at 37°C in 5% CO_2 the position of the wound on the grid was noted and the cells along the wound edge were micro-injected.

Micro-injection was performed using a Nikon diaphot 200 with an eppendorf micromanipulator 5171 and an eppendorf transjector 5246. Cells were injected using sterile micro-injection capillaries (femtotip, eppendorf). Femtotips were filled using a micro-loading pipette (eppendorf). Cells were micro-injected at a pressure of 150 hPA for 0.1-0.2 seconds. Typically 20-50 cells were micro-injected in 30 minutes. All proteins were micro-injected at a concentration of 2mg/ml in microinjection buffer (75mM Potassium Chloride, 10mM Potassium phosphate).

2.33 Apoptosis assay

One of the first detectable nuclear change in apoptotic cells is the condensation of the nucleus. The condensed nucleus then becomes fragmented into small dense particles. These changes can be visualised in cells stained with the DNA intercalating Hoechst 33342 dye. Non apoptotic cells stained with the dye display blue nuclei while apoptotic cells have smaller more intensely stained blue nuclei reflecting the nuclear chromatin condensing during early apoptosis. In addition, apoptotic cells exhibiting nuclear fragmentation are also observed with dense blue particles.

To examine the effects of FAK mutants on apoptosis, BAE cells were grown in 35mm dishes and cotransfected with 1.25μg of FAK mutant and 1.25μg of pGreen Lantern-1

(pGL) or cotransfected with the empty pFlag vector and 1.25μg of pGL. The pGreen lantern-1 encodes a modified version of the green fluorescent protein and allows visualisation of the transfected cells by fluorescence microscopy. Eighteen to twenty hours post-transfection the medium was gently removed and replaced with medium containing 10μg/ml of Hoechst dye and cells incubated at 37°C for 30-60 minutes. Cells were then analysed using a fluorescence microscope for nuclear morphology (Hoechst excitation 350nm, emission 461nm) and green lantern expression (excitation 488nm, emission 530nm). Using fluorescence it was possible to identify the transfected cells and by changing the filter, the transfected cell were categorised as apoptotic or normal cells according to their nuclear morphology. Five random fields of cells were viewed on each slide and the fraction of green lantern expressing cells undergoing apoptosis determined.

2.34 Densitometric scanning

Densitometric scanning was carried out using a Sharp JX-330 scanner and analysed with ImageMaster 1D software (Pharmacia Biotech UK Ltd., Buckinghamshire, UK).

2.35 Statistical analysis

Statistical analysis was performed using the paired or unpaired Students t-test as stated in the figure legends. An unpaired test compares groups when the individual values are not paired or matched with one another. A paired test is appropriate for repeated experiments run at different times, each with its own control.

CHAPTER THREE

Modulation of FAK phosphorylation in response to VEGF and adhesion

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Introduction

Vascular endothelial growth factor is an angiogenic factor (Connolly *et al.* 1989, Ferrara and Henzel 1989, Levy *et al.* 1989, Leung *et al.* 1989). It also is mitogenic for endothelial cells and mediates changes to the cytoskeleton, inducing membrane ruffles, actin stress fibre formation, cell shape change and migration (Ferrara and Henzel 1989, Plouet *et al.* 1989, Waltenberger *et al.* 1994, Yoshida *et al.* 1996, Senger *et al.* 1996, Abedi and Zachary 1997). In addition, VEGF also increases vascular permeability (Senger *et al.* 1983 and 1990), an effect also likely to involve the cytoskeleton. Knowledge of the signalling pathways activated by VEGF is limited. VEGF has two main receptors, VEGFR-1 and 2 (de Vries *et al.* 1992, Terman *et al.* 1992). VEGFR-2 is efficiently autophosphorylated in response to ligand binding whereas autophosphorylation of VEGFR-1 is almost undetectable (Waltenberger *et al.* 1994, Yamane *et al.* 1994, De Vries *et al.* 1992, Seethram *et al.* 1995). VEGF has been reported to induced tyrosine phosphorylation of numerous proteins. Some of the proteins identified include phospholipase Cγ (PLCγ), Ras GTPase activating protein (GAP), the oncogenic adaptor protein Nck and phosphatidylinositol 3-kinase (P1-3 kinase) (Guo *et al.* 1995).

Permeability is dependent on modulation of cell:cell interactions whereas efficient migration requires modulation of the cell:substrate adhesions to obtain optimum adhesiveness. Potential candidates for mediation of permeability, changes in the cytoskeleton and migration induced by VEGF would be proteins localised to cell:substrate and cell:cell adhesions. One such protein is FAK, which has a vital role in migration and is involved in cytoskeletal organisation. FAK is tyrosine phosphorylated in response to adhesion (Burridge *et al.* 1992, Guan and Shalloway 1992, Hanks *et al.* 1992, Kornberg *et al.* 1992). Cells from FAK deficient mice exhibit increased numbers of adhesions, disorganisation of the cortical cytoskeleton and reduced migration (Ilic *et al.* 1995). Microinjection of a truncated FAK, which displaces endogenous FAK from focal adhesions, inhibited migration (Gilmore and Romer, 1996) whereas overexpression of

FAK stimulated cell migration (Cary *et al.* 1996). The tyrosine phosphorylation state of FAK appears to be important in its capacity to modulate cell migration. FAK is tyrosine phosphorylated in migrating cells (Romer *et al.* 1994) and deletion of its autophosphorylation site inhibits the increase in migration observed in FAK overexpressing cells (Cary *et al.* 1996).

Focal adhesion kinase is a tyrosine kinase and possesses phosphoacceptor sites and two proline rich regions (Calab *et al.* 1995 and 1996, Schaller *et al.* 1994, Schlaepfer et al. 1994, Hildebrand *et al.* 1995 and 1993, Tachibana *et al.* 1995). Phosphorylation of the tyrosine residues in the catalytic domain of FAK increase its tyrosine kinase activity (Calab *et al.* 1995). The phosphoacceptor sites, when phosphorylated, allow signalling proteins containing SH2 domains to bind. Proteins reported to bind to FAK include the Src family kinases (Cobb *et al.* 1994), Grb2 (Schlaepfer *et al.* 1994), Grb7 (Han and Guan 1999), PI3K (Chen and Guan 1994) and shc (Schlaepfer *et al.* 1998). Proteins containing SH3 domains are able to bind to the two proline rich domains in FAKs C-terminal domain and include p130Cas (Polte and Hanks 1995 and 1997, Harte *et al.* 1996), p85 subunit of PI3K (Guinebault *et al.* 1995) and Graf (Hildebrand *et al.* 1996).

The initial aim of this study was to investigate whether FAK played a role in mediating signalling pathways activated by VEGF. If FAK did mediate signals induced by VEGF, which VEGF receptor mediated the phosphorylation of FAK and was this activation direct or modulated via other signalling molecules. In addition, modulation of the tyrosine phosphorylation of FAK and its involvement of other signalling proteins in the signalling cascade were investigated.

Modulation of FAK phosphotyrosine in response to VEGF and adhesion.

3.1 Adhesion induces tyrosine phosphorylation of FAK.

Initial experiments aimed to determine conditions under which FAK could be immunoprecipitated and show changes in tyrosine phosphorylation status in response to known stimuli. The best characterised stimulus for activation of tyrosine phosphorylation of FAK is cell adhesion (Burridge *et al.* 1992, Guan and Shalloway 1992, Hanks *et al.* 1992, Kornberg *et al.* 1992). To determine the effect of adhesion on FAK phosphorylation in endothelial cells Human Umbilical Vein Endothelial (HUVE) cells from an $80cm^2$ flask were trypsinised, resuspended and either plated onto fibronectin and allowed to spread for 60 minutes, or held in suspension for the same period of time. Focal adhesion kinase was immunoprecipitated from the cell lysates. Immunoprecipitates were subjected to SDS-PAGE electrophoresis and western blotting followed by analysis of FAK phosphotyrosine level. Phosphorylation status of immunoprecipitated FAK was analysed by probing blots with anti phosphotyrosine RC20. In HUVE cells adherent to fibronectin FAK was tyrosine phosphorylated (figure 3.1). In contrast, cells held in suspension had a markedly decreased level of FAK tyrosine phosphorylation. These experiments confirmed previously published data on endothelial (Romer *et al.* 1994, Sankar *et al.* 1995, Williams *et al.* 1996) and other cell types such as fibroblasts (Burridge *et al.* 1992, Guan and Shalloway 1992, Hanks *et al.* 1992) and provided a demonstration that the conditions used for cell lysis, immunoprecipitation and phosphorylation in these experiments could detect FAK phosphorylation. It was not possible to analyse these blots by densitometric scanning as re-probing for FAK did not provide clear enough blots for normalisation with respect to the amount of FAK.

3.2 VEGF stimulates tyrosine phosphorylation of FAK in human endothelial cells.

To examine the effects of the pro-angiogenic and permeability factor, VEGF, on tyrosine phosphorylation state of FAK, endothelial cells were challenged with the growth factor at a concentration of 2.5nM for 10 minutes. Cells were then lysed and FAK immunoprecipitated. Immunoprecipitates were resolved by SDS PAGE and the

phosphorylation state of FAK assessed by probing western blots of the transferred proteins with anti-phosphotyrosine antibodies (figure 3.2A left panel). Blots were stripped and re-probed with an antibody against FAK (figure 3.2A right panel). As shown in figure 3.2A, VEGF induced a marked increase in the phosphorylation state of focal adhesion kinase compared to control treated cells. Similar analysis of FAK phosphorylation in a series of experiments and quantitation of tyrosine phosphorylation and FAK levels by densometric scanning of western blots was performed. The relative phosphorylation states were normalised with respect to FAK level in each lane. This revealed VEGF increased FAK phosphorylation 4.4 fold compared to control treated cells (see figure 3.2B).

3.3 Fibroblast Growth Factor (FGF) stimulates tyrosine phosphorylation of FAK in human endothelial cells.

In addition to VEGF, basic Fibroblast Growth Factor (bFGF) is a potent activator of angiogenesis, endothelial migration and cytoskeletal re-organisation (Montesano *et al.* 1986, Baffour *et al.* 1992, Presta *et al.* 1986, Sato and Rifkin 1988). It was of interest therefore to determine whether this agent also modulated FAK phosphorylation. HUVE cells were therefore challenged with control vehicle or bFGF for 10 minutes before lysis and immunoprecipitation of FAK. The tyrosine phosphorylation state of FAK was then determined by phosphotyrosine immunoblotting. As with VEGF, the mitogen bFGF, also induces an increase in the tyrosine phosphorylation state of FAK (figure 3.3). It was not possible to analyse these blots by densitometric scanning as re-probing for FAK did not provide clear enough blots for normalisation with respect to the amount of FAK.

3.4 Time course of VEGF stimulated tyrosine phosphorylation of FAK

To examine the time course of VEGF activation of FAK, HUVE cells were treated with VEGF for various times and immunoprecipitated FAK analysed for tyrosine phosphorylation by western blotting. Following probing, bands on western blots were quantitated by densitometric scanning and the time course of VEGF activity shown in figure 3.4. The blots demonstrated there was only a slight increase in tyrosine phosphorylation after 30 seconds and maximal tyrosine phosphorylation was observed at

10 minutes. This phosphorylation level subsequently declined although it was still above control levels after 30 minutes.

3.5 Concentration dependence of VEGF stimulated FAK phosphorylation.

HUVE cells express two receptor populations having markedly different affinities for VEGF, (Waltenburger *et al.* 1994). These receptors are reported to have Kd values for VEGF of approximately 9pM/L and 770pM/L for VEGFR-1 and 2 respectively. In order to gain an insight into which receptor population was mediating the effect of VEGF on FAK, the concentration dependence of VEGF activation of FAK was investigated. HUVE cells were treated with VEGF at various concentrations for ten minutes and immunoprecipitated FAK analysed for tyrosine phosphorylation by western blotting. Following probing, bands on western blots were quantitated by densitometric scanning and the concentration dependency of VEGF activity shown in figure 3.5. FAK phosphorylation in response to VEGF was concentration dependent. The peak activation was observed at 2.5nM/L and concentrations above this level did not further enhance this phosphorylation. The half maximal stimulation of FAK phosphorylation was induced by a VEGF concentration of 120pM/L.

3.6 Signalling pathway activated by VEGF involved in the induction of tyrosine phosphorylation of FAK.

3.6.1 The VEGF receptors do not directly interact with FAK to induce activation and intermediate signalling molecules lie between VEGF receptors and activation of FAK.

The VEGF receptors have tyrosine kinase activity and therefore could directly phosphorylate FAK. The possibility, therefore, that FAK and the VEGF receptors interact directly was examined by analysing FAK recovered from control and VEGF stimulated cells for the presence of bound VEGF receptors.

Blots demonstrating the increase in FAK phosphorylation in response to VEGF were stripped and re-probed with antibodies to the VEGF receptors, VEGFR-1 and 2. If these

receptors directly interact with FAK, it will be bound to them and therefore they will coimmunoprecipitate when FAK is immunoprecipitated. Western blot membranes were re-probed with anti-VEGFR-1 or anti-VEGFR-2. As shown in figure 3.6, although FAK was recovered from cells and demonstrated increased tyrosine phosphorylation in response to VEGF, re-probing blots with antibodies to VEGFR-1 and 2 failed to demonstrate bound VEGF receptor co immunoprecipitating with FAK. It is important to note however that there was no positive control to ensure that the antibodies for the two VEGF receptors were working satisfactorily. It is possible that FAK does associate with the VEGF receptors but the immunoprecipitation method used in this system disrupts any association. The time course demonstrated that maximal activation of FAK did not occur for 10 minutes which suggested that intermediate signalling molecules lay between FAK and the VEGF receptors. The time course and immunoblotting data together suggest the VEGF receptors did not directly interact with FAK. In addition, proteins of the molecular weight predicted for VEGFR-1 and 2 did not appear when the membranes were probed with anti-phosphotyrosine antibodies. VEGFR-1 and VEGFR-2 become tyrosine phosphorylated upon VEGF stimulation of HUVE cells (Kanno *et al.* 2000) and if these receptors where co-immunoprecipitated with FAK they would appear on the anti-phosphotyrosine blots.

3.6.2 Involvement of phosphatidylinositol 3'-kinase in VEGF stimulated tyrosine phosphorylation of FAK.

The amino acid sequence of VEGF has a limited but significant homology to the A and B chains of PDGF. Both growth factors are dimers and their folding could be similar as all eight cysteine residues involved in the intra and inter chain disulphides in PDGF B are conserved in VEGF. Clusters of basic amino acids in the COOH terminal halves of human VEGF and PDGF B commonly occur (Keck *et al.* 1989, Tischer *et al.* 1989, Conn *et al.* 1990). PDGF, like VEGF, also increases the tyrosine phosphorylation level of FAK (Rankin and Rozengurt 1994) and it has more recently been demonstrated that PI-3 kinase activation is necessary for PDGF stimulated tyrosine phosphorylation of FAK (Rankin *et al.* 1996, Saito *et al.* 1996). Due to the similarities between PDGF and VEGF it is possible PI-3 kinase activation is also necessary for VEGF stimulated tyrosine phosphorylation of FAK. The involvement of PI-3 kinase in VEGF stimulated tyrosine phosphorylation of FAK was investigated to determine whether PI-3 kinase activity is a general requirement

for receptor tyrosine kinase activation of FAK phosphorylation. Wortmannin was used to inactivate PI 3-kinase as it directly binds to and inhibits its catalytic subunit (Yano *et al.* 1993, Arcaro and Wymann 1993, Okada *et al.* 1994).

Quiescent cells were pre-incubated for 15 minutes with 100nM wortmannin before the addition of 2.5nM VEGF for 10 minutes. Quantitative analysis of tyrosine phosphorylation and FAK levels was performed by densometric scanning. The relative phosphorylation states were normalised with respect to the FAK level in each lane. As shown in figure 3.7A and C, the level of tyrosine phosphorylation of FAK upon VEGF stimulation was slightly reduced but not abolished in the presence of wortmannin. This suggests that PI-3 kinase may have a minor role in VEGF activated FAK phosphorylation. As shown in figure 3.7C, in this series of experiments there was a large variation in the degree to which VEGF activated FAK phosphorylation. More detailed investigation will be required to assess the degree to which VEGF induced FAK phosphorylation is wortmannin sensitive.

In order to determine whether wortmannin could inhibit FAK phosphorylation induced by other agonists at the concentration used in HUVE cells, the effect of the inhibitor on lysophosphatidic acid induced FAK phosphorylation in these cells was examined. Lysophosphatidic acid (LPA) induces FAK phosphorylation in mouse fibroblasts, (Kumagai *et al.* 1993). The effects of LPA and wortmannin on FAK phosphorylation in HUVE cells was therefore examined. As shown in figure 3.7B (lanes one and two), LPA induced FAK phosphorylation in HUVE cells as assessed by anti-phosphotyrosine immunoblotting of immunoprecipitated FAK following challenge with 2μM LPA. Preincubation of cells with 100nM wortmannin for 15 minutes inhibited the effect of LPA on FAK phosphorylation in HUVE cells (figure 3.7B lanes two and four), demonstrating wortmannin was effective in HUVE cells at the concentration used. Quantitative analysis of tyrosine phosphorylation and FAK levels was performed by densometric scanning. The relative phosphorylation states were normalised with respect to the FAK level in each lane. As shown in figure 3.7D, compared to control treated cells, LPA stimulated a 2.6 fold increase in FAK tyrosine phosphorylation which was completely inhibited by incubation with wortmannin.

3.6.3 Wortmannin inhibits VEGF induced membrane ruffling.

PDGF induced membrane ruffling has been shown to be dependant on wortmannin inhibitable PI-3 kinase activity in other cell types (Rankin *et al.* 1996). As a further test that wortmannin had effects on HUVE cells, therefore, the effects of the inhibitor on VEGF induced ruffling was examined. HUVE cells were grown on coverslips and treated with control vehicle or 2.5nM VEGF in the presence or absence of wortmannin for 10 minutes. Cells were fixed and stained for filamentous actin as described in materials and methods. The effect of VEGF on the endothelial cytoskeleton is shown in figure 3.8B. To determine the effects of wortmannin the percentage of cells exhibiting edge ruffles was determined for 10 randomly selected fields on each coverslip. Means and deviation from the mean is presented for duplicate experiments. As shown in figure 3.8A, VEGF stimulated ruffling in HUVE cells. In the presence of the PI3K inhibitor, wortmannin, a decrease the effect of VEGF on membrane ruffling was seen.

These data suggest that there may be differences between induction of FAK tyrosine phosphorylation by PDGF, as reported in fibroblasts (Rankin and Rozengurt 1994) and the effects of VEGF on FAK tyrosine phosphorylation. Rankin et al (1996) report that wortmannin abolishes PDGF stimulated tyrosine phosphorylation. If further investigation confirm a partial or minor effect of wortmannin on VEGF induced FAK tyrosine phosphorylation it would suggest differences in the intracellular signalling pathways by which VEGF and PDGF for stimulate tyrosine phosphorylation of FAK.

3.6.4 Interaction of FAK with Src and Fyn.

The lack of any apparent direct interaction between VEGF receptors and FAK suggested that following VEGF activation, FAK tyrosine phosphorylation results from stimulation of an intracellular tyrosine kinase active on FAK, inhibition/release of a tyrosine phosphatase or induction of FAK trans or autophosphorylation. The intracellular tyrosine kinases of the Src family have previously been shown to associate with FAK and are therefore candidates for mediating the effects of VEGF on FAK tyrosine phosphorylation. The possibility that VEGF modulates association of Src family kinases with FAK was therefore examined.

HUVE cells were stimulated with VEGF or control vehicle and FAK was immunoprecipitated using Backer buffer so as to minimise disruption of any bound proteins. After probing the nitrocellulose membrane with anti phosphotyrosine and FAK antibodies the membrane was stripped and re-probed with antibodies to Fyn and Src. As shown in figure 3.9A, increased levels of Src and Fyn co-immunoprecipitated with FAK stimulated with VEGF compared with control. Quantitative analysis of Src, Fyn and FAK levels was performed by densitometric scanning of the western blots from a series of experiments. The relative amounts of Src or Fyn were normalised with respect to FAK levels in each lane. As demonstrated in figure 3.9B immunoprecipitated FAK from cells stimulated with VEGF revealed a 2.4 and 3.1 fold increase in association with Src and Fyn respectively compared to control treated cells.

FAK does not possess any SH2 domains itself but it contains several tyrosine residues, which upon phosphorylation, provide binding sites for SH2 domain containing proteins including Src and Fyn (Schaller *et al.* 1994, Cobb *et al.* 1994, Xing *et al.* 1994). The far western technique was employed to establish whether Fyn bound directly to FAK via its SH2 domain.

HUVE cells were stimulated with VEGF, FAK was immunoprecipitated and detected by western blotting. Slightly less FAK was immunoprecipitated from the VEGF stimulated cells however VEGF stimulated a slight increase in tyrosine phosphorylation of FAK. The blot was then stripped and incubated with the SH2 domain of Fyn (fused with GST) at a concentration of 2μg/ml. Any bound fusion protein was then detected with an anti-GST antibody. As shown in figure 3.10A and B, the Fyn SH2 domain binds to FAK demonstrating Fyn interacts directly with FAK and via its SH2 domain and not via another protein. It did not appear that the level of Fyn-SH2 domain bound to FAK was affected by the phosphorylation state of FAK although the increase in FAK tyrosine phosphorylation was not great enough to reach a clear conclusion. Further experiments are therefore required to determine whether increased binding of Fyn/Src to FAK in response to VEGF was as a result of increased binding sites on FAK. VEGF stimulated recruitment to FAK would allow Src family kinases to stimulate tyrosine phosphorylation of FAK thereby mediating the effects of VEGF on FAK tyrosine phosphorylation.

To test this hypothesis further a mutant form of Fyn was used that lacked kinase activity. In addition a number of mutant forms of FAK were generated to probe the mechanism of VEGF induced FAK tyrosine phosphorylation and recruitment of Src family kinases. These experiments are described in chapter four.

3.6.5 Interaction of FAK with the p85 subunit of PI-3 kinase and Grb2

In addition to Src and Fyn, FAK has been reported to recruit the p85 subunit of PI-3 kinase and the adapter protein Grb2. These signalling intermediates have been shown to associate with FAK in response to cell adhesion (Chen and Guan 1994a, Chen *et al.* 1996, Schlaepfer *et al.* 1994). It was of interest therefore to determine whether VEGF modulated FAK recruitment of the p85 subunit of PI-3 kinase or Grb2. HUVE cells were stimulated with VEGF or control vehicle and FAK was immunoprecipitated using Backer buffer so as to minimise disruption of any bound proteins. After probing the nitrocellulose membrane with anti phosphotyrosine and FAK antibodies the membrane was stripped and re-probed with anti bodies to Grb2 and the p85 subunit of PI-3 kinase. Under the conditions used, no detection of the p85 subunit of PI-3 kinase was observed after immunoprecipitation, suggesting VEGF activation of FAK does not result in this molecule associating with FAK (figure 3.11 A). The p85 subunit was detected in whole cell lysates demonstrating the antibody was functional (figure 3.11 B, right panel). The recruitment of Grb2 to FAK was unclear. In a number of experiments VEGF was not consistently shown to stimulate Grb2 binding to FAK. As shown in figure 3.11 B (left panel), Grb2 was detected in whole cell lysates demonstrating the antibody was functional.

3.7 Bovine endothelial cells.

To investigate the mechanism of VEGF induced FAK tyrosine phosphorylation and involvement of Src and Fyn, a dominant negative approach was planned. This entailed expression of mutated signalling constructs in endothelial cells. A number of different conditions were tested for transfection of these cells. The transfection efficiencies were determined by expression of a green fluorescent marker protein by the cells. Despite

several attempts it was not possible to establish transfection conditions which resulted in more than 2% of HUVE cells expressing the GFP marker protein.

It was considered impractical to obtain meaningful data from immunoprecipitation and blots from a population of cells in which only 2% of the cells expressed the test construct. To pursue the dominant negative approach therefore an alternative endothelial background was chosen. Transfection of BAE cells resulted in a higher percentage transfection efficiency and more consistent levels of expression.

BAE cells express both VEGFR-1 and 2 (Pepper *et al.* 1998) however changing to BAE cells for dominant negative experiments necessitated characterization of FAK phosphorylation in these cells. To do this the effects of adhesion and VEGF on FAK tyrosine phosphorylation was examined in BAE cells. Unfortunately at this stage in the study the commercially available antibody used for FAK immunoprecipitation ceased to be effective. Despite numerous attempts it was not possible any longer to consistently immunoprecipitate FAK from the HUVE or BAE cells with this antibody. A series of experiments were performed to test the ability of DRC anti FAK and a number of commercially available antibodies for their ability to immunoprecipitate FAK. A suitable antibody was not found.

To over come this problem, phosphorylated FAK was immunoprecipitated with anti phosphotyrosine antibody linked to agarose beads and the amount of FAK immunoprecipitated determined by anti-FAK immunoblotting. This method was not so desirable as proteins associated with FAK cannot be studied as all tyrosine phosphorylated proteins are immunoprecipitated. Another problem with this method is proteins which are not tyrosine phosphorylated can be immunoprecipitated if they are associated with other tyrosine phosphorylated proteins. This can give a false impression of the tyrosine phosphorylation level of immunoprecipitated proteins. However, this can be overcome by reprobing blots with phosphotyrosine antibodies to ensure the immunoprecipitated protein of interest is tyrosine phosphorylated but unfortunately this does introduce another step in the procedure. Another drawback is only the tyrosine phosphorylated population of the protein of interest is immunoprecipitated which makes analysis of the relative amounts of phosphorylation to total protein unobtainable.

3.7.1 Effect of adhesion on FAK tyrosine phosphorylation in BAE cells.

Initial experiments with BAE cells sought to confirm previous demonstrations that adhesion stimulated tyrosine phosphorylation of FAK in BAE cells. BAE cells grown in 90mm plates were trypsinised and half kept in suspension while the rest were replated onto fibronectin as described in materials and methods. After 90 minutes the cells were washed, lysed in Backer buffer and the tyrosine phosphorylated proteins were immunoprecipitated with monoclonal anti phosphotyrosine-agarose beads, subjected to SDS-PAGE electrophoresis and western blotting. Tyrosine phosphorylation levels were detected with anti phosphotyrosine antibody and the blot stripped and reprobed to demonstrate the protein was FAK using DRC anti-FAK. As shown in figure 3.12, tyrosine phosphorylated FAK was present in the lysates of the cells held in suspension at similar levels to the replated cells. This suggests that the FAK in the adherent cells and the cells in suspension are tyrosine phosphorylated and adhesion does not induce additional phosphorylation as the cells in suspension are already phosphorylated. This observation may be an artefact of the indirect method used to examine FAK tyrosine phosphorylation that had to be adopted in the absence of immunoprecipitation competent anti-FAK antibody. This possibility is discussed in section 3.7. Alternatively the high level of FAK tyrosine phosphorylation in the cells in suspension may be due to activation of cell:cell adhesion receptors caused by the cells clumping together stimulating tyrosine phosphorylation of FAK. This experiment was repeated three times and no consistent effect of adhesion on FAK phosphorylation was observed. It was not possible to quantitate the relative tyrosine phosphorylation state of FAK in these experiments as FAK was immunoprecipitated with anti-phosphotyrosine antibody.

3.7.2 Effect of VEGF stimulation on FAK tyrosine phosphorylation in BAE cells.

BAE cells were grown in 90mm plates, at confluency, were incubated in serum free media for 18 hours before stimulation with 2.5nM VEGF or control vehicle for 10 minutes. Cells were washed, then lysed with Backer buffer and immunoprecipitated with monoclonal anti phosphotyrosine-agarose, subjected to SDS-PAGE electrophoresis and western blotting. Tyrosine phosphorylation levels were detected with anti phosphotyrosine antibody and the blot stripped and reprobed to demonstrate the protein was FAK using DRC anti FAK. Although BAE cells stimulated with VEGF did show a slight increase in tyrosine

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phosphorylation of FAK, there was a high level of tyrosine phosphorylation in the non VEGF stimulated cells (figure 3.13). These experiments demonstrated that in contrast to HUVE cells, basal levels of FAK tyrosine phosphorylation were elevated in BAE cells preventing examination of the effects of VEGF. It was not possible to quantitate the relative tyrosine phosphorylation state of FAK in experiments in which FAK was immunoprecipitated with anti-phosphotyrosine antibody.

Results from VEGF and adhesion activated FAK phosphorylation demonstrated relatively high levels of FAK tyrosine phosphorylation in BAE cells held under basal conditions in the absence of agonist or serum. Although this precluded examination of the mechanism of VEGF effects on FAK in BAE cells it was considered these cells would allow determination of the involvement of Src and other signalling intermediates in FAK phosphorylation status. Furthermore the ease of transfection of BAE cells would allow a structure/function analysis of FAK in control of endothelial cell function.

3.7.3 Far westerns demonstrate Fyn and Grb2 binding sites are available on FAK in BAE cells

In order to demonstrate that Fyn and Grb2 binding sites are available on FAK in serum activated BAE cells the far western technique was employed. This method was also used to investigate if Fyn and Grb2 bound directly to FAK or bound indirectly via association with another protein. During this technique the western blot membrane was probed with the SH2 domain of Fyn and the SH2 domain of Grb2 which directly binds to the denatured FAK on the western blot if the appropriate binding sites are present.

Quiescent BAE cells were activated with serum for 10 or 20 minutes before the cells were lysed with Backer buffer and FAK immunoprecipitated using anti phosphotyrosine-agarose beads. The lysates were subjected to SDS-PAGE electrophoresis and western blotting and the presence of FAK acknowledged with DRC FAK antibody. The blot was stripped and incubated with the SH2 domain of Fyn (fused with GST) at a concentration of 2μg/ml. Any bound fusion protein was then detected with an anti-GST antibody and visualised using chemiluminescence. This process was repeated with the SH2 domain of Grb2 and the p85 subunit of PI3K (both fused to GST) also at a concentration of 2μg/ml.

Figure 3.14 showed that binding sites are available for the signalling molecules Fyn and Grb2 on FAK in BAE cells as well as previously shown in HUVE cells and that these cells would be suitable for further investigations into the role of FAK in endothelial signalling. The p85 subunit of PI3K was not found to be associated with FAK in BAE cells in this system.

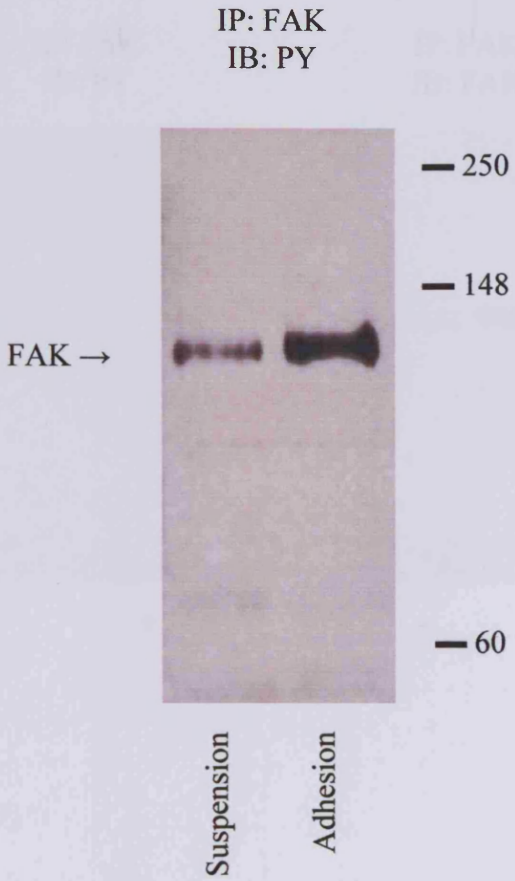


Figure 3.1. Adhesion stimulates tyrosine phosphorylation of FAK in HUVE cells. HUVE cells were trypsinised, resuspended and either plated onto fibronectin and allowed to spread for 60 minutes (adhesion), or held in suspension for the same period of time (suspension) as described in section 2.13. Focal adhesion kinase was immunoprecipitated from the cell lysates and immunoprecipitated protein was resolved by SDS-PAGE and subjected to immunoblot analysis (as described in section 2.6.1, 2.7 and 2.8). The tyrosine phosphorylation state of FAK was determined by probing with antiphosphotyrosine antibody (PY) as described in section 2.9.1. The position of the molecular mass markers are indicated in kDa on the right. This figure is representative of three experiments. IP, immunoprecipitate; IB, immunoblot.

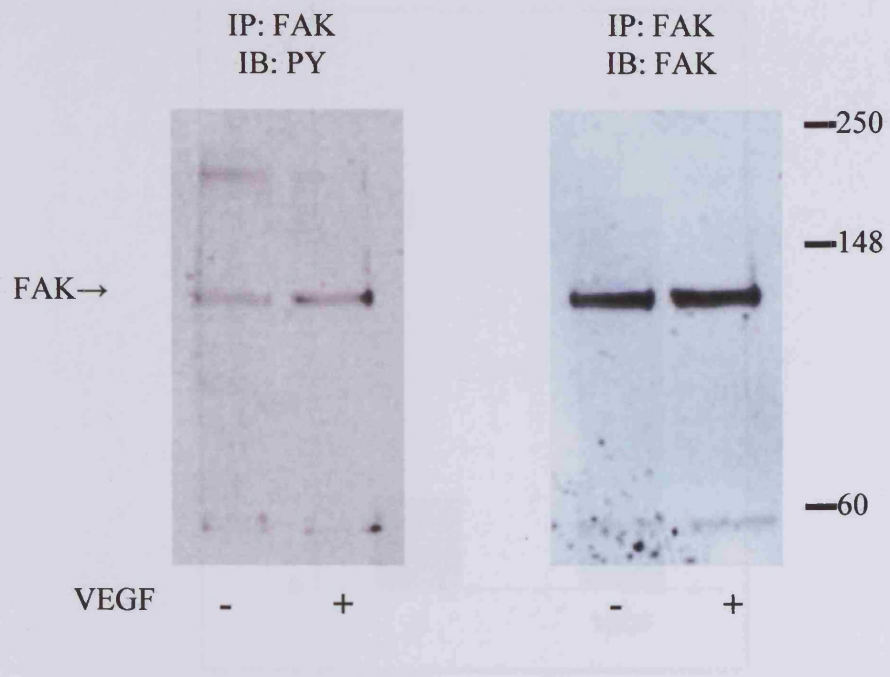


Figure 3.2A. VEGF stimulates tyrosine phosphorylation of focal adhesion kinase in human endothelial cells. HUVE cells were challenged with 2.5nM VEGF (+) or control vehicle (-) for 10 minutes lysed and FAK was immunoprecipitated with an anti-FAK monoclonal antibody coupled to agarose beads as described in section 2.6.1. Immunoprecipitated protein was resolved by SDS-PAGE and transferred to blotting membranes as described in section 2.7 and 2.8. The tyrosine phosphorylation state of FAK was determined by antiphosphotyrosine immunodetection (PY) (left panel) as described in section 2.9.1. Blots were stripped and reprobed with DRC FAK polyclonal antibody (right panel) as described in section 2.9.2. The position of the molecular mass markers are indicated in kDa on the right. This figure is representative of nine experiments. IP, immunoprecipitate; IB, immunoblot.

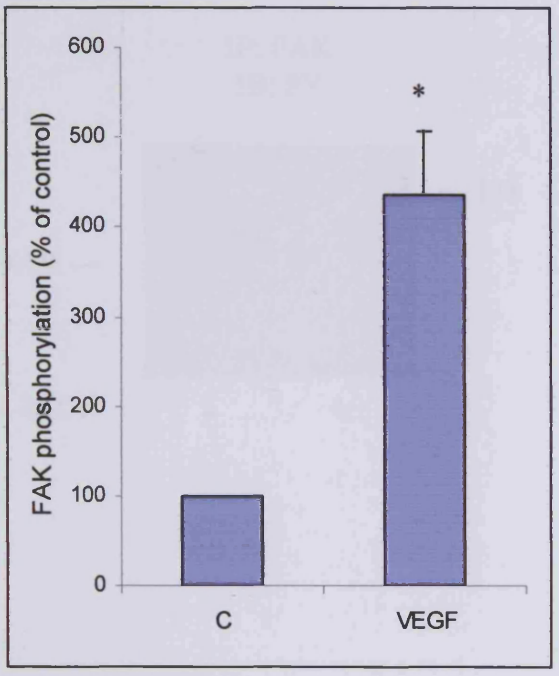


Figure 3.2B. Quantitative analysis of VEGF stimulated FAK tyrosine phosphorylation. A series of VEGF stimulated FAK tyrosine phosphorylation experiments were performed as described in figure 3.2A. For each experiment the level of tyrosine phosphorylation of immunoprecipitated FAK was determined by densitometric scanning of blots probed for tyrosine phosphorylation and FAK (described in section 2.34). The relative phosphorylation states were normalised with respect to FAK levels in each lane of each blot. Results are expressed as a percentage of tyrosine phosphorylation in control treated cells (C). Data is presented as mean and standard error of the mean for nine experiments. Data was analysed using the paired Students t-test, * indicates $p<0.002$, relative to control treated cells (Bailey, Statistical methods in biology, 1979).

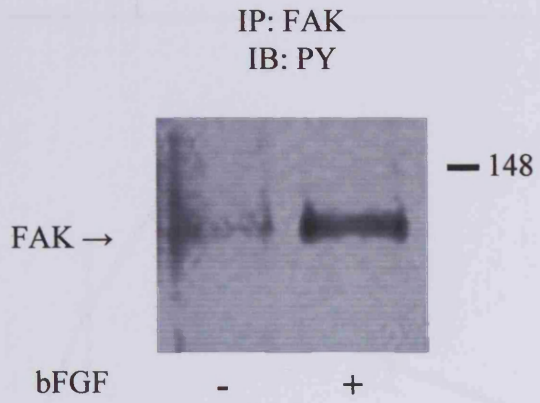


Figure 3.3. bFGF induces tyrosine phosphorylation of FAK in human endothelial cells. HUVE cells were challenged with control vehicle (-) or 5μg/ml of bFGF (+) for 10 minutes, lysed and FAK immunoprecipitated as described in section 2.6.1. Immunoprecipitated protein was resolved by SDS-PAGE and transferred to blotting membrane as described in section 2.7 and 2.8. The tyrosine phosphorylation state of FAK was determined by antiphosphotyrosine immunodetection (PY) as described in section 2.9.1. The position of the molecular mass markers are indicated in kDa on the right. IP, immunoprecipitate; IB, immunoblot.

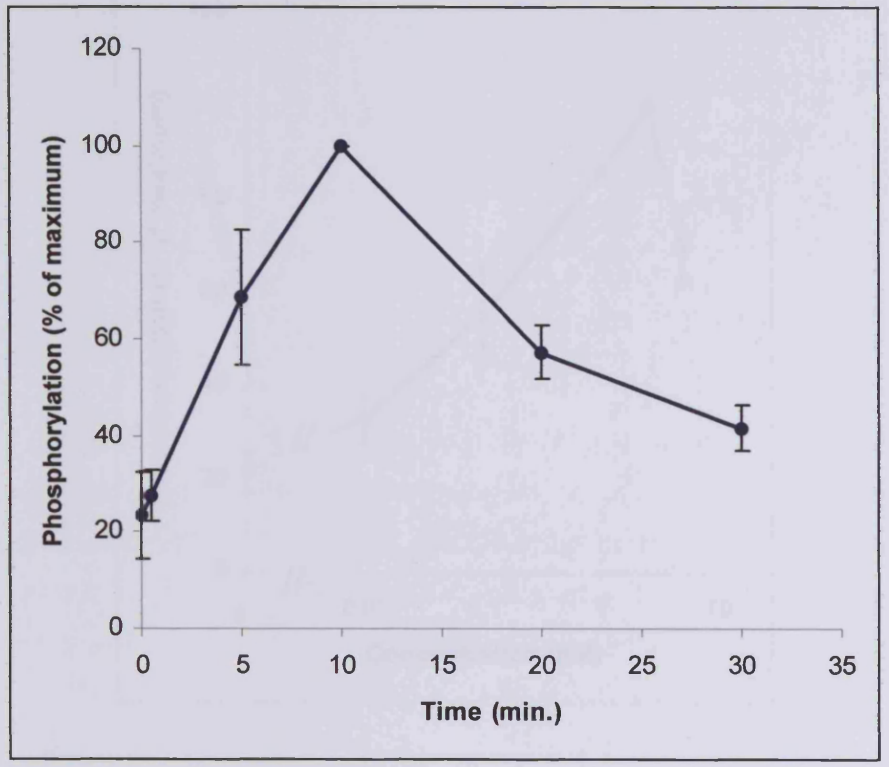


Figure 3.4. Time course of VEGF stimulated tyrosine phosphorylation of FAK. HUVE cells were challenged with 2.5nM VEGF or control vehicle for the times indicated before lysis and immunoprecipitation of FAK as described in section 2.6.1. Immunoprecipitated proteins were resolved by SDS-PAGE and subjected to immunoblot analysis as described in materials and methods. The tyrosine phosphorylation state of immunoprecipitated FAK was detected by probing blots with antiphosphotyrosine antibody (as described in section 2.9.1). Blots were stripped and reprobed with a polyclonal antibody to FAK (as described in section 2.9.2). Following chemiluminescent detection phosphorylation was quantified as peak area optical density of bands obtained by densitometric scanning of blots (described in section 2.34). Tyrosine phosphorylation of FAK is expressed as a percentage of the maximal response. Data is from a minimum of three experiments and mean and standard error of the mean are given.

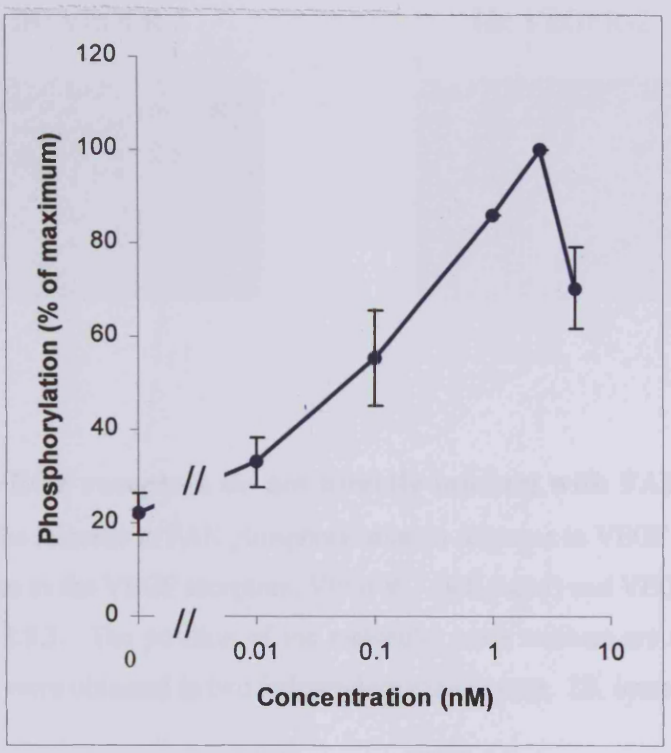


Figure 3.5. Concentration dependence of VEGF stimulated FAK phosphorylation. HUVE cells were challenged with the indicated concentrations of VEGF for 10 minutes before lysis and immunoprecipitation of FAK (as described in section 2.6.1). Immunoprecipitated proteins were resolved by SDS-PAGE and subjected to immunoblot analysis as described in materials and methods. The tyrosine phosphorylation state of immunoprecipitated FAK was detected by probing blots with antiphosphotyrosine antibody (as described in section 2.9.1). Blots were stripped and reprobed with a polyclonal antibody to FAK as described in section 2.9.2. Following chemiluminescent detection phosphorylation was quantified as peak area optical density of bands obtained by densitometric scanning of blots (described in section 2.34). Tyrosine phosphorylation of FAK is expressed as a percentage of the maximal response. Data is from a minimum of three experiments and mean and standard error of the mean are given.

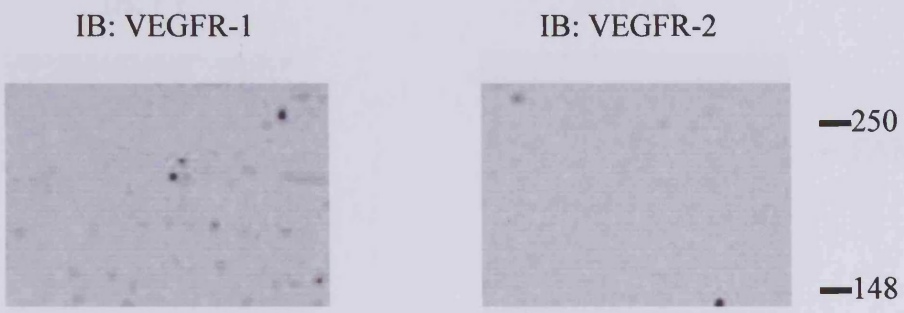


Figure 3.6. The VEGF receptors do not directly interact with FAK. Blots from figure 3.2A demonstrating the increase in FAK phosphorylation in response to VEGF were stripped and re-probed with antibodies to the VEGF receptors, VEGFR-1 (left panel) and VEGFR-2 (right panel) as described in section 2.9.3. The position of the molecular mass markers are shown in kDa on the right. Similar results were obtained in two independent experiments. IB, immunoblot.

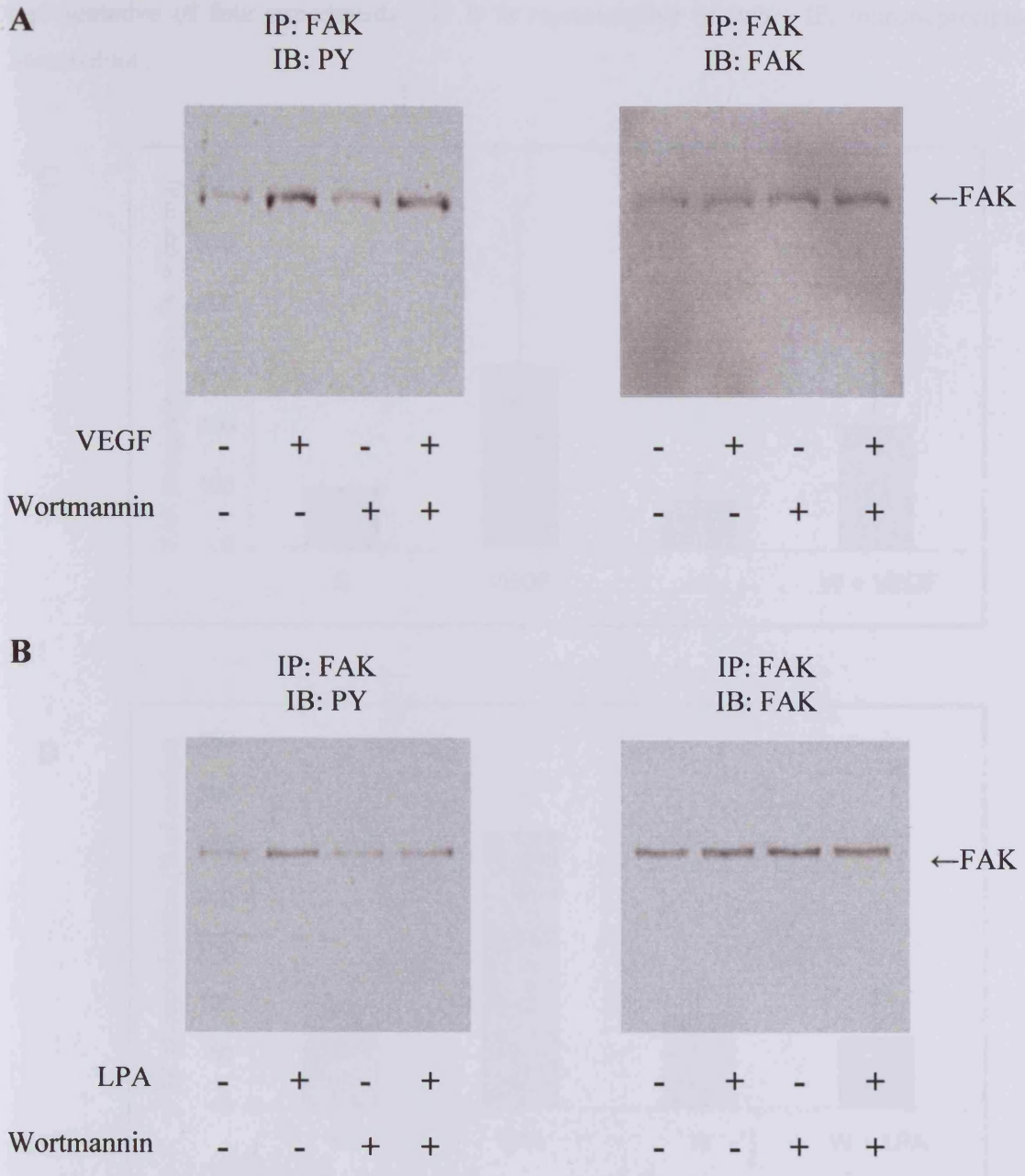
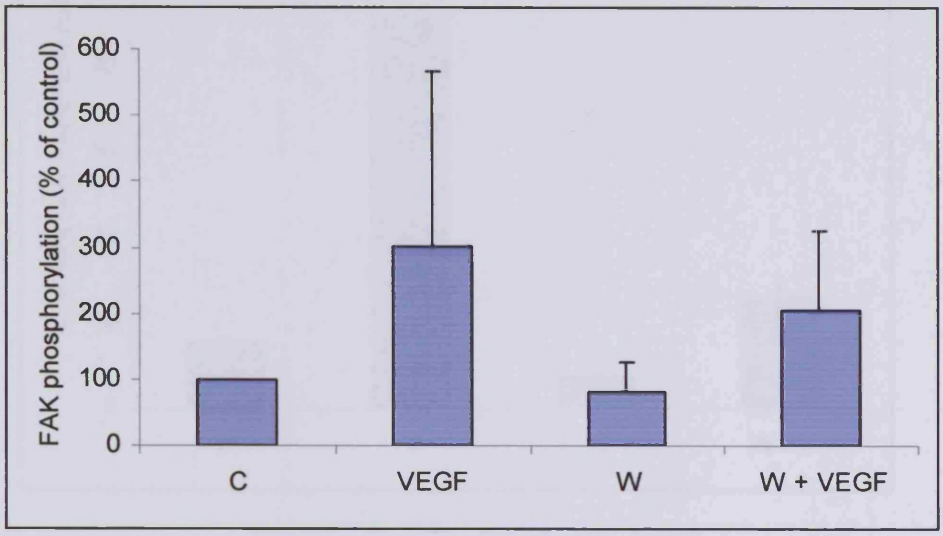


Figure 3.7A and B. Effect of wortmannin on VEGF and LPA stimulated tyrosine phosphorylation of FAK in human endothelial cells. HUVE cells were challenged with (A) 2.5nM VEGF or (B) 2μM LPA in the absence or presence of 100nM wortmannin as indicated. After 10 minutes the cells were lysed and FAK immunoprecipitated with an anti-FAK monoclonal antibody coupled to agarose beads as described in section 2.6.1. Immunoprecipitated protein was resolved by SDS-PAGE and transferred to blotting membranes as described in materials and methods. The tyrosine phosphorylation state of immunoprecipitated FAK was detected by probing blots with antiphosphotyrosine antibody (PY) (left panels) as described in section 2.9.1. Blots were stripped and reprobed with a polyclonal antibody to FAK (right panels) as described in section 2.9.2. Figure A is

representative of four experiments and B is representative of two. IP, immunoprecipitate; IB, immunoblot.

C



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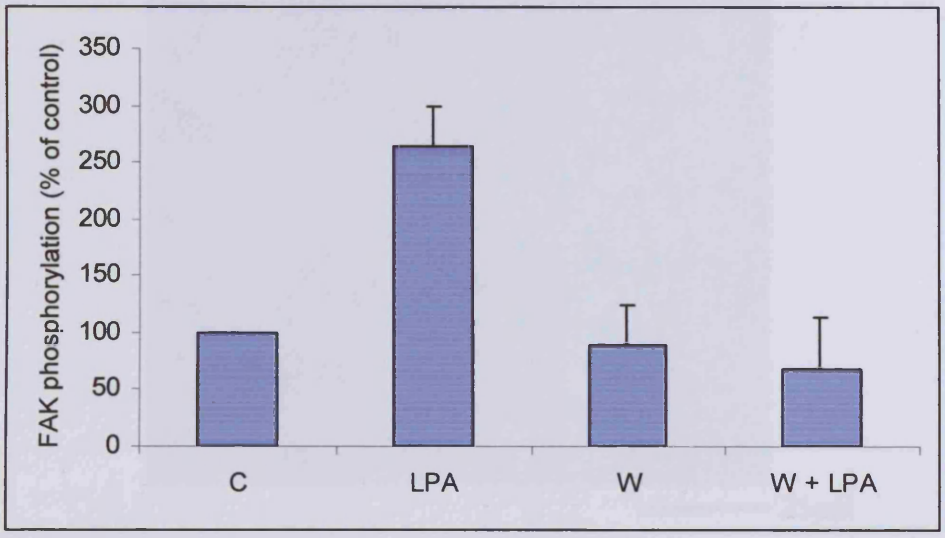
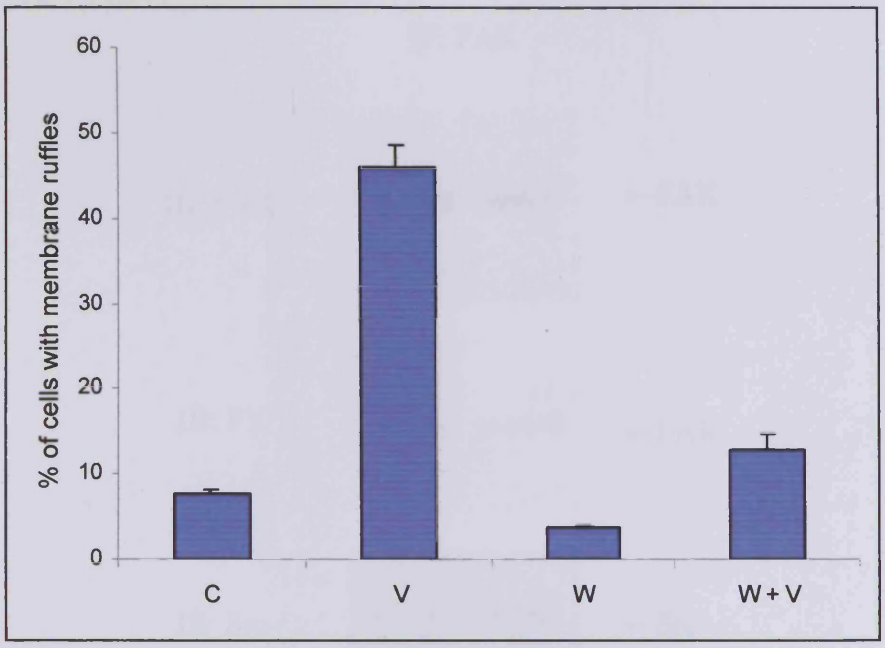


Figure 3.7 C and D. Quantitative analysis of the effect of wortmannin on VEGF and LPA activation of FAK tyrosine phosphorylation. A series of VEGF (figure 3.7 C) or LPA (figure 3.7 D) stimulated FAK tyrosine phosphorylation experiments were performed as described in figure 3.7 A and B. For each experiment the level of tyrosine phosphorylation of immunoprecipitated FAK was determined by densitometric scanning of blots probed for tyrosine phosphorylation levels and FAK (described in section 2.34). The relative tyrosine phosphorylation states were normalised with respect to FAK in each lane of each blot. Results are expressed as a percentage of tyrosine phosphorylation in control treated cells (C). Data from duplicate experiments are presented as mean and deviation from the mean. W, wortmannin.

A



B

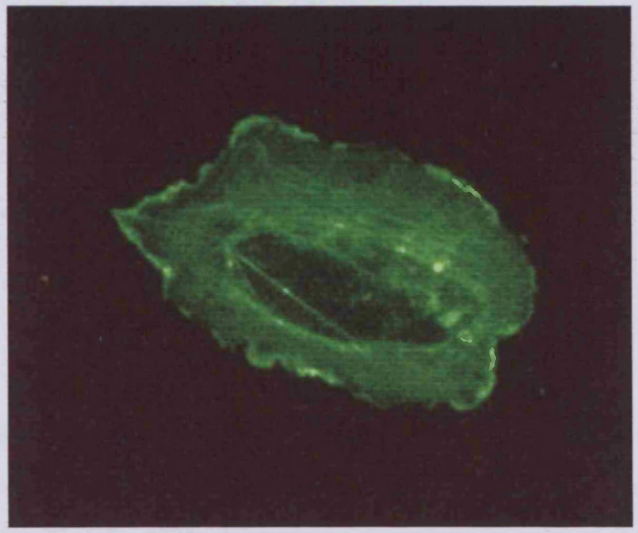


Figure 3.8. Effects of wortmannin on VEGF induced membrane ruffling in human endothelial cells. (A) HUVE cells were challenged with control vehicle (C) or 2.5nM VEGF (V) in the absence or presence of 100nM wortmannin (W) for 10 minutes. Cells were fixed and filamentous actin visualised by FITC-conjugated phalloidin as described in materials and methods section 2.14.1. The percentage of cells exhibiting edge ruffles were determined for 10 randomly selected fields on each coverslip. Cells were considered as ruffling if any part of the cell perimeter exhibited concentrated filamentous actin staining, typical of membrane ruffling, regardless of the variation in size or intensity. Means and deviations from the mean are presented for duplicate experiments. (B) An example of VEGF induced membrane ruffling in HUVE cell from A.

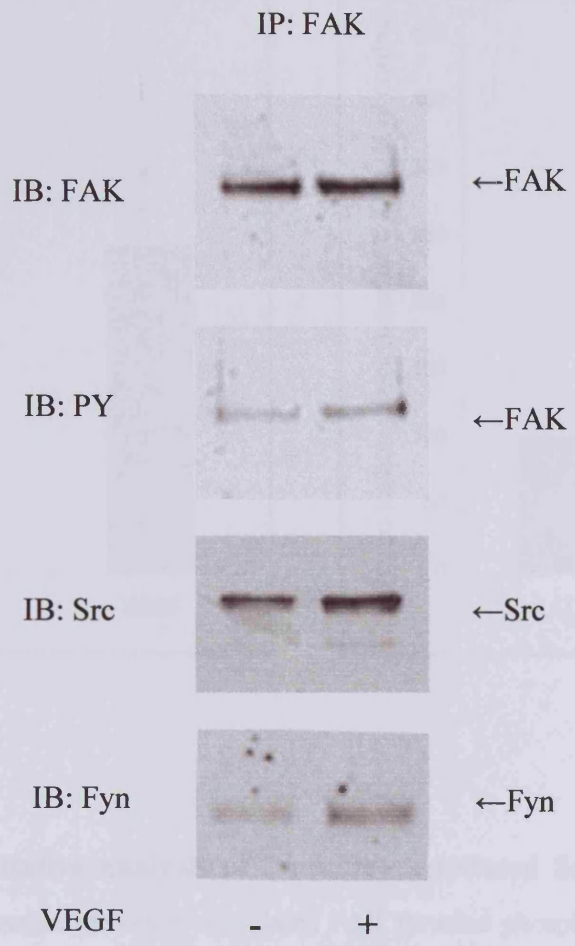


Figure 3.9A. Interaction of FAK with Src and Fyn. HUVE cells were challenged with 2.5nM VEGF (+) or control vehicle (-) for 10 minutes, lysed and FAK immunoprecipitated with an anti-FAK monoclonal antibody coupled to agarose beads as described in section 2.6.1. Immunoprecipitated protein was resolved by SDS-PAGE, transferred to blotting membranes for immunoblot analysis as described in materials and methods. Tyrosine phosphorylation of FAK was determined by antiphosphotyrosine immunodetection (PY) as described in section 2.9.1. Blots were stripped and reprobed with antibodies to FAK, Src and Fyn as indicated (as described in section 2.9.2 and 2.9.4). Figures are representative of four experiments. IP, immunoprecipitate; IB, immunoblot.

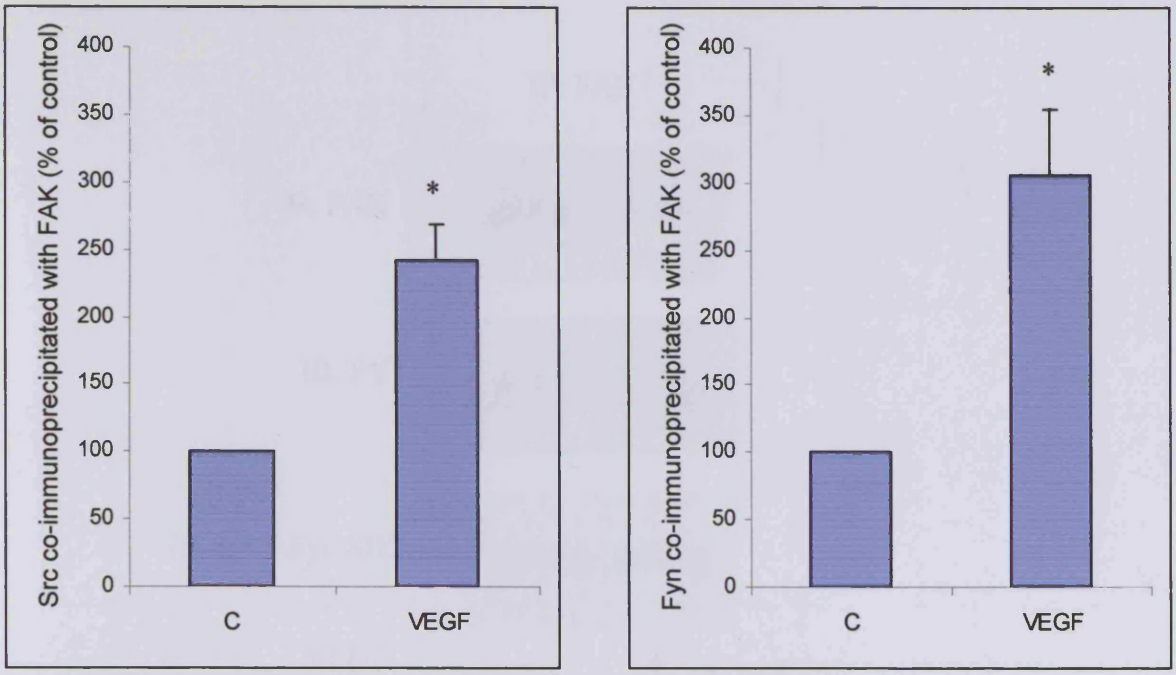


Figure 3.9B. Quantitative analysis of immunoprecipitated Src and Fyn with VEGF stimulated FAK. A series of VEGF stimulated FAK tyrosine phosphorylation experiments were performed and the association of Src and Fyn with immunoprecipitated FAK investigated as described in figure 3.9A. For each experiment the relative amount of Src (left panel) or Fyn (right panel) immunoprecipitated with control or VEGF stimulated FAK was determined by densitometric scanning of blots probed for FAK and Src or Fyn (described in section 2.34). The amount of Src or Fyn bound was normalised with respect to FAK levels in each lane of each blot. Results are expressed as a percentage of Src or Fyn bound in control treated cells (C). Data is presented as mean and standard error of the mean for three experiments. Data was analysed using the paired Students t-test, * indicates $p<0.05$, relative to control treated cells (Bailey, Statistical methods in biology, 1979).

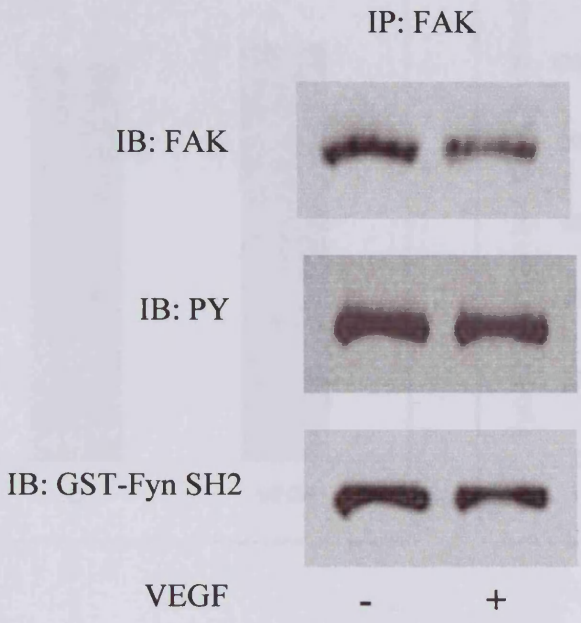


Figure 3.10A. Association of Fyn SH2 domain with FAK. Quiescent HUVE cells were challenged with 2.5nM VEGF (+) or control vehicle (-) for 10 minutes, lysed and FAK was immunoprecipitated with an anti-FAK monoclonal antibody coupled to agarose beads as described in section 2.6.1. Immunoprecipitated protein was resolved by SDS-PAGE, transferred to blotting membranes for immunoblot analysis as described in materials and methods. Tyrosine phosphorylation of FAK was determined by antiphosphotyrosine immunodetection (PY) as described in section 2.9.1. Blots were stripped and reprobed with DRC FAK polyclonal antibody as described in section 2.9.2. The blot was then stripped and incubated with the SH2 domain of Fyn (fused with GST) at a concentration of 2μg/ml (as described in section 2.12). Any bound fusion protein was then detected with an anti-GST antibody (as described in section 2.12). IP, immunoprecipitate; IB, immunoblot.

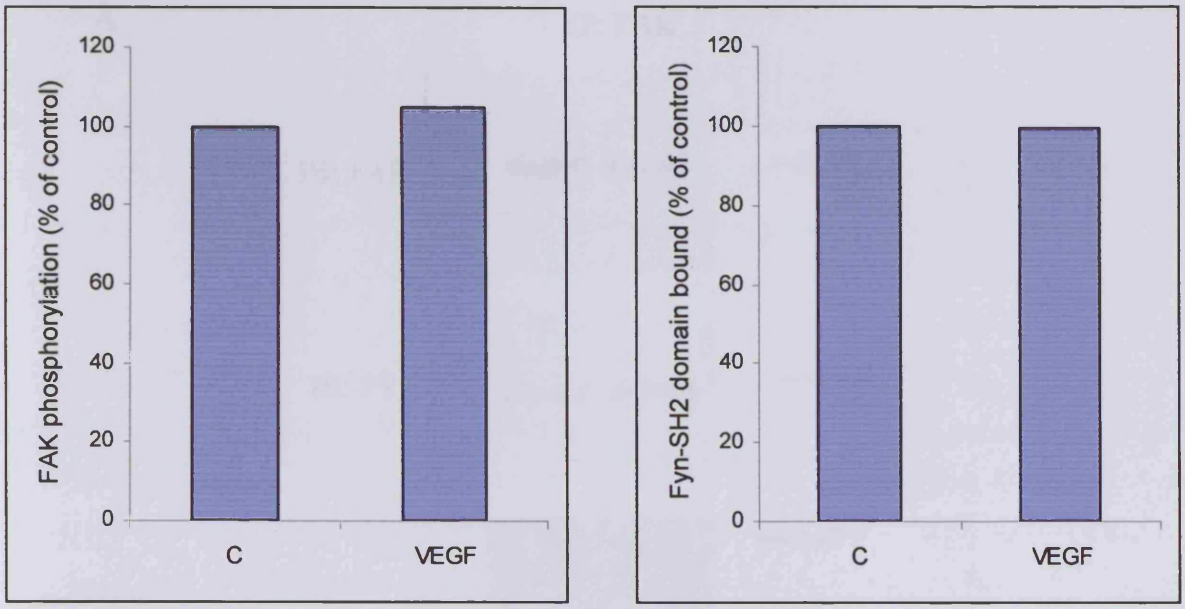


Figure 3.10B. Quantitative analysis of the association of Fyn-SH2 domain with FAK. The association of Fyn-SH2 domain with immunoprecipitated FAK from control (C) or VEGF stimulated cells was investigated as described in figure 3.10A. The relative amount of FAK tyrosine phosphorylation and Fyn-SH2 domain bound to FAK was determined by densitometric scanning of blots probed for FAK, tyrosine phosphorylation levels and Fyn-SH2 domain (described in section 2.34). The amount of tyrosine phosphorylation and Fyn-SH2 domain bound was normalised with respect to FAK levels in each lane. Data is presented as a percentage of tyrosine phosphorylation (left panel) or Fyn-SH2 domain bound (right panel) in control treated cells.

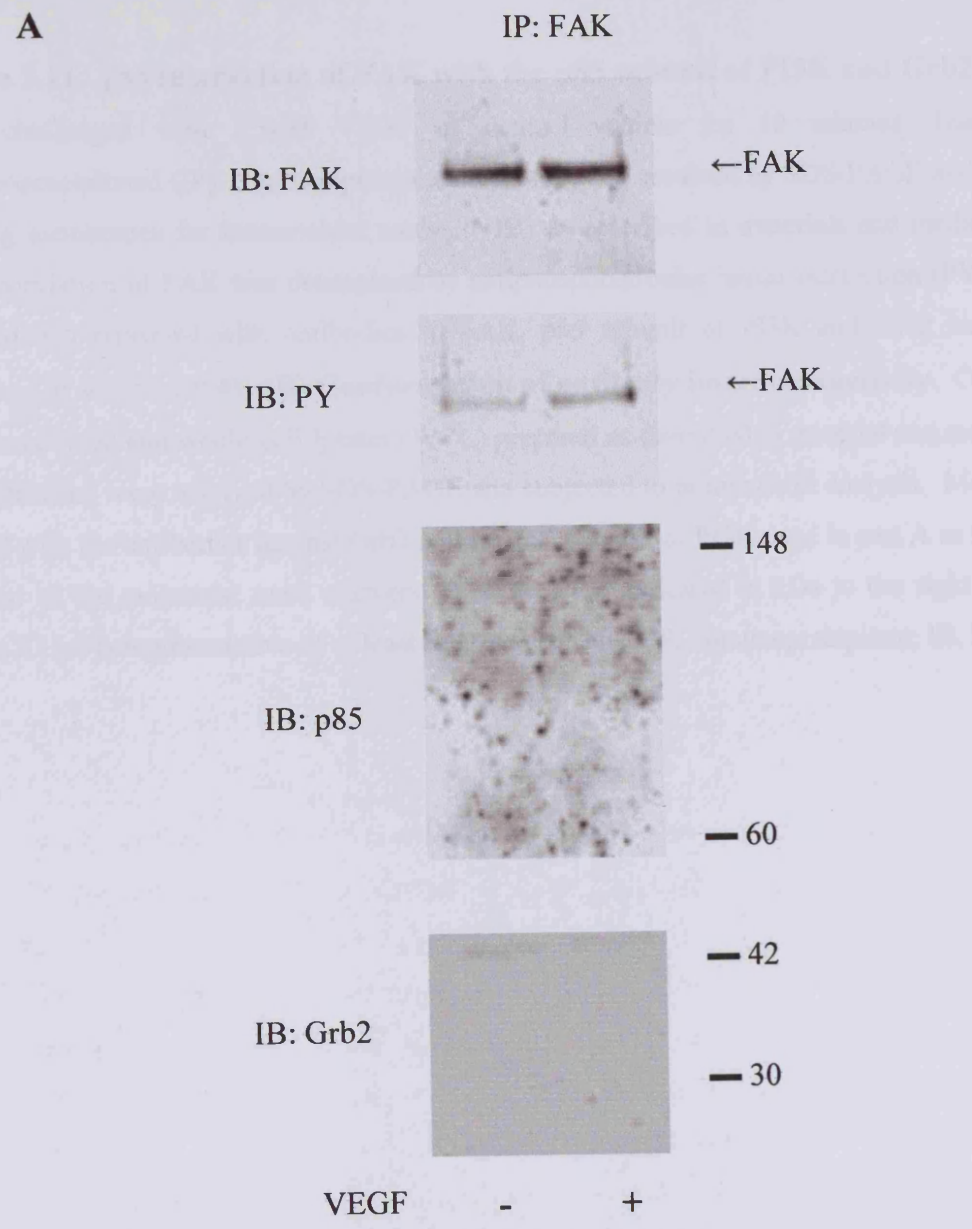

A
IP: FAK
IB: FAK
←FAK
IB: PY
←FAK
148
IB: p85
60
42
IB: Grb2
30
VEGF
-
+
B
IB: Grb2
60
42
Grb2→
30
WCL
IB: p85
148
p85→
60
42
WCL

Figure 3.11. (A) Interaction of FAK with the p85 subunit of PI3K and Grb2. HUVE cells were challenged with 2.5nM VEGF or control vehicle for 10 minutes, lysed and FAK immunoprecipitated (IP). Immunoprecipitated protein was resolved by SDS-PAGE and transferred to blotting membranes for immunoblot analysis (IB) as described in materials and methods. Tyrosine phosphorylation of FAK was determined by antiphosphotyrosine immunodetection (PY). Blots were stripped and reprobed with antibodies to FAK, p85 subunit of PI3K and Grb2 as indicated (as described in section 2.9.4). **(B) Conformation of antibody immunoreactivity.** Cultured HUVE cells were lysed and whole cell lysates (WCL) prepared as described in material and methods (section 2.5). Proteins were resolved by SDS-PAGE and subjected to immunoblot analysis. Membranes were probed with the antibodies against Grb2 and the p85 subunit of PI3K used in part A as indicated. The position of the molecular mass markers (if shown) are indicated in kDa to the right of the panels. Figure 3.11A is representative of at least four experiments. IP, immunoprecipitate; IB, immunoblot.

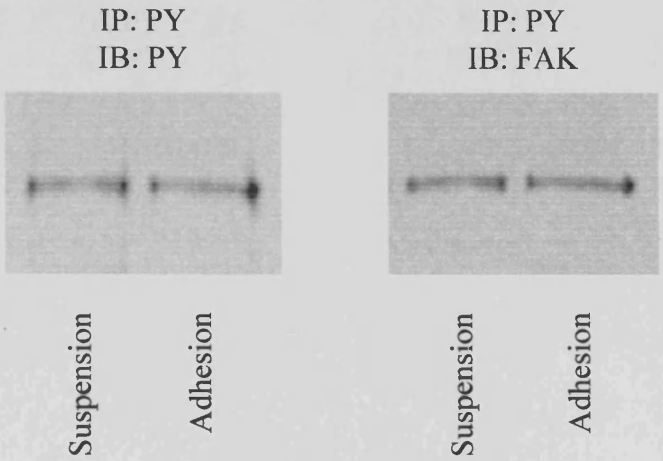


Figure 3.12. Effect of adhesion on FAK tyrosine phosphorylation in BAE cells. BAE cells were tyripsinised, resuspended and either plated onto fibronectin and allowed to spread for 60 minutes (adhesion), or held in suspension for the same period of time (suspension) as described in section 2.13.. The tyrosine phosphorylated proteins were immunoprecipitated with monoclonal antiphosphotyrosine-agarose beads from cell lysates as described in section 2.6.3. Immunoprecipitated protein was resolved by SDS-PAGE, transferred to blotting membranes for immunoblot analysis (IB) as described in materials and methods. Tyrosine phosphorylation levels were detected with anti-phosphotyrosine antibody (left panel) and the blot stripped and reprobed to demonstrate the protein was FAK using DRC anti-FAK antibody (right panel) as described in sections 2.9.1 and 2.9.2. Figure is representative of three experiments. IP, immunoprecipitate; IB, immunoblot.

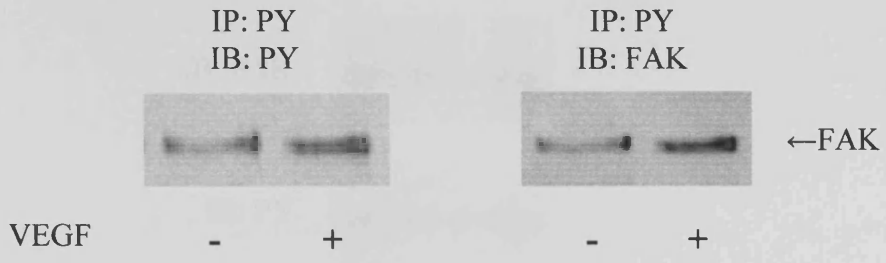


Figure 3.13. Effect of VEGF on FAK tyrosine phosphorylation in BAE cells. BAE cells were challenged with 2.5nM VEGF or control vehicle for 10 minutes as indicated. The tyrosine phosphorylated proteins were immunoprecipitated with monoclonal antiphosphotyrosine-agarose beads from cell lysates (as described in section 2.6.3). Immunoprecipitated protein was resolved by SDS-PAGE and transferred to blotting membranes for immunoblot analysis (IB) as described in materials and methods. Tyrosine phosphorylation levels were detected with anti-phosphotyrosine antibody (left panel) and the blot stripped and reprobed to demonstrate the protein was FAK using DRC anti-FAK antibody (right panel) as described in sections 2.9.1 and 2.9.2. Figure is representative of three experiments. IP, immunoprecipitate; IB, immunoblot.

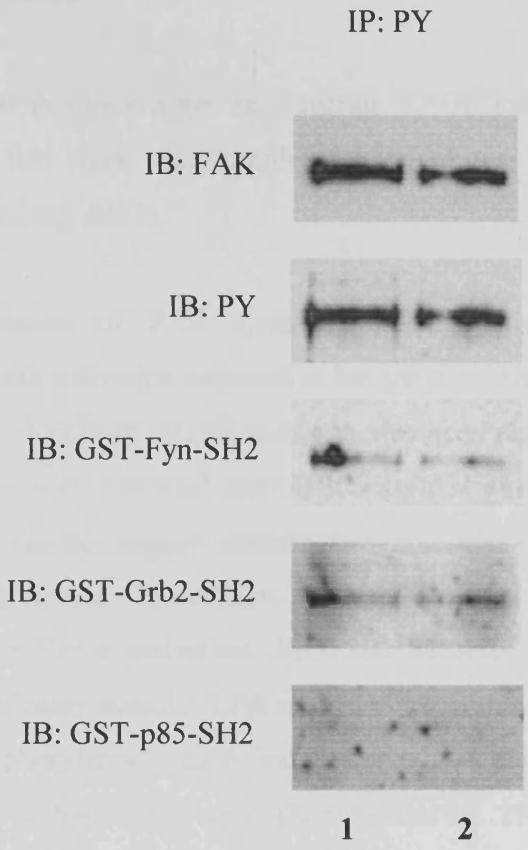


Figure 3.14. Fyn and Grb2 binding sites are available on FAK in BAE cells. Quiescent BAE cells were activated with serum for 10 minutes (lane 1) or 20 minutes (lane 2). The tyrosine phosphorylated proteins were immunoprecipitated with monoclonal antiphosphotyrosine-agarose beads from cell lysates as described in section 2.6.3. Immunoprecipitated protein was resolved by SDS-PAGE and transferred to blotting membranes for immunoblot analysis as described in materials and methods. Tyrosine phosphorylation levels were detected with anti-phosphotyrosine antibody (PY) and the blot stripped and reprobed to demonstrate the protein was FAK using DRC anti-FAK antibody (FAK) as described in sections 2.9.1 and 2.9.2. The blot was stripped again and incubated with the SH2 domain of Fyn (fused with GST) at a concentration of 2μg/ml. Any bound fusion protein was then detected with an anti-GST antibody (as described in section 2.12). This process was repeated with the SH2 domain of Grb2 and the p85 subunit of PI3K as indicated (both fused to GST) also at a concentration of 2μg/ml. IP, immunoprecipitate; IB, immunoblot.

3.8 Discussion

Data presented in this chapter demonstrate VEGF increases tyrosine phosphorylation of FAK. After this work was completed a report was published confirming this finding (Abedi and Zachary 1997).

VEGF stimulation of FAK tyrosine phosphorylation was time and concentration dependent. Peak activation occurred at ten minutes with a concentration of 2.5nM VEGF. Stimulation by VEGF of HUVE cells has also been reported to stimulate phospholipase C and D activity with maximal stimulation seen at similar time to FAK (Seymour *et al.* 1996). These kinetics suggest intermediate molecules lie between the VEGF receptors and FAK phosphorylation. In addition, the VEGF receptors were not found to be associated with FAK upon VEGF activation. FAK also becomes tyrosine phosphorylated in response to a number of other stimuli. LPA and HGF show similar kinetics to VEGF induced FAK tyrosine phosphorylation. LPA induces maximal phosphorylation of FAK ten minutes after addition to Swiss 3T3 cells (Kumagai *et al.* 1993) with hepatocyte growth factor stimulation of human oral squamous carcinoma cells being activated by three minutes with maximal activation seen between 10 and 30 minutes (Matsumoto *et al.* 1994).

Bombesin tyrosine phosphorylation of FAK was detectable after seconds and caused a maximal stimulation of FAK after one minute (Sinnet-Smith *et al.* 1993). In addition, Bradykinin, a small peptide mitogen, also stimulated rapid and transient FAK tyrosine phosphorylation in Swiss 3T3 cells, peaking after only one minute (Leeb-Lundberg *et al.* 1994). In contrast, sphingosine stimulated FAK phosphorylation in Swiss 3T3 cells with the maximal stimulation 60 minutes after addition (Seufferlein and Rozengurt 1994). PDGF stimulation of FAK persists for as long as four hours in rabbit vascular smooth muscle cells (Abedi *et al.* 1995).

VEGF has been shown to induce membrane ruffles as early as five minutes which are still present after 15 minutes and to also induce cell shape changes after 15 minutes (Waltenberger *et al.* 1994, Rousseau *et al.* 1997). This is consistent with the time course of FAK tyrosine phosphorylation observed and therefore possible FAK is involved with modulation of the cytoskeleton upon VEGF stimulation.

Porcine aortic endothelial (PAE) cells are reported not to posses VEGFR-1 and 2 however when expressed individually the Kd of VEGFR-1 and VEGFR-2 for VEGF is 16pM and 760pM respectively (Waltenberger *et al.* 1994). Waltenberger *et al.* (1994) demonstrated HUVE cells were found to have two distinct receptor populations with Kd values of 9 and 770pM whereas Olander *et al.* (1991) demonstrated Kd values of 9pM and 1.5 nM in HUVE cells. The difference in the lower affinity receptor may be due to Olander using HUVE cells with a relatively high passage number. However Bikfalvi *et al.* (1991) found Kd values for VEGF binding to HUVE cells of 9pM and 179pM (+/-101pM). VEGF stimulation of FAK tyrosine phosphorylation was half maximal at a concentration of 120pM/L. The high affinity receptors would be saturated at a lower concentration than was necessary for half maximal activation of FAK tyrosine phosphorylation. It is probable therefore that VEGF acts via the lower affinity receptor (VEGFR-2) to activate FAK tyrosine phosphorylation. VEGF could also act via a heterodimer of the lower and higher affinity receptors. There is some evidence indicating VEGFR-1 and 2 can form heterodimers as the soluble form of VEGFR-1 has been shown to form a VEGF-stabilised complex with the extracellular domain of VEGFR-2 *in vitro* (Kendall *et al.* 1996). The arrangement of the receptor binding sites on VEGF indicates a VEGF dimer may be able to bind and link together two VEGF receptors to form homo or heterodimers of receptors (Wiesmann *et al.* 1997, Fuh *et al.* 1998). Recently Kanno *et al.* (2000) reported VEGF induced tyrosine phosphorylation of FAK via VEGFR-2. Kanno *et al.* (2000) deduced this by stimulating HUVE cells with VEGF in the presence of blocking antibodies against each VEGF receptor. The tyrosine phosphorylated proteins were immunoprecipitated and the blots were probed to detect FAK. However, as FAK was not directly immunoprecipitated but detected from anti-phosphotyrosine immunoprecipitations this may not truly reflect the tyrosine phosphorylation state of FAK. The highest concentrations of VEGFR-2 blocking antibody used (30μg/ml) completely inhibit the presence of FAK in the immunoprecipitations which suggests VEGF signals through VEGFR-2 to induce tyrosine phosphorylation of FAK. In addition however, 10μg/ml of VEGFR-2 blocking monoclonal antibody did not completely abolish the immunoprecipitation of FAK although 10μg/ml of VEGFR-2 blocking monoclonal antibody in conjunction with 1μg/ml of VEGFR-1 blocking antibody reduced the amount of immunoprecipitated FAK to almost undetectable levels. This could indicate heterodimerisation of the two receptors is partially involved in signalling to FAK as well as signalling through VEGFR-2 alone.

Interestingly VEGFR-2 is the receptor which has mostly been associated with transducing the effects of VEGF on migration, actin reorganisation and mitogenicity. This has been demonstrated in transfected PAE cells stably expressing VEGFR-1 or 2 (Waltenburger *et al.* 1994, Seetharam *et al.* 1995) and also in BAE cells after treatment with antisense sequence to VEGFR-1 mRNA (Bernatchez *et al.* 1999). However more recently, blocking monoclonal antibodies against VEGFR-1 and 2 used in HUVE cells have demonstrated the VEGFR-1 blocking antibody completely abolished migration of HUVE cells whereas the VEGFR-2 blocking antibody only demonstrated 50% inhibition (Kanno *et al.* 2000). Further analysis revealed VEGF induced actin reorganisation and cell spreading on type-1 collagen via VEGFR-1 and not VEGFR-2 whereas VEGF induced vinculin assembly in focal adhesions via VEGFR-2 and not VEGFR-1. Kanno *et al.* (2000) proposed VEGFR-1 and 2 both contributed to enhance migration but by different mechanisms. That is, VEGFR-1 mediated signals preferentially modulating actin reorganisation (via activation of p38 MAP kinase) whereas VEGFR-2 regulates cell adhesion by mediating vinculin assembly in focal adhesions and tyrosine phosphorylation of FAK and paxillin. This indicates VEGF tyrosine phosphorylation of FAK may be involved in modulation of cell adhesion rather than actin reorganisation.

Maximal effects of FAK tyrosine phosphorylation were seen with 2.5nM VEGF and demonstrated a typical bell shaped response curve with higher concentrations stimulating FAK tyrosine phosphorylation to a lesser extent. This is probably due to VEGF receptor internalisation (Bikfalvi *et al.* 1991). Interestingly, Abedi and Zachary (1997) stated VEGF at a concentration of 0.25nM demonstrated maximal stimulation of FAK tyrosine phosphorylation in HUVE cells which is ten fold lower than demonstrated in this thesis. However, Abedi and Zachary did not directly measure the tyrosine phosphorylation of FAK as they immunoprecipitated with an anti-phosphotyrosine antibody and then probed with FAK. This does not account for FAK being immunoprecipitated due to association with another tyrosine phosphorylated protein.

It has been reported using the yeast two-hybrid system that the p85 subunit of PI3K interacts via a SH2 domain with a VEGFR-1 tyrosine autophosphorylation site (Cunningham *et al.* 1995). The p85 regulatory subunit of PI3K has been demonstrated by immunoprecipitation to be constitutively associated with VEGFR-2 but not VEGFR-1 in HUVE cells (Thakker *et al.* 1999). Guo *et al.* (1995) reported that VEGF induced tyrosine

phosphorylation of both the p110 and p85 subunits of PI3K in cultured bovine aortic endothelial (BAE) cells and Thakker *et al.* (1999) demonstrated treatment of HUVE cells with VEGF induced an increase in PI3K activity. Conversely, VEGF has also been shown not to increase PI3K activity in HUVE cells (Abedi and Zachary 1997). Waltenberger *et al.* (1994) also observed no effects on tyrosine phosphorylation of PI3K in PAE cells transfected with either VEGFR-1 or 2. The discrepancy could be due to the PAE cells used by Waltenberger lacking a component of the signalling pathway required in certain signal transduction events. It was of interest therefore to examine if PI3K had a role in VEGF-induced tyrosine phosphorylation of FAK in HUVE cells. To do this the inhibitor wortmannin was used. However, due to the large variation in levels of VEGF induced FAK phosphorylation observed in these experiments it was not possible to draw any clear conclusions about the degree of involvement of PI3K in VEGF stimulated FAK tyrosine phosphorylation

The neuropeptides bombesin and endothelin act via different G protein coupled receptors to modulate FAK phosphorylation and do so independently of PI3K (Rankin *et al.* 1996). It was of interest therefore to note that LPA which also acts via a G protein coupled receptor to phosphorylate FAK was inhibited by wortmannin and therefore PI3K dependent.

The demonstration that VEGF induced membrane ruffling in HUVE cells was wortmannin sensitive suggests PI3K is involved in this effect of VEGF. Similarly, membrane ruffling induced by PDGF and EGF are both inhibited by wortmannin (Wennstrom *et al.* 1994, Rankin *et al.* 1996). Conclusive data on the degree to which PI3K participates in VEGF induced FAK phosphorylation will allow determination of whether the growth factor regulates the focal adhesion and cortical actin components of the cytoskeleton via at least two different pathways. In this regard it is interesting to note that Kanno *et al.* (2000) demonstrated VEGFR-2 modulates focal adhesions while VEGFR-1 modulates actin reorganisation in HUVE cells.

Numerous studies have demonstrated Src family kinases bind to FAK. In Src transformed CE cells and NIH3T3 cells, immunoprecipitation of Src from the cell lysates revealed the presence of FAK in the Src immune complexes (Cobb *et al.* 1994, Schaller *et al.* 1994, Xing *et al.* 1994). In addition, immunoprecipitation of overexpressed tagged FAK from

Cos cells demonstrated the presence of Src in the immune complex (Eide *et al.* 1995). The interaction between Src family kinases and FAK has also been demonstrated in non-transfected or non-transformed cells. In normal adherent subconfluent CE cells various Src family kinase members were immunoprecipitated from the cell lysates and the presence of FAK investigated. Interestingly, FAK was demonstrated to be associated with Fyn but not with the Src family members Src, Yes, Lyn, or Hck (Cobb *et al.* 1994). Although the association of Src family kinases with FAK has been established in adherent and transfected cells less has been reported about the association when FAK becomes tyrosine phosphorylated during stimulation by ligands/factors. Here, it has been demonstrated that stimulation of HUVE cells with the ligand, VEGF, also induces an increase in association of Src and Fyn with FAK of 2.4 and 3.1 fold respectively. In addition, Zhang *et al.* (1996) demonstrated amyloid ß peptide stimulation of neuronal cells induces the tyrosine phosphorylation of FAK and an increase the stable association of FAK with Fyn.

Having demonstrated using the far western technique that Fyn binds directly with FAK via its SH2 domain, possible mechanisms whereby increased binding of Src family kinases to FAK could occur include conformational changes in FAK or Fyn. It has been proposed FAK is phosphorylated on tyrosine 397 in serum starved cells even though Src family kinases are not associated with FAK under these conditions (Schlaepfer and Hunter 1996, Schlaepfer *et al.* 1998). A conformational change has also been suggested by Schlaepfer and Hunter (1996) as FAK deletion mutants which do not posses the first 100 residues were highly tyrosine phosphorylated and found to be associated with Src in serum starved human 293 cells. The deletion of these residues may affect the folding of FAK while it is in an inactive state and allow the exposure of tyrosine 397 to which Src family kinases can bind. The recruitment of Fyn/Src to FAK induced by VEGF stimulation would allow the Src family kinases to tyrosine phosphorylate other sites on FAK. The FAK tyrosine phosphorylation sites 407, 576, 577, 861 and 925 have been shown to be preferentially phosphorylated by v-Src family PTK *in vivo* (Calalb *et al.* 1995 and 1996, Schlaepfer and Hunter 1996).

In addition to Src and Fyn, FAK has been reported to recruit the p85 subunit of PI-3 kinase in response to cell adhesion and PDGF stimulation (Chen and Guan 1994b, Chen *et al.* 1996) and the adapter protein Grb2 in response to cell adhesion to fibronectin (Schlaepfer

et al. 1994). Phosphorylation of FAK tyrosine 925 creates an SH2 binding site for the small adapter protein Grb2 (Schlaepfer *et al.* 1994) while the p85 subunit has been reported to bind to tyrosine 397 in NIH 3T3 cells (Chen *et al.* 1996). In the system used here, VEGF did not stimulate the association of PI3K with FAK. In NIH3T3 cells adhesion to fibronectin or Src transformation leads to the association of Grb2 with tyrosine residue 925 of FAK via its SH2 domain (Schaepfer *et al.* 1994). However, as demonstrated here, stimulation of HUVE cells with VEGF did not induce the same clear response. It is probable that activation of FAK via different mechanisms leads to activation of different signalling pathways.

Data presented in this chapter demonstrated that VEGF stimulated tyrosine phosphorylation of FAK in a time and concentration dependent manner in human endothelial cells. Peak activation occurred at 10 minutes with a concentration of 2.5nM/L VEGF. The PI3K inhibitor, wortmannin may partially inhibit the VEGF induced tyrosine phosphorylation of FAK and blocks FAK phosphorylation in response to lysophosphatidic acid and VEGF induced membrane ruffling.

The tyrosine kinases Src and Fyn were associated with FAK upon VEGF stimulation of HUVE cells. The ability of Fyn SH2 domain to bind FAK immunoprecipitated from endothelial cells treated with VEGF was examined by far-western blotting. It should be noted that the basal level of FAK tyrosine phosphorylation in the single experiment that was performed was high and not further increased by VEGF. Although this experiment did demonstrate that Fyn could bind directly to FAK via its SH2 domain, clearly further experiments are required to confirm this and determine whether VEGF induced FAK phosphorylation creates new Fyn binding sites on FAK. VEGF stimulated recruitment to FAK would allow Src family kinases to stimulate tyrosine phosphorylation of FAK thereby mediating the effects of VEGF on FAK tyrosine phosphorylation. Investigation into the association of other signalling molecules with FAK revealed VEGF activation of HUVE cells did not induce the association of the p85 subunit of PI3K with FAK and association of the adapter protein Grb2 with FAK was not consistent.

In order to further analyse the role of FAK, mutant FAK constructs would be utilised. Due to the poor transfection levels of HUVE cells however, another endothelial cell line, BAE cells were examined. BAE cells held under basal conditions exhibit relatively high levels

of FAK tyrosine phosphorylation which would preclude the examination of the mechanism of VEGF effects on FAK in BAE cells. The ease of transfection of BAE cells however would allow a structure/function analysis of FAK in control of endothelial cell function. As demonstrated by the far western technique, Fyn and Grb2 binding sites are present on BAE cells.

CHAPTER FOUR

Generation, expression and characterisation of FAK mutant constructs

Chapter Four

Generation, expression and characterisation of FAK mutant constructs

Introduction

FAK becomes tyrosine phosphorylated in response to adhesion to ECM proteins and transformation by v-Src and this phosphorylation can correlate with increased FAK tyrosine kinase activity (Guan and Shalloway 1992, Burridge *et al.* 1992, Kornberg *et al.* 1992, Calalb *et al.* 1995). Detachment of cells by trypsinisation reduces the phosphotyrosine content of FAK (Hanks *et al.* 1992). There have been six FAK tyrosine phosphorylation sites identified to date, which have been mapped to residues 397, 407, 576, 577, 861 and 925 (Calalb *et al.* 1995 and 1996, Schaller *et al.* 1994, Schlaepfer *et al.* 1994). The phosphorylation of protein tyrosine residues is commonly involved in regulating their interaction with SH2 domain containing proteins. Cytoplasmic signalling proteins recognise phosphorylated tyrosine residues and interact through Src homology 2 domains (SH2 domains) and PTB domains.

Tyrosine 397 is the major site of FAK autophosphorylation (Schaller *et al.* 1994). In Src transformed cells FAK and Src are stably associated and an intact Src SH2 domain is necessary for this association (Cobb *et al.* 1994). In cells overexpressing Src and tyrosine 397 mutated FAK, Src and FAK failed to form a stable complex (Schaller *et al.* 1994), suggesting Src binds to FAK at tyrosine 397 via its SH2 domain. Several other proteins have been reported to bind to the autophosphorylation site of FAK via SH2 domains. These include Fyn, another Src family kinase (Cobb *et al.* 1994, Polte and Hanks 1995), the p85 subunit of PI3K (Chen and Guan 1994, Chen *et al.* 1996), the adapter protein Shc (Schlaepfer *et al.* 1998), the signalling protein Grb7 (Han and Guan 1999) and the γ1 isoform of phospholipase C (Zhang *et al.* 1999). Schlaepfer *et al.* (1994) demonstrated adhesion induced association of Src and the adapter protein Grb2, to FAK. The Grb2 association was via its SH2 domain. Schlaepfer and Hunter (1996) discovered that mutation of FAK tyrosine 397, the Src binding site, disrupted not only Src binding but also the association of Grb2 with FAK *in vivo*. They proposed Src family protein tyrosine

kinases are recruited to FAK following integrin induced tyrosine phosphorylation and bind via tyrosine 397. Src then phosphorylates FAK at tyrosine 925 creating a binding site for Grb2 via its SH2 domain. In addition, tyrosines 407, 576, 577 and 861 have also shown to be phosphorylated by Src (Calab *et al.* 1995 and 1996).

The phosphorylation state of FAK not only allows other signalling protein to bind but also affects its own tyrosine kinase activity. Tyrosines 576 and 577 lie within the catalytic domain of FAK and maximal kinase activity towards exogenous substrates was observed when these tyrosines were phosphorylated (Calab *et al.* 1995).

Having demonstrated VEGF stimulated tyrosine phosphorylation of FAK and induced Src family association with FAK this mechanism was investigated further by the generation of various FAK mutants. A Fyn kinase negative construct was also used as a dominant negative to investigate the role of Fyn in the tyrosine phosphorylation of FAK.

The mechanism of FAK activation is not clear. One speculative model suggests that FAK may be inactive due to interaction between its N and C terminal domains as a deletion of the first 100 residues of the N-terminal region of FAK enhance its activity (Schlaepfer and Hunter 1996). This would suggest a conformational change was required to activate FAK however it is not known how the conformational change is induced or whether another mechanism entirely is responsible for activation. In this chapter the mechanism of FAK activation was investigated further by use of the mutant FAK clones.

4.1 Generation and expression of full length flag tagged FAK

4.1.1 Cloning of focal adhesion kinase into the pFLAG-CMV2 expression vector.

cDNA corresponding to mouse FAK was obtained from ATCC and subcloned into a flag expression vector (pFLAG-CVM2 was obtained from Sigma-Aldridge, Poole, Dorset, UK). Epitope tagging of FAK enabled specific analysis of exogenously expressed protein and also allowed efficient immunoprecipitation and visualisation. A flag tag was chosen due to its small size, which would be expected to have minimal effect on FAK function.

A Not1 restriction site was introduced at the 5' end of the full length FAK insert in the PT7-7-FAK clone (ATCC, clone code 63207) using site directed mutagenesis. Primers were designed and mutagenesis performed as described in materials and methods section 2.28.

Digestion of the mutated PT7-7-fak with Not1 and Bgl2 restriction enzymes demonstrated that a Not1 site had been introduced to the plasmid (see figure 4.1). When the unmodified PT7-7-Fak was digested with Bgl2 and Not1 restriction enzymes, two fragments (3.5kb and 2.5kb in size) were produced due to the two Bgl2 sites present in PT7-7-Fak. When the modified PT7-7-Fak containing the newly introduced Not1 site was digested with Not1 and Bgl2, this produced two fragments of 3.2kb and 2.5kb. This was due to the Not1 site in the 3.5kb fragment, which was digested into two fragments of 3.2kb and 0.3kb. The 0.3kb fragment was not visible on the gel as it was too small.

Both the Flag plasmid and the mutated PT7-7-Fak containing the newly introduced Not1 site were digested with Blg2 and Not1. The fragments from the digests were separated by agarose gel electrophoresis and the appropriate band was cut out and gel extracted. The FAK fragment was ligated into the flag vector via the Not1 and Bgl2 site with the resulting construct containing an N-terminally flag tagged FAK which was assigned the name pFFfl and corresponding expressed protein, FFfl (see figure 2.1 for schematic representation of FFfl). The entire FAK sequence except for the start methionine was inserted into the flag vector in frame with the flag tag.

4.1.2 Expression of the pFFfl construct.

Initial expression of pFFfl was demonstrated as shown in figure 4.2. BAE cells grown in 6 well plates were transfected with 2μg of the pFFfl construct as described in materials and methods. The cells were lysed with reducing sample buffer, boiled, sonicated and the proteins separated by SDS-PAGE electrophoresis and then subjected to western blotting. The western blots were probed with anti-flag and anti-FAK antibody. The DRC anti-FAK blot showed a prominent band of the size expected for FAK (125kDa) in the transfected cells (lanes 3 and 4) which was present in the lysates of non transfected cells at a much lower concentration (lanes 1 and 2). The band in the nontransfected cells was thought to be endogenous FAK. It was evident however that the pFFfl construct was expressed in BAE cells.

In order to investigate the proteins associated with FAK, the pFFfl construct was transfected into cells and expressed flag-tagged FAK protein immunoprecipitated 22 hours later. Blots of immunoprecipitated FFfl were probed for associated proteins. For these experiments BAE cells grown in 90mm dishes were transfected with 7.5μg of DNA as described in materials and methods. The cells were lysed with Backer buffer and the expressed FAK immunoprecipitated using anti-flag antibody before being subjected to SDS-PAGE electrophoresis and western blotting. The western blots were probed with antibodies recognising candidate FAK binding partners (figure 4.3A and B). The flag antibody detected a band of 125kDa which was demonstrated to be highly tyrosine phosphorylated when probed with the anti-phosphotyrosine antibody. Further probing of the blots demonstrated that Src and Fyn bound to FFfl however p85 did not. As shown in figure 4.3B a small amount of background Src was immunoprecipitated by the flag antibody. Probing with the Grb2 antibody failed to produce conclusive results. The Grb2 antibody was not able to detect its respective target protein in whole cell lysates. Previous experiments with this antibody had shown clear immunoreactivity (see chapter 3 figure 3.11 B). A number of different conditions were used for probing blots with this antibody however it was not possible to obtain conclusive data with it.

4.2 Generation and expression of mutant FAK constructs.

4.2.1 Generation of mutant FAK constructs

Mutant forms of FAK were constructed by site directed mutagenesis in order to further investigate the role of its tyrosine phosphorylation state and the significance of the signalling molecules binding. FAK possesses six identified phosphoacceptor sites and it is well established which tyrosine residues bind which signalling molecules. With the knowledge that Src and Fyn had been demonstrated to bind to residue 397 and Grb2 to residue 925 these residues were mutated to prevent the binding of these signalling molecules (Schaller *et al.* 1994, Schlaepfer and Hunter 1996). The kinase activity of FAK was disrupted to investigate its role in signalling and a truncated form of FAK containing the focal adhesion targeting sequence constructed to 'block' full length FAK from focal adhesions.

The pFF397F and pFF925F constructs were generated by mutating the original pFFfl construct using site directed mutagenesis to change the tyrosine residue 397 or 925 respectively to a phenylalanine (as described in section 2.29.1 and 2.29.2). This mutation would prevent proteins containing SH2 domains binding to these residues. Proteins containing SH2 domains can bind to phosphorylated tyrosine residues, via their SH2 domains, so by mutating these sites from tyrosine to phenylalanine this interaction is prevented and the significance of the interaction of these signalling molecules can be tested. See figure 2.1 for schematic representation of FF397F and FF925F.

pFF454R was generated from the pFFfl clone using site directed mutagenesis to incorporate an arginine in place of the lysine at residue 454 (as described in section 2.29.3). This point mutation renders FAK kinase deficient (Hildebrand *et al.* 1993) as this residue is within the ATP-binding site of the catalytic domain and is conserved in the eukaryotic protein kinase superfamily (Hanks *et al.* 1988). This construct could be used to investigate the role of the kinase activity of FAK. See figure 2.1 for a schematic representation of FF454R.

pFF792 encodes a C-terminal truncated form of FAK starting at the 792 residue. The pFF792 construct was generated by introducing a Not1 restriction site into the pFFfl plasmid at residue 792 using site directed mutagenesis (as described in section 2.29.4).

The construct was then digested with Not1 restriction enzyme which resulted in the production of two fragments which were separated by agarose gel electrophoresis (see figure 4.4). The smaller fragment comprised of the first two-thirds of FAK and the larger fragment contained the flag plasmid and the third C-terminal part of FAK. The larger band of interest was cut out of the gel and extracted. A simple re-ligation of the fragment was performed as described in materials and methods (see figure 2.1 for schematic representation of FF792). This construct was predicted to be localised to focal adhesions as it still contains the FAT (focal adhesion targeting) sequence. This construct contains the tyrosine residues 861 and 925 and the second proline rich region although the first proline rich region is absent. In the rest of this thesis the proteins expressed from the constructs described above are designated by the same name as the constructs but lacking the prefix p, for example FF792 corresponds to the protein expressed from the plasmid pFF792.

4.2.2 Expression of the mutant FAK constructs.

The mutant constructs were expressed in BAE cells and immunoprecipitated with a flag antibody. BAE cells grown in 90mm plates were transfected with 7.5μg of DNA. After 22 hours the cells were lysed with Backer buffer and the expressed proteins immunoprecipitated with an anti-flag antibody before being subjected to SDS-PAGE electrophoresis and western blotting. Probing of the western blots with anti-flag showed varying levels of expression between the mutant FAK proteins however these expression levels were consistent over several experiments (Figure 4.5A and B, top panels).

All the mutant FAK proteins appeared the same size of 125KDa except, as expected, the truncated form of FAK (FF792) which appeared about 35KDa in size. Of the same sized constructs, the pFF397F consistently showed the highest level of expression and the full length FAK, pFFfl demonstrated the lowest. It is not clear why there was a difference in expression levels but it is possibly due to the number of transfected cells rather than the level of expression in each cell. It is possible that the various mutant FAK proteins affect cell survival and cell death. The full length FAK however demonstrated the lowest expression levels even though full length FAK is thought to be a protective factor against cell death. Another possibility for the different amounts of FAK mutants on the blots is that the highly phosphorylated full length FAK was degraded as a way of removing

activated FAK. As the other FAK mutants were not as phosphorylated they may not have been broken down as quickly.

Differences in expression levels between different forms of FAK could reflect differences in transfection efficiency. Supercoiled plasmid DNA is most efficiently transfected into cells. The distribution of DNA forms between supercoiled, coiled and linear were not routinely analysed for each plasmid prior to transfection. It is possible therefore that the differences in expression levels may have been caused by variations in transfection efficiencies, due to the different forms of DNA produced during plasmid preparations.

4.3 Analysis of tyrosine phosphorylation state and proteins associated with mutant forms of FAK.

Before investigating the effects of the full length and mutant FAK constructs on cell function the expressed proteins were further characterised. The tyrosine phosphorylation state and proteins associated with the mutant forms of FAK were examined.

4.3.1 Tyrosine phosphorylation levels of mutant FAK proteins.

Probing the blots with a phosphotyrosine antibody demonstrated the *in vivo* tyrosine phosphorylation state of each mutant FAK protein. FFfl demonstrated an extremely high level of tyrosine phosphorylation especially considering the low expression level. However the FF397F demonstrated virtually no tyrosine phosphorylation suggesting that tyrosine 397 is the major phosphorylation site and is possibly required to be phosphorylated before other tyrosines are able to become phosphorylated. The kinase deficient FAK protein demonstrated a small amount of tyrosine phosphorylation which was more than seen with FF397F. This tyrosine phosphorylation could have been caused by transphosphorylation by endogenous FAK. FF792 contains two tyrosines 861 and 925 which could potentially become phosphorylated but this C-terminal FAK showed no tyrosine phosphorylation. FF925F, which lacks one tyrosine residue at 925, demonstrated similar phosphorylation levels to the full length FAK as might be expected (figure 4.5A and B).

Quantitative analysis of the tyrosine phosphorylation state of the FAK mutants in a series of experiments was performed. For each experiment the level of tyrosine phosphorylation of the immunoprecipitated FAK mutant was determined by densitometric scanning of the blots probed for tyrosine phosphorylation and Flag (described in section 2.34). The relative phosphorylation states were normalised with respect to Flag-FAK in each lane. As shown in figure 4.5C, FF397F, FF454R and FF925F demonstrated tyrosine phosphorylation levels of 9, 22 and 90 percent of FFfl respectively.

4.3.2 Association of Src with mutant forms of FAK.

The association of Src with each expressed protein was investigated to indicate the potential signalling pathways activated. Quantitative analysis of the amount of Src associated with the mutant FAK proteins in a series of experiments was performed. For each experiment the amount of Src co-immunoprecipitated with each FAK mutant was determined by densitometric scanning of the blots probed for Src and Flag. The amount of Src bound was normalised with respect to Flag-FAK in each lane. It appeared some Src was associated with FF397F and FF454R (figure 4.5D, left panel) however when non-transfected controls were performed and non specific binding of Src taken into account it was apparent that Src did not bind to FF397F, FF454R or FF792 (figure 4.5D, right panel). The highest association of Src was found to be with full length FAK, with slightly less binding to FF925F although the difference was not statistically significant.

4.3.3 Association of the p85 subunit of PI3K with mutant forms of FAK.

No p85 was found to associate with any of the mutant forms of FAK or the full length FAK (figure 4.5B). This is consistent with the previous results obtained with endogenous FAK (section 3.6.5) which also demonstrated no association of the p85 subunit of PI3K with FAK.

4.3.4 Association of Fyn and Grb2 with mutant forms of FAK.

These experiments were not conclusive due to problems with the detecting antibodies.

4.4 Immunofluorescence staining to detect localisation of epitope tagged mutant FAK proteins.

4.4.1 Immunofluorescence staining for flag epitope tagged mutant FAK proteins and vinculin.

The mutant forms of FAK were expressed and cells analysed by immunofluorescence dual staining for flag and vinculin. Staining for flag would reveal the localisation of the flag-FAK mutant proteins. The flag tag is very small and as all the constructs contained the focal adhesion targeting (FAT) sequence it was expected that the expressed proteins would be localised to the focal adhesions. Vinculin is a protein localised to focal adhesions and was used as a probe to detect the focal adhesions.

BAE cells grown on coverslips were transfected with pFFfl. Twenty-four hours post transfection the cells were fixed and analysed by immunofluorescence dual staining to show FFfl and vinculin distribution (see materials and methods sections 2.14.2, 2.14.3 and 2.14.5). Probing with anti-flag antibody revealed staining throughout the cell indicating the presence of FFfl throughout the cell (see figure 4.6). This most likely results from saturation of available binding sites at focal adhesions by the levels of FAK expressed. Vinculin localisation was undetectable in the transfected cells due to 'bleed through' from the high levels of Flag expression. In non-transfected cells, vinculin immunostaining demonstrated the formation and location of focal adhesions. BAE cells transfected with the other FAK mutant construct demonstrated the same high expression level and undetectable vinculin localisation due to 'bleed through' (data not shown). A confocal laser scanning microscope would have been beneficial in this experiment as it would have enabled viewing of cross-sectional layers of the cell. By selecting cross-sectional views of the cytoplasmic face of the plasma membrane adjacent to the adhesive substrate it may have been possible to detect any FAK localised to focal adhesions.

4.4.2 Immunofluorescence staining for flag epitope tagged mutant FAK proteins and F-actin.

The mutant forms of FAK were expressed and cells analysed by immunofluorescence dual staining for flag and F-actin. As FAK can effect cell shape and migration the cells were probed for actin to investigate if the mutants had any effect on actin stress fibres. Staining for flag would reveal the localisation of the mutant FAK proteins.

BAE cells grown on coverslips were transfected with FFfl. Twenty-four hours post transfection the cells were fixed and analysed by immunofluorescence dual staining to show FFfl and F-actin distribution. Cells were probed for filamentous actin using a FITC conjugated phalloidin antibody before staining for flag (see materials and methods section 2.14.1, 2.14.3 and 2.14.5). Probing to flag revealed similar results as in section 4.4.1. Staining throughout the cell indicated the presence of FFfl throughout the cell (see figure 4.7). This most likely results from saturation of available binding sits at focal adhesions by the levels of FAK expressed. Actin localisation was undetectable in transfected cells due to high levels of Flag. In non-transfected cells the F-actin staining demonstrated the formation of stress fibres in the BAE cells. BAE cells transfected with the other FAK mutant construct demonstrated the same high expression level and undetectable actin staining due to 'bleed through' (data not shown). The use of a confocal laser scanning microscope may have enable localisation of the FAK mutants in this experiment by viewing cross-sectional sections of the cell as discussed in section 4.4.1.

4.5 Use of Fynk$^-$ as a dominant negative to investigate the role of Fyn in the tyrosine phosphorylation of FAK.

A kinase inactive Fyn construct, Fynk$^-$ was obtained as a kind gift from Dr. Sarah Courtneidge. In order to further investigate the signalling pathway of FAK activation the full length FAK and the kinase inactive Fyn were coexpressed. The Fynk$^-$ would act as a dominant negative, binding to FAK but not tyrosine phosphorylating it and preventing the association of endogenous Src family kinases. The coexpresion of these two constructs could give more information on the role of Fyn in downstream signalling.

4.5.1 Initial expression of Fynk$^-$.

BAE cells were transfected in 6 well plates with 2μg of pFynk$^-$, the cells lysed with reducing sample buffer and the whole cell lysates subjected to SDS-PAGE electrophoresis and immunoblotted for the detection of Fynk$^-$ using a Fyn antibody. As shown in figure 4.8, cells transfected with pFynk$^-$ demonstrated high levels of Fyn immunoreactivity indicating expression of Fynk$^-$.

4.5.2 Co-transfection of Fynk$^-$ and full length flag tagged FAK.

BAE cells grown in 6 well plates were transfected with 1.25μg of pFynk$^-$ and 1.25μg of pFFfl and the whole cell lysates subjected to SDS-PAGE electrophoresis and immunoblotted for the detection of Fyn and FAK. Both of the constructs were present in the whole cell lysates as shown in figure 4.9.

4.5.3 Effect of Fynk$^-$ on FAK tyrosine phosphorylation in response to adhesion.

BAE cells grown in 80cm^2 flasks were cotransfected, with either 4μg of pFFfl and 4μg of pFlag or 4μg of pFynk$^-$ and 4μg of pFFfl. Twenty-four hours after transfection the cells were treated with trypsin-EDTA and half of the cells re-plated onto fibronectin coated plates while the other half were kept in suspension. After 60 minutes the fibronectin coated plates were rinsed with PBS to remove any non adherent cells and the adherent cells lysed. The cells in suspension were pelletted, rinsed in PBS and lysed. The full length Flag-tagged FAK was immunoprecipitated from the cell lysates using a flag antibody, subjected to SDS-PAGE electrophoresis and immunoblotted for the detection of tyrosine phosphorylation levels. As shown in figure 4.10, the phosphorylation level of flag tagged full length FAK was not altered if the cells were held in suspension or were allowed to adhere and under both conditions FAK was highly phosphorylated.

Kinase negative Fyn should act as a dominant negative, binding to FAK but not tyrosine phosphorylating it while preventing endogenous Fyn or Src from binding and inducing further tyrosine phosphorylation. The kinase negative Fyn, however did not appear to affect the tyrosine phosphorylation state of FAK suggesting that the high level of phosphorylation seen was due to autophosphorylation of tyrosine 397 and not further

phosphorylation of other tyrosine sites by endogenous Fyn or Src. It must be noted however, that due to problems with the Fyn antibody (see above) it was not possible to confirm Fyn binding to FAK in these co-expression experiments.

4.5.4 Investigating the effect of Fynk⁻ on the ability of VEGF to activate endogenous FAK.

In order to investigate the role of Fyn in VEGF induced tyrosine phosphorylation of endogenous FAK, BAE cells were transfected with kinase inactive Fyn. BAE cells plated in 90mm plates were transfected with 7.5μg pFynk⁻ or pFLAG-CMV-2 as a control. After 20 hours the cells were washed twice in PBS before the addition of serum free DMEM to quiesce the cells. After 24 hours the BAE cells were stimulated with control vehicle or 2.5nM of VEGF for 10 minutes. Fyn was immunoprecipitated from the cell lysates, subjected to SDS-PAGE electrophoresis and western blot analysis. As shown in figures 4.11A and B, no FAK was detected on the blots but was detected in the lysates after immunoprecipitation of Fyn. This indicated FAK did not remain associated with Fyn under these conditions so its phosphorylation state could not be examined. Ideally FAK would have been immunoprecipitated to investigate its tyrosine phosphorylation level in the presence of kinase inactive Fyn but unfortunately the FAK antibody was no longer suitable for immunoprecipitations. As FAK could no longer be immunoprecipitated directly, a flag tagged FAK construct could have been utilised but as demonstrated in the previous experiment (section 4.5.3).

4.6 Investigation of the mechanism of FAK tyrosine phosphorylation.

As expression of the kinase inactive Fyn did not affect tyrosine phosphorylation of FAK it was hypothesized that FAK phosphorylation observed in these experiments resulted from transphosphorylation between FAK molecules. This could occur due to clustering of FAK at focal adhesions. pFF792 contains the FAT sequence and was used as a dominant negative to compete with full length FAK for the FAK binding site in focal adhesions.

To investigate if excluding FAK from the focal adhesion effected its tyrosine phosphorylation level, both pFFfl and pFF792 were coexpressed and phosphorylation levels compared to expression of full length FAK alone. BAE cells grown in 90mm plates

were transfected with 4μg of pFFfl and 4μg of pFF792 or 4μg of pFFfl and 4μg of the empty pFLAG vector. Cells were lysed in Backer buffer and the Flag-tagged FAK proteins immunoprecipitated from the cell lysates, subjected to SDS-PAGE electrophoresis and western blotted to detect the tyrosine phosphorylation level of the full length Flag-FAK (see figure 4.12A). The full length FAK which was coexpressed with the truncated C-terminal FAK demonstrated substantially reduced levels of phosphorylation compared with the full length FAK expressed with flag. Quantitative analysis of a series of experiments was performed by densitometric scanning for tyrosine phosphorylation and FFfl levels. The relative phosphorylation states were normalised with respect to FFfl levels in each lane. As shown in figure 4.12B co-expression of FF792 with FFfl reduced the tyrosine phosphorylation state of FFfl by 4.8 fold (see figure 4.12B).

This suggests that FAK phosphorylation may depend on its ability to localise to focal adhesions and that when FAK is restricted from localising to focal adhesions, due to the presence of the C-terminal FAK, phosphorylation cannot occur. Alternatively it may be that FAK phosphorylation results from dimerisation or interaction with another protein and this is prevented by the presence of truncated FAK. If FAK does need to dimerise in order for phosphorylation to occur, the C-terminal FAK must posses the sequence required for dimerisation but not phosphorylation as coexpression with C-terminal FAK reduces phosphorylation of full length FAK.

4.6.1 Coexpression of mutant FAK constructs with full length FAK.

To further investigate whether FAK undergoes transphosphorylation it was necessary to co-express kinase-negative FAK and wild type FAK and to be able to specifically recover the kinase-negative protein and examine its phosphorylation status. To do this wild type FAK was subcloned into pCR3 and coexpressed with the flag tagged FAK mutant constructs. Once the flag mutants were immunoprecipitated the effect of full length FAK on their phosphorylation level was examined to investigate if any loss of phosphorylation could be rescued.

To generate pCR3-FAK, full length FAK was digested out of pFFfl using Not1 and Xba1. pCR3 was also digested with Not1 and Xba1 and the products separated on an agarose gel

and the appropriate bands cut out and purified. The full length FAK was ligated into pCR3 to generate a non tagged full length FAK construct (described in section 2.30). To analyse expression of pCR3-FAK, whole cell lysates of BAE cells either transfected with pCR3-FAK or empty vector were subjected to SDS-PAGE and western blotting analysis. Probing with antiphosphotyrosine antibody revealed the presence of a tyrosine phosphorylated protein the size of FAK which was present to a greater extent in the pCR3-FAK transfected cells compared to the cells transfected with empty vector (figure 4.13A). This suggested transfection of BAE cells with pCR3-FAK lead to expression of FAK.

To investigate the effect of full length FAK on the FAK mutants BAE cells grown in 90mm plates were cotransfected with 4μg of pCR3-FAK and 4μg of a flag tagged mutant FAK construct or 4μg of the empty pCR3 vector and 4μg of a flag tagged mutant FAK construct. The flag tagged FAK was then immunoprecipitated using an anti-flag antibody, subjected to SDS-PAGE electrophoresis and western blotting. The western blots were probed to detect the tyrosine phosphorylation level of the FAK mutants. As shown in figure 4.13B, the phosphorylation level of FF792 was not effected by the presence of full length FAK. The presence of full length FAK increased the phosphorylation level of FF454R compared with FF454R expressed without exogenous FAK. The phosphorylation level of FF397F was not particularly altered by co-expression of wild-type FAK.

Quantitative analysis of a series of experiments to establish the tyrosine phosphorylation levels of FF397F and FF454R when co-expressed with full length FAK were performed. For each experiment the level of tyrosine phosphorylation of immunoprecipitated Flag-FAK was determined by densitometric scanning of blots probed for tyrosine phosphorylation and Flag. The relative phosphorylation states were normalised with respect to Flag-FAK levels in each lane. As shown in figure 4.13C, co-expression of full length FAK with FF397F had little effect on FF397F tyrosine phosphorylation whereas co-expression of full length FAK with FF454R resulted in a 4.5 fold increase in tyrosine phosphorylation of FF454R compared to its expression with empty vector. This increase was not statistically significant when analysed by the paired students t-test (Bailey, Statistical Methods in Biology, 1979). However on each occasion, at least a 2.3 fold increase in tyrosine phosphorylation of FF454R was seen when co-expressed with full

length FAK and therefore more detailed experiments are required to establish any statistical significance.

The increase in phosphorylation of FF454R, the kinase negative FAK, in the presence of wild type FAK suggests the kinase negative FAK is transphosphorylated by wild-type FAK. The evidence that FF397F phosphorylation level was not altered by full length FAK would suggests that the main residue of phosphorylation by FAK is tyrosine 397 and that this residue must be phosphorylated before other tyrosines can become phosphorylated perhaps because Fyn, Src or another kinase bind to it via their SH2 domain. FF792 may also not become phosphorylated, as it does not posses the tyrosine 397 residue to allow a kinase to bind.

Together these data are consistent with FAK being able to undergo transphosphorylation at tyrosine 397 when in close proximity with another FAK molecule.

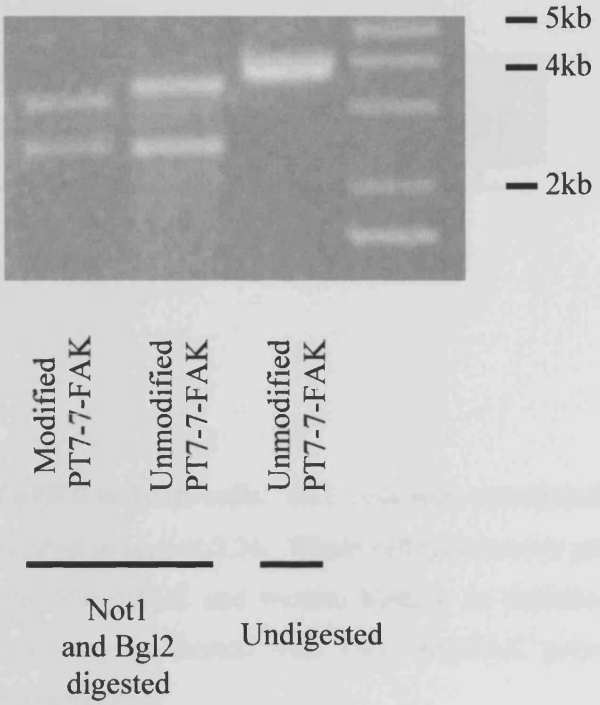


Figure 4.1. Verification of the introduction of a Not1 site into PT7-7-FAK. PT7-7-FAK which had a Not1 site introduced by site directed mutagenesis (modified PT7-7-FAK) as described in section 2.28 and unmodified PT7-7-FAK were digested with restriction enzymes Not1 and Bgl2 as described in section 2.21. A sample from each digest, as well as an undigested sample of PT7-7-FAK was run on a 1% agarose gel and visualised by UV illumination as described in section 2.20. The position of the 1kb DNA ladder molecular weight markers are indicated to the right of the panel.

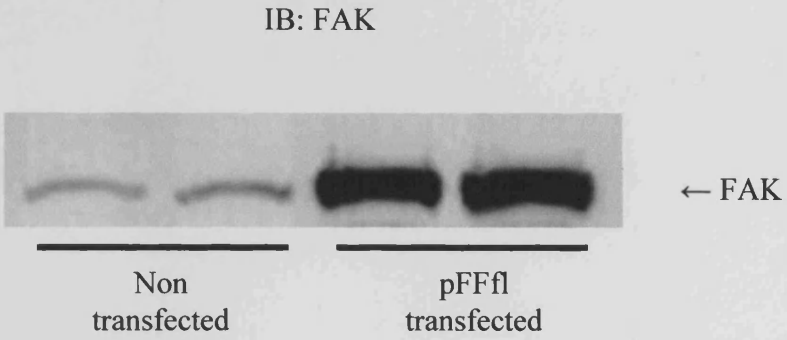


Figure 4.2. Expression of pFFfl in BAE cells. BAE cells were transfected with pFFfl or non-transfected as a control as described in section 2.26. Whole cell lysates were prepared as described in section 2.5 and analysed by SDS-PAGE and western blotting as described in materials and methods. Immunoblot analysis was performed with DRC anti-FAK polyclonal antibody as described in section 2.9.2. IB, immunoblot.

A

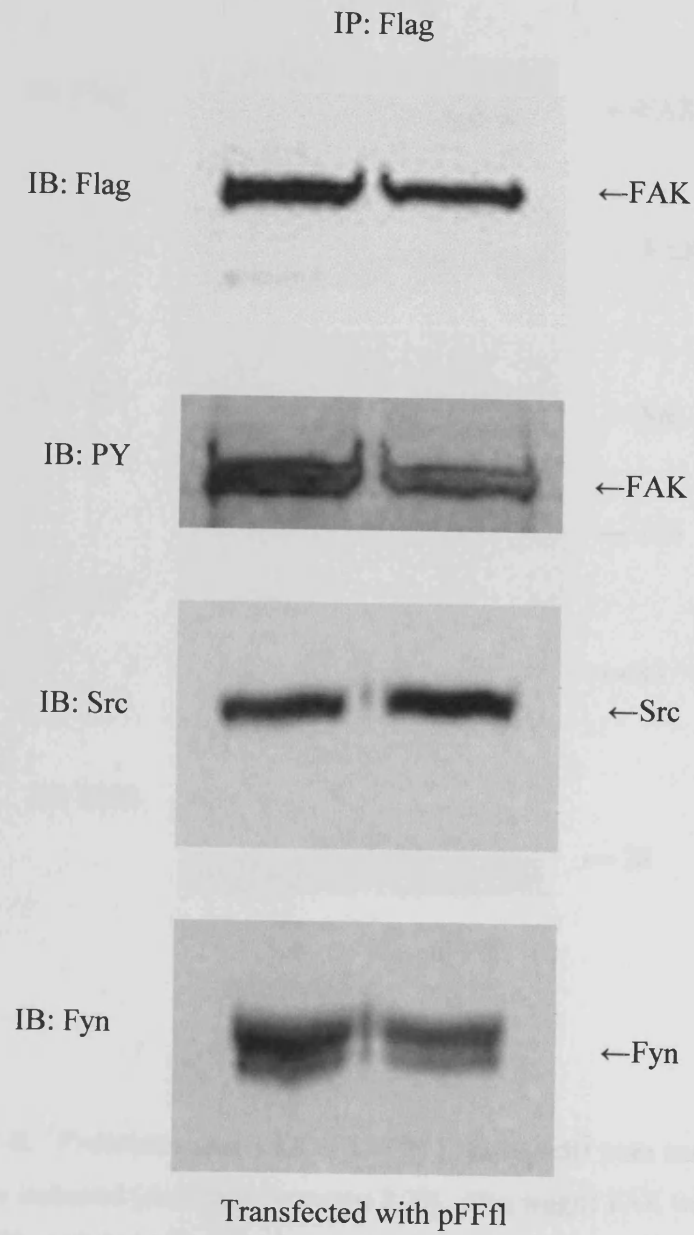

IP: Flag
IB: Flag
←FAK
IB: PY
←FAK
IB: Src
←Src
IB: Fyn
←Fyn
Transfected with pFFfl

Figure 4.3A.

B

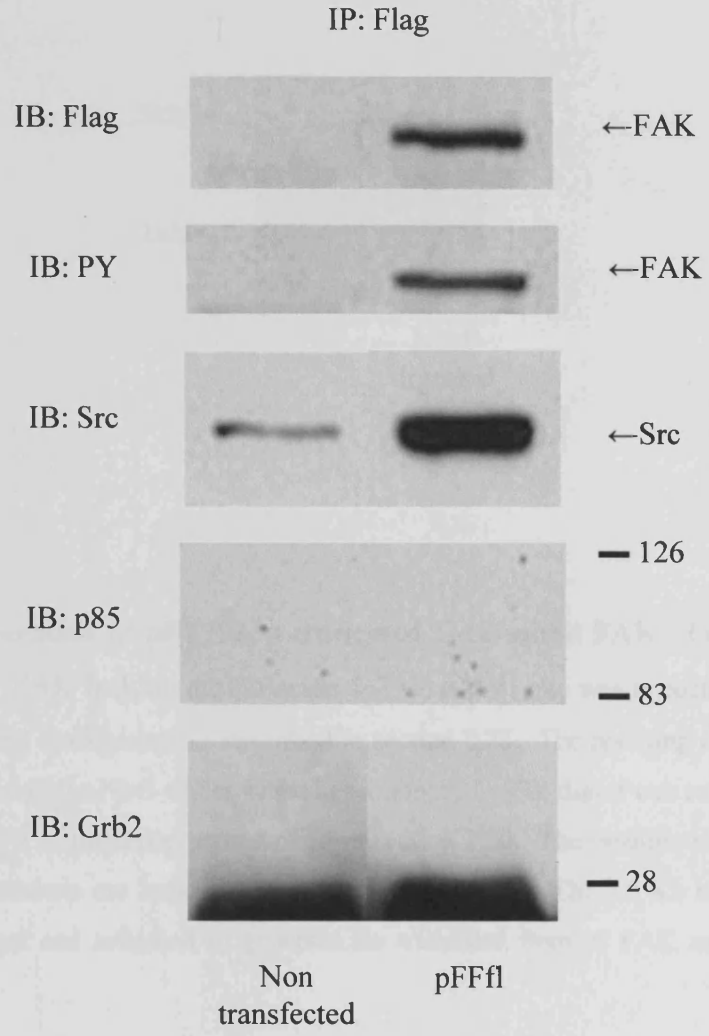


Figure 4.3A and B. Proteins associated with FFfl. BAE cells were transfected with pFFfl or left untransfected as indicated (described in section 2.26). Flag tagged FAK was immunoprecipitated from cell lysates with anti-flag M2 antibody covalently attached to agarose (as described in section 2.6.4). Immunoprecipitated proteins were resolved by SDS-PAGE and subjected to immunoblot analysis as described in section 2.7 and 2.8. Blots were probed to detect immunoprecipitated FFfl with anti-flag antibody (Flag) and phosphotyrosine levels with anti-phosphotyrosine antibody (PY) (described in section 2.9.1 and 2.9.5). The presence of co-immunoprecipitated Src (Src), Fyn (Fyn), the p85 subunit of PI3K (p85) and Grb2 (Grb2) was tested by re-probing blots with the relevant antibodies as indicated (described in section 2.9.4). The position of the molecular mass markers (if shown) are indicated in kDa to the right of the panels. IP, immunoprecipitate; IB, immunoblot. A and B are from separate experiments and are representative of five experiments except for Fyn blot which was performed once.

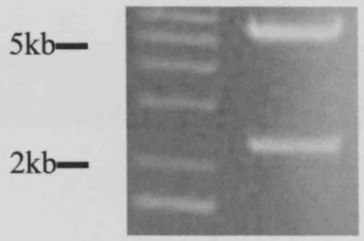


Figure 4.4. Generation of pFF792, a truncated C-terminal FAK. In order to generate a truncated form of FAK lacking amino acids 1-791, a Not1 site was introduced at residue 792 of pFFfl by site directed mutagenesis as described in section 2.25. The resulting construct was digested with the restriction enzyme Not1 as described in section 2.21. The digest was run on a 1% agarose gel and visualised by UV illumination as described in section 2.20. The position of the 1kb DNA ladder molecular weight markers are indicated on the left of the panel. The 5.5 Kb band was subsequently excised from the gel and religated to generate the truncated from of FAK as described in section 2.29.4.

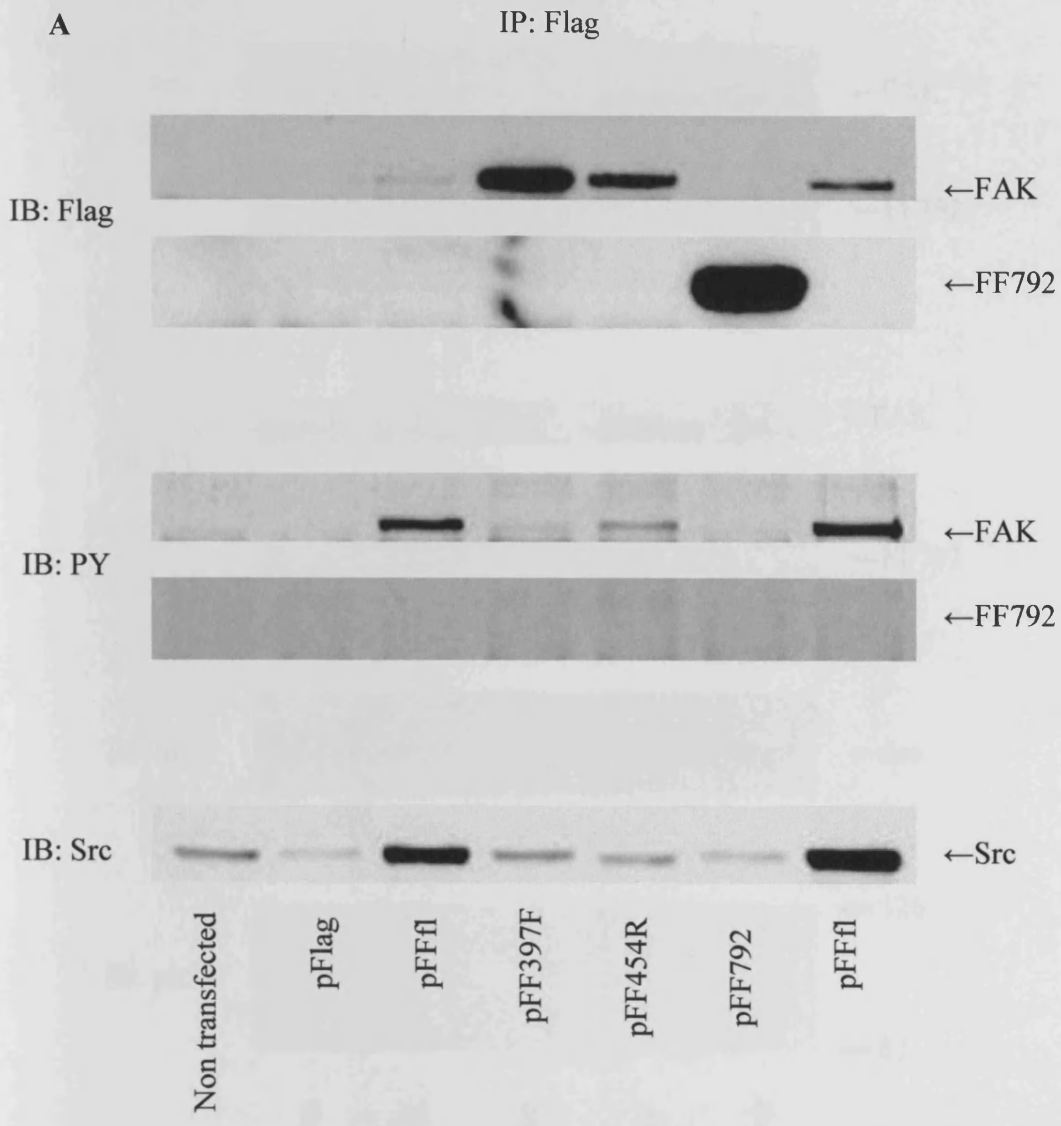

A
IP: Flag
IB: Flag
←FAK
←FF792
IB: PY
←FAK
←FF792
IB: Src
←Src
Non transfected
pFlag
pFFfl
pFF397F
pFF454R
pFF792
pFFfl

Figure 4.5A

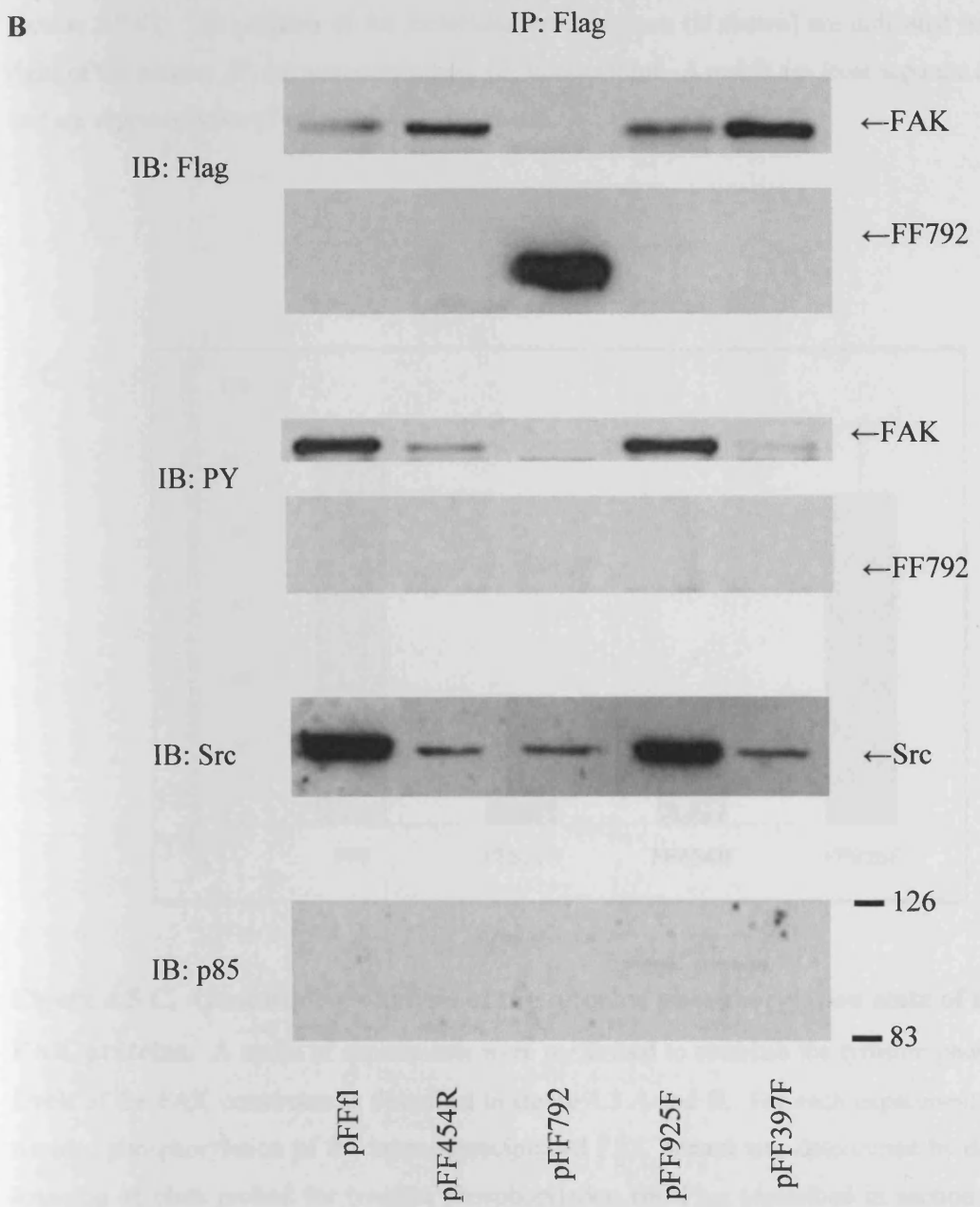


Figure 4.5 A and B. Tyrosine phosphorylation state and proteins associated with FAK mutants. BAE cells were transfected with the various FAK mutants or left untransfected as indicated (described in section 2.26). Flag tagged FAK mutants were immunoprecipitated from cell lysates with anti-flag M2 antibody covalently attached to agarose (as described in section 2.6.4). Immunoprecipitated proteins were resolved by SDS-PAGE and subjected to immunoblot analysis as described in section 2.7 and 2.8. Blots were probed to detect FAK immunoprecipitated with anti-flag antibody (Flag) and phosphotyrosine levels with anti-phosphotyrosine antibody (PY) (described in section 2.9.1 and 2.9.5). The presence of co-immunoprecipitated Src (Src) and the p85 subunit of PI3K (p85) was tested by re-probing blots with the relevant antibodies as indicated (described in

section 2.9.4). The position of the molecular mass markers (if shown) are indicated in kDa to the right of the panels. IP, immunoprecipitate; IB, immunoblot. A and B are from separate experiments and are representative of at least four experiments.

C

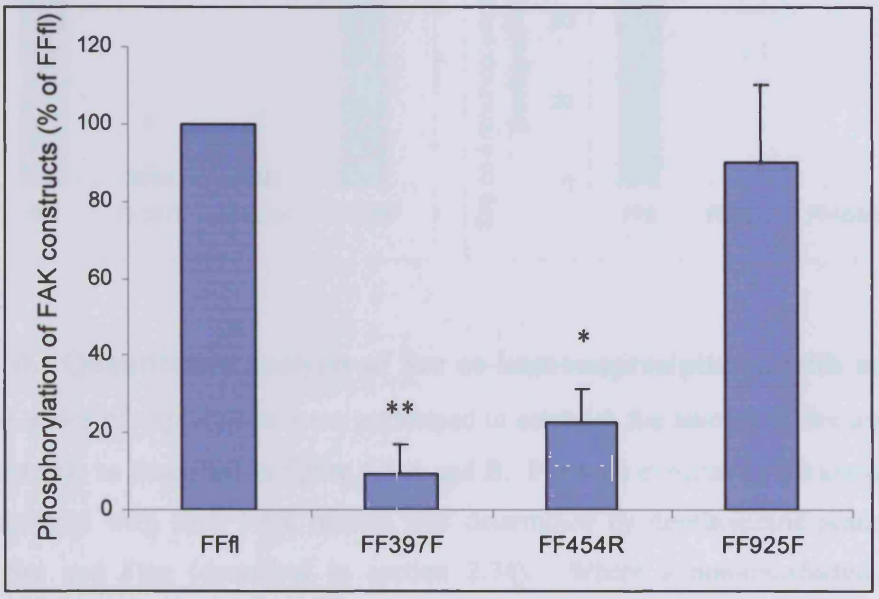


Figure 4.5 C. Quantitative analysis of the tyrosine phosphorylation state of the mutant FAK proteins. A series of experiments were performed to establish the tyrosine phosphorylation levels of the FAK constructs as described in figure 4.5 A and B. For each experiment the level of tyrosine phosphorylation of the immunoprecipitated FAK mutant was determined by densitometric scanning of blots probed for tyrosine phosphorylation and Flag (described in section 2.34). The relative phosphorylation states were normalised with respect to FAK levels (Flag probe) in each lane of each blot. Results are expressed as a percentage of tyrosine phosphorylation of FFfl. Data is presented as mean and standard errors about the mean for at least five experiments. Data was analysed using the paired Students t-test, * indicates $p<0.002$, ** indicates $p<0.001$ compared with FFfl (Bailey, Statistical Methods in Biology, 1979).

D

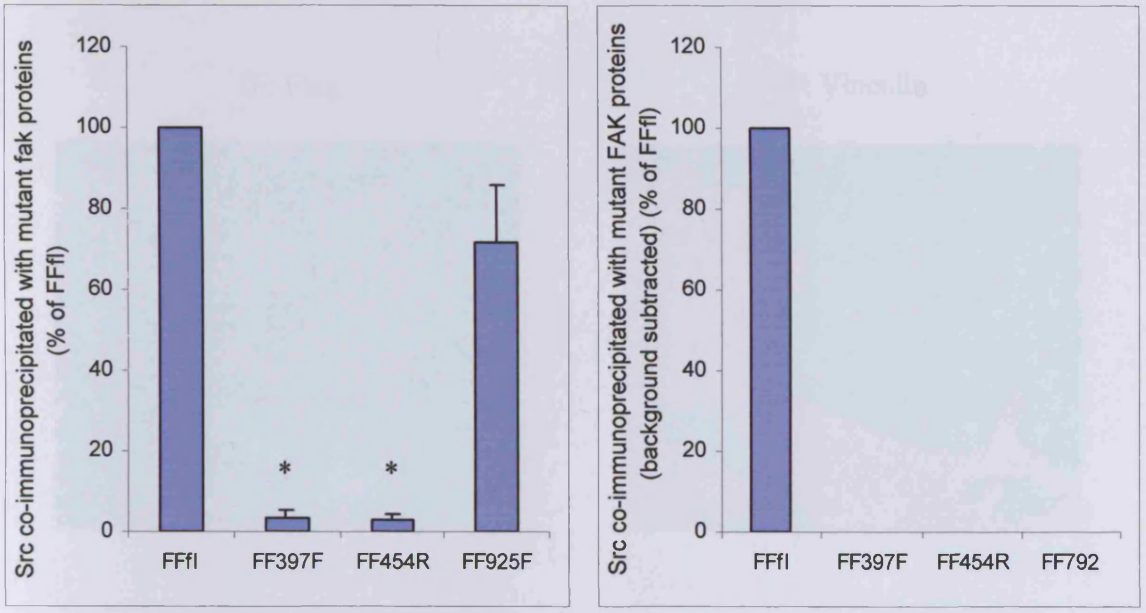


Figure 4.5 D. Quantitative analysis of Src co-immunoprecipitated with mutant FAK proteins. A series of experiments were performed to establish the amount of Src associated with the FAK constructs as described in figure 4.5 A and B. For each experiment the amount of Src co-immunoprecipitated with each FAK mutant was determined by densitometric scanning of blots probed for Src and Flag (described in section 2.34). Where a non-transfected control was performed, the background level of immunoprecipitated Src (non-specific binding) was deducted from the results prior to the data being analysed (right panel). Where a non-transfected control was not performed (left panel) data was analysed including non-specific binding. The amount of Src was normalised with respect to FAK levels (Flag probe) in each lane of each blot. Results are expressed as a percentage of Src association with FFfl. Data is presented as mean and standard errors about the mean for three experiments. Data was analysed using the paired Student's t-test, * indicates $p<0.001$, compared to cells transfected with FFfl (Bailey, Statistical Methods in Biology, 1979).

IF: Flag

IF: Vinculin

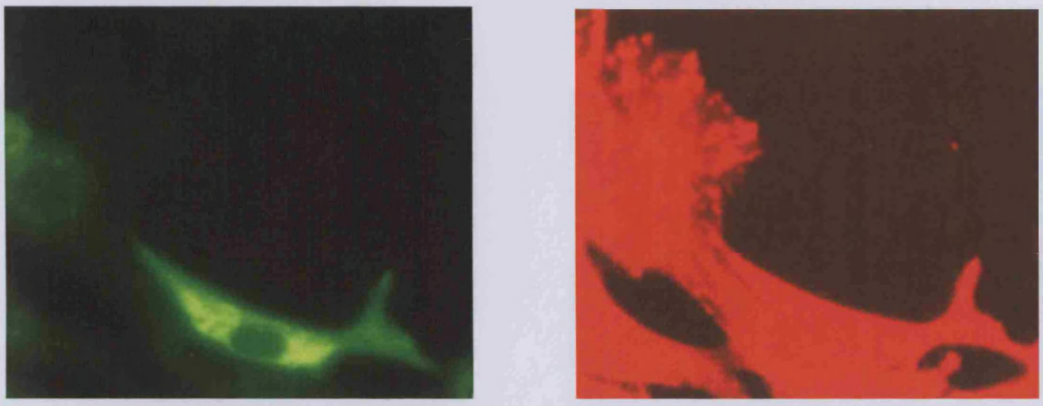


Figure 4.6. Immunofluorescence staining for flag epitope tagged FAK and vinculin. BAE cells grown on coverslips were transfected with FFfl and analysed by immunofluorescence (IF) dual staining to examine FFfl distribution with anti-flag monoclonal antibody (left panel) as described in section 2.14.3 and vinculin localisation with anti-vinculin monoclonal antibody (right panel) as described in section 2.14.2 and 2.14.5.

IF: Flag

IF: F-actin

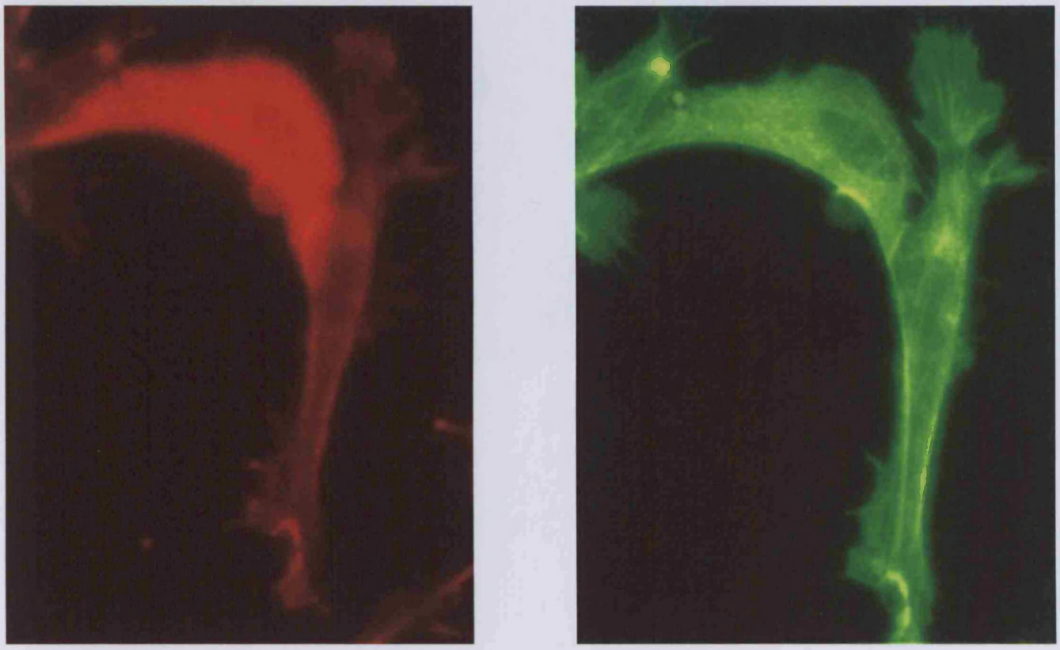


Figure 4.7. Immunofluorescence staining for Flag epitope tagged FAK and F-actin. BAE cells grown on coverslips were transfected with FFfl and analysed by immunofluorescence (IF) dual staining to examine FFfl distribution with anti-flag monoclonal antibody (left panel) as described in section 2.14.3 and F-actin distribution with FITC phalloidin (right panel) as described in section 2.14.1 and 2.14.5.

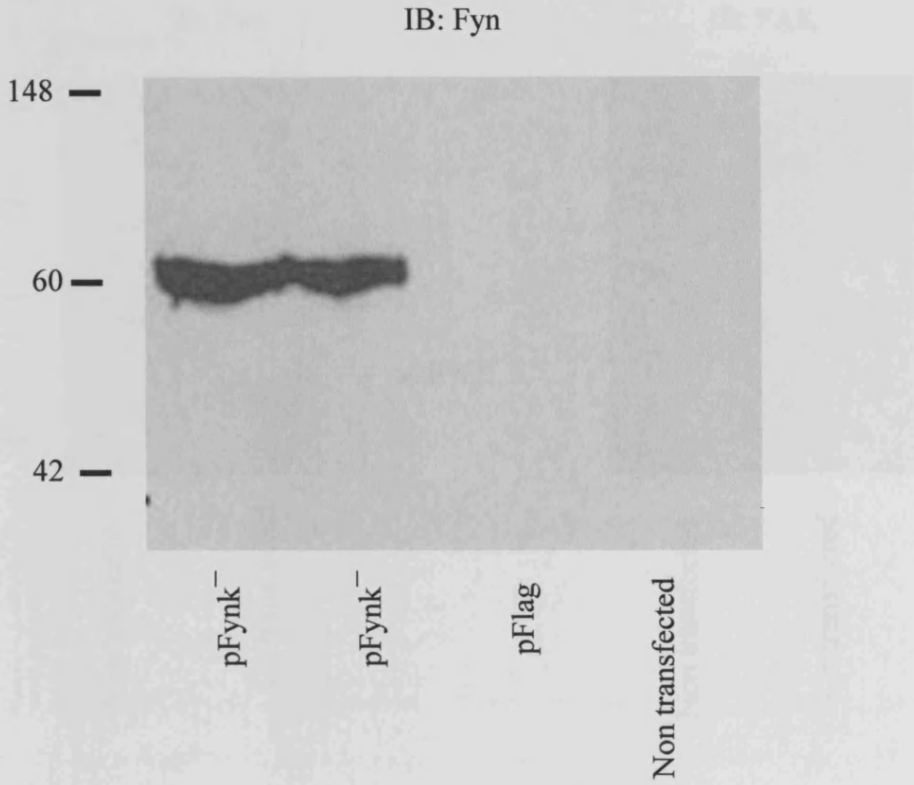


Figure 4.8. Expression of Fynk⁻ in BAE cells. BAE cells were transfected with pFynk⁻, empty flag vector (pFlag) or left untreated as described in section 2.26. Whole cell lysates were prepared as described in section 2.5 and analysed by SDS-PAGE and western blotting as described in section 2.7 and 2.8. Immunoblotting analysis was performed with anti-Fyn monoclonal antibody as described in section 2.9.4. IB, immunoblot. The position of the molecular mass markers are indicated in kDa to the left of the blot. Similar results were obtained in two independent experiments.

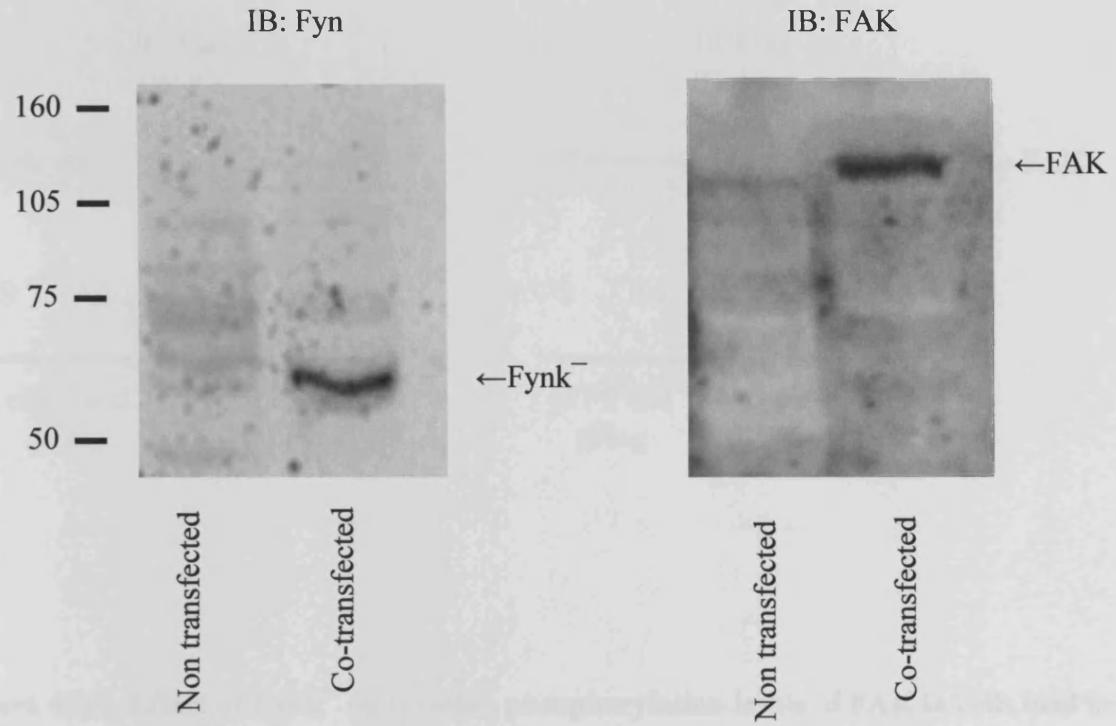


Figure 4.9. Co-transfection of pFFfl and Fynk⁻. BAE cells were co-transfected with pFFfl and pFynk⁻ (described in section 2.26) or not transfected as indicated. Whole cell lysates were prepared as described in section 2.5 and analysed by SDS-PAGE and western blotting as described in materials and methods. Immunoblot analysis was performed with anti-Fyn monoclonal antibody (left panel) as described in section 2.9.4. The membrane was stripped and re-probed with DRC anti-FAK polyclonal antibody (right panel) as described in section 2.9.2. IB, immunoblot. The position of the molecular mass markers are indicated in kDa to the left of the blot.

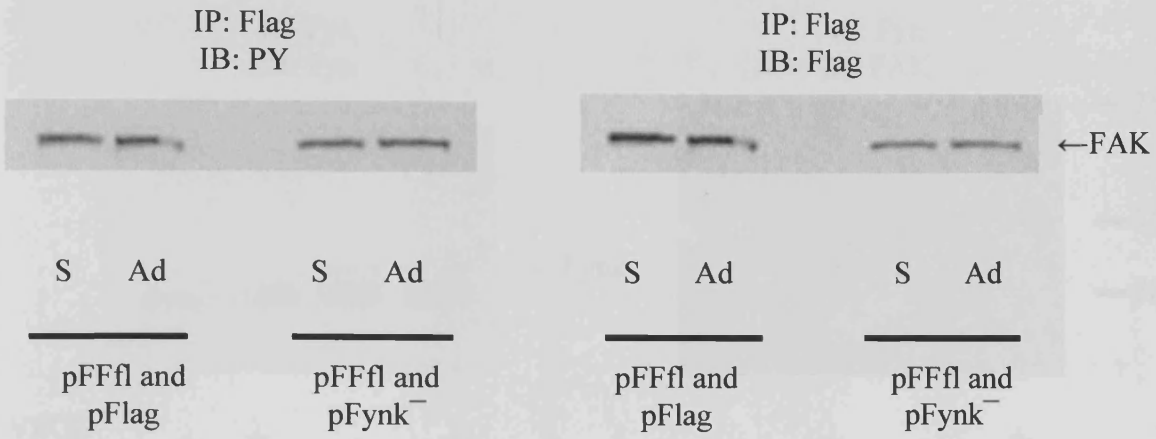


Figure 4.10. Effect of Fynk⁻ on tyrosine phosphorylation levels of FAK in cells held in suspension or on adhesion to fibronectin. BAE cells were transfected with pFFfl and empty Flag vector or pFFfl and pFynk⁻ as indicated. Twenty four hours after transfection, cells were trypsinised and either held in suspension (S) or plated onto fibronectin for 60 minutes (Ad) as described in materials and methods section 2.13. Flag tagged FAK was immunoprecipitated from cell lysates with anti-flag M2 antibody covalently attached to agarose (as described in section 2.6.4). Immunoprecipitated proteins were resolved by SDS-PAGE and subjected to immunoblot analysis as described in section 2.7 and 2.8. Tyrosine phosphorylation levels were determined by antiphosphotyrosine (PY) immunodetection (left panel) as described in section 2.9.1. Blots were stripped and reprobed with flag antibody to verify expression levels (right panel) as described in section 2.9.5. IP, immunoprecipitate; IB, immunoblot. Similar results were obtained in two independent experiments.

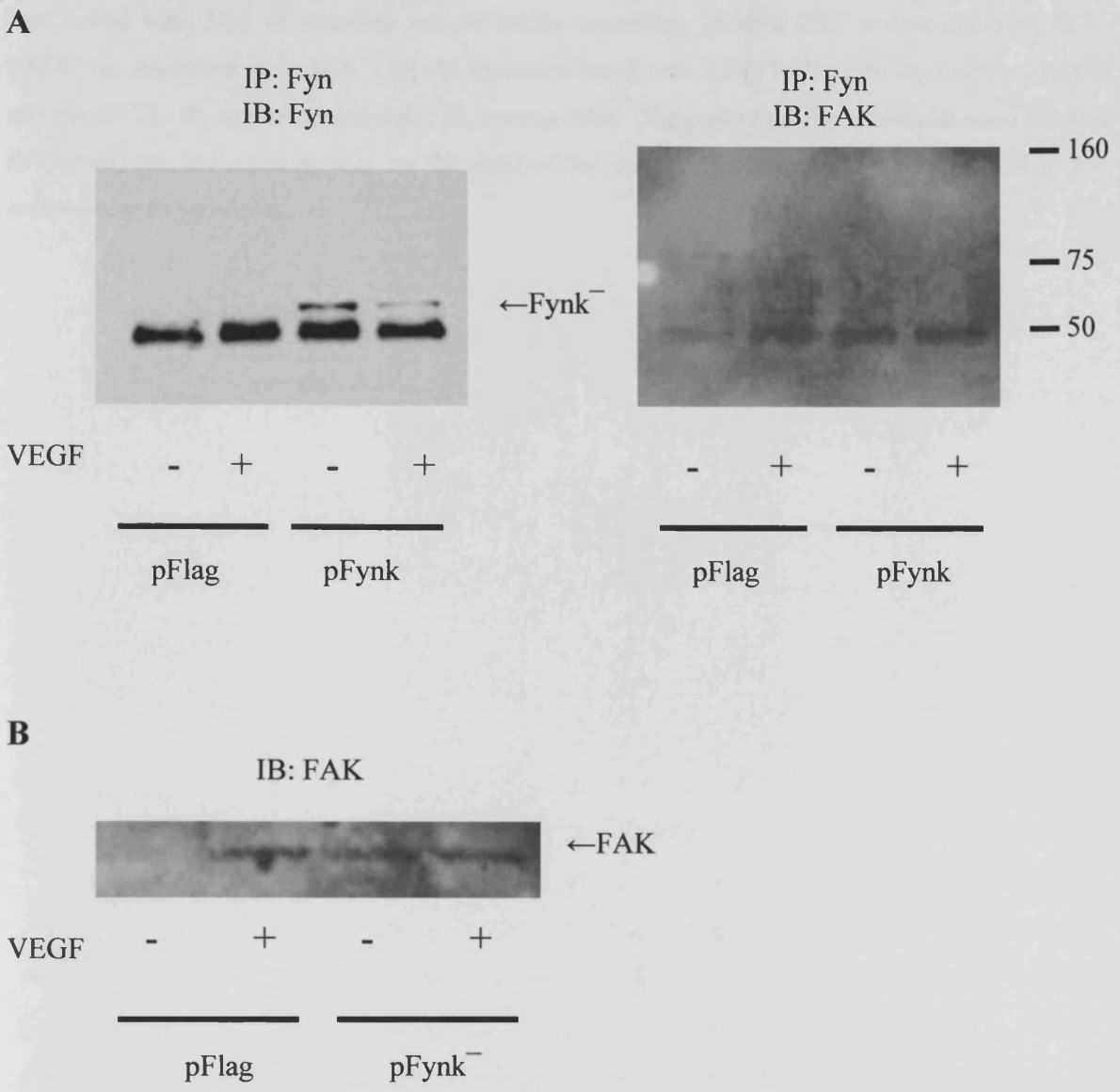


Figure 4.11. (A) Investigating the effect of Fynk⁻ on the ability of VEGF to activate endogenous FAK tyrosine phosphorylation. BAE cells were transfected with Fynk⁻ or empty flag vector (pFlag) as indicated (described in section 2.26). The cells were quiesced for 24 hours before being challenged with control vehicle or 2.5nM of VEGF for 10 minutes. Fyn was immunoprecipitated from the cell lysates with anti-Fyn monoclonal antibody as described in section 2.6.5. Immunoprecipitated proteins were resolved by SDS-PAGE and subjected to immunoblot analysis as described in section 2.7 and 2.8. Blots were probed using anti-Fyn monoclonal antibody (left panel) before being stripped and reprobed with DRC anti-FAK antibody (right panel) as described in section 2.9.4, 2.11 and 2.9.2. Molecular mass markers are indicated on the right in kDa. **(B) Detection of FAK in the lysates after immunoprecipitation of Fyn.** After immunoprecipitation of Fyn, from the lysates in A, 50 µl from 0.5ml of the remaining cell lysate

was boiled with 50μl of reducing sample buffer containing 200mM DTT and analysed by SDS-PAGE (as described in section 2.7) and immunoblotted with DRC FAK antibody (as described in section 2.9.2). IP, immunoprecipitate; IB, immunoblot. The position of the molecular mass markers (if shown) are indicated in kDa on the right of the panels Similar results were obtained in two independent experiments.

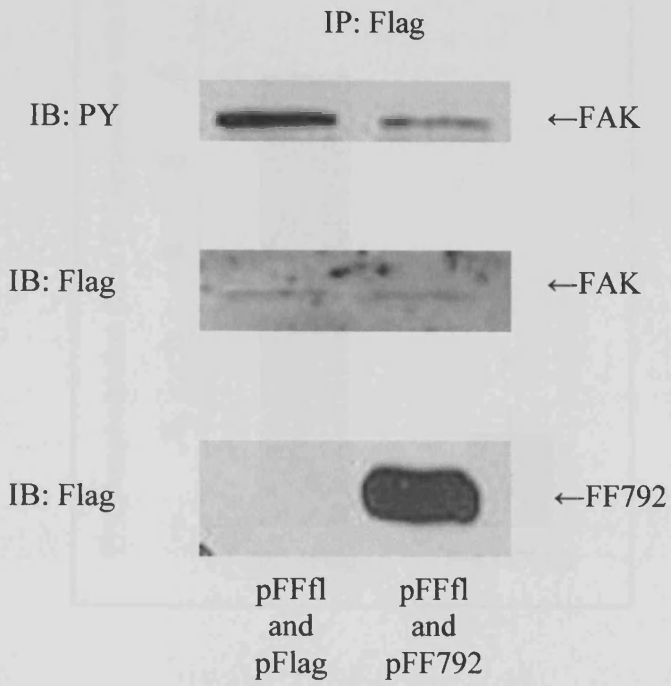


Figure 4.12A. Expression of pFF792 with pFFfl reduces the tyrosine phosphorylation level of FAK. BAE cells were transfected with pFFfl and pFlag or pFFfl and pFF792 as indicated. The flag tagged FAK constructs were immunoprecipitated from the cell lysates with anti-Flag M2 antibody covalently attached to agarose (as described in section 2.6.4). Immunoprecipitated proteins were resolved by SDS-PAGE and subjected to immunoblot analysis as described in section 2.7 and 2.8. Membranes were probed with anti-phosphotyrosine antibody (top panel) before stripping and reprobing with anti-Flag antibody to detect the epitope tagged full length FAK (middle panel) and the truncated C-terminal FAK (bottom panel) as described in section 2.9.1 and 2.9.5. IP, immunoprecipitate; IB, immunoblot; PY, phosphotyrosine. The figures are representative of four experiments.

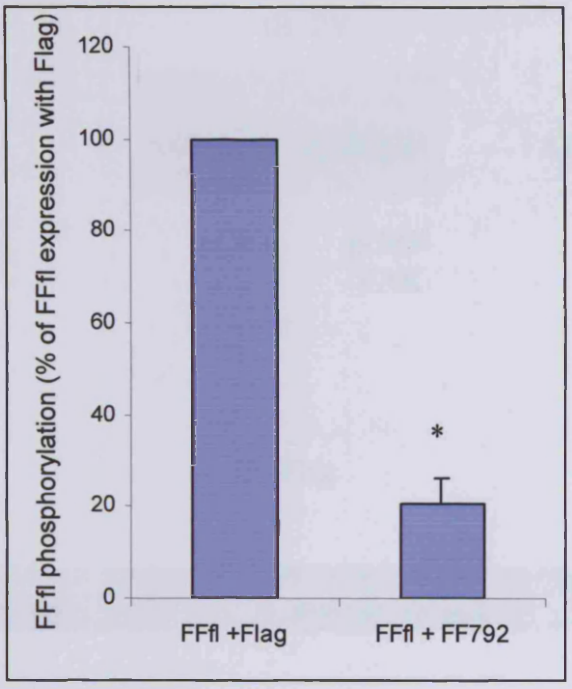


Figure 4.12B. Quantitative analysis of the tyrosine phosphorylation level of FFfl when co-expressed with FF792. A series of co-expression experiments of FFfl with FF792 were performed as described in figure 4.12A. For each experiment the level of tyrosine phosphorylation of the immunoprecipitated FFfl was determined by densitometric scanning of blots probed for tyrosine phosphorylation and Flag (described in section 2.34). The relative phosphorylation states were normalised with respect to FFfl levels in each lane of each blot. Results are expressed as a percentage of tyrosine phosphorylation of FFfl expressed with Flag. Data is presented as mean and standard errors about the mean for four experiments. Data was analysed using the paired Student's t-test, * indicates $p<0.001$, compared to cells transfected with FFfl and empty flag vector (Bailey, Statistical Methods in Biology, 1979).

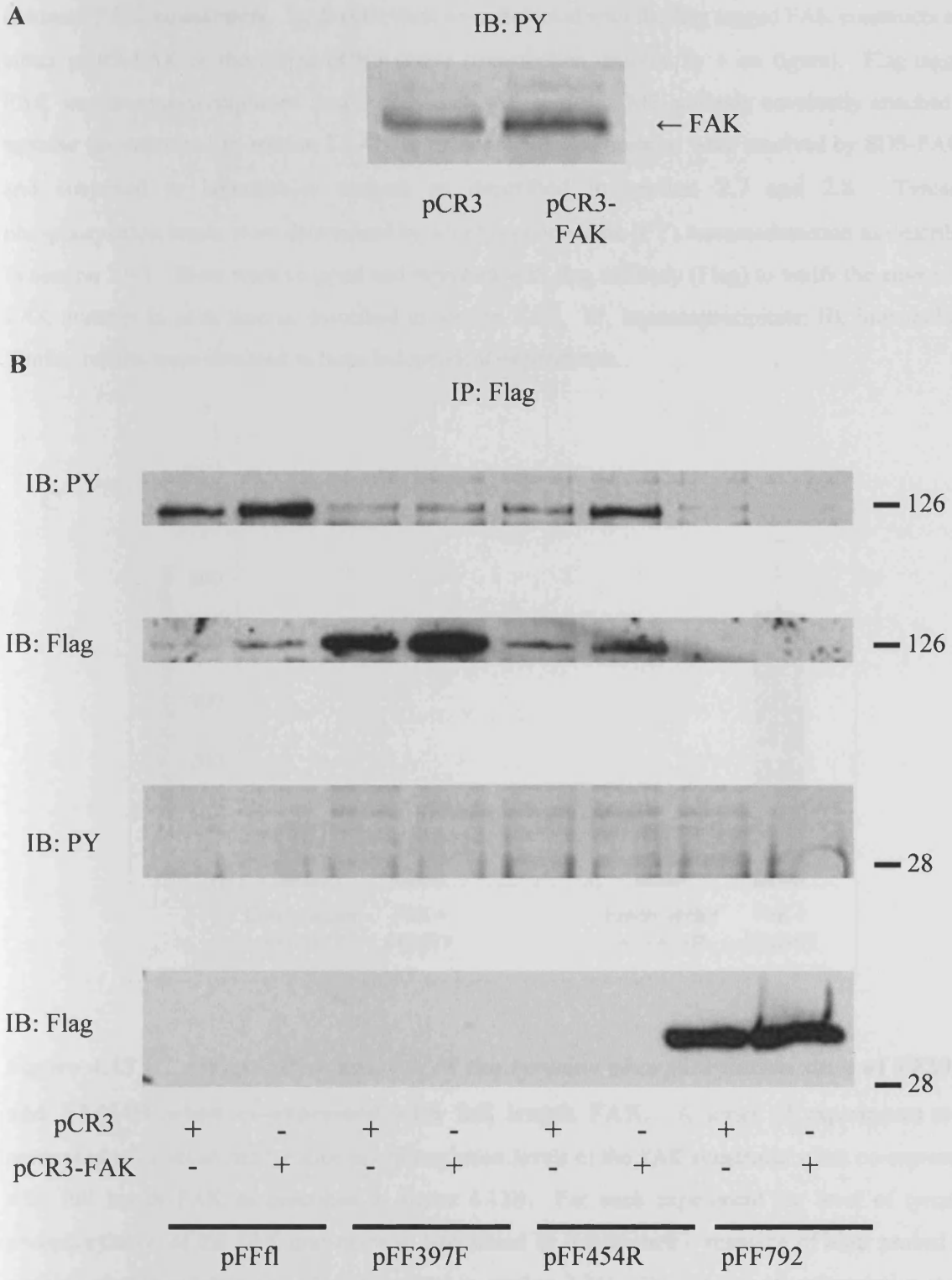


Figure 4.13. (A) Expression of pCR3-FAK in BAE cells. BAE cells were transfected with either the empty pCR3 vector or pCR3-FAK as indicated. Twenty four hours later the cells were lysed and proteins subjected to SDS-PAGE and immunoblot analysis with anti-phosphotyrosine antibody as described in section 2.7, 2.8 and 2.9.1. **(B) The effect of full length FAK on the**

mutant FAK constructs. BAE cells were co-transfected with the flag tagged FAK constructs and either pCR3-FAK or the empty pCR3 vector (transfection denoted by + on figure). Flag-tagged FAK was immunoprecipitated from cell lysates with anti-flag M2 antibody covalently attached to agarose (as described in section 2.6.4). Immunoprecipitated proteins were resolved by SDS-PAGE and subjected to immunoblot analysis as described in section 2.7 and 2.8. Tyrosine phosphorylation levels were determined by antiphosphotyrosine (PY) immunodetection as described in section 2.9.1. Blots were stripped and reprobed with flag antibody (Flag) to verify the amount of FAK mutants in each lane as described in section 2.9.5. IP, immunoprecipitate; IB, immunoblot. Similar results were obtained in three independent experiments.

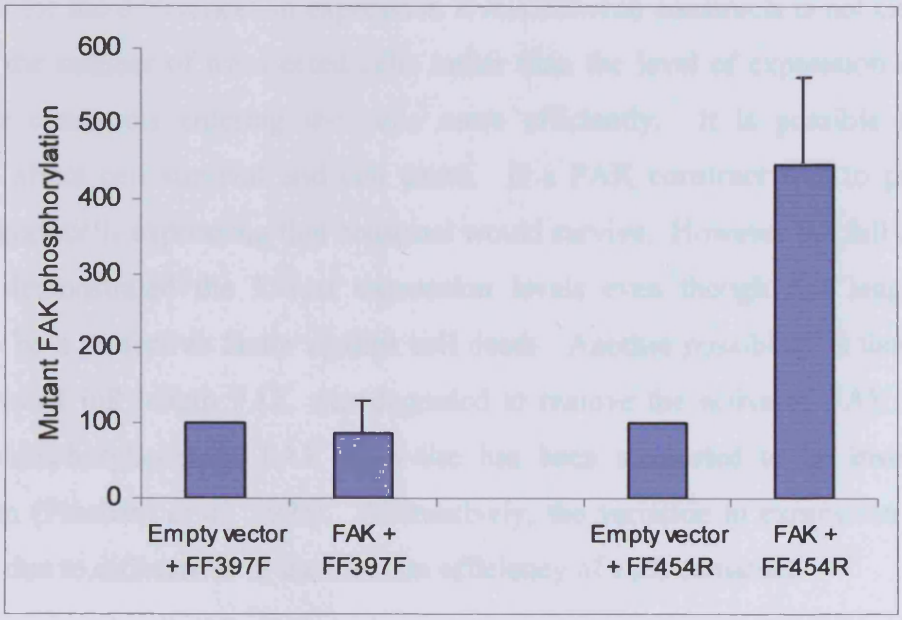


Figure 4.13 C. Quantitative analysis of the tyrosine phosphorylation state of FF397F and FF454R when co-expressed with full length FAK. A series of experiments were performed to establish the tyrosine phosphorylation levels of the FAK constructs when co-expressed with full length FAK as described in figure 4.13B. For each experiment the level of tyrosine phosphorylation of the FAK mutants was determined by densitometric scanning of blots probed for tyrosine phosphorylation and Flag (described in section 2.34). The relative phosphorylation states were normalised with respect to the amount of FAK mutant in each lane of each blot. Results are expressed as a percentage of tyrosine phosphorylation of each construct expressed with empty vector. Data is presented as mean and standard errors about the mean for three experiments.

4.7 Discussion

Expression levels

The pFF397F consistently demonstrated the highest level of expression compared to other constructs of a similar size. It is interesting to note that Owen *et al.* (1999) found a similar results in stably transfected cells. Owen *et al.* (1999) induced expression of 397 mutated FAK for a quarter of the length of time of wild type FAK in order to achieve similar levels of expression. FAK mutated at 397 also demonstrated a higher expression level in CHO cells compared to wild type and kinase deficient FAK (Cary *et al.* 1996).

The reason for the differences in expression levels between constructs is not clear but may be due to the number of transfected cells rather than the level of expression in each cell with some constructs entering the cells more efficiently. It is possible the various constructs affect cell survival and cell death. If a FAK construct was to promote cell survival, more cells expressing that construct would survive. However the full length FAK construct demonstrated the lowest expression levels even though full length FAK is thought to be a protective factor against cell death. Another possibility is that the highly phosphorylated full length FAK was degraded to remove the activated FAK. Extensive tyrosine phosphorylation of FAK by v-Src has been suggested to be involved in its degradation (Fincham *et al.* 1995). Alternatively, the variation in expression levels may have been due to differences in transfection efficiency of each construct.

Tyrosine phosphorylation levels

Despite the low levels of FFfl present on the blots this construct demonstrated the highest level of tyrosine phosphorylation. Highly phosphorylated full length FAK was also seen when overexpressed in CHO cells (Cary *et al.* 1996). The single residue substitution at tyrosine 397 to phenylalanine virtually abolished tyrosine phosphorylation of the construct as previously demonstrated in CHO cells (Cary *et al.* 1996) and CE cells (Schaller *et al.* 1994). Interestingly, Schlaepfer and Hunter (1996) found some increase in tyrosine phosphorylation of this mutant form of FAK when serum starved human kidney epithelial 293 cells were replated onto fibronectin. Even with the kinase domain of the FF397F intact this protein demonstrates tyrosine phosphorylation levels which were 9 percent of

FFfl. This suggests the tyrosine 397 is required to be phosphorylated to enable other sites to become phosphorylated. Src family kinases bind to this residue it has been demonstrated Src facilitates tyrosine phosphorylation of other FAK tyrosine residues (Calalb *et al.* 1995).

The kinase deficient FAK, FF454R, did demonstrate tyrosine phosphorylation but only 22 percent of the phosphorylation seen with wild type FAK. Previously FAK kinase deficient mutants have also demonstrated tyrosine phosphorylation in CHO and epithelial cells (Cary *et al.* 1996, Schlaepfer and Hunter 1996). It is possible that kinase deficient FAK can be transphosphorylated by endogenous FAK.

Schlaepfer and Hunter (1996) showed FAK mutated at tyrosine 925 showed higher levels of tyrosine phosphorylation than wild type FAK in serum starved cells, however once activated by adhesion to fibronectin both showed similar high levels of tyrosine phosphorylation. Both FFfl and FF925F demonstrated similar levels of tyrosine phosphorylation in BAE cells. FF792, which consists of the C-terminal part of FAK including residues 792 to 1052, demonstrated no tyrosine phosphorylation but this is not surprising as it does not contain the autophosphorylation site at residue 397 or the kinase domain tyrosines 576 and 577.

Association with Src and Fyn

As expected Src was associated with full length FAK and FF925F. Src was not associated with FF397F and others have shown previously that loss of this autophosphorylation site abolishes this binding capacity (Schaller *et al.* 1994, Cary *et al.* 1996, Schlaepfer and Hunter 1996). Tyrosine 397 is considered the primary *in vivo* site of Src association with FAK (Schaller *et al.* 1994, Eide *et al.* 1995). The truncated FAK was also not associated with Src in this system probably due to the absence of tyrosine 397. The kinase inactive FAK, FF454R was also not associated with Src.

Although the Fyn antibody was no longer functional Cary *et al.* (1996) demonstrated in CHO cells that wild type and kinase defective FAK were associated with Src and Fyn but a Y397 mutant was not. The kinase inactive FAK R454 associated with Src seen by Cary *et*

al. (1996) is possibly mediated through tyrosine 397 transphosphorylation by endogenous FAK.

Association with the p85 subunit of PI3K and Grb2

PI3K has been reported to bind to tyrosine 397 however in this study no association was found with any of the mutant forms of FAK or wild type FAK. It has been established that Grb2 binds to tyrosine 925 upon adhesion to fibronectin (Schlaefper *et al.* 1994). Unfortunately as the Grb2 antibody was not functional it was not possible to asses the binding of Grb2 to the FAK mutants generated. Schlaepfer and Hunter (1996) demonstrated that on adhesion to fibronectin Grb2 bound to wildtype FAK but not to FAK mutated at residue 397 or 925. In addition, kinase defective FAK did not bind Grb2 even though Src kinase was bound which indicates tyrosine 925 was not phosphorylated. As *in vitro* FAK does not appear to phosphorylate tyrosine 925 but Src does this result was interpreted by Schlaepfer and Hunter (1996) that FAK kinase activity was required for full Src activation.

The use of dominant negative Fyn kinase ($Fynk^-$).

Fibroblasts isolated from transgenic mice that lack either Src or Fyn still induce tyrosine phosphorylation of FAK to the same level as control cells. This is most likely due to other Src family members compensating for the loss (Bockholt and Burridge 1995). Another method to examine the role of the Src family kinases is the use of a dominant negative approach. The kinase deficient Fyn construct has previously been shown to act as a dominant negative reagent in PDGF receptor tyrosine phosphorylation by Twamley *et al.* 1992). The kinase deficient Fyn construct would act as a dominant negative by binding to FAK, preventing any endogenous Src family kinases from associating with and tyrosine phosphorylating FAK. When $Fynk^-$ was coexpressed with full length flag tagged FAK the tyrosine phosphorylation level remained unaltered compared to FAK expression alone. Even holding the cells in suspension in the presence of $Fynk^-$ did not alter the high tyrosine phosphorylation level of FAK. Eide *et al.* (1995) reported full length FAK expressed in COS cells remain tyrosine phosphorylated when the cells were detached. This was thought to be due to the level of expression as when FAK was expressed at lower levels in CHO cells the cells showed normal FAK dephosphorylation on detachment. As

the Fynk^{-} did not affect the level of tyrosine phosphorylation of FAK it was considered that FAK tyrosine phosphorylation seen in these experiments did not result from Fyn mediated phosphorylation. The high level of autophosphorylation may have been induced by the transfected cells possessing large amounts of FAK bringing it into close proximity with other FAK resulting in its activation.

Mechanism of FAK activation

The mechanism of FAK activation is not clear. An emerging picture is when FAK becomes tyrosine phosphorylated at tyrosine 397, its autophosphorylation site, this enables Src family kinases to bind which are able to tyrosine phosphorylate tyrosine 576 and 577 which are in the FAK activation loop. As with other kinases this appears to enhance the kinase activity of FAK. This increases FAK autophosphorylating and transphosphorylating abilities and in turn leads to enhanced phosphorylation of tyrosine 397. Src family kinases are also able to tyrosine phosphorylate other tyrosine residues on FAK including 407, 861 and 925 (Schaller *et al.* 1994, Calalb *et al.* 1995 and 1996).

Schlaepfer *et al.* (1996), demonstrated that FAK containing a deletion of the first 100 amino acids displayed enhanced tyrosine phosphorylation levels when expressed in fibroblast cells held in suspension compared with wild type FAK. In addition, he found FAK is tyrosine phosphorylated in serum starved fibroblast cells at tyrosine 397 but Src family kinases are not associated with FAK. These observations led Schlaepfer *et al.* (1996) to propose FAK was tyrosine phosphorylated in serum starved cells but Src family kinases were unable to associate with tyrosine 397 due to binding between the C and N terminal domains of FAK preventing the availability of tyrosine 397. Activation of FAK would induce a conformational change thus exposing tyrosine phosphorylated residue 397 to allow Src kinases to bind.

Evidence against this model is provided by Shen and Schaller (1999), who demonstrated mutant FAK constructs with a deletion of the first 361 residues or a deletion of resides 51-377 display similar tyrosine phosphorylation levels to wild type FAK when plated onto poly-l-lysine or fibronectin in serum free medium or grown in culture. Therefore the association between the C-terminal and N-terminal of FAK may not be the inhibiting factor of FAK activation.

The expression of the Fyn dominant negative kinase with full length FAK does not alter tyrosine phosphorylation level of FAK. Assuming the Fynk^{-} binds to FAK this suggests the primary route of phosphorylation is autophosphorylation or phosphorylation by another kinase, which could include FAK. Close association of FAK, such as may occur in focal adhesions, could allow transphosphorylation of FAK. Expression of full length FAK with the truncated C-terminal FAK reduced the tyrosine phosphorylation level of full length FAK by 4.8 fold. As the truncated C-terminal FAK would compete with full length FAK for localisation at focal adhesion this could be the reason for the reduced tyrosine phosphorylation. Therefore FAK phosphorylation could be induced by localisation to focal adhesions. The reduced state of tyrosine phosphorylation seen in full length FAK could be caused by the C-terminal truncated FAK preventing access of full length FAK to focal adhesions. At physiological levels of FAK, the localisation of FAK at focal adhesions could allow molecules to cluster and therefore induce transphosphorylation. The overexpression of FAK in this system may mimic clustering of FAK induced by localisation to focal adhesions. Further evidence that FAK undergoes transphosphorylation is provided by the finding that phosphorylation of kinase inactive FAK is at least partially restored by co-expression of wild-type protein.

Recently Shen and Schaller (1999) have presented evidence which suggests localisation of FAK to focal adhesions is critical for its tyrosine phosphorylation during adhesion. FAK mutants with partial deletions of the FAT domain failed to locate to focal adhesions or become tyrosine phosphorylated when re-plated onto fibronectin after being held in suspension. When the FAT sequence of FAK was replaced with a focal adhesion targeting sequence from vinculin the chimera exhibited similar tyrosine phosphorylation as wild type FAK. Interestingly the FAT domain FAK mutants were tyrosine phosphorylated when grown in culture and demonstrated similar tyrosine phosphorylation levels to full length FAK. It would seem cells in culture are able to tyrosine phosphorylate FAK which is not localised to focal adhesions.

Data in this chapter demonstrated co-expression of full length FAK with kinase-negative Fyn, in adhesion experiments, did not reduced the high level of tyrosine phosphorylation of FAK suggesting the tyrosine phosphorylation observed was due to autophosphorylation and not further phosphorylation of other tyrosine sites by endogenous Fyn or Src. Co-

expression of full length FAK with the truncated C-terminal FAK (FF792) demonstrated substantially reduced levels of tyrosine phosphorylation of full length FAK compared to expression of full length FAK alone. This suggests FAK may be required to localise to focal adhesions for phosphorylation to occur as the C-terminal FAK may restrict the localisation of full length to focal adhesions. Alternatively it may be FAK phosphorylation results from dimerisation and the C-terminal FAK binds with FAK but is unable to induce phosphorylation of full length FAK.

CHAPTER FIVE

Effect of the FAK mutants on cell function

Chapter Five

Effect of the FAK mutants on cell function

Introduction

FAK has been implicated in controlling several cellular responses including cell spreading, migration, survival and proliferation. The FAK mutants generated in chapter four were used to examine involvement of specific regions of the protein in mediating effects on endothelial cell migration and endothelial cell survival.

Anoikis is a term which describes apoptosis which occurs due to loss of attachment from the ECM (Frisch and Francis, 1994). A number of studies have established FAK has a role in apoptosis/anoikis. Inhibition of FAK by microinjection of antibodies to an epitope near the FAT sequence resulted in apoptosis in fibroblasts (Hungerford *et al.* 1996). Expression of a constitutively activated form of FAK prevents epithelial cells from undergoing apoptosis upon cell detachment (Chan *et al.* 1994). In addition, both tyrosine 397 and the kinase activity of FAK were necessary to suppress anoikis in cells in suspension (Frisch *et al.* 1996). FAK appears to play a role in providing an anti-apoptotic signal during integrin engagement. The mutant FAK clones were utilised to explore what signals FAK generates during attachment which may prevent apoptosis. The apoptosis inducing potential of the mutant FAK clones were explored to try to establish the anti-apoptotic signals generated by wild type FAK in adherent cells.

As well as suppressing anoikis FAK is also involved in cell migration as demonstrated by several studies. Overexpression of FAK in Chinese hamster ovary cells has been shown to stimulate cell migration (Cary *et al.* 1996). FAK deficient cells exhibited reduced mobility compared to wild type cells (Ilic *et al.* 1995). Microinjection of the C-terminal portion of FAK to displace endogenous FAK from focal adhesions, caused the microinjected cells to display decreased migration (Gilmore and Romer 1996). Tyrosine phosphorylation of FAK has been reported to be increased during migration (Romer *et al.* 1994) and indeed, mutation of the major FAK phosphorylation site abolishes FAK induced increases in cell migration (Cary *et al.* 1996).

Cells migrate by structures at the front of the cell protruding out and forming adhesions with the substratum. These protrusions can be cylinder like structures called filapodia and pseudopodia or thin protruding sheets termed lamellipodia. After stable adhesion at the front of the cell has been established the cell body and nucleus moves forward with deadhesion and retraction of the rear of the cell. Although it is known FAK affects cell migration, how it does this is less clear. Some information has come from studies with FAK deficient cells which displayed increased numbers of adhesions and disorganisation of the cortical cytoskeleton (Ilic *et al.* 1995). Analysis of the role of FAK in modulation of these cell protrusions may give a greater insight into how FAK effects migration and adhesion at the cell periphery.

The aim of the studies described in this chapter was to define the roles of specific domains and residues within FAK in control of endothelial migration and survival.

Effect of the FAK mutants on cell function

5.1 Micro-injection of dominant negative GST-FAK fusion protein and its effect on cell migration.

In order to determine whether FAK is involved in VEGF induced endothelial cell migration a GST-FAK fusion protein consisting of the C-terminal FAT domain was microinjected into endothelial cells. This construct lacks kinase activity and as it contains the focal adhesion targeting sequence it will be localised to focal adhesions and displace endogenous FAK from the adhesions (Hildebrand *et al.* 1993).

5.1.2 Expression and purification of GST-FAK fusion protein.

The pGEX C-terminal FAK was a gift from Prof. D. R. Critchley, Dept. of Biochemistry, University of Leicester. This construct encoded the third terminal end of FAK, residues 693-1052, fused at the amino terminus to GST.

E. Coli JM101 cells transformed with the pGEX C terminal FAK plasmid were induced to express the fusion protein as described in materials and methods section 2.31. To obtain GST protein, for use as a control in microinjection, the pGEX vector containing no insert was also expressed and the GST purified in the same way as the GST-C terminal FAK. The purity of the expressed GST and GST-C terminal FAK fusion protein was assessed by SDS-PAGE analysis followed by staining with Coomassie brilliant blue. GST is 26 KDa and the resulting GST-C terminal FAK fusion protein was 66 KDa as shown in figure 5.1A. Several other bands in addition to GST-C terminal FAK were observed on the gel which were thought to be degradation products. To try to overcome this, competent E. Coli BL21 cells were used to express the plasmid instead of E. Coli JM101 as BL21 cells produce less proteolytic enzymes which cause degradation of proteins. The same procedure was followed for expression and purification of the pGEX C-terminal FAK plasmid and pGEX after competent E. Coli BL21 cells were transformed with the pGEX C-terminal FAK and pGEX plasmid. Similar levels of degradation products were seen after expression in E. Coli BL21 cells. In an attempt to eliminate some of the smaller proteins the GST-FAK was subjected to centrifuged filtration using 50KDa pore sized filters. This was not effective. Analysis by SDS-PAGE followed by western blotting and

probing with the DRC FAK antibody revealed the addition products were detected by the FAK antibody and were therefore breakdown products of FAK (see figure 5.1B). These truncated forms of FAK, where they contained the FAT sequence, would be expected to displace endogenous FAK from adhesions in the same way as full length GST-C terminal FAK. It was therefore not envisaged the degradation products would affect the study.

5.1.3 Micro-injection of GST-C terminal FAK.

Overexpression of FAK in Chinese hamster ovary cells had been shown to stimulate cell migration (Cary *et al.* 1996) whereas FAK deficient cells demonstrated reduced migration (Ilic *et al.* 1994). FAK is clearly involved in cell migration and so in order to investigate its role further a GST-C terminal FAK fusion protein and GST alone was microinjected into HUVE cells and the effects on cell migration induced by VEGF recorded.

In order to investigate the role of microinjected FAK fusion protein the technique of microinjecting endothelial cells was first investigated. Endothelial cells are relatively 'flat' cells and do not protrude from the coverslip very much. Microinjection of this cell type is therefore difficult and requires extensive practise in order that many cells may be injected within a short period of time. A cell migration assay was also established to observe the effect on cell migration of microinjecting GST-C terminal FAK and performed as described below.

HUVE cells used for microinjection studies were grown on gridded coverslips. When the cells reached confluency they were serum starved overnight before one-half of the cells were removed from the coverslip using a clean cell scraper. The position of the wound on the gridded coverslip was noted and the cells along the wound edge were micro-injected with either GST-C terminal FAK fusion protein or GST alone at a concentration of 2mg/ml in microinjection buffer (as described in section 2.32). The cells were incubated at 37°C for 16 hours in the presence of 100ng/ml VEGF before fixing and staining cells for GST in order to visualise the location of GST-C terminal FAK or GST micro-injected cells (as described in section 2.14.4). Figures 5.2 A and B demonstrate the GST stained cells and the corresponding phase contrast.

By changing between the phase contrast which showed the grid lines and the fluorescence filter, which indicated the position of the microinjected cells, it was possible to determine the distance of the micro-injected cell from the wound edge. As shown in figure 5.3A, the number of stained cells within 40μm sections from the wound edge were recorded (i.e. the number of stained cells between 0-40μm, 40-80μm, 80-120μm, 120-160μm, 160-200μm and 200-240μm from the wound edge were recorded). The numbers of cells micro-injected with the C-terminal domain FAK were compared to those cells micro-injected with GST alone. Overall the graph demonstrated over half of the C-terminal FAK injected cells were present within the first 40μm section from the wound edge. The number of C-terminal FAK injected cells decreased the further the section was from the wound edge so the regions furthest from the wound edge contained the fewest cells with none of the cells migrating over 200μm. The GST injected cells however were found equally distributed in each 40μm section up to 200μm from the wound edge with a few cells migrating over 200μm. In the 40μm nearest to the wound edge 52.3% of the C-terminal FAK injected cells were found compared to only 27.6% of the GST injected cells which was a statistically significant difference in the numbers of each cells ($p<0.001$). There was no statistical difference between the numbers of C-terminal FAK injected cells compared to GST injected cells in the 2nd and 3rd section from the wound edge. However in the 4th and 5th sections, (120- 160μm and 160-200μm) from the wound edge more GST injected cells were present compared to the C-terminal FAK injected cells ($p<0.05$ and $p<0.001$ respectively). As shown in figure 5.3B, the mean distance of the GST-C terminal FAK injected cells from the wound edge was 75.8μm (+/- 6.8μm) compared to 120.3μm (+/- 4.2μm) for the GST injected cells and this was a significant difference in the distance from the wound edge ($p<0.001$). This demonstrated the C-terminal FAK injected cells did not migrate as far from the wound edge as the cells injected with GST alone and implies they were impaired in their migration.

5.2 Initial investigation into the role of VEGF in cell survival

In addition to suppression of endothelial cell migration, an apparent decrease in cell survival was observed in endothelial cells microinjected with GST-C terminal FAK. VEGF modulation of FAK in cell survival was to be investigated using the various FAK

constructs generated in chapter four. Initial experiments aimed to establish the role of VEGF in cell survival and the time course over which VEGF had an effect.

HUVE cells were seeded in gridded dishes and once they had reached 50% confluency, four randomly selected squares within the grid were clearly marked and the number of cells within each square was recorded. The cells were then washed and placed either in serum free media, serum free media containing 100μg/ml VEGF or HUVE cells culturing media (see section 2.3.1 for culturing media). The number of cells within the same four squares were counted over a 72 hour time period at 8, 21, 29, 50 and 72 hours. The percentage of cells surviving at each time point were recorded for each type of media and displayed in figure 5.4. As demonstrated in figure 5.4 VEGF maintained the number of cells for up to 21 hours with a slight decline in cell numbers by 29 hours.

Unfortunately it transpired the HUVE cells were unsuitable for transfection, as discussed in section 3.7, and therefore BAE cells were used. In addition, to more accurately analyse the decrease in cell survival, the incidence of apoptosis was investigated. The various FAK constructs generated in chapter four were used to investigate if the cell death was caused by apoptosis and if the tyrosine phosphorylation state of FAK played a role in the cell death. In addition, the constructs were used to investigate which regions of FAK might be involved.

5.3 Effect of FAK mutant constructs on apoptosis

The earliest detectable nuclear change in apoptosis is the condensation of nuclear chromatin along the nuclear membrane. The whole nucleus becomes condensed and fragments into smaller dense particles which are packaged and taken up by other cells. The morphological changes which occur in the nucleus can be detected by the use of a DNA-intercalating dye, such as Hoechst dye, which enables visualisation of the nucleus.

In order to investigate the effect of each FAK construct on apoptosis BAE cells were grown in 35mm dishes and transfected with 1.25μg of FAK mutant construct or empty pFlag vector. 1.25μg of the plasmid pGreen Lantern-1 (pGL) was cotransfected with the FAK constructs. pGreen Lantern-1 encodes a modified version of the green fluorescent

protein (GFP) and therefore enabled visualisation of the transfected cells by fluorescence microscopy (excitation 488nm, emission 530nm). After eighteen to twenty hours the cells were incubated with 10μg/ml Hoechst dye for 30-60 minutes. When the Hoechst stained cells were viewed, non apoptotic cells displayed a blue nucleus. Cells which were undergoing early apoptosis had condensed intensely stained blue nuclei or exhibited fragmented nuclear staining. Five random fields from each dish were viewed and the apoptotic state of the transfected cells was recorded. According to the morphology of their nucleus cells were classed as either normal or apoptotic and the percentage of apoptotic cells for each FAK mutant is displayed in figure 5.5.

The level of apoptosis in full length FAK (FFfl) expressing cells was similar to those cells expressing GFP alone (18.1% (+/- 3.8) compared to 21.6% (+/- 3.8)). The highest level of apoptosis was seen in the C-terminal truncated FAK expressing cells (FF792). 38.0% (+/- 2.0) of these cells underwent apoptosis compared to only 18.1% (+/- 3.8) of cells transfected with pGreen Lantern alone ($p<0.001$). This demonstrated that the expression of C-terminal truncated FAK induces apoptosis.

The C-terminal construct is known to displace endogenous FAK from adhesions (Hildebrand *et al.* 1993, Gilmore and Romer 1996). This suggests loss of full-length FAK from adhesions induces apoptosis in endothelial cells. The anti-apoptotic effect of adhesion localised full-length FAK could be mediated by its kinase activity and/or ability to associate with/recruit other proteins. Therefore to investigate involvement of FAK kinase activity experiments were performed with the kinase inactive FF454R. Expression of FF454R did not affect the percentage of cells undergoing apoptosis. The levels of apoptosis were 15.3% (+/- 5.3) and 18.1% (+/- 3.8) respectively and this difference was not statistically significant. This would suggest kinase activity is not required for the anti-apoptotic effect of FAK.

As shown in chapter 4, mutation of tyrosine 397 results in loss of Src and Fyn binding to FAK and virtually abolished the tyrosine phosphorylation level of FAK. Therefore to examine whether recruitment of Src/Fyn by full-length FAK was involved in the anti-apoptotic signalling from this protein the FF397F construct was expressed in endothelial cells and the percentage of apoptotic cells determined. FF397F was expressed to assess the

role of Src/Fyn binding to 397 in the increase in apoptosis seen when the C-terminal FAK was expressed. Again this construct produced similar levels of apoptosis as the GST alone expressing cells, namely 12.9% (+/- 4.1) and 18.1% (+/- 3.8) respectively which was not a statistically significant difference. This suggests full length FAK lacking Src/Fyn binding is still able to protect endothelial cells from apoptosis.

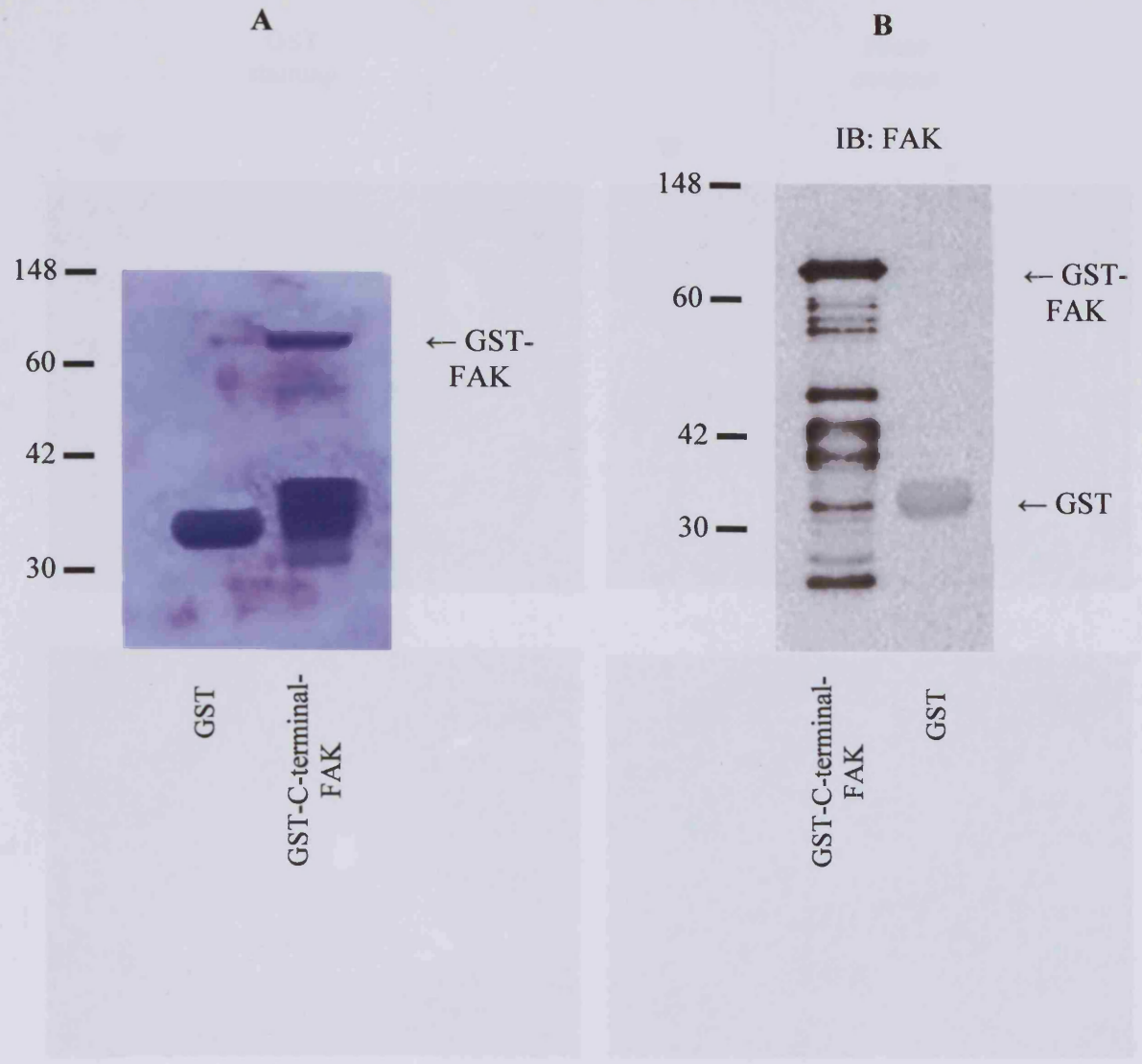


Figure 5.1. Assessment of the purity of the expressed GST and GST-C terminal FAK. Bacterially expressed GST and GST-FAK were prepared as described in section 2.31 **(A)**. A sample of each of the purified expressed proteins were resolved by SDS-PAGE and visualised by staining with Coomassie brilliant blue as described in section 2.7. **(B)** A sample of the purified protein was analysed by SDS-PAGE followed by western blotting and probing with the DRC anti-FAK antibody as described in section 2.7, 2.8 and 2.9.2. The position of the molecular mass markers is indicated in kDa on the left.

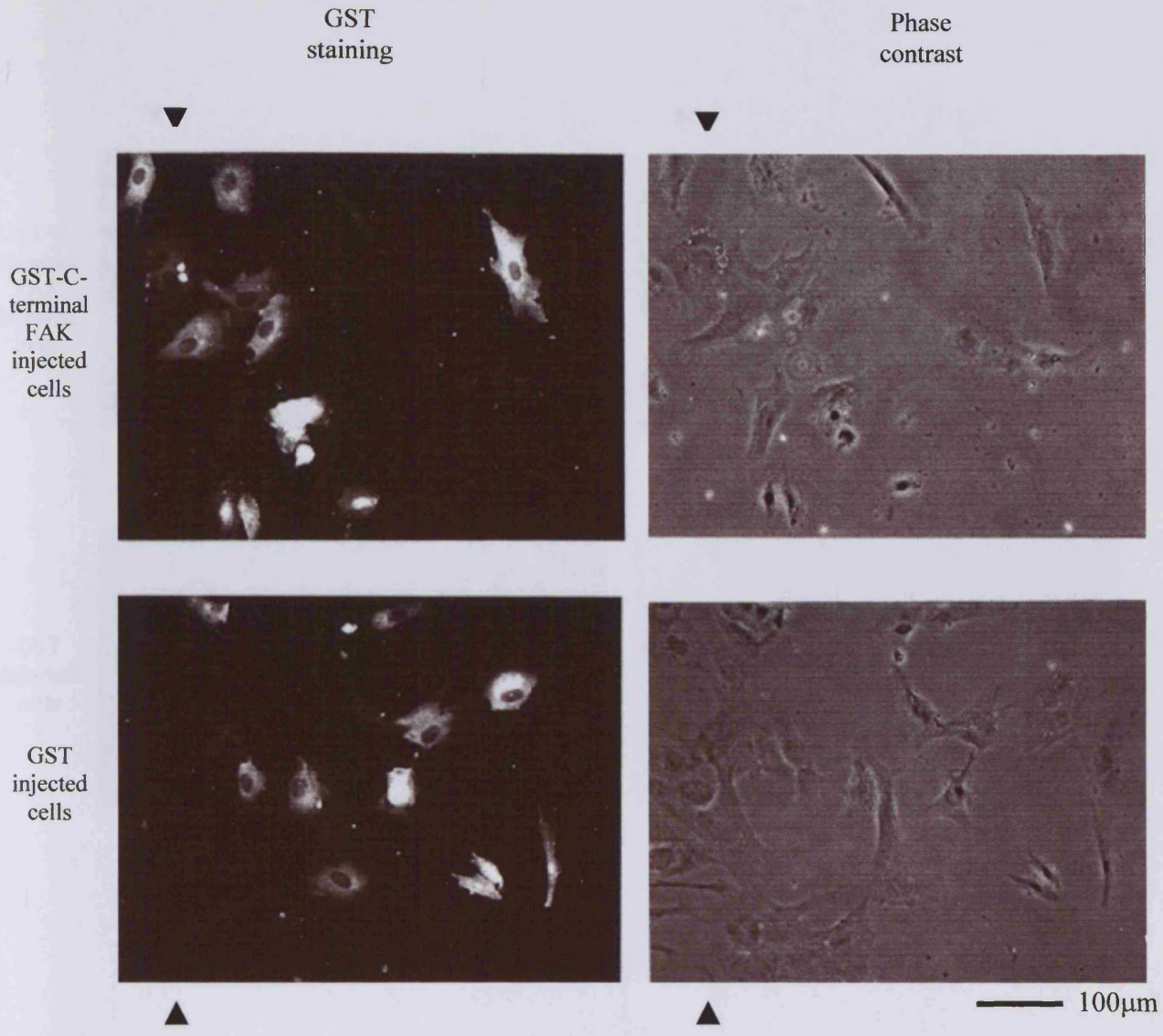

GST
staining
Phase
contrast
GST-C-terminal FAK injected cells
GST injected cells
100μm

Figure 5.2A

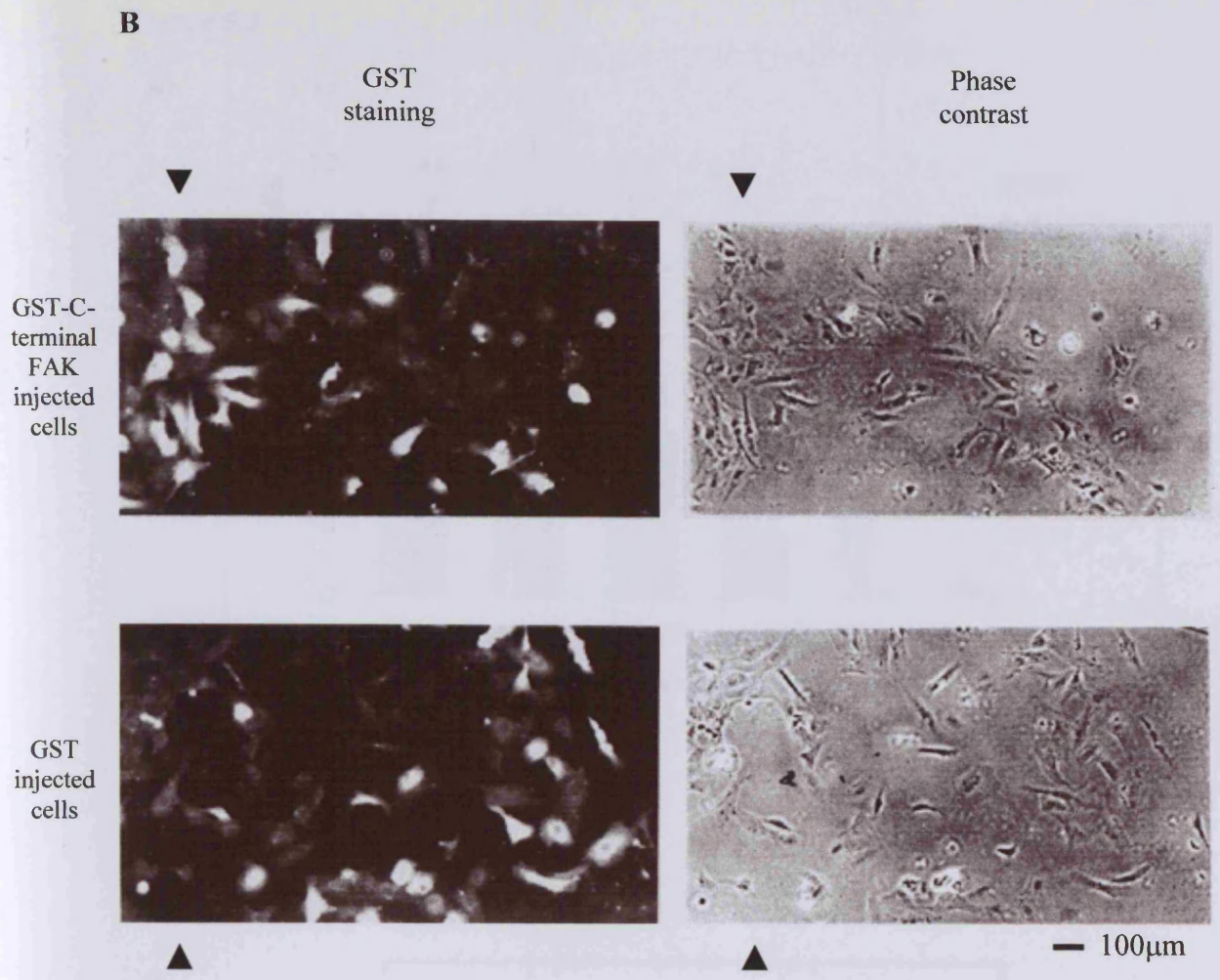
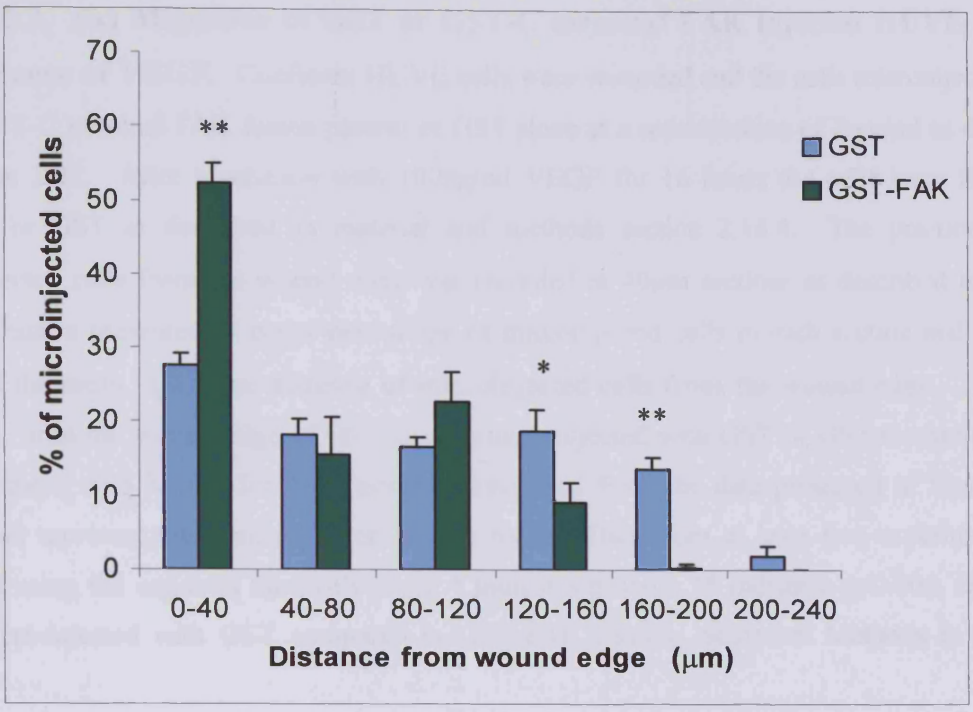


Figure 5.2 A and B. Immunofluorescence of HUVE cells microinjected with GST-C-terminal FAK and GST. HUVE cells were grown to confluence on glass coverslips before scraping one half of the coverslip clear of cells. Cells at the wound edge were then micro-injected with GST-C terminal FAK (top panel) or GST (lower panel) (as described in section 2.32). After incubation for 16 hours in the presence of 100ng/ml VEGF the cells were fixed and stained with anti-GST (as described in section 2.14.4) to detect the microinjected cells. Photographs demonstrate the GST stained cells (left panels) and the corresponding phase contrast image (right panels). Arrows ▲ and ▼ indicate the wound edge. A and B represent different powers of magnification as indicated by the bar size.

Figure 5.3

A


70
60
50
40
30
20
10
0
% of microinjected cells
**
*
**
GST
GST-FAK
0-40
40-80
80-120
120-160
160-200
200-240
Distance from wound edge (μm)

B

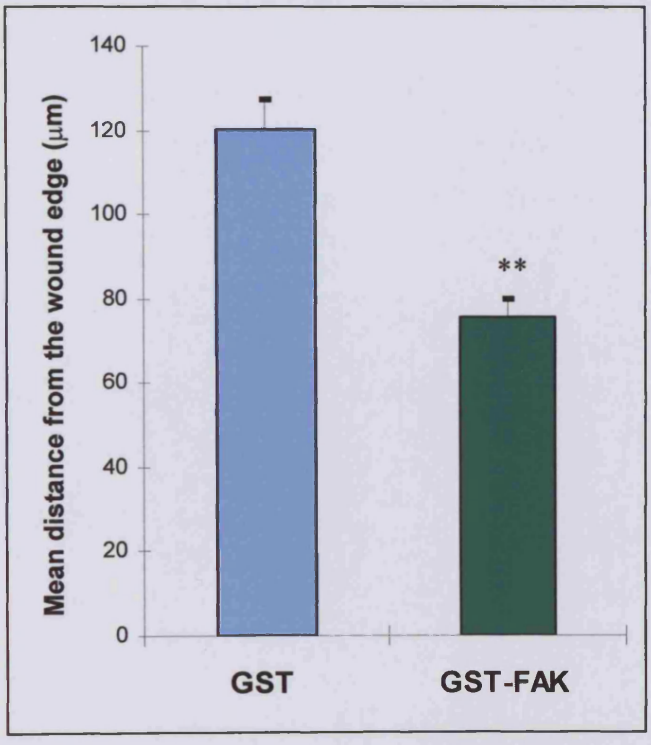

140
120
100
80
60
40
20
0
Mean distance from the wound edge (μm)
**
GST
GST-FAK

Figure 5.3. (A) Migration of GST or GST-C terminal FAK injected HUVE cells in the presence of VEGF. Confluent HUVE cells were wounded and the cells microinjected with either GST-C terminal FAK fusion protein or GST alone at a concentration of 2mg/ml as described in section 2.32. After incubation with 100ng/ml VEGF for 16 hours the cells were fixed and stained for GST as described in material and methods section 2.14.4. The position of the microinjected cells from the wound edge was recorded in 40μm sections as described in section 5.1.3. Data is presented as mean percentage of microinjected cells in each section and standard errors of the mean. **(B) The distance of microinjected cells from the wound edge.** The mean distances from the wound edge of HUVE cells microinjected with GST or GST-C terminal FAK and incubated with VEGF for 16 hours was calculated from the data presented in figure 5.3A. Error bars represent the standard errors of the mean. Data from at least five experiments was analysed using the unpaired Student's t-test, * indicates $p<0.05$, ** indicates $p<0.001$, relative to cells micro-injected with GST compared to GST-FAK (Bailey, Statistical Methods in Biology, 1979).

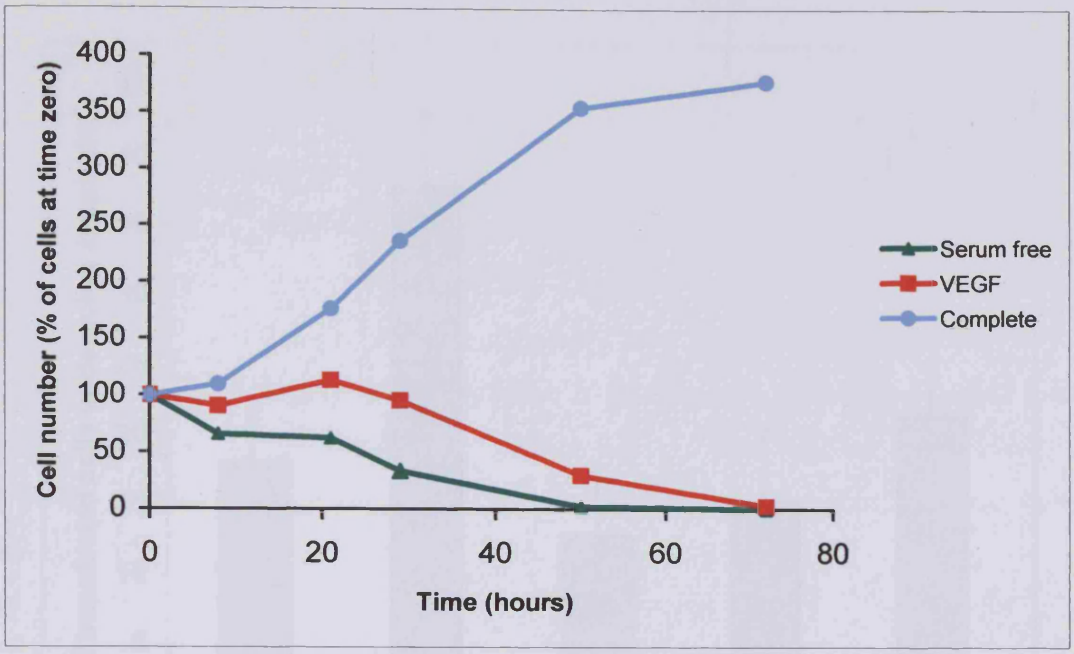


Figure 5.4. Effect of VEGF on survival of HUVE cells in the absence of serum. HUVE cells were incubated in serum free media (serum free), serum free media containing 100ng/ml of VEGF (VEGF) or HUVE cell culturing media (complete). Four randomly selected squares within the gridded dish the cells were grown on were clearly marked and the number of cells within each square was recorded. The percentage of cells at each of the time points for each type of media were recorded and are expressed relative to cell numbers at time zero. Data is presented for two independent experiments.

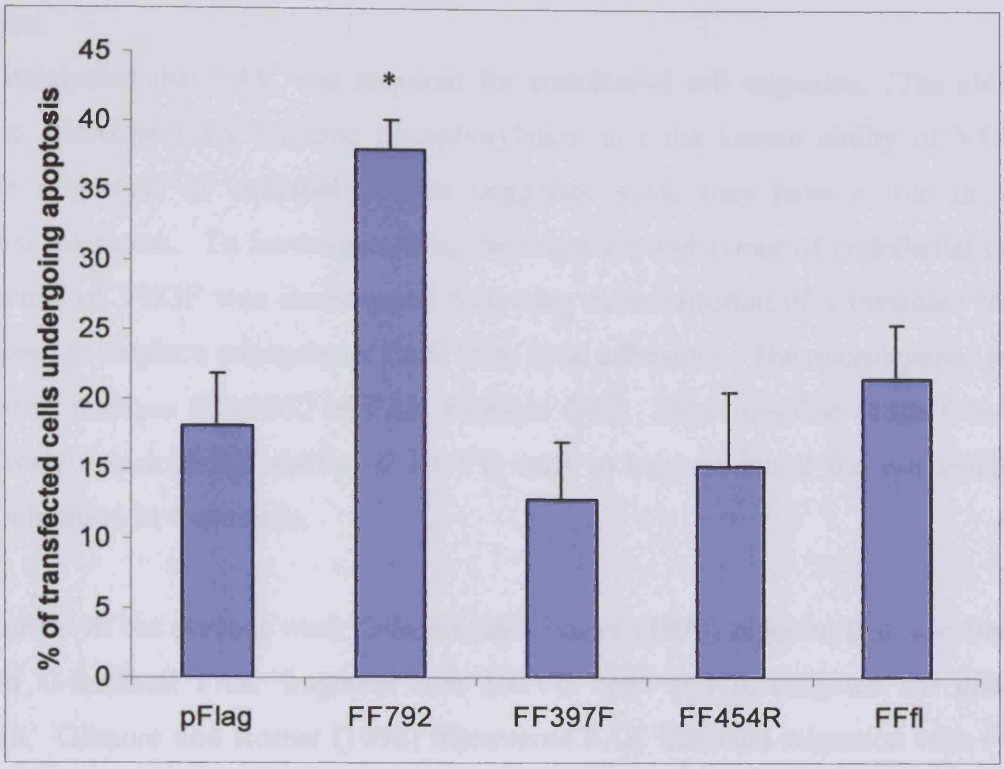


Figure 5.5. Apoptosis induced by expression of various FAK mutant proteins. BAE cells were transfected with a FAK mutant or empty flag vector (pFlag) and co-transfected with pGreen Lantern-1 which enabled visualisation of the transfected cells. Eighteen to twenty hours after transfection the cells were incubated with Hoechst dye for 30-60 minutes to enable visualisation of apoptotic cells as described section 2.33. A random field was viewed by fluorescence microscopy to identify the transfected cells. The same field of cells were then viewed with filters which enabled the visualisation of Hoechst dye and by changing between the two filters the transfected cells were identified as either normal cells or apoptotic as described in section 2.33. This was repeated for five random fields in each dish. Results are expressed as the percentage of transfected cells undergoing apoptosis in the presence of each FAK mutant. Data is presented as mean and standard errors of the mean for at least five experiments. Data was analysed using the unpaired Student's t-test, * indicates $p<0.001$ relative to cells transfected with empty pFlag vector (Bailey, Statistical Methods in Biology, 1979).

5.4 Discussion

Migration.

It was anticipated that FAK was required for endothelial cell migration. The ability of VEGF to stimulate FAK tyrosine phosphorylation and the known ability of VEGF to stimulate migration of endothelial cells suggested FAK may have a role in VEGF stimulated migration. To investigate this, the migratory behaviour of endothelial cells in the presence of VEGF was investigated following microinjection of a truncated form of FAK shown to displace endogenous FAK from focal adhesions. The microinjected protein consisted of residues 693-1052 of FAK fused to GST. Microinjection of the C-terminal FAK clearly impaired the ability of HUVE cells to migrate but it did not completely abolish migration in these cells.

In the course of the present work Gilmore and Romer (1996) reported that injection of a truncated C-terminal FAK fragment into HUVE cells and investigated the effect on migration. Gilmore and Romer (1996) discovered FAK inhibited migration with 64% of the HUVE cells microinjected with 2ug/ml of their C-terminal FAK remaining at the wound edge displaying no migration at all. A third of the microinjected cells just entered the migrating pack. The results of Gilmore and Romer (1996) displayed more severely impaired migrating abilities of HUVE cells microinjected with C-terminal FAK compared to the results presented in this thesis. This possibility could be due to the difference in the microinjected C-terminal FAKs. Gilmore and Romer (1996) injected a C-terminal portion of FAK encompassing residues 765-1052 which does not contain the proline rich region-1. The FAK C-terminal fusion protein used in this thesis consisted of residues 693-1052 of FAK which includes the proline rich region-1 (spanning amino acids 712-718). p130Cas has been demonstrated to bind to the proline rich region-1 of FAK and therefore migration in HUVE cells may involve this signalling molecule. Other domains and residues of FAK not present in the C-terminal FAK are clearly essential for migration and loss of these due to displacement of endogenous FAK from focal adhesions impairs cellular migration.

Cary *et al.* (1996) demonstrated stable overexpression of FAK in CHO cells resulted in increased migration. Mutation of tyrosine 397 however abolished FAKs ability to stimulate migration, reporting tyrosine phosphorylation of tyrosine 397 and subsequent Src

binding essential for the increase in cell migration observed. Recently Cary *et al.* (1998) investigated the effect of p130Cas in promoting cell migration. Expression of p130Cas with FAK further increased the effect of FAK on cell migration. A FAK mutant with impaired ability to bind p130Cas demonstrated migration levels similar to control levels as did the expression of the SH3 domain of p130Cas with FAK. It appears there is role for p130Cas in FAK mediated cell signalling although other sites on FAK also contribute.

Apoptosis

By expressing mutant forms of FAK in adherent cells the role of FAK in transducing survival signals was investigated. The control level of apoptosis induced by transfection of BAE cells with GFP alone was 18%. This figure compares with 25% in fibroblast cells adherent to fibronectin in a serum free medium and 21% when MDCK cells were held in suspension for seven hours in serum containing medium (Ilic *et al.* 1998, Frisch *et al.* 1996). The control levels of apoptosis in BAE cells was lower than in the other cells described possibly because the BAE cells were both adherent and in the presence of serum.

In MDCK cells held in suspension, the expression of constitutively activated FAK decreased the apoptotic levels in these cells from 21% by 10 fold (Frisch *et al.* 1996). In chapter four it was demonstrated FFfl expressed in BAE cells was highly tyrosine phosphorylated (section 4.3.1) but this activated FAK did not produced a protective effect in adherent BAE cells in serum as the apoptotic levels in FFfl expressing cells were similar to control. In addition, fibroblasts transfected with wild type FAK which were adherent to fibronectin but in a serum-free environment did not demonstrate a difference in apoptotic level compared to control cells (Ilic *et al.* 1998). Activated FAK does not appear to provide an additional protective effect over control cells when cells are adherent but can protect cells from undergoing apoptosis due to loss of attachment. In addition, transfection with GFP-FAK did not provide a protective effect in the absence of serum (Ilic *et al.* 1998).

Transfection of adherent BAE cells with FF397F did not induce apoptosis above control levels. In MDCK cells held in suspension, expression of FAK mutated at 397 did not rescue cells from apoptosis whereas expression of activated FAK did. This led Frisch *et al.* (1996) to conclude that this tyrosine residue was essential for resistance to apoptosis in

non-adherent cells. Expression of FAK mutated at tyrosine 397 would have blocked signalling molecules binding to residue 397 via their SH2 domains. As demonstrated in chapter four, Src was unable to bind to FF397F and disruption of this tyrosine would also prevent association with the p85 subunit of PI3K which has also been reported to bind to tyrosine 397 (Chen *et al.* 1996). As constitutively activated PI3K blocked anoikis in MDCK cells (Khwaja *et al.* 1997), it is possible that the p85 subunit of PI3K binding to tyrosine 397 of FAK is involved in this activation. However, as the BAE cells which were adherent did not undergo apoptosis when FF397F was expressed, loss of signalling through tyrosine 397 does not induce apoptosis in adherent endothelial cells.

Loss of the kinase activity of FAK did not induce apoptosis in adherent BAE cells. In MDCK cells in suspension, expression of FAK mutated at residue 454 to abolish its kinase activity did not prevent the fibroblasts from undergoing apoptosis whereas activated FAK did prevent cells undergoing apoptosis (Frisch *et al.* 1996). The kinase activity of FAK appeared to be necessary for FAK to protect cells in suspension from undergoing apoptosis but in adherent cells, lack of FAK kinase activity does not induce apoptosis.

Expression of FF792 in BAE cells induced an increase in apoptosis from 18% in control cells to 38%. This would suggest that essential sequences involved in signalling to prevent apoptosis were not present in the truncated C-terminal FAK and the N-terminal two-thirds of FAK contained these sequences. As demonstrated previously however neither tyrosine 397 or FAK kinase activity were required as loss of these signalling capacities did not induce apoptosis. Frisch *et al.* (1996) demonstrated tyrosine 397 and the kinase activity of FAK necessary to rescue cells from anoikis but in adherent BAE cells these sequences did not appear necessary. This difference could be explained as in adherent cells other proteins may be present in the focal adhesion which can compensate for the lack of FAK kinase activity or loss of tyrosine 397 but not for the loss of other sequences within FAK. Cells in suspension do not posses focal adhesions and therefore other proteins which can compensate for lack of FAK kinase activity or loss of tyrosine 397 are not localised near FAK and the cells undergo apoptosis. Interestingly the activated FAK used by Frisch *et al.* (1996) was membrane bound.

Ilic *et al.* (1998) demonstrated 75% of fibroblasts in serum-free medium transfected with the FAT sequence of FAK underwent apoptosis compared to 25% of GFP transfected cells.

The FAT sequence they used induced a higher level of apoptosis than the truncated C-terminal FAK expressed in BAE cells. As FF792 is 49 amino acids longer, these residues may contribute to help stabilise interaction with other proteins, alternatively the presence of serum may have been responsible for the differences in apoptosis levels.

Recently Almeida *et al.* (2000) investigated the role of the proline rich region-1 of FAK in anchorage-dependent survival. The prolines at residues 712 and 713 were mutated to alanines in FAK and FRNK and expressed in rabbit synovial fibroblasts (RSF). The fibroblasts were plated on fibronectin in serum free medium to examine the effect of survival signals from fibronectin. Expression of these mutant proteins resulted in high levels of apoptosis similar to that of GFP-FAT. As it has been demonstrated p130Cas binds to proline rich region-1 of FAK via its SH3 domain (Polte and Hanks 1995) Almeida *et al.* (2000) identified p130Cas as a potential candidate for transmission of survival signals from FAK.

Overexpression of the SH3 domain of p130Cas and GFP-FRNK resulted in apoptosis levels which were similar to GFP-FAT. In this system the SH3 domain of p130Cas acts as a dominant negative binding to FRNK and therefore not allowing p130Cas to bind. They concluded p130Cas was required for the transmission of matrix survival signals from fibronectin via interaction with the proline rich region-1 of FAK.

The results presented in this thesis are consistent with the involvement of p130Cas in transducing survival signals via FAK in adherent cells. Whereas in non adherent cells other regions of FAK such as tyrosine 397 and its kinase activity are required for cell survival, in adherent cells these regions are not required. p130Cas also appears to be involved in signalling via FAK during endothelial cell migration although clearly other region of FAK are also involved. Microinjection of the C-terminal GST fusion protein compromised endothelial cell migration but did not abolish it. The C-terminal FAK microinjected in this thesis contained the proline rich region-1 to which p130Cas binds. The C-terminal FAK microinjected by Gilmore and Romer (1996) did not posses the proline rich region-1 and demonstrated much more severely impaired migration.

CHAPTER SIX

General Discussion

Chapter six

6. General Discussion

Focal adhesion kinase is a tyrosine kinase which is localised to focal adhesions in adherent cells. Migration is influenced by the strength of the adhesions between the cell and the substratum. FAK is localised to focal adhesions and becomes tyrosine phosphorylated upon adhesion and it is therefore possible that FAK could play a role in modulation of migration. Indeed, cells from FAK deficient mice displayed reduced migration and enhanced focal adhesions formation (Ilic *et al.* 1995). In addition, overexpression of FAK in CHO cells increased the cells migration (Cary *et al.* 1996). The angiogenic factor vascular endothelial growth factor induces endothelial cell migration and cytoskeletal changes, which may suggest it signals via an adhesion associated protein such as FAK. As demonstrated in this thesis, VEGF induced the tyrosine phosphorylation of FAK in HUVE cells. This was later confirmed by Abedi and Zachary (1997).

Half maximal stimulation of FAK tyrosine phosphorylation occurred at a VEGF concentration of 120pM/L. The high affinity VEGF receptor (VEGFR-1) has a Kd value of 9pM and would be saturated at a lower concentration than was necessary for half maximal activation of FAK tyrosine phosphorylation. It is probable therefore that VEGF acts via the lower affinity receptor (VEGFR-2) to activate FAK tyrosine phosphorylation. VEGF could also act via a heterodimer of the lower and higher affinity receptors. This finding has been confirmed by Kanno *et al.* (2000) who used blocking antibodies to VEGFR-1 and 2 and reported a high concentration of the VEGFR-2 blocking antibody abolished FAK tyrosine phosphorylation.

The VEGF receptor stimulates tyrosine phosphorylation of FAK partially via PI3K as the PI3K inhibitor, wortmannin, reduced but did not abolish FAK phosphorylation induced by VEGF. The VEGF induced membrane ruffling in HUVE cells was wortmannin sensitive and suggests PI3K is involved in this effect of VEGF. Further studies on the degree to which PI3K participates in VEGF induced FAK phosphorylation will allow determination of whether the growth factor regulates the focal adhesion and cortical actin components of the cytoskeleton via at least two different pathways.

The tyrosine kinases, Src and Fyn were both found to associate with FAK upon VEGF stimulation. Once Src/Fyn was bound to FAK they could facilitate the phosphorylation of other tyrosine residues. Tyrosines 407, 576, 577 and 861 are phosphorylated by Src *in vitro* and tyrosines 407, 576 and 577 are phosphorylated by v-Src *in vivo* (Calalb *et al.* 1995 and 1996) and therefore it is conceivable once Src/Fyn has bound to FAK they facilitate further tyrosine phosphorylation of FAK.

The downstream function of VEGF induced FAK tyrosine phosphorylation was investigated. VEGF stimulates endothelial cell migration and FAK is localised to focal adhesions and is therefore a potential candidate for involvement in VEGF stimulated migration. Microinjection of a truncated C-terminal FAK showed reduced, migration in endothelial cells stimulated with VEGF demonstrating that VEGF signalled via FAK to induce endothelial cell migration. This data is supported by Kanno *et al.* (2000) who demonstrated VEGF induced migration of endothelial cells is influenced by signalling through the VEGFR-2 which leads to tyrosine phosphorylation of FAK. Interestingly, signals via VEGFR-1 also effect migration by modulating actin reorganisation.

To further investigate the role of FAK, mutant proteins were generated and expressed in BAE cells due to low transfection levels in HUVE cells. Unfortunately the high basal levels of FAK tyrosine phosphorylation in BAE cells precluded the investigations into the effects of VEGF however the role of FAK in adherent cells was investigated. An insight into the mechanism of FAK activation was given by expression of full length FAK with the truncated C-terminal FAK (FF792). Full length FAK tyrosine phosphorylation was reduced when expressed with the truncated C-terminal FAK. This could suggest localisation to focal adhesions is responsible for tyrosine phosphorylation of FAK as competition with the truncated C-terminal FAK could displace the full length FAK from focal adhesions. Shen *et al.* (1999) suggested FAK localisation to focal adhesions was critical for FAK regulation and reported FAK localisation to focal adhesions was necessary for potential downstream signalling of FAK such as tyrosine phosphorylation of paxillin. In addition, FAK mutated in the FAT domain did not behave like wild type FAK when held in suspension or replated. Conversely, Richardson and Parson (1996) did not believe the reduction seen in FAK tyrosine phosphorylation when FRNK was expressed was due to

competition for localisation to focal adhesions as they did not observe a significant reduction in the intensity of staining of wild type FAK at focal adhesions.

Alternatively dimerisation may be the cause of FAKs autophosphorylation. Immunofluorescence studies to detect localisation of the mutant proteins demonstrated high expression levels of the mutant FAK proteins demonstrating much of the expressed protein located in the cytoplasm. Even though not all the expressed protein was localised to focal adhesions the full length FAK demonstrated high tyrosine phosphorylation levels. In addition, cells in suspension also displayed high levels of FAK tyrosine phosphorylation even though focal adhesions are not present in suspended cells. Expression of kinase deficient Fyn with full length FAK did not affect the tyrosine phosphorylation levels of FAK and this suggests the high level of tyrosine phosphorylation was caused by autophosphorylation and not further phosphorylation of other residues on FAK by Src or Fyn. One possible way the expressed FAK which is not localised to focal adhesions becomes autophosphorylated is by dimerisation with other FAK molecules in close proximity. Indeed it may be the clustering of FAK at focal adhesions which enables dimerisation and transphosphorylation. If dimerisation was the route of FAK transphosphorylation, expression of the truncated C-terminal FAK with full length FAK suggests the C-terminal FAK contains the sequence to enable dimerisation with FAK but not the tyrosine phosphorylation capacity. Any full length FAK which dimerised with the truncated C-terminal FAK would not undergo transphosphorylation. Further evidence that FAK is transphosphorylated by other FAK molecules is the increase in tyrosine phosphorylation of the kinase inactive FAK observed when coexpressed with full length FAK.

In addition to investigating the mechanism of FAK activation, the mutant FAK proteins were utilised to investigate FAKs role in endothelial functions. It had been observed that on microinjection of the C-terminal FAK, a decrease in cell survival was also seen as well as reduced cell migration. Investigation into the role of FAK in cell survival was addressed by the expression of FAK mutants to assess the role of various regions of FAK. In adherent endothelial cells, loss of the autophosphorylation site (tyrosine 397) or loss of the kinase ability of FAK did not induce cells to undergo apoptosis. The truncated C-terminal

FAK however did induce a significant increase in apoptosis. Recently Almeida *et al.* (2000) identified p130Cas, binding to the proline rich region-1 of FAK, as playing a role in transmission of survival signals from FAK. The data presented in this thesis is in agreement with this as the mutant FAK protein, FF792, the C-terminal FAK, induced apoptosis. This construct lacks the proline rich region-1 and therefore blocks signalling via p130Cas.

Future work

Although all the FAK mutants used in this thesis contained the FAT sequence and should therefore be localised to focal adhesions, immunofluorescence studies to confirm this would be beneficial. Analysis of the localisation and affect on the actin cytoskeleton of FAK and FAK mutants by immunofluorescence was not possible due to the high expression levels. Use of a confocal laser scanning microscope may overcome this problem. A confocal laser scanning microscope takes images of cross-sectional layers of the cell and therefore the sections of the cell containing the focal adhesions can be viewed. By visualisation of cross-sectional layers, rather than the entire cell immunofluorescence, any localisation of proteins is not obscured by the fluorescence throughout the cell. In addition, it is possible to view only the most intense staining in the cell due to imaging equipment which blocks out fluorescence below a certain intensity. Alternatively it may be possible to use an expression vector which results in lower expression levels of the FAK mutant proteins. A cell containing lower concentrations of the expressed protein would not have such a high level of fluorescence and enable visualisation of the localisation of FAK.

In order to continue the study with the HUVE cells originally studied rather than BAE cells, the use of retroviral transfection of FAK mutants could be employed. This method would also achieve 100% transfection level and therefore make analysis of any cell function easier as all cells express the mutant protein and it is not necessary to first identify transfected cells.

Beneficial future studies would involve further analysis into the mechanism of FAK activation, specifically focusing on the autophosphorylation and transphosphorylation of

FAK and events which enable Src family kinases to bind to FAK tyrosine 397. Investigation into the possibility of FAK activation induced by dimerisation could be performed by expressing full length FAK, containing an epitope tag other than FLAG with the FLAG tagged FAK mutants. Immunoprecipitation of full length tagged FAK would reveal how the tyrosine phosphorylation state of full length FAK was affected by the mutant FAK proteins. In addition, if the FAK mutants were co-immunoprecipitated with the full length FAK it would demonstrate an association between FAK molecules. This would warrant further analysis to discover the dimerisation sequence(s) in FAK.

It is now quite clear that FAK plays a role in cell migration at least partly through the interaction of p130Cas and Src family kinases binding to FAK. Further analysis of the specific role of FAK in endothelial cell migration would be an interesting area to examine. Migration however is a complex process. Cells migrate by structures at the front of the cell protruding out and forming adhesions with the substratum. These protrusions can be cylinder like structures called filapodia and pseudopodia or thin protruding sheets termed lamellipodia. After stable adhesion at the front of the cell has been established, the cell body and nucleus moves forward with deadhesion and retraction of the rear of the cell. The effect of each FAK region on specific parts of migration are not known. Further analysis of the role of FAK in migration by in-depth analysis of the effect of FAK mutants which are unable to bind Src family kinases or p130Cas on more specific parts of migration would be extremely informative. Processes such as lameilapodia and filapodia extension and retraction rates and the number and type of cellular protrusions could be studied. These processes could possibly be quantitatively analysed using time lapse video microscopy and high resolution imaging techniques.

Acknowledgements

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APPENDIX ONE

Antibodies.

FAK

DRC FAK, anti-focal adhesion kinase polyclonal antibody (kind gift from Prof. D. Critchley, Dept. of Biochemistry, Leicester University). Anti-FAK monoclonal antibody coupled to Protein A agarose, (Upstate biotechnology, Lake Placid, NY, USA).

Filamentous Actin

FITC labelled phalloidin (Sigma-Aldridge, Poole, Dorset, UK), used at 2μg/ml.

Flag

Anti-FLAG BioM2 monoclonal antibody, murine IgG_1. (Sigma-Aldridge, Poole, Dorset, UK), used at 10μg/ml. Anti-FLAG M2 monoclonal antibody covalently attached to agarose beads (Sigma-Aldridge, Poole, Dorset, UK). Anti-FLAG M2 monoclonal antibody, (Sigma-Aldridge, Poole, Dorset, UK), used at 10μg/ml.

Fyn

Anti-Fyn ($p59^{fyn}$), mouse monoclonal antibody (NeoMarkers, Union City, CA, USA), used 1:200 for immunoblotting and 1:60 for immunoprecipitations. Fyn-SH2-GST (Santa Cruz Biotechnology, California, USA), used at 2μg/ml.

Grb2

Anti-Grb2 (C-23) rabbit polyclonal antibody (Santa Cruz Biotechnology, California, USA), used 1:500. Grb2 (54-164)-SH2-GST (Santa Cruz Biotechnology, California, USA), used at 2μg/ml.

GST

Anti-Glutathione-S-Transferase (GST), (Sigma-Aldridge, Poole, Dorset, UK), used 1:2000.

PI3K

Anti-PI 3-kinase p85α rabbit polyclonal antibody (Santa Cruz Biotechnology, California, USA), used 1:500. PI 3-kinase p85α (333-430) SH2-GST (Santa Cruz Biotechnology, California, USA), used at 2μg/ml.

Phosphotyrosine

Monoclonal anti-phosphotyrosine-agarose, Clone PT-66, (Sigma-Aldridge, Poole, Dorset, UK). RC20 Recombinant anti-phosphotyrosine antibody conjugated to HRP, (Transduction Laboratories, Lexington, KY, USA), used 1:2500. Monoclonal anti-phosphotyrosine Clone PT-66, (Sigma-Aldridge, Poole, Dorset, UK), used 1:1000.

Src

Anti-Src ($pp60^{src}$), rabbit polyclonal antibody, (Santa Cruz Biotechnology, California, USA), used 1:500.

VEGF receptors

Flt-1 (VEGFR-1) rabbit polyclonal antibody (Santa Cruz Biotechnology, California, USA), used 1:500. Flk (VEGFR-2) rabbit polyclonal antibody (Santa Cruz Biotechnology, California, USA), used 1:500.

Vinculin

Anti-Vinculin VIN-11-5 monoclonal antibody (Sigma-Aldridge, Poole, Dorset, UK), used 1:100.

Secondary antibodies

Anti-Rabbit horseradish peroxidase conjugate (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK), used 1:2500.

Anti-Mouse horseradish peroxidase conjugate (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK), used 1:2500.

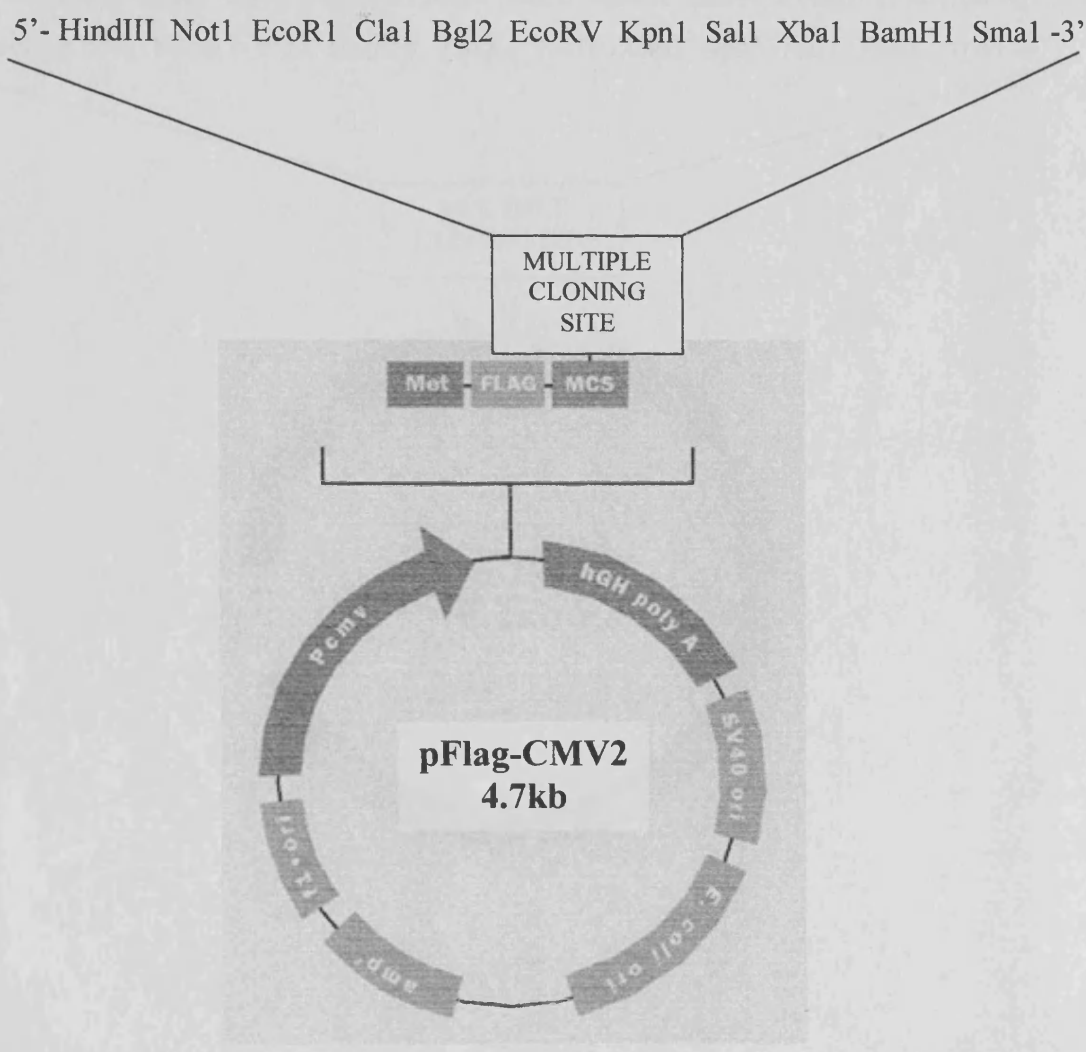
Anti-mouse Ig Cy3 conjugate (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK), used 1:250.

ExtrAvidin-FITC conjugate (Sigma-Aldridge, Poole, Dorset, UK), used 1:200.

Anti-rabbit IgG FITC conjugate (Sigma-Aldridge, Poole, Dorset, UK), used 1:200.

APPENDIX TWO

pFlag-CMV2 vector map and multiple cloning site



G

pCR3 vector map and multiple cloning site

5'- HindIII Kpn1 Sac1 BamH1 Spe1 Eag1/XmaIII BstXI EcoR1 (TA cloning insertion site) EcoR1 Pst1 EcoRV BstX1 Not1 Xho1 Sph1 Nsi1 Xba1 Apa1 -3'