INTERACTIONS THAT CONTRIBUTE TO FAK ACTIVATION

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ABSTRACT

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(Under the direction of Michael D Schaller)

Focal adhesion kinase (FAK) is an important regulatory protein that integrates signals from integrins and growth factor receptors to control cell growth, survival, and migration. FAK function is tightly regulated and the complete mechanism of activation remains to be resolved. The FAT domain of FAK associates with the scaffolding protein paxillin as a major mechanism of FAK localization. Structural insights have been used to design point mutations to disrupt FAK binding to paxillin and resulting defects in FAK function have been characterized. The FERM domain of FAK is thought to play an important role in relaying biological signals. This domain is involved in an intramolecular interaction with the FAK catalytic domain that severely inhibits activity. I hypothesize that FERM domain associations disrupt the inhibitory association between the FERM and catalytic domains, thus activating FAK. A mutagenesis strategy has been used to identify binding sites on the FERM domain.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CAM	chick chorioallantoic membrane
EC	endothelial cell
ECM	extracellular matrix
EGFR	epidermal-derived growth factor receptor
ERK	extracellular signal-related kinase
FAK	focal adhesion kinase
FAT	focal adhesion targeting
FERM	band 4.1protein/ERM (ezrin, radixin, moesin)
FGF	basic fibroblast growth factor
FRNK	FAK-related non-kinase
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
Grb	growth factor receptor bound protein
GST	glutathione S-transferase
HUVEC	human umbilical vein endothelial cells
IGF	insulin-like growth factor
LIM	Lin-11, Isl-1, Mec-3
MAP kinase	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
NMR	nuclear magnetic resonance

PDGFR	platelet-derived growth factor receptor
PH	plexdrin homology
PI3K	phosphatidylinositol 3-kinase
SH	Src homology
TRANCE	TNF-related activation-induced cytokine
VEGF	vascular endothelial growth factor

CHAPTER ONE

BACKGROUND AND SIGNIFICANCE

1.1 FAK AND ANGIOGENESIS

Angiogenesis- the formation of new blood vessels- is an important process during development and throughout life. Alterations in normal levels of angiogenesis play a role in several pathological conditions such as stroke, coronary artery disease, rheumatoid arthritis, psoriasis, diabetic retinopathy, age-related macular degeneration, and cancer (Eliceiri and Cheresh, 2001). During angiogenesis, the new blood vessels are formed by vascular endothelial cells (EC), which integrate environmental stimuli to regulate vessel formation (Daniel and Abrahamson, 2000;Orr and Murphy-Ullrich, 2004). The initial step in this process is the sprouting of the ECs, which requires migration into the extracellular matrix (ECM) (Peng *et al.*, 2004). This migration is mediated by signals from both integrins and growth factor receptors (Eliceiri and Cheresh, 2001;Eliceiri, 2001;Schwartz and Ginsberg, 2002). These signals cause the ECs to loosen cell-cell contacts and secrete enzymes that degrade the subendothelial basement membrane. After migration, the ECs assemble a lumen, completing the vessel formation; although maturation of the vessels requires further processes such as smooth muscle recruitment and organization (Orr and Murphy-Ullrich, 2004). EC spreading, migration, and morphogenesis are essential for angiogenesis (Kim *et al.*, 2000). Accordingly, gene targeting studies have revealed roles in vasculogenesis and

angiogenesis for angiogenic growth factors, ECM components, and integrin ECM receptors (Ilic *et al.*, 2003).

FAK is activated by many angiogenic stimuli such as insulin-like growth factor (IGF)-1, basic fibroblast growth factor (FGF), angiopoietin-1, EphrinA1, thrombospondin, and TNF-related activation-induced cytokine (TRANCE). In many of these cases, FAK activation is thought to play an important role in relaying downstream signals (Carter *et al.*, 2002;Kim *et al.*, 2002;Orr and Murphy-Ullrich, 2004;Duxbury *et al.*, 2004;Orr *et al.*, 2004). For example, FAK activation has been shown to be required for angiopoietin-1 stimulated EC sprouting (Kim *et al.*, 2000). The most compelling evidence for an essential role for FAK during angiogenesis comes from studies of VEGF (Hood *et al.*, 2003;Avraham *et al.*, 2003). Stimulation of ECs with VEGF induces Src-dependent phosphorylation of FAK at tyrosine 861, an event that is essential for VEGF stimulated effects on cell migration and survival (Abu-Ghazaleh *et al.*, 2001;Eliceiri *et al.*, 2002). Using a chick chorioallantoic membrane (CAM) angiogenesis assay, inhibition of FAK was shown to disrupt VEGF-mediated Ras and c-Raf activity in a Src-dependent manner (Avraham *et al.*, 2003). It has also been shown that the tubulogenesis activity associated with an activated form of VEGFR-1 is dependent upon FAK (Maru *et al.*, 2001). The same study found an association between VEGFR-1 and FAK following VEGF stimulation that correlated with tubulogenic ability, further supporting a role for FAK during VEGF stimulated angiogenesis (Maru *et al.*, 2001).

FAK is important for regulating several processes integral to angiogenesis, such as cell growth, survival, and migration (Hanks *et al.*, 2003). The first insight into a role for FAK in the vasculature came from studies which identified a high level of FAK staining in the vasculature of the developing mouse embryo (Polte *et al.*, 1994). The importance of FAK in

vascular development was verified upon development of knockout mice that die at embryonic day 8.5 due to major defects in vascular development, both in vasculogenesis and angiogenesis (Ilic *et al.*, 1995). There have also been several studies that have manipulated FAK signaling in ECs, most commonly by expression of FAK or the FAK dominant negative variant FRNK. The observed effects on angiogenesis or processes important for angiogenesis have implicated FAK in the control of EC migration and tubulogenesis.

1.2 FAK REGULATION OF CELLULAR PROCESSES

FAK plays an important role in the regulation of several cellular processes including cell spreading, cell migration, cell proliferation, cell survival, and apoptosis. Regulation of these processes is important not only for angiogenesis, but also for development and cancer progression. For these reasons, many studies have investigated the role of FAK in the regulation of these processes. These studies indicate that FAK signaling is mediated by binding of FAK to various interacting partners.

Several studies have demonstrated a role for FAK in cell spreading on fibronectin. *fak*$^{-/-}$ MEFs have defects in attachment to fibronectin and reportedly exhibit rounded morphologies and are poorly spread compared to *fak* $^{+/+}$ MEFs or *fak*$^{-/-}$ MEFs re-expressing FAK (Westhoff *et al.*, 2004). Furthermore overexpression of the FAK dominant negative FRNK delays cell spreading in CE cells (Richardson and Parsons, 1996). These results suggest a role for FAK in the regulation of this important cellular process.

FAK has long been implicated in integrin-mediated cell motility. Overexpression of the FAK dominant negative FRNK inhibits migration of endothelial cells in a wound healing assay (Gilmore and Romer, 1996). Alternatively, overexpression of FAK increases cell

migration in CHO cells (Cary *et al.*, 1998). Finally, *fak*$^{-/-}$ MEFs show reduced rates of cell migration (Ilic *et al.*, 1995) that is restored upon FAK re-expression (Owen *et al.*, 1999;Sieg *et al.*, 2000). FAK is thought to regulate cell migration via multiple signaling partners. p130 CAS has been shown to regulate migration independently of FAK and a FAK mutant defective for p130CAS binding failed to induce cell migration (Cary *et al.*, 1998;Sieg *et al.*, 2000). PI3K has also been suggested to mediate FAK effects on cell migration and a FAK mutant defective for PI3K binding also failed to induce cell migration (Reiske *et al.*, 1999). Finally, Grb7 has been proposed to be required for FAK effects on migration as this protein has been independently linked to regulation of cell migration and can bind FAK (Han and Guan, 1999;Han *et al.*, 2000). These studies indicate that FAK plays a critical role in regulation of cell migration, possibly via multiple pathways.

Several lines of evidence link FAK to regulation of cell proliferation. Inhibition of FAK by expression of FRNK (Gilmore and Romer, 1996) or by injection of FAK monoclonal antibody (Hungerford *et al.*, 1996) both result in inhibition of DNA synthesis and cell cycle arrest. Alternatively, expression of FAK positively regulates cell cycle progression following serum-stimulation (Zhao *et al.*, 1998b). FAK is thought to regulate cell cycle progression via MAPK signaling to the ERK and JNK MAPKs (Zhao *et al.*, 1998a;Oktay *et al.*, 1999).

Detachment from the ECM induces apoptosis in many cells types. Apoptosis due to loss of cell attachment is termed anoikis and is regulated by integrins and FAK signaling. This role for FAK in providing resistance to anoikis is supported by several studies. A membrane-targeted, activated form of FAK expressed in epithelial cells provided protection from apoptosis in these cells (Frisch *et al.*, 1996). Alternatively, inhibition of FAK by antisense

(Xu *et al.*, 1996) or injection of monoclonal FAK antibody (Hungerford *et al.*, 1996) has been shown to induce apoptosis in different cell types. Finally, FAK proteolysis at specific sites has been demonstrated in various cell lines undergoing apoptosis, indicating that FAK signaling needs to be inhibited in order for apoptosis to proceed in these cells (Crouch *et al.*, 1996;Wen *et al.*, 1997;Levkau *et al.*, 1998). FAK associations with both p130CAS and PI3K are thought to be important for FAK's regulation of cell survival (Chan *et al.*, 1999;Bellas *et al.*, 2002). Recent studies have also identified an association between FAK and p53. This association suppresses p53-mediated apoptosis and inhibits p53 transcriptional activity, representing an independent pathway by which FAK controls apoptosis (Ilic *et al.*, 1998;Golubovskaya *et al.*, 2005).

1.3 FAK ACTIVATION

FAK has long been known to play an important role in mediating integrin signaling (Hanks *et al.*, 2003;Orr and Murphy-Ullrich, 2004). When integrins bind to ECM proteins, they cluster on the cell surface resulting in clustering of FAK at these sites of adhesion. FAK clustering induces autophosphorylation of Y397, which creates docking sites for proteins with SH2 domains, specifically Src, the p85 subunit of PI3K, Grb7, and phospholipase C-? (Turner, 2000;Schaller, 2001a). Once Src binds, it phosphorylates other tyrosine residues in FAK resulting in further activation. Specifically, Y576 and Y577 are located within the activation loop of the kinase and phosphorylation of these sites is necessary for maximal catalytic activity (Calalb *et al.*, 1995). Src can also phosphorylate Y861 and Y925 in the C-terminus of FAK. Phosphorylation of Y861 downstream of VEGF stimulation contributes to the formation of a complex between FAK and the avß5 integrin, necessary for VEGF-

stimulated migration (Eliceiri *et al.*, 2002). Phosphorylation of Y925 creates a binding site for the SH2 domain of Grb2, which may be important for activation of ERK (extracellular signal-related kinase) and for dynamin-mediated focal adhesion disassembly (Hanks *et al.*, 2003;Orr and Murphy-Ullrich, 2004;Ezratty *et al.*, 2005). These phosphorylation events are required for FAK activation downstream of integrin attachment and are required for FAK downstream signaling.

1.4 FAK DOMAINS

FAK is composed of three major domains as shown in Figure 1 (Hanks *et al.*, 2003). The C-terminal FAT domain is necessary and sufficient for the localization of FAK to focal adhesions by binding to other focal adhesion proteins like paxillin (Schaller, 2001a). This domain of FAK and its association with paxillin is discussed further in Chapter 2. An alternately spliced form of FAK termed FRNK (FAK-related non-kinase) is composed of the C-terminal domain, including the FAT sequences. FRNK has been shown to act as a dominant negative in several systems, disrupting endogenous FAK function. FAK also contains a central catalytic domain that is responsible for phosphorylating various focal adhesion proteins, such as paxillin and p130CAS. The function of the N-terminal FERM domain of FAK has until recently remained a mystery. There have been reports of proteins binding to the FAK FERM domain, but the relevance of these interactions has not been determined (Schaller, 2001a;Liu *et al.*, 2002). New insights suggest that this domain is involved in an intramolecular interaction with the FAK catalytic domain that negatively regulates FAK activity. This domain of FAK is discussed further in Chapter 3.

1.5 SUMMARY

Angiogenesis is an important process during development and throughout life. Deviations from normal levels of angiogenesis play a role in several pathological conditions, such as stroke and coronary artery disease (Eliceiri and Cheresh, 2001). During angiogenesis, new blood vessels arise from vascular endothelial cells (EC), which integrate signals from integrins and growth factor receptors to regulate cell growth, survival, and migration (Daniel and Abrahamson, 2000;Eliceiri and Cheresh, 2001;Eliceiri, 2001;Orr and Murphy-Ullrich, 2004). An important protein known to play a role in the regulation of these EC processes is the focal adhesion kinase (FAK). FAK is activated by several angiogenic stimuli and FAK is important for regulating several processes integral to angiogenesis, such as cell growth, survival, and migration. FAK function is regulated in several ways and although much is known about phosphorylation events that play a role in FAK's activation, the complete mechanism of activation still remains to be resolved.

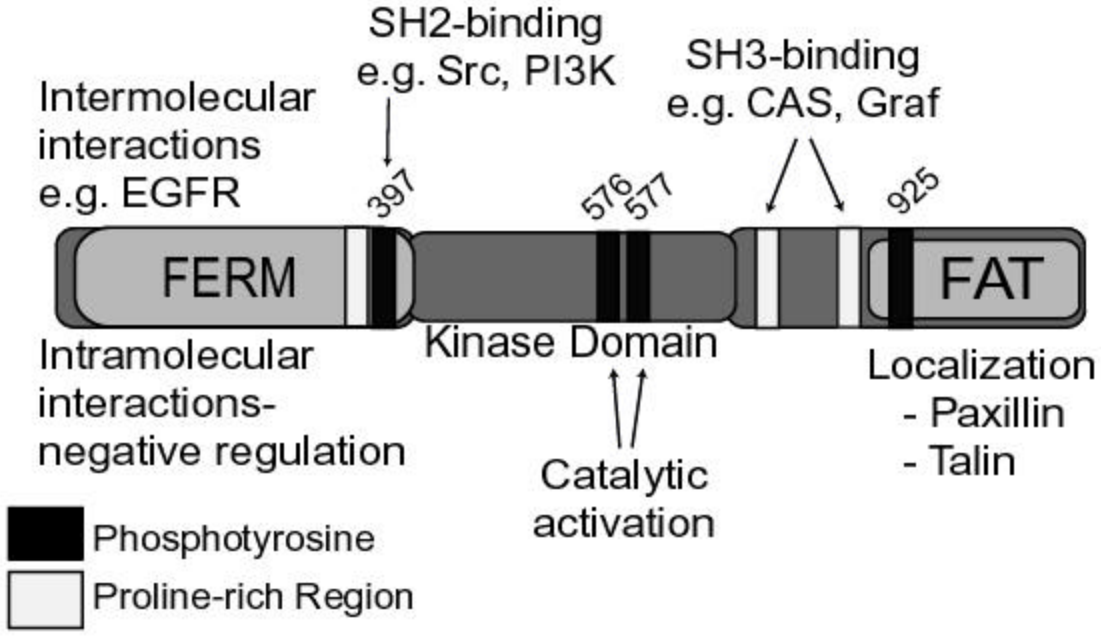

Figure 1. Cartoon representation of FAK. FAK is composed of an N-terminal FERM domain, a central kinase domain responsible for substrate phosphorylation, and a C-terminal focal adhesion targetting (FAT) domain.

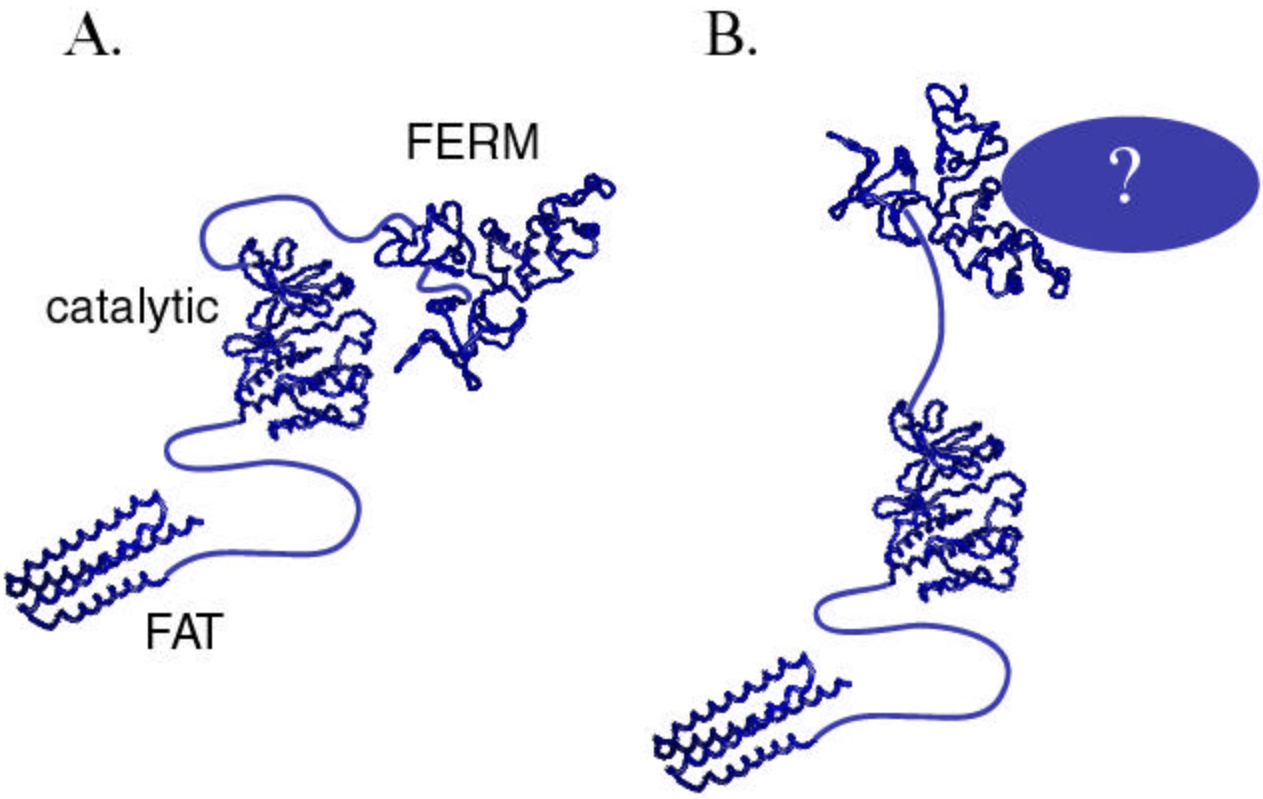

Figure 2. Model for the role of the FERM domain in FAK activation. The FERM domain of FAK has recently been reported to bind to the FAK catalytic domain, resulting in an inhibited form of the kinase (A). Binding by some unknown protein(s) to the FERM domain is thought to disrupt the FERM-catalytic domain interaction, thus activating FAK (B).

CHAPTER TWO

PAXILLIN BINDING IS IMPORTANT FOR FAK LOCALIZATION, BIOCHEMICAL, AND BIOLOGICAL FUNCTION

2.1 INTRODUCTION

Focal adhesion kinase is a non-receptor tyrosine kinase that plays an important role in mediating essential cellular processes, such as cell growth, survival, and migration. FAK knockout mice die at E8.5, exhibiting developmental defects similar to those seen in FN-deficient and ß1 integrin knockout mice (Watt and Hodivala, 1994;Hirsch *et al.*, 1996). Fibroblasts isolated from *fak*$^{-/-}$ mice are more rounded or pancake-shaped, have more focal adhesions, and have a migratory defect as compared to cells from wild type littermates (Ilic *et al.*, 1995). In accordance with the phenotype of the *fak*$^{-/-}$ mice, FAK has long been known to play an important role in mediating integrin signaling (Schaller, 2001a;Hanks *et al.*, 2003).

When integrins bind to ECM proteins, they cluster on the cell surface resulting in clustering of FAK at these sites of adhesion, called focal adhesions. FAK clustering induces autophosphorylation of Y397, which creates docking sites for proteins with SH2 domains, specifically Src, the p85 subunit of PI3K, Grb7, and phospholipase C-? (Turner, 2000). Src binding is considered an important event as it subsequently phosphorylates other tyrosine residues in FAK. Y576 and Y577 are located within the activation loop of the kinase and phosphorylation of these sites by Src is necessary for maximal catalytic activity of FAK

(Calalb *et al.*, 1995). Src can also phosphorylate Y861 and Y925 in the C-terminus of FAK. Phosphorylation of Y861 downstream of VEGF stimulation promotes the formation of a complex between FAK and the avß5 integrin, which is necessary for VEGF-stimulated migration (Eliceiri *et al.*, 2002). Phosphorylation of Y925 creates a binding site for the SH2 domain of Grb2 and may be important for activation of ERK (extracellular signal-related kinase) (Chen *et al.*, 1995;Schlaepfer and Hunter, 1996;Schaller, 2001a;Hanks *et al.*, 2003). Grb2 binding to Y925 also recruits dynamin to focal adhesions, which is important for focal adhesion disassembly (Ezratty *et al.*, 2005). Thus, these Src-dependent phosphorylation events play an important role in regulation of FAK signaling downstream of integrin attachment.

FAK is composed of three major domains (Schaller, 2001a;Hanks *et al.*, 2003). The N-terminal FERM domain of FAK contains binding sites for a number of proteins and has recently been reported to interact with the catalytic domain of FAK to inhibit catalytic activity (Cooper *et al.*, 2003;Dunty *et al.*, 2004). FAK contains a central catalytic domain that is responsible for phosphorylating various focal adhesion proteins, such as paxillin, p130CAS, and tensin. The C-terminal focal adhesion targeting (FAT) domain is required for the localization of FAK to focal adhesions and binds to two focal adhesion associated proteins, paxillin and talin (Hildebrand *et al.*, 1993;Hildebrand *et al.*, 1995).

Paxillin is a scaffolding protein composed of multiple protein-protein interaction domains, including five N-terminal LD motifs, four C-terminal LIM domains, and SH2 and SH3 domain binding sites (Schaller, 2001b). The second and fourth LD motifs of paxillin have been identified as FAK-binding sites and each of these sites binds to FAK with similar affinity (Thomas *et al.*, 1999). Paxillin is localized to focal adhesions by interactions

mediated by its C-terminal LIM domains (Schaller, 2001b) and paxillin is known to be one of the first proteins recruited into these structures as they are formed (Webb *et al.*, 2004). Binding to paxillin is proposed as one mechanism of localization of FAK to focal adhesions and association with talin has been suggested as an alternative mechanism of localization. Results from paxillin knockout studies show reduced phosphorylation of FAK in *paxillin*$^{-/-}$ embryonic stem cells (Wade *et al.*, 2002) and in *paxillin*$^{-/-}$ cultured mouse embryonic fibroblasts (Hagel *et al.*, 2002). This indicates that paxillin binding may function in the regulation of FAK activity, in addition to its proposed role in regulating localization.

Recent reports have used both NMR and crystallographic approaches to determine the structure of the C-terminal FAT domain of FAK as a four-helix bundle (Arold *et al.*, 2002;Hayashi *et al.*, 2002;Liu *et al.*, 2002;Gao *et al.*, 2004;Bertolucci *et al.*, 2005). Additional structural studies have also revealed the structure of the FAT domain of FAK in complex with peptides mimicking the LD2 peptide of paxillin (Gao *et al.*, 2004;Bertolucci *et al.*, 2005). The striking finding from these studies was the identification of two paxillin-binding sites on the FAT domain of FAK. This finding is particularly intriguing, given the presence of two FAK-binding sites in the N-terminus of paxillin. Each paxillin-binding site in the FAT domain is composed of a surface exposed hydrophobic patch, adjacent to a series of basic residues. One binding site lies at the interface of α-helices 2/3 and the other at the interface of α-helices 1/4 and thus the two sites are on opposite sides of the four-helix bundle. While evidence suggests that the LD2 peptide of paxillin interacts with both of these sites with similar affinity (Gao *et al.*, 2004), the results of a paramagnetic labeling experiment suggests that the paxillin LD4 peptide has a preference for one site over the other (Bertolucci *et al.*, 2005).

These studies raise an interesting question of whether the two paxillin-binding sites of FAK are simply redundant interaction surfaces that strengthen the association between these two proteins, or alternatively, whether paxillin binding to each site might mediate a distinct function. To address these important questions, site-directed mutagenesis of the paxillin-binding regions on FAK was used to disrupt binding to the α-helix 2/3 paxillin binding site (E949A/K956A/R963A or EKR) or to the α-helix 1/4 paxillin binding sites (I937A), respectively. Combining these mutations together (E949A/K956A/R963A/I937A or EKR/I937A) completely abolished paxillin binding as demonstrated *in vitro* by GST pulldown and *in vivo* by co-immunoprecipitation as previously reported (Gao *et al.*, 2004). These mutants have been characterized to determine the role of paxillin binding to each site individually or to both binding sites in the function of FAK.

2.2 MATERIALS AND METHODS

Molecular biology. FAK mutants were engineered using pBluescript-FAK as a template (Schaller *et al.*, 1992). Point mutations were engineered using the QuikChange mutagenesis kit (Stratagene, La Jolla CA). Sequence analysis was performed to verify the intended point mutations and that no unintended mutations were present. These analyses were performed in the UNC-CH Genome Analysis Facility on a model 3730 DNA Analyzer (Perkin Elmer, Applied Biosystems Division) using the ABI PRISM^{TM} Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer, Applied Biosystems Division). Full-length FAK cDNAs were subcloned into RCAS A as described previously (Hildebrand *et al.*, 1993). Full-length FAK cDNAs were subcloned into pEGFP as described previously (Cooley *et al.*, 2000). Full-length FAK cDNAs were excised from pBluescript using BamHI and SalI and inserted into the multiple cloning site of the pBABE vector. All subcloned constructs were verified by sequence analysis. Point mutations were engineered into pEGFP-EKR/I937A using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla CA). Sequence analysis was performed on each mutant to verify the intended point mutations and that no unintended mutations were present.

Cells and viruses. Chicken embryo (CE) cells were prepared and maintained as described previously (Reynolds *et al.*, 1989). These cells were transfected using Lipofectamine PLUS (Life Technologies, Gaithersburg, MD) with RCAS A retroviral vectors encoding wild type or mutant FAK proteins (Gao *et al.*, 2004). To address the regulation of FAK phosphorylation in response to adhesion, cells were trypsinized, washed in 0.5 mg/mL soybean trypsin inhibitor (Sigma, St. Louis, MO) in PBS, held in suspension for 30 minutes,

and replated onto dishes coated with fibronectin (50 µg/ml) for 45 minutes. In order to enhance the phosphotyrosine content of cellular proteins in some experiments, cells were treated with 50 µM sodium vanadate for 16 hrs prior to lysis.

CHO cells were maintained as described (Webb *et al.*, 2004). To generate stable populations of CHO cells expressing GFP tagged constructs, the cells were transfected with plasmids encoding wild type FAK or mutants fused to GFP using Lipofectamine PLUS (Life Technologies), followed by selection and maintenance in 1 mg/ml G418 (Gibco BRL). Populations of G418 selected CHO cells were then sorted by FACS to enrich for GFP expression. Trypsinized CHO cells were washed once in PBS then resuspended in 0.5 ml PBS. Flow cytometry was performed on a Becton Dickinson FACScan interfaced to a Cytomation, Inc. Cicero data acquisition system in the UNC Flow Cytometry Facility.

Fak$^{-/-}$ MEFs were maintained as described (Westhoff *et al.*, 2004). In order to generate populations of *fak*$^{-/-}$ MEFs expressing wild type or FAK mutants, the pBABE retroviral vector was used. To generate virus to infect the *fak*$^{-/-}$ MEFs, Phoenix cells were transfected with the various pBABE constructs. Phoenix cells were maintained in DMEM F12 (Gibco BRL, Rockville, MD) supplemented with 10% FBS (Gibco BRL), penicillin, streptomycin, gentamicin and kanamycin (Sigma). Phoenix cells were transfected with 8 µg pBABE DNA using Lipofectamine PLUS (Life Technologies). After 48 hrs, virus-containing media was collected and filtered through a 0.45 µm syringe filter. For infection, each dish of *fak*$^{-/-}$ MEFs was incubated for 24 hrs with 4 mL filtered virus-containing media, 4 µg/mL polybrene (Sigma), and 5 mL complete media. At 24 hrs, the infection media was replaced with complete media. At 48 hrs post-infection, media was replaced with complete media

containing 2 µg/mL puromycin (Sigma) for selection and maintenance of expressing populations.

Cell adhesion assay. Cell adhesion assays were performed as described previously (Westhoff *et al.*, 2004). Drug selection media was removed from cells overnight. Cells were trypsinized, washed once with PBS containing 0.5 mg/mL soybean trypsin inhibitor (Sigma), once with serum-free DMEM, and resuspended in serum-free DMEM containing 0.5 mg/mL lipid-free BSA (Sigma). Cells were held in suspension for 1 hr at 37°C prior to plating onto fibronectin-coated surfaces. Bacterial 60 mm dishes were coated at 4°C with 50 µg/mL bovine plasma fibronectin (Sigma) overnight then blocked for 1 hr with DMEM containing 0.5 mg/mL lipid-free BSA (Sigma). $1x10^5$ cells were added to each 60 mm dish and incubated at 37°C for 60 minutes, followed by fixation and staining using a Hema3 kit (Fisher Diagnostics, Middletown, VA) according to manufacturer's instructions. The number of cells in five random fields was counted for each experiment.

Protein analysis. Cells were washed twice with PBS and lysed in modified RIPA buffer (50 mM Tris [pH 7.3], 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate) containing protease and phosphatase inhibitors as described previously (Gao *et al.*, 2004). Protein concentrations were determined with the bicinchoninic acid assay (Pierce, Rockford, Ill.). The FAK phosphorylation site-specific antibodies (Biosource International, Camarillo, CA) and paxillin, p130CAS, and PY20 phosphotyrosine antibodies (BD Biosciences, San Diego, CA) were purchased commercially. The BC4 polyclonal antiserum has been described

previously (Schaller *et al.*, 1992). Immunoprecipitations and Western blotting were performed as previously described (Gabarra-Niecko *et al.*, 2002).

Immunofluorescence. Glass coverslips were coated with 50 µg/mL bovine plasma fibronectin (Sigma) in PBS for 1 hr at 37 °C. Cells were plated onto the coated coverslips and maintained at 37°C for 16 hr. Cells were fixed in 3.7% formaldehyde and permeabilized with 0.5% Triton X-100. FAK was detected using BC4 and rhodamine-conjugated anti-rabbit antibody (Jackson ImmunoResearch Labs). For co-staining studies, a paxillin monoclonal antibody (BD Biosciences) and rhodamine conjugated anti-mouse secondary antibodies (Jackson Labs) were used to visualize focal adhesions while an avian GFP antibody (Chemicon International, Temula, CA) and FITC anti-chicken secondary antibodies (Jackson Labs) were used to detect GFP-tagged proteins. Cells were visualized using a Leitz Orthoplan fluorescence microscope, and images captured with a Hamamatsu digital camera and Metamorph imaging software (Universal Imaging Corporation, West Chester, PA). Images were taken with identical exposure times. Paxillin staining was used to identify focal adhesions and cells were scored for co-localization of paxillin and GFP staining, indicating FAK localization at these sites. The results were expressed as percentage of cells exhibiting focal adhesion localization of GFP-FAK to focal adhesions. ANOVA analyses with Dunnett post-tests were performed using GraphPad software (San Diego, CA) to identify statistically significant differences in localization efficiency. For live cell imaging, EGFP N-terminally tagged forms of wild type FAK or the FAK mutants were expressed in CE cells using Lipofectamine PLUS (Invitrogen). At 24 hrs, the transfected cells were plated in 35 mm glass bottom dishes (MatTek, Ashland, MA) and incubated overnight at 37^{o}C. At 48 hrs,

cells were viewed using an Olympus IX81 microscope by epiflourescence and TIRF microscopy.

Protein preparation and in vitro binding experiments. GST fusion proteins were expressed in *E. coli* and purified as described (Gabarra-Niecko *et al.*, 2002). Briefly, expression was induced with 0.1 mM isopropyl 1-thio-ß-D-galactopyranoside, followed by incubation for 2 hrs at 37^{o}C. The bacteria were harvested and sonicated in TETN buffer (1% Triton X-100, 20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA) plus protease inhibitors (1 mM PMSF, 10 µg/mL leupeptin, 10 Ug/mL aprotinin). Clarified supernatants were bound to glutathione-agarose beads (Sigma) for 1 hr at 4^{o}C, washed three times with PBS, and resuspended in equal volumes PBS. Fusion proteins were quantified by SDS/PAGE and Coomassie blue staining. For binding experiments, 1 mg of vanadate-treated CE lysate was precleared by incubation with 100 µg of GST bound to glutathione-agarose beads for 1 hr at 4^{o}C, then incubated with 25 µg GST-Grb2SH2 immobilized on beads for 2 hrs at 4^{o}C. The beads were washed twice with modified RIPA buffer, twice with PBS, eluted in sample buffer, and analyzed by western blotting.

2.3 RESULTS

To determine if there is a specific role for each paxillin-binding site of the FAT domain of FAK, mutants that disrupt binding to paxillin at each site individually or to both sites simultaneously have been generated. Wild type FAK or the FAK mutants were expressed in primary chicken embryo (CE) cells using the RCAS A retroviral vector, in *fak*$^{-/-}$ MEFs using the pBABE retroviral vector, and as GFP fusion proteins in CHO cells. Cells were lysed and expression levels examined by western blotting. Expression of each mutant was comparable to that of wild type FAK in each of these cell systems, as shown in Figure 3. Further, expression levels in CHO cells and the MEFs was similar to the normal level of expression of endogenous FAK.

Paxillin is known to have two binding sites for FAK and mutants of paxillin that disrupt binding to FAK have been previously described (Thomas *et al.*, 1999). Recent studies have revealed that FAK also has two binding sites for paxillin. To assess the ability of individual binding sites on paxillin and FAK to interact, a GST pulldown approach was utilized. GST fusion proteins, shown in Figure 4A, were used to bind to FAK variants expressed in CE cells. The GST fusion proteins contained the N-terminal portion of paxillin containing both FAK binding sites, i.e. LD 2 and LD 4 (GST-PaxN1C3). GST fusion proteins with point mutations that disrupt FAK binding to LD2 (D146A) or LD4 (D268A) were also used, as well as a double mutant that abolishes FAK binding to both LD2 and LD4 (D146A/D268A). Fusion proteins, immobilized on glutathione beads, were incubated with lysates of CE cells expressing FAK variants and bound FAK assessed by western blotting. Wild type FAK bound to the wild type recombinant paxillin, exhibited slightly reduced binding to both the D146A and D268A mutants, and bound poorly to the D146A/D268A double mutant. These

observations are similar to published results using these paxillin mutants (Thomas *et al.*, 1999). The same trend is observed with the I937A FAK mutant, i.e. reduced binding to the D146A and D268A mutants and poor binding to the D146A/D268A double mutant. In contrast, the EKR FAK mutant exhibits a different pattern of binding. This FAK mutant binds the wild type paxillin fusion protein and shows reduced binding to the D268A mutant. This FAK mutant binds very poorly to the D146A mutant and the D146A/D268A double mutant. This differential binding reveals that the two FAK-binding sites of paxillin do not interact with FAK in precisely the same way. While the LD2 motif of paxillin (this site is intact in the D268A mutant) interacts with FAK mutants defective for paxillin binding at either site in the FAT domain, the LD4 motif of paxillin (this site is intact in the D146A mutant) preferentially interacts with the I937A mutant of FAK. Thus the LD4 motif preferentially associates with the intact paxillin-binding site in this mutant, which is at the interface of α-helices 2 and 3.

The subcellular localization of FAT domain mutants was assessed by immunofluorescence of exogenously expressed protein. CE cells expressing FAK were plated onto fibronectin-coated coverslips and allowed to adhere overnight. Coverslips were fixed, permeabilized, and stained with a FAK polyclonal antibody and FITC-anti-rabbit secondary antibody. The exogenous proteins are expressed at high levels making it easy to distinguish their expression. Examples of cells exhibiting focal adhesion localization of each of the FAK mutants are shown in Figure 5A. To validate these findings, a complimentary approach using GFP-tagged FAK variants was used to assess localization. GFP fused to wild type FAK or the FAK mutants were transiently expressed in CE cells. At 24 hrs post-transfection, the cells were plated onto glass-bottom dishes and 24 hours later, the cells were viewed by

epiflourescence and TIRF microscopy. Although transfection efficiency is low in these cells, sufficient numbers of cells could be analyzed, as shown in Figure 5B. For each of the mutants, there were cells exhibiting focal adhesion localization of FAK by both epiflourescence and TIRF microscopy. These experiments revealed that mutants of FAK that disrupt paxillin binding to either site on the FAT domain or to both sites can still localize to focal adhesions. However, during the course of imaging the transfected CE cells it was apparent that some of the mutants were more difficult to find at focal adhesions, suggesting there might be a quantitative difference in efficiency of localization. To further investigate the subcellular localization of these mutants, stable populations of CHO cells expressing GFP-FAK fusion proteins at a similar level as endogenous FAK protein were established. Cells were plated on glass coverslips and allowed to spread overnight before being fixed and stained using an avian GFP antibody (Chemicon International) and FITC secondary antibody. Focal adhesions were stained using a paxillin monoclonal antibody and a rhodamine-labeled secondary antibody. Fixed cells were imaged (data not shown) and paxillin staining was used to identify focal adhesions. Cells were scored for co-localization of paxillin and GFP staining, indicating FAK localization at these sites. The results were expressed as percentage of cells exhibiting focal adhesion localization of GFP-FAK (Figure 6A). Like wild type FAK, the EKR FAK mutant was targeted to focal adhesions in about 90% of the cells. The I937A mutant was localized to focal adhesion in about 55% of the cells, indicating that this paxillin-binding site may be more important for focal adhesion targeting than the other paxillin-binding site. Although some cells expressing the mutant EKR/I937A, which has defects in both paxillin-binding sites, exhibit focal adhesion localization of the FAK mutant, the efficiency of targeting was severely decreased as compared to wild type FAK. These

results indicate that although paxillin binding is not absolutely required for FAK localization, this association does play a role in FAK's ability to localize to focal adhesions.

Although the above experiments indicated that paxillin plays a major role in the regulation of FAK targeting to focal adhesions, the FAK mutant defective for paxillin binding still correctly localized in approximately 10% of cells. This mutant presents a suitable background for additional mutagenesis studies to identify residues outside of the paxillin-binding regions of the FAT domain that are important for FAK localization. Additional alanine substitutions at conserved residues on the surface of the FAT domain were made and the subcellular localization of the resulting mutants was assessed upon transient expression as GFP fusion proteins in CE cells. Using this approach, E997 was identified as a residue important for FAK localization to focal adhesions in the absence of paxillin binding (Figure 6B). This mutant was further analyzed by stable expression of the GFP fusion protein in CHO cells. The E997A mutation in combination with the EKR/I937A mutations resulted in a complete absence of the FAK mutant from focal adhesions. However, the E997A mutant in an otherwise wild type background, i.e. with intact paxillin-binding sites, was able to localize to focal adhesions nearly as well as wild type FAK (Figure 6A). This result indicates that E997 is important for the localization of FAK to focal adhesions in the absence of paxillin binding.

FAK phosphorylation is important for its activation and downstream signaling. CE cells expressing either wild type FAK or FAK mutants were used to assess defects in FAK phosphorylation when paxillin binding to either or both paxillin-binding sites is disrupted. Phospho-specific antibodies were used to assess the activation state of FAK in cells growing in culture and of cells stimulated by cell adhesion to fibronectin. Once expression of the

mutants was confirmed, cell lysates were used for western blotting using phospho-specific antibodies to FAK PY397, PY576, PY577, and PY861, as shown in Figure 7A. These results indicate that Y397 is phosphorylated comparably on wild type and mutant FAK proteins. This site is the major autophosphorylation site of FAK. This experiment indicates that paxillin binding is not required for FAK to autophosphorylate itself in cells growing in culture. Phosphorylation of Y861, like that of Y397 is not dependent upon paxillin binding as mutants that disrupt binding to individual or both sites have no effect on phosphorylation of Y861. In contrast, phosphorylation of Y576 and Y577 in cells growing in culture is dependent upon paxillin binding. Both of these sites in the activation loop of the kinase show reduced phosphorylation when paxillin binding is disrupted. In fact, phosphorylation at these sites is reduced in mutants with a single functional paxillin-binding site.

Phosphorylation of FAK was also measured from cells stimulated by cell adhesion. Cells were trypsinized, held in suspension, and plated onto a plastic dish coated with fibronectin. Cell lysates were analyzed using phospho-specific antibodies. These results, shown in Figure 7A, indicate that Y397 and Y861 are phosphorylated in each of the mutants comparably to wild type FAK following cell adhesion. Phosphorylation of Y576 and Y577 is dependent upon paxillin binding following cell adhesion since phosphorylation was reduced in all mutants that disrupt paxillin binding to at least a single paxillin-binding site in FAK.

The FAT domain of FAK contains one known phosphorylated tyrosine at residue 925. This site is known to bind Grb2 to signal to the MAPK pathway or recruit dynamin into complex. A GST pulldown approach was taken to assess phosphorylation of Y925. CE cells expressing wild type or mutant forms of FAK were treated overnight with 50 µM sodium vanadate before lysis in order to prevent dephosphorylation of cellular proteins. Lysates

were used to bind GST-fusion proteins containing the SH2 domain of Grb2 as shown in Figure 7B. FAK protein from lysates expressing wild type FAK bind GST-Grb2SH2 well, while all mutants that disrupt paxillin binding dramatically reduce Grb2SH2 binding to background levels. This experiment indicates that paxillin binding is required for Src phosphorylation of Y925, as mutations that disrupt paxillin binding to individual or both paxillin-binding sites abolished Grb2SH2 binding to FAK.

To assess downstream signaling of the various FAK FAT domain mutants, phosphorylation of FAK substrates was measured as previously described (Gabarra-Niecko *et al.*, 2002). Briefly, CE cells expressing wild type or mutant forms of FAK were vanadate treated to prevent dephosphorylation of cellular proteins. Lysates were used to immunoprecipitate FAK substrates p130CAS and paxillin. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a phosphotyrosine antibody, as shown in Figure 8. The results indicate a further role for paxillin binding in FAK downstream signaling since phosphorylation of FAK substrates was decreased when paxillin binding defective mutants were expressed. In this experiment, mutants at either paxillin-binding site exhibited reduced phosphorylation of FAK substrates, while mutants with disrupted paxillin binding to both sites failed to induce phosphorylation of both p130CAS and paxillin. This experiment did not reveal any differences in signaling downstream of the EKR and I937A mutants of FAK, suggesting that both paxillin-binding sites are equally important for phosphorylation of these two substrates when FAK is expressed in cells.

To assess biological effects of these mutations on FAK function, *fak*$^{-/-}$ MEFs stably expressing the various mutants or wild type FAK were used in cell attachment assays as

described in the materials and methods. Briefly, cells were held in suspension, followed by plating on fibronectin for 60 minutes. Cells were fixed and attached cells were counted. These experiments revealed a defect in attachment in *fak*$^{-/-}$ MEFs expressing each of the FAK mutants that disrupt paxillin binding. Mutation of either individual paxillin-binding site or of both binding sites simultaneously resulted in a defect in adhesion to FN-coated dishes. This experiment indicates that both paxillin-binding sites play an important role in FAK's ability to control cell attachment to FN.

2.4 DISCUSSION

This study has provided interesting insights into the association between FAK and paxillin. Recent structural analyses have revealed that FAK has two binding sites for paxillin. This raises the question of whether these binding sites are simply redundant, whether they act in concert, or whether engagement of distinct binding sites results in different biochemical or biological outcomes. Site directed mutagenesis was used to disrupt paxillin binding to each individual binding site or to both binding sites on the FAK FAT domain. The results indicate that the two binding sites do behave differently to regulate certain FAK functions such as localization, but behave similarly to regulate other FAK functions such as downstream signaling.

A previous study determined that the binding affinities of the two paxillin-binding sites on the FAT domain for a peptide mimicking the LD2 motif of paxillin were similar, approximately 10 micromolar (Gao *et al.*, 2004). Further, ITC analysis demonstrated that the I937A mutation abolished binding of the peptide to one paxillin-binding site and the EKR mutation abolished binding of the peptide to the other paxillin-binding site. It was therefore anticipated that the two binding sites would contribute equally to binding of full-length paxillin. However, binding studies using recombinant GST-paxillin fusion proteins and co-immunoprecipitation experiments demonstrated that the two paxillin-binding sites do not contribute equally to binding to full-length paxillin. Both approaches showed substantially more paxillin binding to the I937A mutant than to the EKR mutant. Thus, the integrity of the paxillin-binding site comprised of α-helices 2/3 of the FAT domain of FAK is more important for the interaction with full-length paxillin than the other binding site. Since the binding affinity of both sites for the LD2 motif was the same, the observation could be

explained if there were differences in the affinity of the two sites for the LD4 motif. In fact, a recent report using paramagnetic labeling demonstrated that the LD4 of paxillin has a preference for the paxillin-binding site between α-helices 2/3 (Bertolucci *et al.*, 2005). Using recombinant fragments of paxillin, with mutations impairing one or the other FAK-binding site, a similar conclusion has been drawn here, i.e. that the LD4 motif preferentially interacts with the paxillin-binding site on the α-helices 2/3 side of the FAT domain. Thus, this binding site can associate with both FAK-binding sites on paxillin, whereas the binding site composed of α-helices 1/4 associates with the LD2 binding site.

Although paxillin binding is not absolutely required for FAK subcellular localization, it is the major mechanism of focal adhesion localization. When paxillin binding is abolished, only about 10% of cells display correct localization of FAK. Further, the I937A FAK mutant was more defective for localization to focal adhesions than the EKR FAK mutant. This seems surprising considering that the EKR FAK mutant exhibits reduced binding to paxillin by GST pulldown or co-immunoprecipitation compared with the I937A FAK mutant (Gao *et al.*, 2004). This indicates that paxillin binding does not precisely correlate with focal adhesion localization. For subcellular localization of FAK, the paxillin-binding site comprised of α-helices 2/3 is more important than paxillin-binding site comprised of α-helices 1/4.

Glutamic acid 997 was identified as an important residue for localization in the absence of paxillin binding. As shown in Figures 6B and C, in the background of a FAK mutant that is unable to bind to paxillin, mutation of this residue to alanine completely abolished focal adhesion localization, but in a wild type FAK background, this mutation does not significantly affect focal adhesion localization. Binding to this residue may represent a

secondary, less efficient mechanism by which FAK can be localized to focal adhesions in the absence of paxillin binding. Talin is a focal adhesion protein that has been reported to bind to the FAT domain of FAK and has been suggested to function as a mechanism for FAK subcellular localization. It is possible that E997 is an important residue for the association between FAK and talin. However, we have been unable to test this hypothesis, as we have been unable to detect the interaction between the FAT domain of FAK and talin by co-immunoprecipitation, GST pulldown or direct binding between purified recombinant proteins. Alternatively, E997 might be important for interaction with another binding partner and the E997A mutant might be a useful tool for the identification of additional physiologically relevant binding partners.

Phosphorylation of FAK is important for association with binding partners and for maximal catalytic activity. Results from paxillin null cells suggest paxillin binding is essential for maximal FAK phosphorylation. The paxillin-binding defective FAK mutants have provided additional insight. When paxillin binding is abolished with the EKR/I937A mutant of FAK and focal adhesion localization is dramatically reduced, the ability of FAK to autophosphorylate on Y397 is not affected. Similarly, phosphorylation of Y861, which is a Src substrate, is not affected. However phosphorylation of Y576 and Y577, the Src phosphorylation sites in the activation loop of FAK, is impaired in this mutant. Several studies have indicated that the Y861 Src phosphorylation site on FAK is regulated differently than phosphorylation of the other Src sites on FAK, so this finding that Y576/Y577 and Y861 are differentially affected is not completely surprising. Interestingly, mutants with defects in a single paxillin-binding site show similar defects in phosphorylation of Y576 and Y577, suggesting that engagement of both paxillin-binding sites is required for maximal

phosphorylation of these sites. The fact that each of the paxillin-binding mutants show similar defects in phosphorylation of FAK indicates that phosphorylation of FAK at the activation loop sites does not precisely correlate with the ability of FAK to localize to focal adhesions. Paxillin binding to both sites on FAK may be required to allow Src binding to FAK, perhaps by tethering FAK to focal adhesions more securely. However, as Y397 phosphorylation is not altered, such a mechanism would indicate a defect in Src recruitment downstream of FAK autophosphorylation. A more likely scenario is that paxillin binding to both sites on FAK may cluster FAK proteins, thus facilitating Src phosphorylation of FAK.

As phosphorylation of the activation loop residues is required for FAK maximal catalytic activity, it is not surprising that phosphorylation of the FAK substrates paxillin and p130CAS is impaired in cells expressing paxillin-binding defective mutants. The more severe defect in substrate phosphorylation observed in cells expressing the EKR/I937A mutant is likely due to the localization defect exhibited by the mutant resulting in the spatial segregation of the kinase and its substrates.

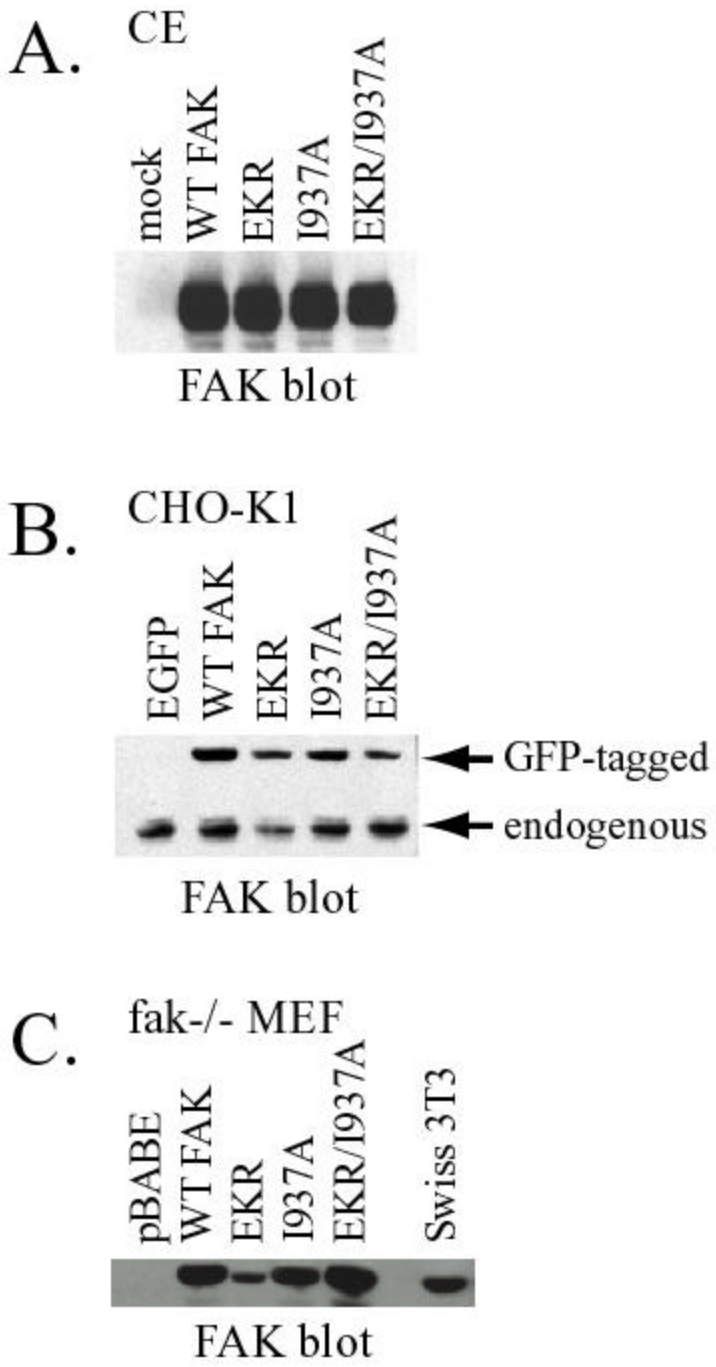

Figure 3. Expression of wild type or mutant FAKs in various cell types. A. CE cells transfected with empty RCAS vector (mock), wild type FAK or FAK mutants were lysed and 25ug of lysate was blotted for FAK expression with BC4 antibody.
B. CHO-K1 cells were transfected with GFP N-terminally tagged versions of wild type or FAK mutants. Stably expressing populations were selected and lysed. C. *fak-/-* MEFs were infected with pBABE virus and selected to stably express wild type or mutant forms of FAK. Cells were lysed and 25 ug of lysate was immunoblotted.

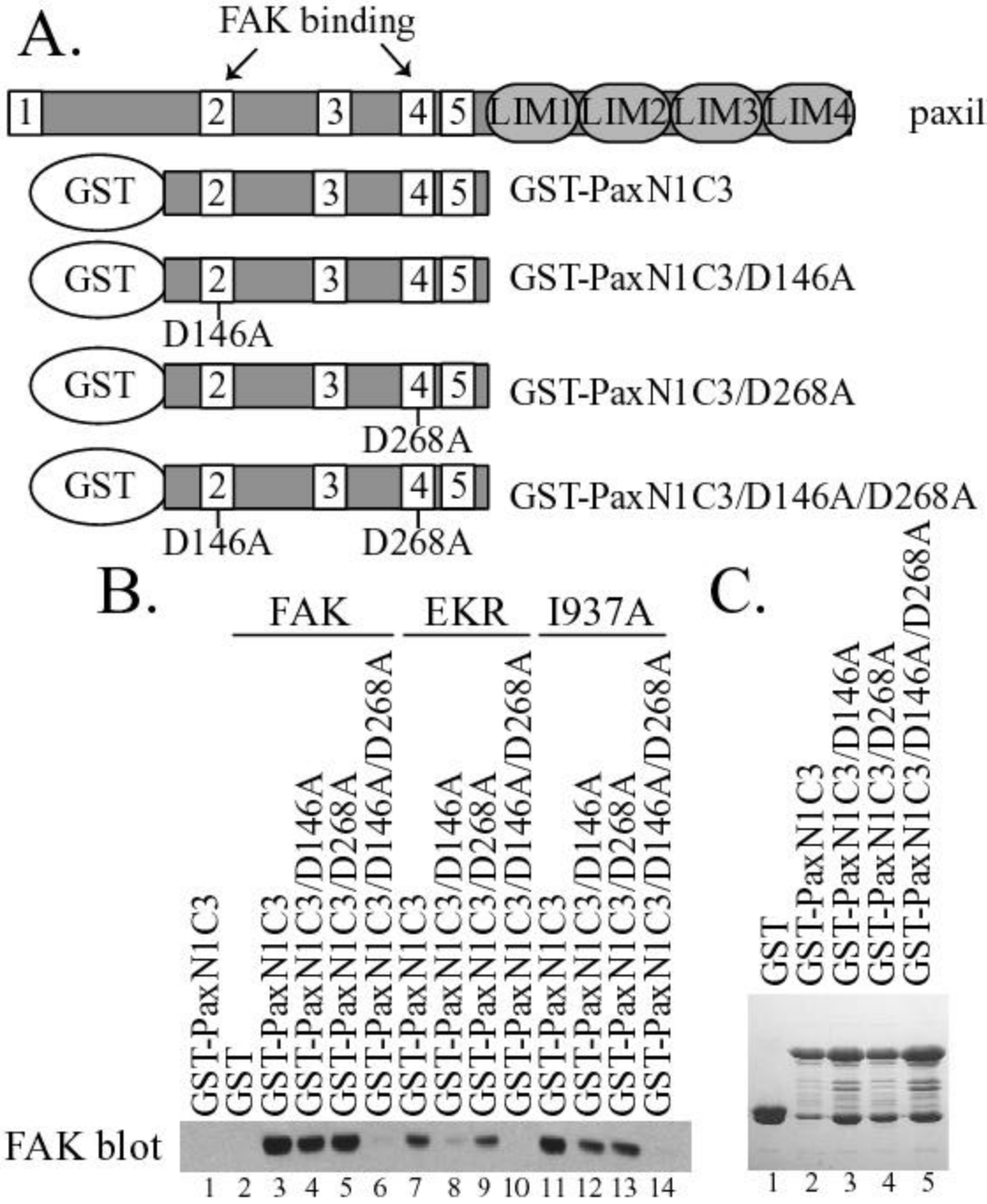

Figure 4. *In vitro* binding experiment to assess relative affinities of paxillin and FAK interactions. A. Schematic representation of paxillin and paxillin GST-fusion constructs. B. CE cells transfected with empty RCAS (lane 1), wild type FAK (lanes 2-6), EKR (lanes 7-10), or I937A (lanes 11-14) were lysed and 1 mg of lysate was bound to GST (lane 2) or GST-fusion constructs of paxillin containing both FAK binidng sites (GST-PaxN1C3; lanes 3, 7, 11) or GST-fusion constructs of paxillin with point mutations to disrupt FAK binding to LD2 (GST-PaxN1C3/D146A; lanes 4, 8, 12), LD4 (GST-PaxN1C3/D268A; lanes 5, 9, 13), or both LD2 and LD4 (GST-PaxN1C3/D146A/D268A; lanes 6, 10, 14). C. GST-fusion proteins were Coomassie stained to ensure equal loading..

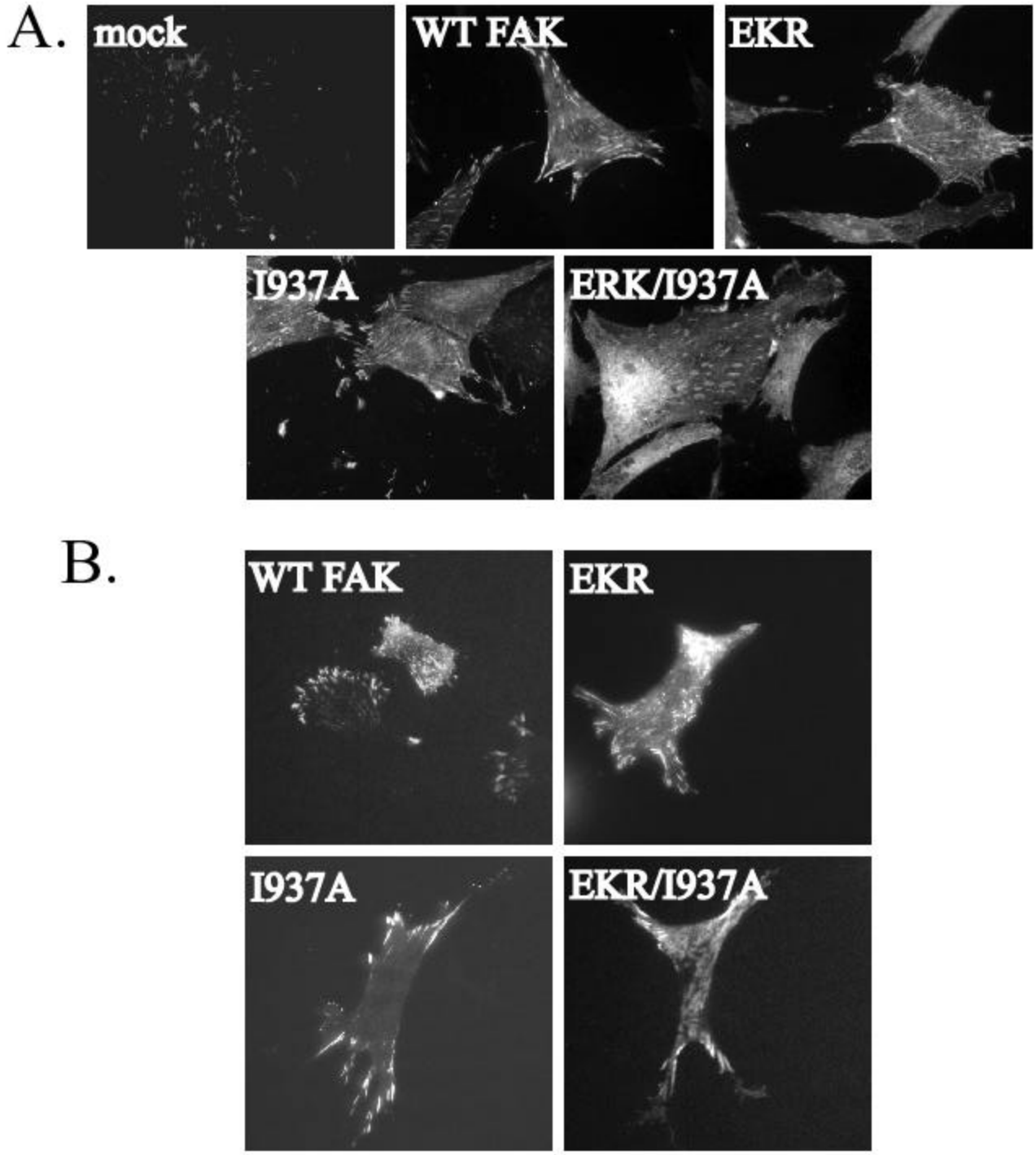

Figure 5. Localization of FAK mutants that disrupt paxillin binding. A. CE transfected with empty RCAS (mock), wild type FAK, or mutants to disrupt paxillin binding were plated onto fibronectin-coated coverslips overnight, then fixed and used for immunofluorescent imaging using FAK BC4 antibody. B. CE cells transfected with GFP-fsuion constructs of wild type FAK or mutants were visualized in live cells by TIRF.

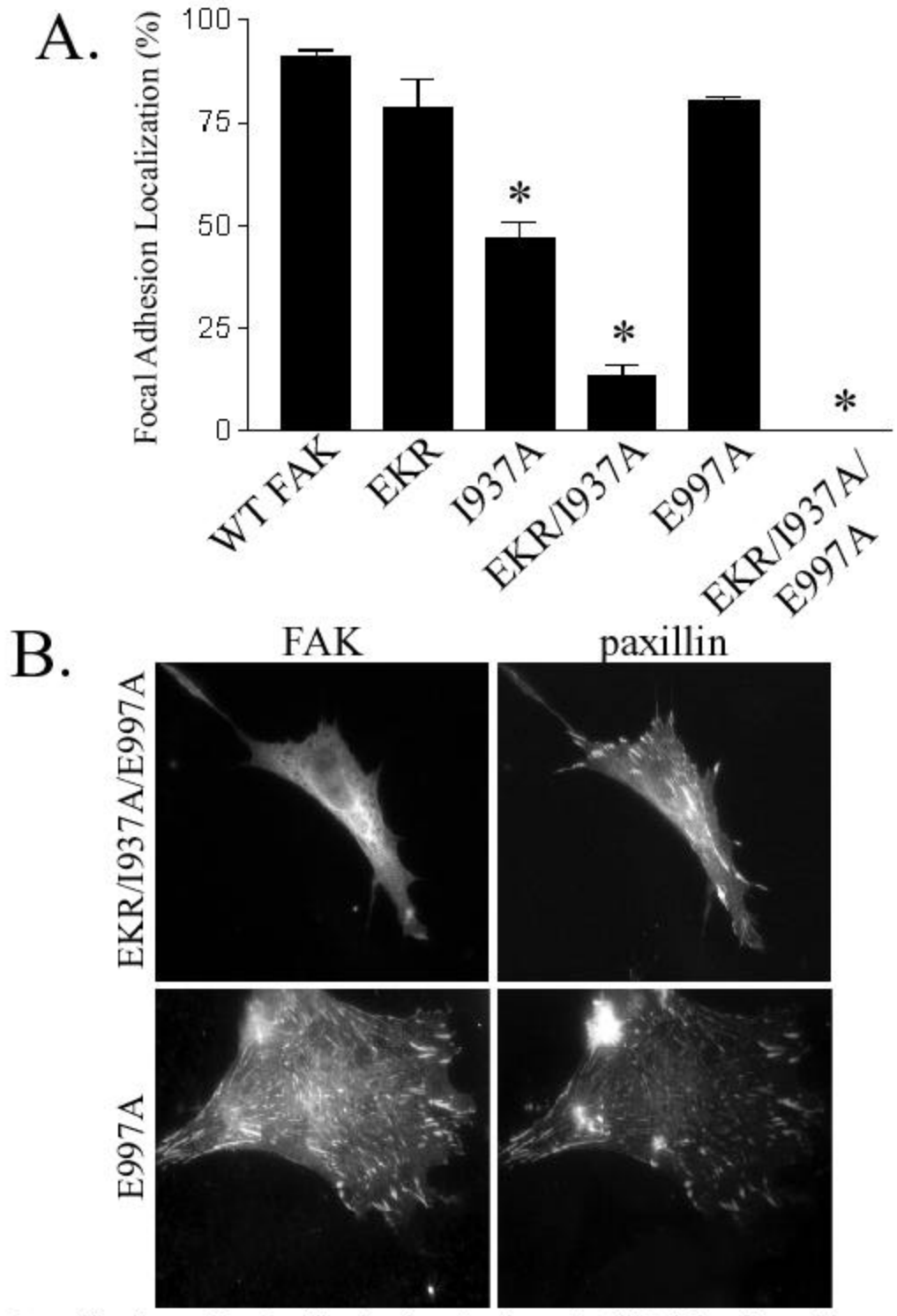

Figure 6. Quantification of focal adhesion localization. A. CHO-K1 cells stably expressing EGFP alone, wild type FAK, or FAK mutants were plated on fibronectin coated coverslips and used for immunofluorescent staining with GFP antibody to enhance GFP signal and paxillin antibody to locate focal adhesions. GFP-positive cells were scored for GFP localization at focal adhesions. $p < 0.01$ for the indicated columns (*). B. CE cells transfected with GFP-fusion constructs of FAK mutants were plated on fibronectin coated coverslips and used for staining with GFP to enhance GFP signal and paxillin antibody to locate focal adhesions.

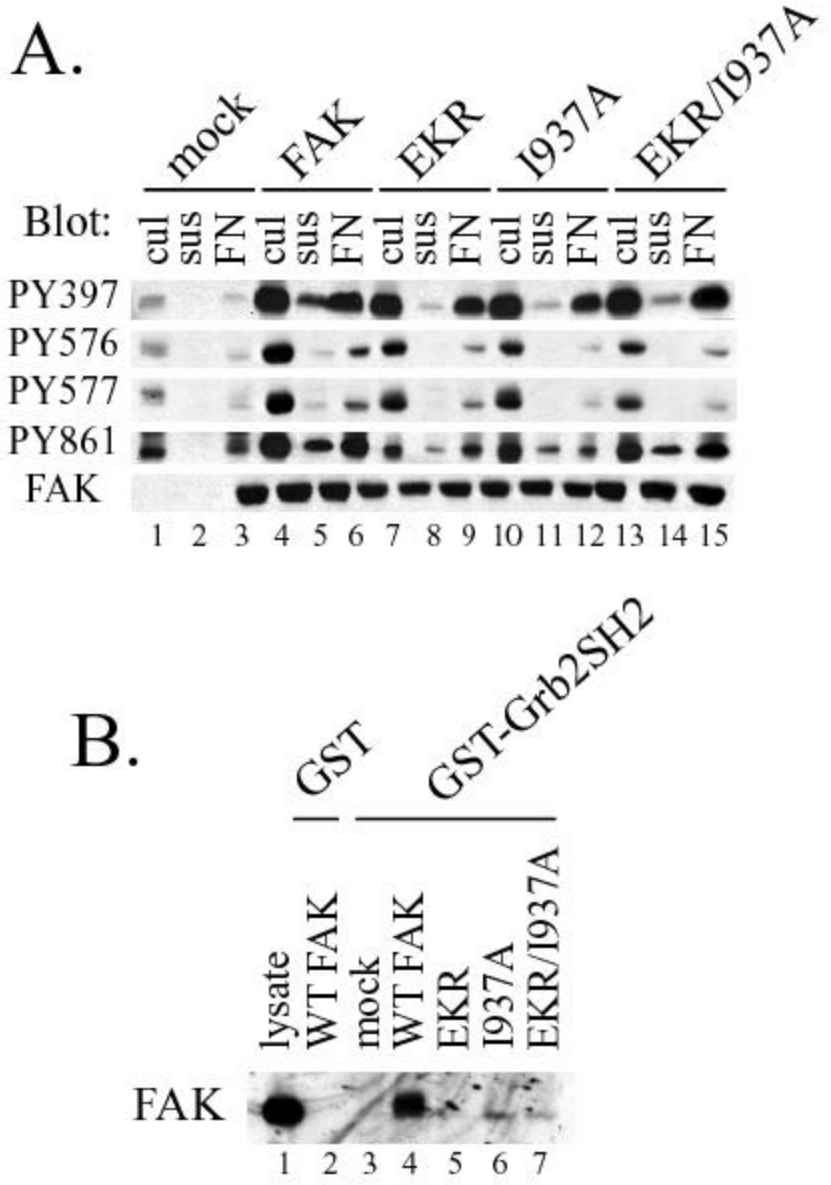

Figure 7. Phosphorylation of FAK at various tyrosine residues. A. CE cells transfected with empty vector RCAS (mock), wild type FAK, or FAK mutants were lysed while growing in culture (lanes 1, 4, 7, 10, 13), held in suspension for 30 minutes (lanes 2, 5, 8, 11, 14), or held in suspension then plated on fibronectin for 45 minutes (lanes 3, 6, 9, 12, 15). 25 µg lysate was immunoblotted for each condition using FAK BC4 antibody or phosphor-specific antibodies to Y397, Y576, Y577, or Y861. B. Vanadate-treated CE cells transfected as above were lysed and 1 mg of lysate was bound to either GST (lane 2) or GST-Grb2SH2 (lanes 3-7) as described in the materials and methods. 25 µg wild type FAK lysate was loaded to show relative binding efficiency.

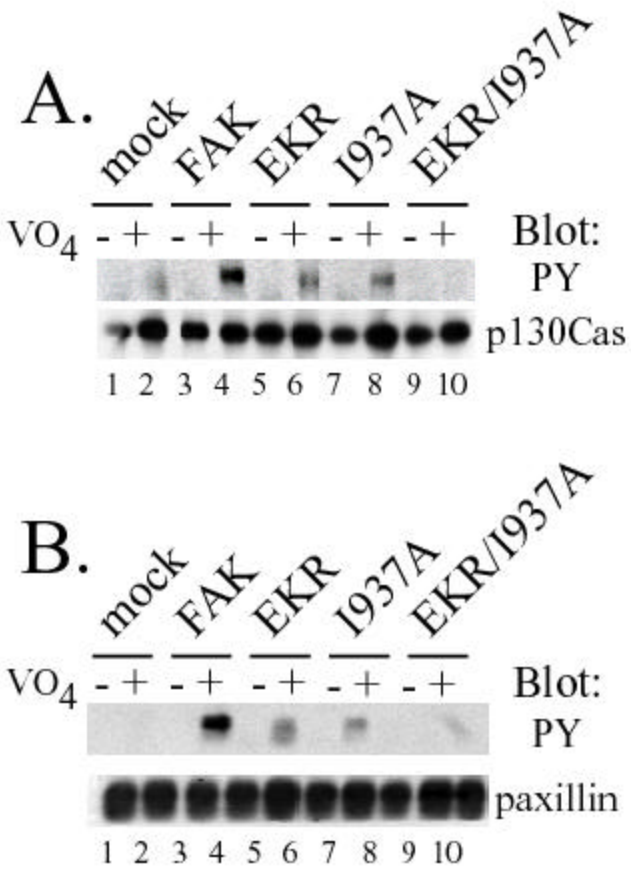

Figure 8. Signaling downstream of FAK in vivo. CE cells transfected with empty RCAS vector (mock), wild type FAK, or FAK mutants were treated with vanadate overnight prior to lysis to boost phosphotyrosine content of cellular proteins. A. p130CAS was immunoprecipitated from 1 mg lysate for each condition as described in the materials and methods. Immunoprecipitated proteins were immunoblotted for phosphotyrosine (upper panel) and p130CAS to reveal loading. B. Paxillin was immunoprecipitated from 1 mg lysate for each condition as described and immunoprecipitated proteins were immunoblotted for phosphotyrosine and paxillin.

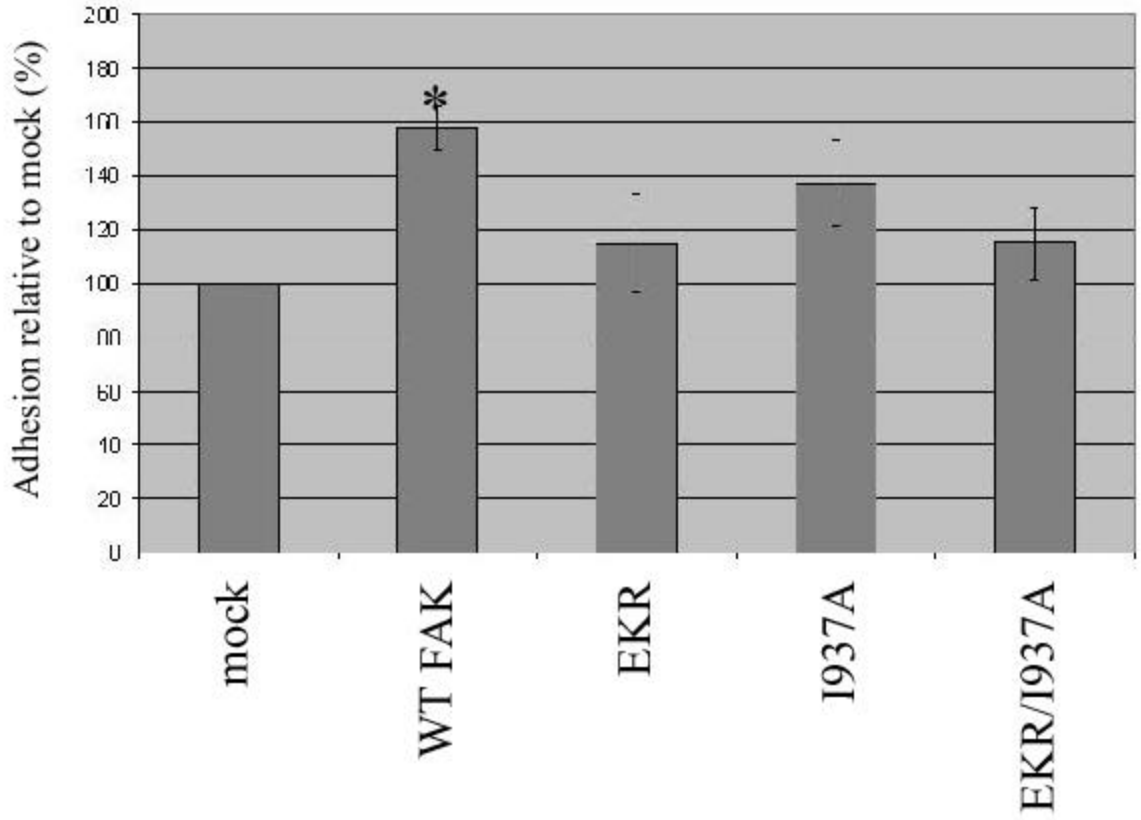

Figure 9. Adhesion of reconstituted fak-/- MEFs. Stably reconstituted fak-/- MEFs were held in suspension for 1 hr before plating on fibronectin for 60 minutes, as indicated, followed by fixation and staining as described in materials and methods. Percent adhesion relative to empty pBABE vector (mock) reconstituted cells is shown. Statistical analysis yielded $p<0.05$(*) as indicated.

CHAPTER THREE

THE FERM DOMAIN OF FAK

3.1 INTRODUCTION

The FERM domain of FAK is composed of three lobes, one ubiquitin-like, one acyl-CoA binding protein-like, and one PH domain-like lobe. Although the folded structure of these lobes resembles the structure of known proteins, the functions of the individual lobes are not conserved. In general, FERM domains in other proteins have been shown to be important for mediating protein-protein interactions of two particular types. 1) These domains have been shown to be important for intramolecular or homophilic intermolecular interactions. 2) FERM domains have been shown to play a role in mediating interactions with cytoplasmic tails of transmembrane proteins, like integrins (Louvet-Vallee, 2000).

The FERM domain of FAK has recently been described to interact with unknown residues in the catalytic domain. This interaction between domains decreases autophosphorylation at Y397 and inhibits phosphorylation of other tyrosine residues, severely inhibiting the biochemical and biological activity of FAK (Toutant *et al.*, 2002;Cooper *et al.*, 2003;Dunty *et al.*, 2004;Cohen and Guan, 2005). The mechanism that relieves this autoinhibition is not clear at this time. A series of point mutations in the FERM domain, termed KAKTLR (K216A/K218A/R222A), results in a form of the kinase that is partially defective for activation following cell adhesion, although this mutation does not affect binding of the

FERM domain to the catalytic domain directly. Presumably, the KAKTLR mutation disrupts some interaction that helps relieve the intramolecular inhibition of FAK (Dunty *et al.*, 2004). This intramolecular inhibition represents a novel mechanism for regulation of FAK activation.

Recently the FERM domain has also been shown to interact with PDGFR and EGFR in fibroblasts (Sieg *et al.*, 2000). This domain was required for EGF-stimulated cell migration in that same study (Sieg *et al.*, 2000). The FERM domain has also been reported to bind to other proteins with established roles in FAK-regulated cellular processes, such as Etk and EphA2. Using large deletion mutants, the interaction between the FERM domain of FAK with the PH domain of Etk was found to be necessary for the migratory potential of HUVECs, thus implying that interactions with the FERM domain can be critical for FAK function (Chen *et al.*, 2001).

Although much is known about phosphorylation events that play a role in FAK's activation, the complete mechanism of FAK activation remains to be determined. The role of the FERM domain of FAK remains to be elucidated. I hypothesize that FERM domain binding to growth factor receptors and/or other cytoplasmic proteins regulates FAK activation by disrupting the inhibitory association between the FERM and catalytic domains, as illustrated in Figure 2. To address these questions, a mutagenesis strategy in conjunction with both biochemical and biological assays has begun to identify binding sites, with the intention to characterize the relevance of these interactions for FAK activation and biological activity.

For this study, point mutations were designed and screened in fibroblasts using biochemical and biological assays to identify mutants that disrupt interactions with known

binding partners and to identify mutants that affect activation or biological activities of FAK. In addition to the novel FERM domain mutants used in these studies, the previously published KAKTLR FERM domain mutant was also further characterized.

Based on evolutionary conservation between species, searches for known binding motifs, and using the structure of the FERM domain of FAK, novel point mutations were designed. These FERM domain mutations are shown in Table I. To study interactions with these various mutants, they were subcloned into multiple expression vectors to use in different systems. These mutants have been expressed as GFP-fusion proteins in 293 cells and CE cells for transient experiments. The mutants have also been stably expressed in *fak*$^{-/-}$ MEFs to assess their abilities to restore FAK function in these cells.

3.2 MATERIALS AND METHODS

Cells and viruses. 293 cells were maintained in DMEM F12 (Gibco BRL, Rockville, MD) supplemented with 10% FBS (Gibco BRL). 293 cells were transfected with 2 µg pEGFP DNA using Lipofectamine PLUS (Life Technologies). *fak*$^{-/-}$ MEFs were used in these studies to address the role of wild type or FAK mutants in cells lacking FAK expression. *fak*$^{-/-}$ MEFs were maintained as described (Westhoff *et al.*, 2004). In order to generate populations of *fak*$^{-/-}$ MEFs expressing wild type or FAK mutants, the pBABE retroviral vector was used. To generate virus to infect the *fak*$^{-/-}$ MEFs, phoenix cells were transfected with the various pBABE constructs. Phoenix cells were maintained in DMEM F12 (Gibco BRL, Rockville, MD) supplemented with 10% FBS (Gibco BRL), penicillin, streptomycin, genamycin and kanamycin (Sigma). Phoenix cells were transfected with 8 µg pBABE DNA using Lipofectamine PLUS (Life Technologies). After 48 hrs, virus-containing media was collected and filtered through a 0.45 µm syringe filter. For infection, each dish of *fak*$^{-/-}$ MEFs was incubated for 24 hrs with 4 mL filtered virus-containing media, 4 µg/mL polybrene (Sigma), and 5 mL complete media. At 24 hrs, the infection media was replaced with complete media. At 48 hrs post-infection, cells were split into complete media containing 2 µg/mL puromycin (Sigma) for selection of expressing populations.

Site-directed Mutagenesis. Point mutations were engineered into pBScript wild type FAK template using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla CA). Sequence analysis was performed on each mutant to verify the intended point mutations and that no unintended mutations were present. These analyses were performed in the UNC-CH Genome Analysis Facility on a model 3730 DNA Analyzer (Perkin Elmer, Applied

Biosystems Division) using the ABI PRISM^{TM} Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer, Applied Biosystems Division).

Protein analysis. Cells were washed twice with PBS and lysed in modified RIPA (50 mM Tris [pH 7.3], 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate) containing protease and phosphatase inhibitors as described previously (Gao *et al.*, 2004). Protein concentrations were determined with the bicinchoninic acid assay (Pierce, Rockford, Ill.). The FAK phosphorylation site-specific antibodies (Biosource International, Camarillo, CA) and paxillin, p130CAS, and PY20 phosphotyrosine antibodies (BD Biosciences, San Diego, CA) were purchased commercially. The BC4 polyclonal antiserum has been described previously (Gabarra-Niecko *et al.*, 2002). Immunoprecipitations and Western blotting were performed as previously described (Gabarra-Niecko *et al.*, 2002).

Immunofluorescence. Glass coverslips were coated with 50 µg/mL bovine plasma fibronectin (Sigma) in PBS for 1 hr at 37°C. Cells were plated onto the coated coverslips and maintained at 37°C for 16 hr. GFP expressing cells were fixed in ice cold 95% ethanol. Cells were visualized using a Leitz Orthoplan fluorescence microscope, and images captured with a Hamamatsu digital camera and Metamorph imaging software (Universal Imaging Corporation, West Chester, PA). Images were taken with identical exposure times.

Modified Boyden Chamber Assay. Subconfluent *fak*$^{-/-}$ MEFs were serum-starved for 20 hrs in DMEM supplemented with 0.5% FBS. The undersides of 8 µm pore transwells were

coated with 10 µg/mL bovine fibronectin for 2 hrs at 37°C. The upper chamber was blocked with 0.05% lipid-free BSA in DMEM. After fibronectin coating, the upper and lower chambers were blocked with 1% lipid-free BSA in DMEM for 1 hr at room temperature. Cells were trypsinized and washed twice with 0.5 mg/mL soy trypsin inhibitor. $1x10^5$ cells were added to the upper chamber of the transwell and allowed to migrate though for 2.5 hrs at 37°C. To terminate the assay, cells were removed from the upper chamber and chambers were washed in PBS, then fixed and stained using Hema3 kit. Stain was eluted using 10% acetic acid and absorbance was measured at 668 nm.

In vitro kinase assay. Transfected 293 cells were lysed in modified RIPA buffer at 72 hrs post-transfection. FAK was immunoprecipitated from 0.2 mg lysate using polyclonal BC4 antibody. Immunoprecipitated protein was washed twice in lysis buffer, twice in PBS, then with kinase reaction buffer (50 mM HEPES [pH 7.4], 10 mM $MnCl_2$). Each reaction was resuspended in 20 µL kinase reaction buffer supplemented with 1 µM ATP and 10 µCi [?-^{32}P] ATP (PerkinElmer Life Sciences). Reactions were incubated at room temperature for 3 or 10 min before addition of 20 µL Laemmli sample buffer and boiling to elute the proteins. Samples were resolved by SDS-PAGE and phosphorylation was revealed by autoradiography.

3.3 RESULTS

Biochemical screening of FERM domain mutants. FERM domain mutants of FAK have been reconstituted in *fak*$^{-/-}$ fibroblasts as described in materials and methods. Expression of the mutants was confirmed by western blotting, as shown in Figure 10. Cell lysates were used for western blotting using phosphospecific antibodies to FAK Y397 and Y576 as shown in Figure 11A. None of the FERM domain mutants screened revealed any differences in phosphorylation of these sites on FAK. Total FAK phosphorylation was also assessed for these mutants. FAK was immunoprecipitated from lysates and immunoblotted for phosphotyrosine. None of the FERM domain mutants screened displayed any defects in total FAK phosphorylation as detected by this method.

Although FAK phosphorylation appeared to be intact with these FERM domain mutants, downstream signaling could still be altered. To assess downstream signaling of these FAK mutants, lysates from these cells were immunoprecipitated for paxillin and immunoblotted for phosphotyrosine, as shown in Figure 11B. There were no measurable differences between paxillin phosphorylation in *fak*$^{-/-}$ MEFs reconstituted with wild type or mutant forms of FAK, indicating that these mutants do not biochemically affect FAK downstream signaling in these cells.

Biological screening of FERM domain mutants. Although there were no striking biochemical differences detected with any of the mutant reconstituted *fak*$^{-/-}$ MEFs, there could still be mutants that affect FAK biological functions. As described previously, the *fak*$^{-/-}$ cells are useful tools to test for biological defects of FAK mutants because these cells are reported to be defective for several FAK-regulated cellular processes. Based on published

reports, it seems that the interactions between the FERM domain of FAK with proteins such as PDGFR, EGFR, and Etk are important for regulating motility, but these findings are based on the analysis of large deletions rather than point mutations. Furthermore, mutations that affect the association between the FERM and catalytic domains have been shown to alter cellular motility (Dunty *et al.*, 2004;Cohen and Guan, 2005). To identify FERM domain mutations of FAK that have defects in FAK biological activity, haptotactic motility was measured using modified Boyden chamber assays.

The FERM domain mutant-reconstituted *fak*$^{-/-}$ MEFs were used in modified Boyden chamber assays to assess haptotaxis as described in the materials and methods. The results of these assays, shown in Figure 12 revealed statistically significant differences between wild type reconstituted cells and several of the mutant reconstituted cell lines. Some of the mutations were unable to restore the motility defect of the *fak*$^{-/-}$ MEFs, such as E245A, K255A, and Q226A. Other mutations increased motility to levels above that of wild type FAK, such as W266A/I268A and V270A/E271A. The locations of these residues on the surface of the FERM domain structure are shown in Figure 13. Mutation of these residues affects FAK biological function, indicating that these residues may be important for interactions that affect FAK signaling.

FAK FERM domain- catalytic domain interaction. Collaborators have recently succeeded at co-crystallizing the FAK FERM and catalytic domains. This has yielded novel structural information about the interface between these two domains of FAK. Their work shows that the FERM domain F2 lobe binds to the C-lobe of the FAK catalytic domain. This interaction is thought to be the major interface for the intramolecular inhibition of FAK. To test whether

this is in fact the case, residues were selected to specifically disrupt this interface, in order to assess whether these mutations result in a constitutively activated form of the kinase as predicted. Based on the co-crystallized structure, F596 and V196 were selected as the most important residues for the interaction in the catalytic and FERM domains, respectively. The structure shows F596 projecting into a pocket on the surface of the FERM domain. To disrupt the association, F596 was mutated to alanine. In the FERM domain, V196 was mutated to phenylalanine to fill the pocket on the FERM domain and potentially occlude binding by the catalytic domain. Both mutations have been tested to assess their biochemical and biological effects.

Biochemical characterization of interface mutants. To assess the biochemical effects of mutations of key residues for the FERM-catalytic domain interaction, mutations were made and expressed as GFP-fusions in 293 cells. Phosphorylation of FAK in these cells was measured using phosphospecific antibodies, as shown in Figure 14A. Mutation of F596A resulted in dramatically increased phosphorylation of FAK at Y861 in cells growing in culture. This mutation or mutation of V196F had little to no effect on phosphorylation of Y397, Y576, or Y577 in cells growing in culture. In order to more clearly assess catalytic activity of these mutants, *in vitro* kinase assays were performed. In cells growing in culture, neither of these mutations had any quantifiable difference in FAK autophosphorylation *in vitro* as shown in Figure 14B.

Given the nature of the interaction between the two FAK domains, it seems likely that the association should be strongest in conditions when the cell would want to most strongly negatively regulate FAK activity. These conditions would include serum-starvation and

suspension. Therefore to try to exacerbate differences between wild type and interface mutants of FAK, the above experiments were repeated following periods of serum-starvation and suspension (data not shown). These experiments have revealed that the GFP-fused exogenous FAK constructs are not regulated by serum-starvation or by suspension in 293 cells.

3.4 DISCUSSION

FERM domain mutants. Two mutations in the FAK FERM domain that yielded similar biological changes in FAK activity were W266A/I268A and V270A/E271A. These mutations are adjacent both in sequence and on the surface of the FERM domain structure. Both mutations increased motility in *fak*$^{-/-}$ MEFs to levels above that of wild type FAK, suggesting that mutations in this region increase FAK function as detected biologically. Neither of these mutations altered FAK biochemically in these experiments, suggesting the effects on FAK function may not be very strong. Mutagenesis of this region could affect FAK function in several ways. This mutation could be non-specifically affecting the structure of FAK, however this is highly unlikely as these mutations are on the surface of the protein. Alternatively, these mutations could disrupt binding of some protein to the FAK FERM domain. This binding could regulate FAK activity in many ways, either by affecting the protein's conformation state, phosphorylation state, or by affecting the binding of some other protein to FAK.

The sequence surrounding these two mutations, SWIISVE, may fit the consensus for a GSK-3 phosphorylation site, SXXXS(P). This is a favorable hypothesis since GSK-3 has recently been identified to phosphorylate FAK in its FAT domain at S722, thus negatively regulating FAK activity (Bianchi *et al.*, 2005). Therefore, it seems possible that GSK-3 also phosphorylates FAK in its FERM domain at S265, negatively regulating FAK activity. Bianchi et al. found that GSK-3 phosphorylation of the FAK FAT domain played an important role in regulating FAK activity during cell spreading and migration (Bianchi *et al.*, 2005). The current mutagenesis findings are consistent with a role for GSK phosphorylation in the negative regulation of FAK-regulated migration.

There are currently no reports of S265 phosphorylation. To test the proposed hypothesis of GSK-3 phosphorylation of FAK, the S265A and S265A/S269A mutations need to be characterized. If these sites are phosphorylated to negatively regulate FAK function, then mutation of these serine residues to alanines should result in the same increases in motility when reconstituted into *fak*$^{-/-}$ MEFs as the W266A/I268A and V270A/E271A mutations.

FAK FERM domain- catalytic domain interaction. Using the structure of the purified FERM domain of FAK bound to the purified catalytic domain of FAK, mutations were designed to disrupt the association. These mutants were expressed as GFP-fusions in 293 cells. The only difference in FAK function for these mutants was an increase in phosphorylation of Y861 when the V596A mutation was expressed. This tyrosine residue is thought to be important for FAK binding to p130CAS and downstream signaling (Lim *et al.*, 2004), indicating a possible activation of FAK function for this mutant in these cells. However, no other effects where observed when these mutants were expressed in these cells. This is in contrast to previous studies that suggest that mutations to disrupt the association between the FAK FERM and catalytic domains should have dramatic effects on FAK catalytic activity and downstream signaling. This may indicate that the single point mutations were inadequate to disrupt the association between the FERM and catalytic domains. To address this concern, the structure of the interface of the domains should be further analyzed to select additional mutations to more dramatically disrupt the association between the domains.

The lack of significant biochemical differences in these studies may also be explained by the use of different expression systems and cell types. The present studies have indicated

that exogenously expressed FAK may exceed the ability of 293 cells to regulate FAK properly when challenged with serum-starvation or suspension. Alternatively, the GFP tag at the N-terminus of FAK may interfere with FAK regulation in these cells by affecting the binding of regulatory proteins. Further, the GFP tag could be disrupting binding between the FERM and catalytic domains of FAK. This would explain why mutations to disrupt this association have no effect on FAK function in these studies. To further study these mutants to understand whether they are able to disrupt binding between the FERM and catalytic domains, untagged constructs will need to be utilized in a cell type where exogenously expressed FAK protein can be regulated properly.

Table I. Designed point mutations of the FAK FERM domain. Based on evolutionary conservation between species, searches for known binding motifs, and using the structure of the FERM domain of FAK, novel point mutations were designed. In addition to these novel FERM domain mutations, other mutations had been generated in the lab previously.

Mutation	Location
P117A/E119A	Acyl-CoA BP-like (F2)
E158A/D161A	Acyl-CoA BP-like
D164A/E166A	Acyl-CoA BP-like
K190A/K191A	Acyl-CoA BP-like
R204A/R205A	Acyl-CoA BP-like
K216A/K218A/R222A	Acyl-CoA BP-like
T219A	Acyl-CoA BP-like
Q226A	Acyl-CoA BP-like
Q230A	Acyl-CoA BP-like
E245A	Hinge between F2-F3
R252A	Hinge between F2-F3
K255A	Hinge between F2-F3
K259A	PH-like (F3)
W266A/I268A	PH-like
V270A/E271A	PH-like
E277A/E278A	PH-like
I320A	PH-like
E338A/D342A	PH-like

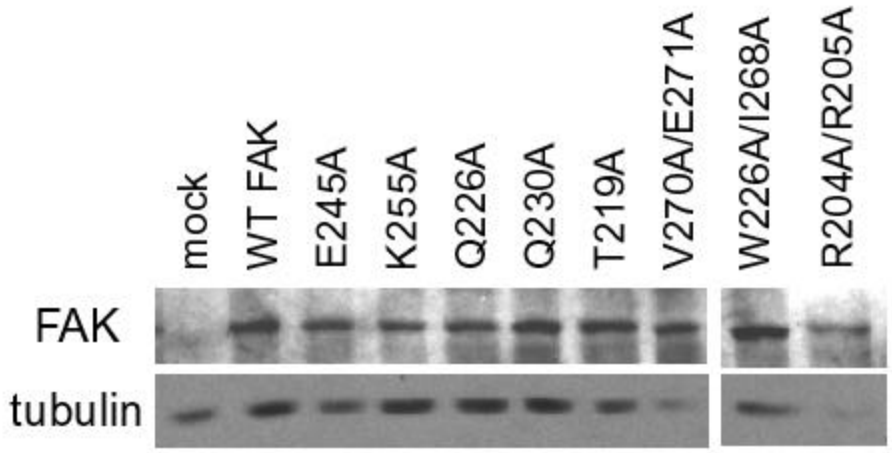

Figure 10. Expression of FERM domain mutants in *fak* -/- MEFs. *fak* -/- MEFs were infected with pBABE virus and selected to stably express wild type FAK or mutant forms of FAK. Cells were lysed and 25 μg of lysate was immunoblotted to check FAK expression.

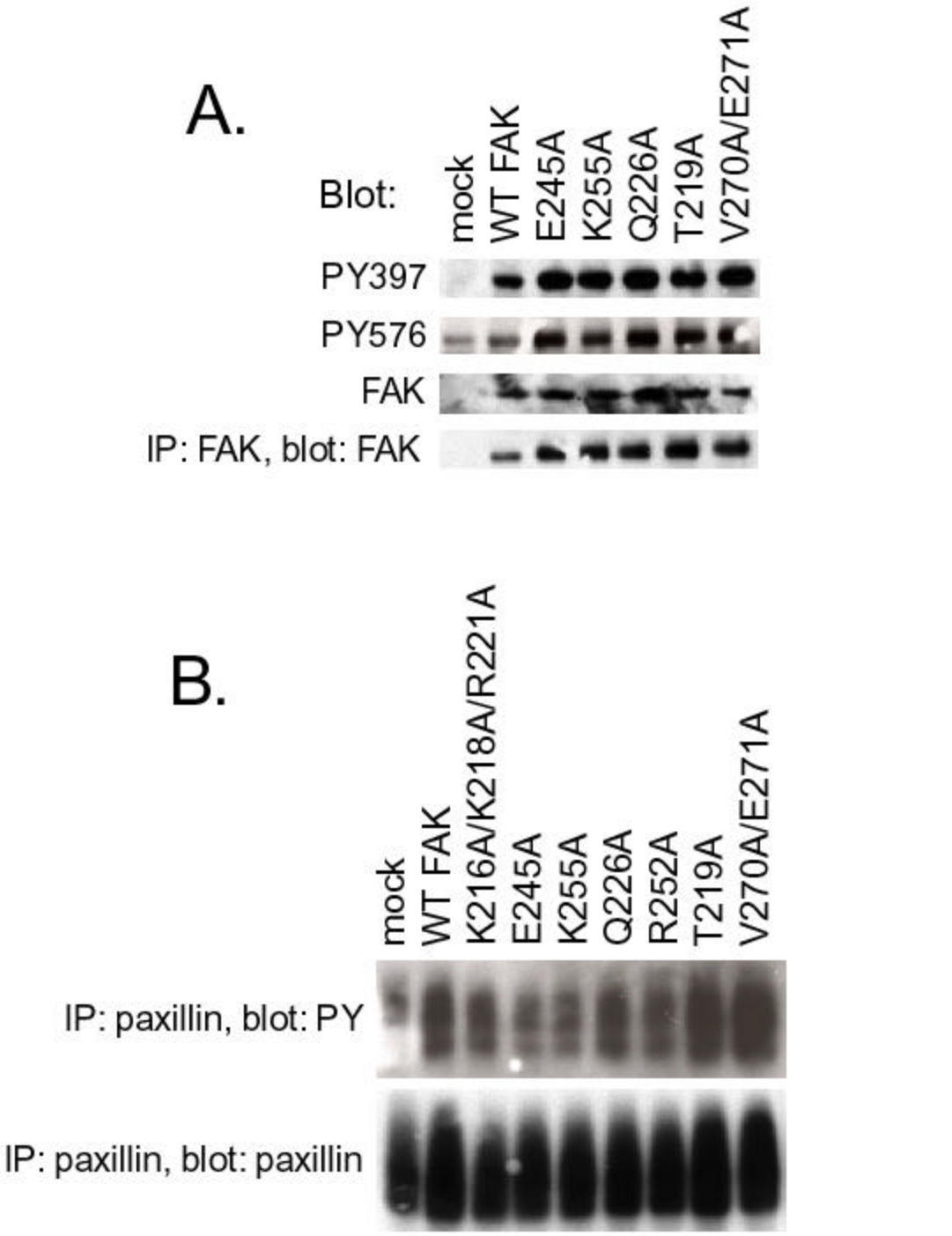

Figure 11. Biochemical measurements of FAK activity in reconstituted *fak* -/- MEFs. A. Reconstituted *fak* -/- MEFs were lysed and 25 μg lysate was immunoblotted using FAK BC4 polyclonal antibody or phosphospecific antibodies to Y397 or Y576. 1 mg lysate was immunoprecipitated, then immunoblotted using FAK polyclonal BC4 antibody (bottom panel). B. Paxillin was immunoprecipitated from 1 mg lysate and bound proteins were immunoblotted for phosphotyrosine or paxillin for loading.

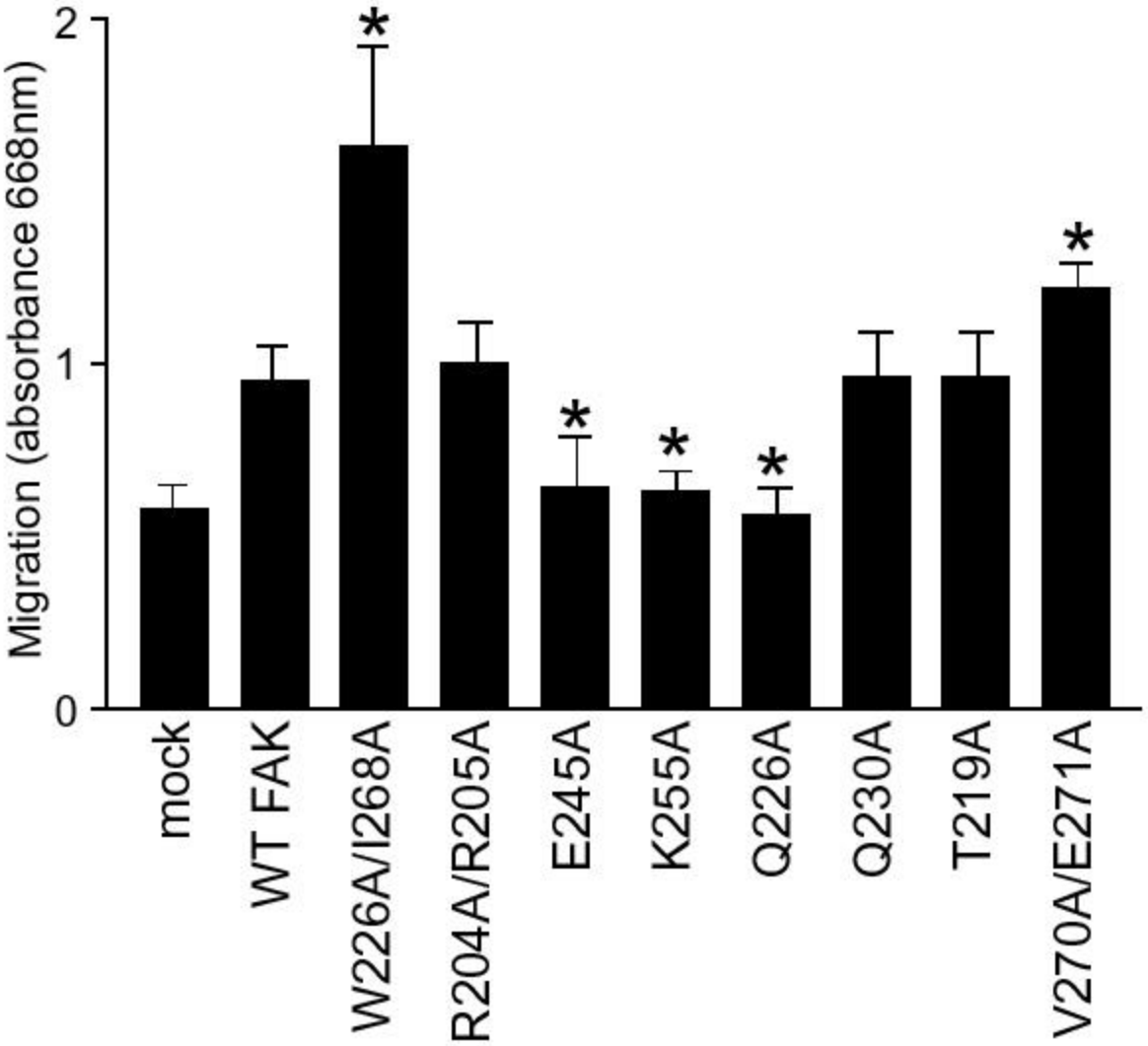

Figure 12. Haptotactic motility of reconstituted *fak* -/- MEFs. Stably reconstituted *fak* -/- MEFs were used in modified Boyden chamber assays as described in the materials and methods to measure migration towards fibronectin. Hema3 stain from motile cells was eluted and absorbance was read. Statistical analysis yielded $p < 0.05$ (*) as indicated.

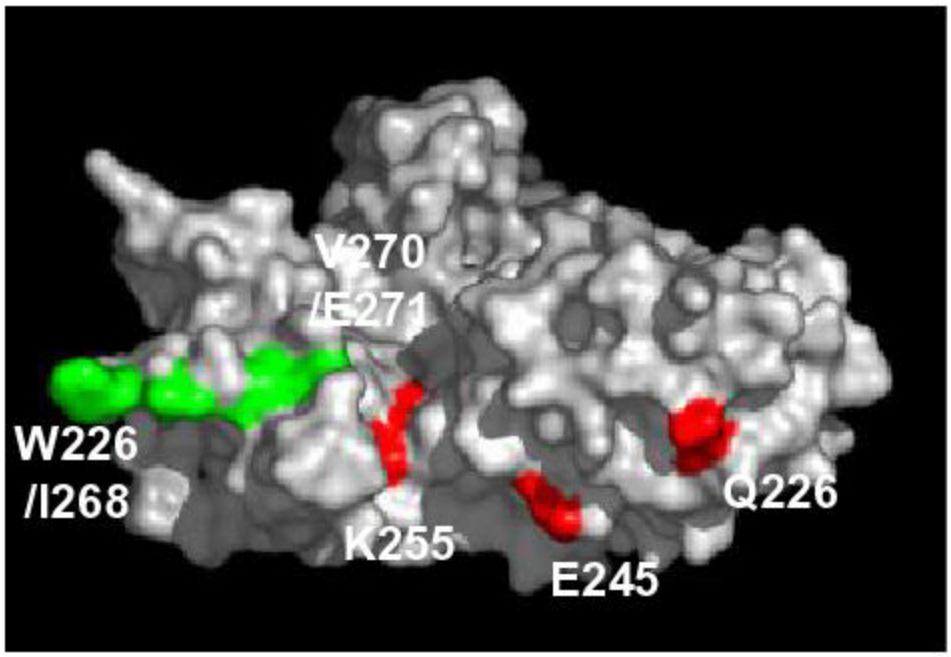

Figure 13. Structure of the FAK FERM domain highlighting biologically interesting mutants. Modified Boyden chamber assays revealed residues that result in increased (green) or decreased (red) motility as compared to wild type FAK when reconstituted in *fak* -/- MEFs.

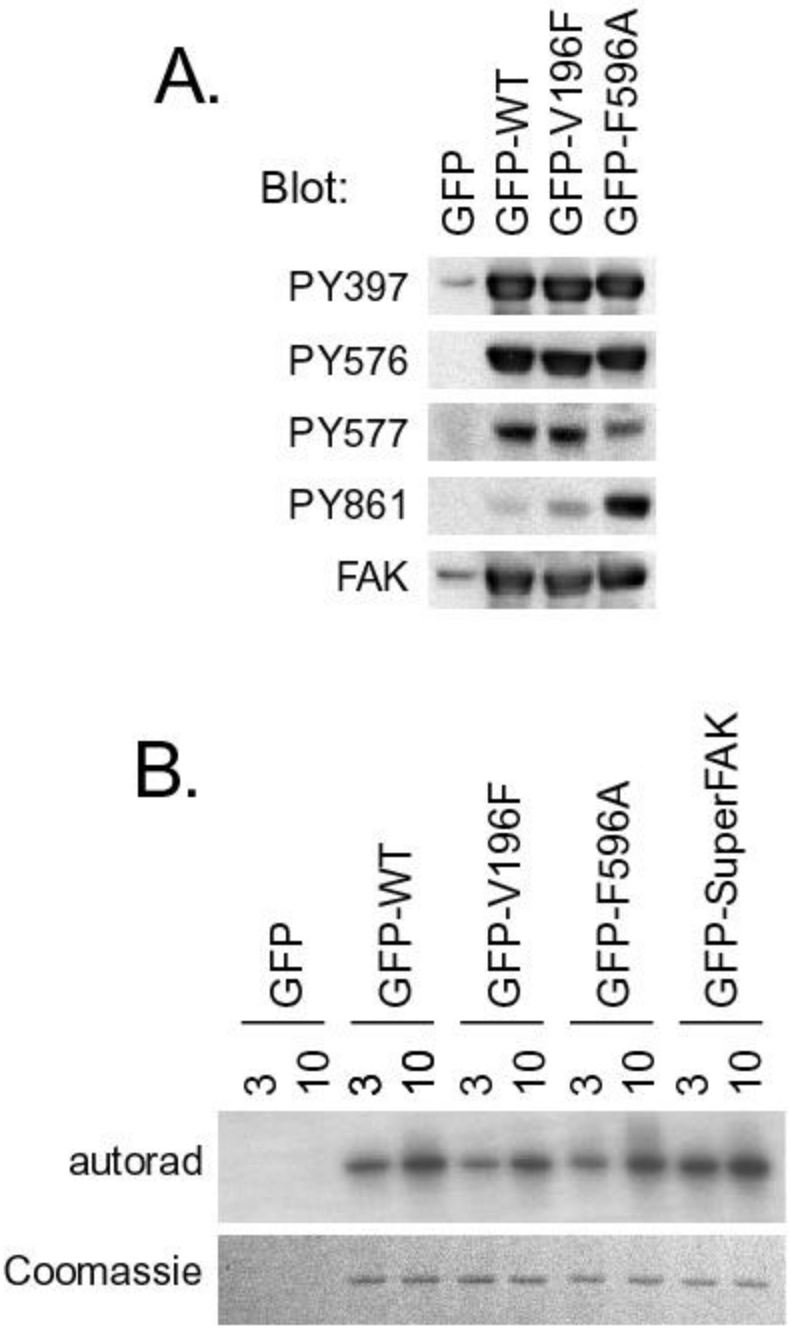

Figure 14. Biochemical measurements of GFP-FAK activity in 293 cells. A. Transiently transfected 293 cells were lysed and 25 μg lysate was immunoblotted using FAK BC4 polyclonal antibody or phosphospecific antibodies as indicated. B. Transiently transfected 293 cells were lysed and 0.2 mg lysate was immunoprecipitated using FAK BC4 polyclonal antibody. Immunoprecipitated proteins were washed and incubated for 3 or 10 minutes with radiolabelled ATP to measure catalytic activity.

CHAPTER FOUR

CONCLUSIONS AND FUTURE DIRECTIONS

Deviations from normal levels of angiogenesis play a role in several pathological conditions, such as stroke and coronary artery disease (Eliceiri and Cheresh, 2001). Angiogenesis requires the proper regulation of several cellular processes including cell growth, survival, and migration (Daniel and Abrahamson, 2000;Eliceiri and Cheresh, 2001;Eliceiri, 2001;Orr and Murphy-Ullrich, 2004). These processes are regulated by signals from integrins and growth factor receptors in vascular endothelial cells and also in fibroblasts. An important protein known to play a role in the regulation of these angiogenic processes is the focal adhesion kinase (FAK). FAK is activated by several angiogenic stimuli and FAK is important for regulating several cellular processes integral to angiogenesis, such as cell growth, survival, and migration. FAK function is regulated in several ways and although much is known about phosphorylation events that play a role in FAK's activation, the complete mechanism of activation still remains to be resolved. The purpose of these studies was to gain further insight into the mechanisms of FAK activation.

FAK subcellular localization is critical for FAK function. Understanding how this localization is mediated could be useful to help design drugs that could displace FAK and therefore disrupt FAK function in cells in which FAK-mediated cellular processes are misregulated. This study has characterized the interaction between FAK and paxillin and the

importance of this association for FAK localization, activation, and downstream signaling. Paxillin binding is the major mechanism by which FAK localizes to focal adhesions, although this association is not absolutely required for FAK localization. This indicates that there is a secondary mechanism by which FAK can target to focal adhesions in the absence of paxillin binding. The present studies have identified a residue outside the paxillin-binding sites on FAK that appears to be responsible for this secondary mechanism of localization; however the protein responsible for binding to this residue remains to be identified. Complete characterization of the mechanisms by which FAK targets to focal adhesions is required before targeted disruption of FAK localization can be achieved.

Phosphorylation of FAK is know to regulate its function is several biological contexts. Although much is known about tyrosine phosphorylation of FAK and the resulting biochemical and biological effects, there have been relatively few studies that have identified or elucidated the functions of serine phosphorylation sites on FAK. The present studies have identified several residues within the FAK FERM domain that seem to play a role in FAK regulation of haptotactic motility. Two of these mutants indicate a possible role for GSK-3 regulation of FAK via serine phosphorylation of the FAK FERM domain. This hypothesis is supported by a recent report of GSK-3 serine phosphorylation of the FAK C-terminus (Bianchi *et al.*, 2005). Although GSK-3 phosphorylation of the FAK FERM domain remains to be demonstrated, GSK-3 regulation of FAK remains an interesting new area of study for FAK regulation.

Finally, the association between the FAK FERM and catalytic domains represents a novel mechanism of regulation of FAK. Mutations to disrupt this association need to be characterized in order to understand the role of this regulatory association. The present

studies have indicated that GFP-tagged constructs are not regulated properly when expressed in 293 cells. In order to understand the role of this association on FAK regulation, untagged forms of the proteins need to be studied in a cell type where they are properly regulated. Understanding this intramolecular inhibition of FAK is important as this could represent a novel regulatory mechanism of FAK that could be manipulated in cells in order to manipulate FAK function when necessary.

These studies have gained interesting insight into the regulation of FAK in fibroblasts. Although these studies have interesting implications for therapeutics, the results from these studies should first be verified in vascular endothelial cells (ECs). Although regulation of many processes is conserved between fibroblasts and ECs, there may be some differences in the responses of the mutant forms of FAK in these different cell types. Therefore, biochemical and biological results obtained in fibroblasts should be confirmed in ECs in order to gain further insight into the regulation of FAK during angiogenesis.

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