Studies of Focal Adhesion Kinase in Epithelial Cells: Involvement in Cell-Cell Adhesion

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This thesis is dedicated to my parents, Ron and Samantha Stewart, who have supported me throughout my entire life and have by their warmth, courage, love and determination provided inspiring examples of the very best of human nature.

Sláinte Mum & Dad!

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Abstract

Epithelial cell-cell adhesion is mediated by tight junctions, adherens junctions and desmosomes. Epithelial cell-matrix adhesion is mediated by hemidesmosomes and focal contacts. These complexes exhibit great plasticity, and each contains molecular components which are able to participate in one or more of the other adhesive complexes. Focal adhesion kinase (FAK/p125^{FAK}) is a non-receptor tyrosine kinase which transduces signals from integrins at sites of focal contact to promote adhesion, spreading and migration. FAK possesses a central kinase domain which is flanked by large, non-catalytic, amino- and carboxy-terminal domains. Whereas the functions of the carboxy-terminal and kinase domains of FAK are well understood, the role of the amino-terminal domain remains unclear. FAK expression was examined in the human epithelial cell line, HEK 293. Amino-terminal FAK immunoreactivity was noted at sites of cell-cell contacts and in the nucleus, in contrast to carboxy-terminal immunoreactivity, which was largely cytoplasmic and perinuclear. Western blot analysis of endogenous FAK revealed expression of a presumptive proteolytic cleavage fragment corresponding to the amino-terminal domain. A series of FAK constructs was generated to test the hypothesis that the observed amino-terminal FAK localisation was due to this proteolytic fragment. Epitope-tagged Amino-Terminal FAK (ATF) constructs localised primarily at areas of cell-cell contact and in the nucleus in HEK 293 cells. This localisation was independent of Tyrosine 397, the major FAK autophosphorylation site. This sub-cellular distribution was confirmed in another epithelial cell line, MDCK, in which transiently transfected ATF constructs also localised primarily to the nucleus and at cell-cell contacts. HEK 293 cells were characterised with respect to expression of adhesive proteins, and ATF was found to co-localise with the tight junction protein occludin, with cortical actin and with junctional β_1 integrin. Immunoprecipitation data suggests that none of these proteins forms a precipitable complex with ATF. These findings indicate that the amino-terminal domain of FAK is capable of localising at epithelial cell-cell contacts and suggest a novel role for FAK in mediating cross-talk between focal contacts and cell-cell contacts through endogenously expressed amino-terminal FAK fragments.

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List of Abbreviations

Abl	ABelson murine Leukaemia virus oncogene
Ad 5	ADenovirus type 5
AJ	Adherens Junction
ASH	Absent, Small or Homeotic
ASIP	Atypical PKC isoform-Specific Interacting Proteins
ATF	Amino-Terminal domain of FAK
BP	Bullous Pemphigus
BSA	Bovine Serum Albumin
E-Cadherin	Epithelial Cadherin
P-Cadherin	Placental Cadherin
N-Cadherin	Neuronal Cadherin
Cas	Crk Associated Substrate
CD	Cluster of Differentiation
C/EBP	cAMP response Element Binding Protein
CentFAK	CENTral domain of FAK
CIAP	Calf Intestinal Alkaline Phosphatase
CH-ILKBP	Calponin Homology ILK Binding Protein
CKII	Casein Kinase II
Crp1	C/EBP Related Protein 1
CS	Connecting Segment
Csk	C-terminal Src Kinase
DAPI	4',6-DiAminidino-2-PhenylIndole
Dfak56	*Drosophila* FAK 56
DlgA	Discs LarGe A
DM	Desmosome
DMEM	Dulbecco's Modified Eagle's Medium
dNTP	DeoxyNucleotide TriPhosphate
DOCK180	180-kDa protein downstream of Crk
DP	DesmoPlakin
Dsh	DiSHevelled
EGFP	Enhanced Green Fluorescent Protein
EGF(R)	Epidermal Growth Factor (Receptor)
ERBIN	ErbB2 INteracting protein
ERK	Extracellular signal Regulated Kinase
Etk	Epithelial and endothelial Tyrosine Kinase
ERM	Ezrin/Radixin/Moesin
F-actin	Filamentous actin
FAK	Focal Adhesion Kinase
FAT	Focal Adhesion Targeting domain
FERM	band Four point 1/Ezrin/Radixin/Moesin
FC	Focal Contact
FGF	Fibroblast Growth Factor
FITC	Fluoresceine IsoThyoCyanate
FNIII	FibroNectin type III
FRNK	FAK-Related Non-Kinase
GAP	GTPase Activating Protein
GDP	Guanosine DiPhosphate
GEF	Guanine nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GFR	Growth Factor Receptor
GJ	Gap Junction
GPCR	G Protein-Coupled Receptor
GPI	GlycosylPhosphatidylInositol
GRAF	GTPase Regulator Associated with FAK
Grb2	Growth factor Receptor Bound protein 2
GSK	Glycogen Synthase Kinase
GTP	Guanosine TriPhosphate
GTPase	Guanosine TriPhosphatase
GUK	GUanylate Kinase
HD	HemiDesmosome
HEK	Human Embryonic Kidney
Hic-5	Hydrogen peroxide-Inducible Clone-5
HRP	Horse Radish Peroxidase
IAP	Integrin Associated Protein
ICC	ImmunoCytoChemistry
Ig	ImmunoGlobulin
IGF-1	Insulin-like Growth Factor-1
ILK	Integrin-Linked Kinase

IP	ImmunoPrecipitation
JAM	Junctional Adhesion Molecule
LAP	LRR And PDZ domain-containing protein
LEF/TCF	Lymphoid Enhancer Factor/T-Cell Factor
LF	Lipofectamine
LF+	LipoFectamine Plus
LF2K	Lipofectamine 2000
LRR	Leucine Rich Repeats
MAGI	Membrane Associate Guanylate kinase Inverted
MAGUK	Membrane-Associated GUanylate Kinase
MAPK	Mitogen Activated Protein Kinase
MDCK	Madin-Darby Canine Kidney
MEM	Minimal Essential Medium
MUPP1	MUltiple PDZ containing Protein 1
NDS	Normal Donkey Serum
NRTK	Non-Receptor Tyrosine Kinase
PAK	p21 Activated Kinase
Par	PARtitioning defective
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF(R)	Platelet Derived Growth Factor (Receptor)
PDZ	PSD95/DlgA/ZO-1
PFA	ParaFormAldehyde
Pfu	*Pyrococcus furiosus*
PG	PlakoGlobin
PH	Pleckstrin Homology domain
PI3K	PhosphatidylInositol-3-Kinase
PI4K	PhosphatidylInositol-4-Kinase
Pilt	Protein Incorporated Later into TJ
PKC	Protein Kinase C
PKL	Paxillin Kinase Linker
PLC	PhosphoLipase C
PP	Plakophilin
PSGAP	PH and SH2 containing GAP
PTP	Protein Tyrosine Phosphatase
Pyk2	Proline rich tYrosine Kinase 2
RACK1	Receptor for Activated C-Kinase 1
RPTP	Receptor Protein Tyrosine Phosphatase
SH2	Src Homology 2
SH3	Src Homology 3
Shc	Src Homology Collagen
SHP-2	SH2 domain Phosphatase-2
SHPS-1	SHP Substrate-1
Sos	Son Of Sevenless
Src	SaRComa gene
TAM	Tyrosine Activation Motif
TBS	Tris Buffered Saline
TER	TransEpithelial Resistance
TJ	Tight Junction
TNFα	Tumour Necrosis Factor α
TRITC	Texas Red IsoThyoCyanate
uPAR	Urokinase Plasminogen Activator Receptor
VASP/ENA	VAsodilator-Stimulated Phosphoprotein / ENAbled
Wg	WinGless
ZA	*Zonula Adherens*
ZO	*Zonula Occludens*
ZONAB	ZO-1 associated Nucleic Acid Binding protein

1 Introduction

1.1 Cellular Contact Structures in Epithelial Cells

Epithelial and endothelial cell monolayers line external body surfaces and blood vessels respectively, providing both barrier and permeability functions separating luminal and serosal compartments. In order to achieve this, they exist as mechanically intact, continuous sheets supported by extracellular matrix, which are capable of restricting the transition of molecules between the respective compartments in a selective manner. This asymmetric function necessitates that the plasma membranes of such monolayers be segregated into apical and basolateral surfaces facing the luminal and serosal compartments respectively.

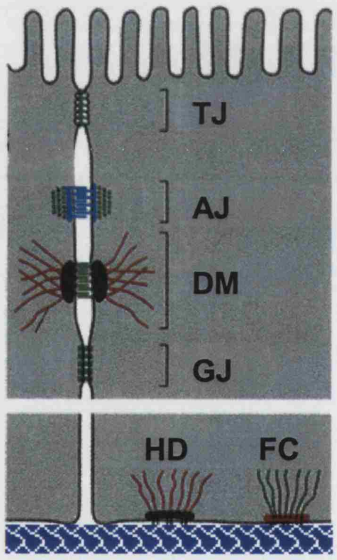

Figure 1.01 The Junctional Adhesion Complexes of Epithelial Cells.

Epithelial cells express a tripartite intercellular adhesion complex consisting of tight junctions (**TJ**), adherens junctions (**AJ**) and desmosomes (**DM**) in the apical aspect of their lateral membranes. TJ mediate restriction of both paracellular transport and apical-basolateral diffusion of membrane components. AJ and DM are responsible for cadherin-mediated cell-cell adhesion and are linked to the actin cytoskeleton (green) and intermediate filaments (red), respectively. Gap junctions (**GJ**) facilitate diffusion of small molecules between cells but are not functionally involved in adhesion. Hemidesmosomes (**HD**) and focal contacts (**FC**) are cell-matrix adhesive junctions, linking matrix components *via* integrins to intermediate filaments and the actin cytoskeleton, respectively.

Construction of epithelial sheets is also dependent upon cell-matrix interactions. In addition to the physical requirements for maintenance of adhesive and barrier functions, epithelia receive information from their cell-cell and cell-matrix contacts, modifying their behaviour appropriately in response to environmental changes. Furthermore, epithelial cells are able to remodel their contact structures in order to permit processes such as migration and transmigration.

These functional requirements of epithelia are fulfilled by highly specialised cell-cell and cell-matrix adhesion complexes. The cell-cell adhesion complexes exist in the apical aspect of the cell membranes and consist of three structurally and functionally distinct entities: tight junctions, adherens junctions and desmosomes. The tripartite intercellular adhesion complex is largely occupied with barrier and intercellular adhesive functions. Adhesion of epithelial cells to matrix is mediated by hemidesmosomes and focal contacts, which are expressed on the basolateral membrane surface. See **Figure 1.01.**

1.1.01 Tight Junctions: Physiology and Morphology

Passage of solutes across epithelial cell monolayers can occur *via* two distinct routes: the transcellular pathway and the paracellular pathway. Transcellular transport refers to the passage of solute macromolecules across the cell, including transport through channels, pumps and transporters, as well as by transcytosis (endocytosis of molecules at one side of a monolayer and exocytosis at the other side). Transcellular transport has been very well characterised and is not thought to involve cellular contacts (for review see Kinne, 1997). Paracellular transport, on the other hand, can be described as the passage of solute molecules across monolayers *via* intercellular pores (**Figure 1.02**). Paracellular transport is mediated by the most apical form of cell-cell contact, the tight junction.

Tight junctions (also referred to as "*zonula occludentes*") were first described morphologically in the 1960s as networks of fibrils containing apparent fusions ("kissing points") between the outer leaflets of the plasma membranes of apposing cells. At these kissing points, the intercellular space was seen to be completely obliterated, in contrast to

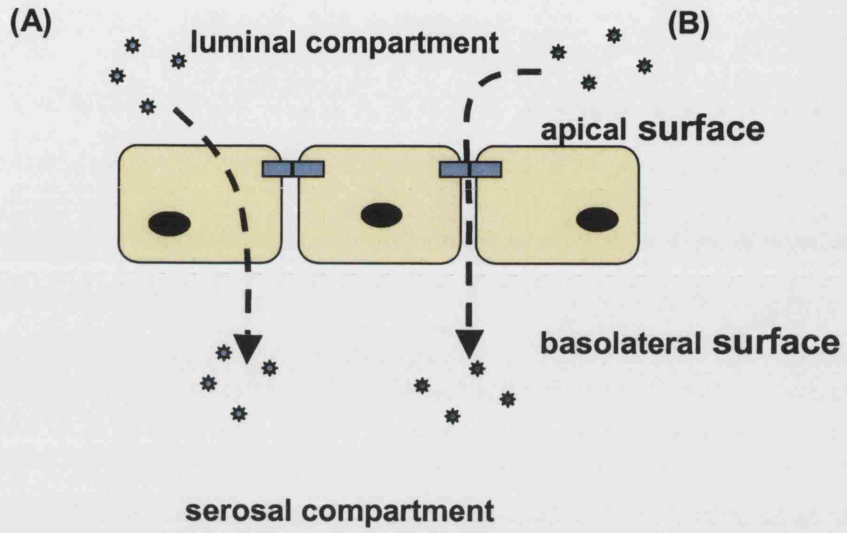

Figure 1.02: Modes of Solute Transfer Across Epithelial Monolayers.

Solute molecules can traverse epithelial monolayers through two distinct pathways. **(A)** Transcellular transport involves the passage of solutes through the cells *via* specialised transporters, pores and pumps as well as by transcytosis. In paracellular transport **(B)**, solutes diffuse across the intercellular junctional complex *via* tight junctions. The paracellular pathway shows both ion and size selectivity, having a preference for cations and an apparent size limit of 30-40Å. The degree of flux through the paracellular pathway varies from tissue to tissue and is also susceptible to modulation in response to environmental change.

adherens junctions where an intercellular gap of 15-20nm can be measured (Cereijido, 1997). Evidence that these morphological features could function as a barrier to paracellular diffusion came from experiments in the early 1960s which demonstrated that electron-dense, macromolecular tracers could diffuse freely along the paracellular pathway up to the level of the tight junction (Cereijido, 1997). This aspect of tight junction behaviour is referred to as "barrier function." Tight junctions were then further functionally defined as semi-permeable, selective ion pores with preference for cations and with an apparent size limit of 30-40Å (Lindeman & Solomon, 1962; van Os *et al.*, 1974). Tight junctions also appear to provide the physical barrier to the apical-basolateral diffusion of membrane components ("fence function") and are thus critical for the establishment and maintenance of epithelial cell polarity (Dragsten *et al.*, 1981; van Meer & Simons, 1986).

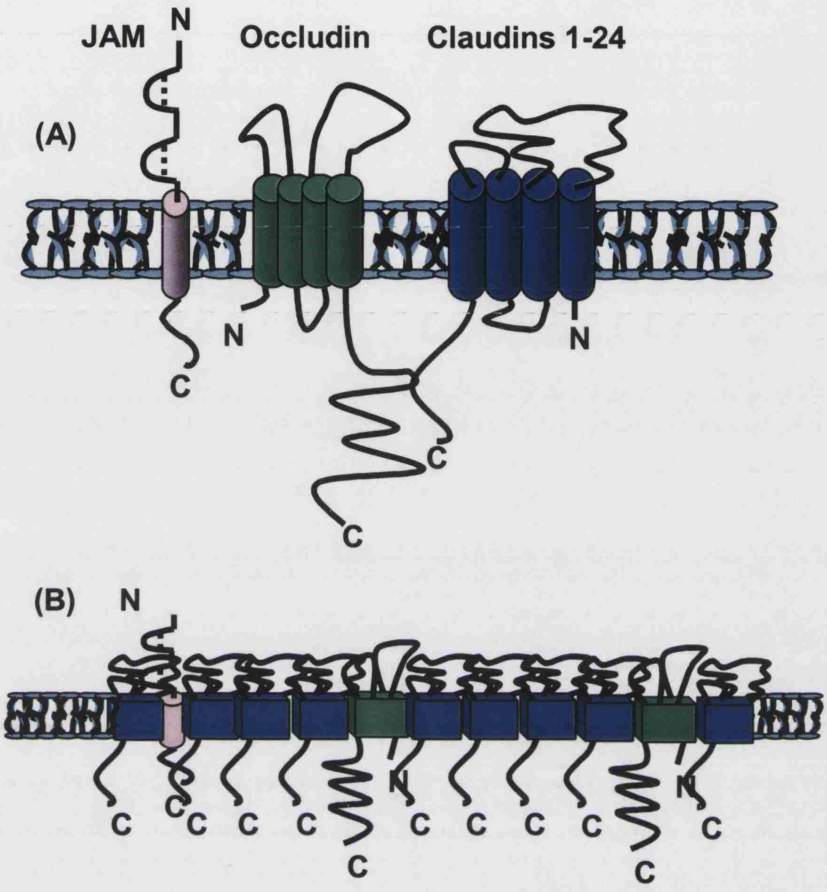

Figure 1.03: Integral Membrane Proteins in Tight Junction Strands.

(A) Three distinct membrane proteins are found in tight junctions. **J**unctional **A**dhesion **M**olecule (JAM) is a member of the immunoglobulin superfamily, possessing two extracellular Ig folds, a single transmembrane domain and an intracellular carboxy-terminal tail ending in valine through which it binds to peripheral membrane PDZ (for **P**SD95/**D**lgA/**Z**O-1) domain-containing proteins. Claudins and occludin share a common domain structure, each possessing four transmembrane domains with (relatively short) intracellular amino-termini and (relatively long) carboxy-termini; however, occludin shares no significant sequence homology with the claudin family. The occludin carboxy-terminus binds directly to the **Gu**anylate **K**inase (GUK) domains of ZO-1 (for ***Z**onula **O**ccludens-1*), ZO-2 and ZO-3. Almost all claudins possess a carboxy-terminal valine residue through which they bind directly to the first PDZ domains of ZO-1, ZO-2 and ZO-3. **(B)** Tight junctions are thought to assemble by hetero-copolymerisation of one or more claudins and lower stoichiometric amounts of occludin. Current evidence suggests that JAM may also participate in these linear copolymers. The copolymers cluster to fom strands ~10 nm in diameter with a cytoplasmic "toothbrush" of protruding, intracellular carboxy-terminal domains, containing multiple docking sites for PDZ domain-containing proteins.

1.1.02 Tight Junctions: Molecular Composition

In the last few years, considerable progress has been made in determining the molecular content of tight junction strands and in understanding how this relates to tight junction function. The backbone of tight junction strands is mainly formed by a relatively new gene superfamily known as the claudins (from Latin *claudere*, "to close"). Claudins are integral membrane proteins, each of which is predicted to possess four transmembrane domains, with both the (relatively short) amino- and (longer) carboxy-termini exposed to the cytoplasm of the cell (**Figure 1.03A**). Members of this family share distant homology with genes functionally associated with cell-cell contacts, including epithelial membrane proteins 1-3, the peripheral myelin protein 22 and the eye lens-specific proteins 19 and 20. It is interesting to note that claudin family members also bear distant homology to calcium channel γ subunits (reviewed in Anderson & van Italie, 1999; Heiskala *et al.*, 2001; Tsukita *et al.*, 2001).

Twenty-four distinct claudin genes have been identified to date, largely through database searching, and at least one of the claudin genes is known to undergo alternative splicing. Claudin sequence conservation is highest in the first and fourth transmembrane segments and in the first and second extracellular loops. The carboxy-terminal tails of claudins exhibit the greatest degree of sequence divergence and possess consensus phosphorylation sites for protein kinase C (PKC), casein kinase II, and cAMP-dependent protein kinase. Almost all of the claudins cloned to date end in a carboxy-terminal valine residue, providing a binding site for PSD95/DlgA/ZO-1 (PDZ) domains, which are discussed in more detail below. Most cells possessing tight junctions express more than two claudin gene products, with the notable exception of oligodendrocytes and Sertoli cells, both of which express only claudin 11 (Morita *et al.*, 1999). The diversity of claudins is thought to be a critical feature in the generation of tissue-specific degrees of junctional "tightness," which is commonly assessed by measuring the potential difference across the epithelial monolayer (transepithelial resistance or TER).

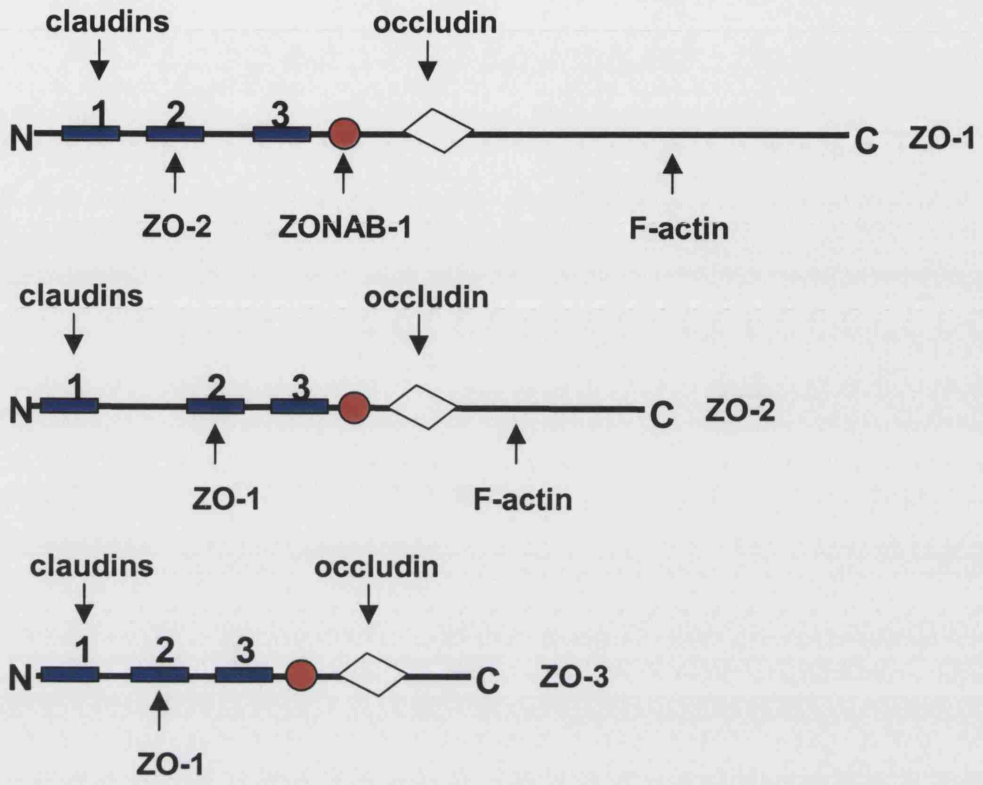

Figure 1.04: The *Zonula Occludens* Family of Peripheral Membrane Proteins.

ZO-1, ZO-2 and ZO-3 are all members of the Membrane Associated Guanylate Kinase (MAGUK) family of proteins. They each possess three amino-terminal PDZ domains (blue boxes), a single SH3 domain (red circles), a catalytically inactive Guanylate Kinase (GUK) domain (white diamonds) and a variable carboxy-terminus. All three proteins bind directly the carboxy-terminal valine residues of claudins through their first PDZ domains and to occludin *via* their GUK domains. ZO-2 and ZO-3 also bind to ZO-1 (but not to each other) through their second PDZ domains. ZO-1 and ZO-2 bind actin filaments with their carboxy-terminal domains, whereas ZO-3 is thought to bind actin *via* an as yet uncharacterised interaction involving the ZO-3 amino-terminal domain. ZO-1 participates in a truly diverse set of protein-protein interactions. Perhaps most interestingly of all, ZO-1 binds to the transcription factor ZONAB-1 (ZO-1-associated Nucleic Acid Binding protein-1), the resulting complex being transported to the nucleus and directly regulating transcription of the ErbB-2 gene.

Occludin is structurally similar to the claudins inasmuch as it is predicted to be an integral membrane protein possessing four transmembrane domains and with both the amino- and carboxy-termini oriented in the intracellular milieu. There is, however, no significant sequence homology between occludin and the claudin family. Occludin

possesses a very large intracellular carboxy-terminal domain, the extreme carboxy-terminus of which is highly conserved across species, and which is thought to possess binding sites for peripheral membrane proteins (**Figure 1.03A**). The rest of the occludin sequence is not well conserved, except that in some areas the overall amino acid composition is maintained. This may indicate that the function of these domains is more dependent on their physicochemical properties rather than on the positioning of specific amino acid residues. Occludin is expressed in all epithelial cells bearing tight junctions, but it is notably absent from some other tight junction-bearing cells such as non-neuronal endothelial cells (Ando-Akatsu *et al.*, 1996).

Recently, another integral membrane protein, Junctional Adhesion Molecule (JAM), has been described that associates functionally with tight junctions. JAM is a member of the immunoglobulin (Ig) superfamily, possessing two amino-terminal Ig-folds, two consensus sequences for N-linked glycosylation, a single transmembrane domain and an intracellular carboxy-terminal tail (**Figure 1.03A**). Whether JAM is actually inserted into tight junctional strands remains unclear at present, but it is known to associate in the same membrane compartment as other tight junction molecules. Recent evidence strongly suggests that JAM may form part of tight junction strands by an interaction with Multiple PDZ Containing Protein 1 (MUPP1), which also interacts with the carboxy-terminal tail of claudin-1 (Williams *et al.*, 1999; Martin-Padura *et al.*, 1998; Hamazaki *et al.*, 2002).

Tight junctions associate with a large number of peripheral membrane proteins, many of which are members of the Membrane Associated Guanylate Kinase (MAGUK) family (**Figure 1.04**). MAGUK family proteins typically possess a number of PDZ homology domains, a catalytically inactive Guanylate kinase (GUK) domain and, often, a Src homology 3 (SH3) domain. The first MAGUK family protein found to be associated with tight junctions was ZO-1 (for *Zonula Occludens*-1). Since then, ZO-2 and ZO-3 have also been discovered and the number of MAGUK and non-MAGUK, PDZ-domain containing proteins found at tight junctions has increased considerably (**Table 1.01**). In general, MAGUK and other PDZ domain-containing proteins are though to act as adaptor molecules. For example, ZO-1, ZO-2 and ZO-3 all bind to the C-termini of claudins *via*

their PDZ domains, to F-actin *via* their carboxy-terminal domains (ZO-1 and ZO-2) and amino-terminal domain (ZO-3), and to occludin *via* their guanylate kinase domains. ZO-1 and ZO-2 can also form heterodimers by interaction of their PDZ domains (**Figure 1.04**; Itoh *et al.*, 1999; Wittchen *et al.*, 1999; Wittchen *et al.*, 2000).

1.1.03 Tight Junctions: Integration of Form and Function

According to the current model of tight junction assembly, claudins are the most critical element in establishing tight junction strands. According to this model, tight junction strands are formed by the hetero-copolymerisation of claudins, into which occludin is inserted in a lower stoichiometry (reviewed in Tsukita *et al.*, 2001; Tsukita *et al.*, 1999). Occludin content within strands seems to be fairly consistent since the number of tight junction strands correlates well with the total amount of expressed cellular occludin (Saitou *et al.*, 1997). These linear hetero-copolymers of claudins, occludin and possibly JAM (**Figure 1.03B**), aggregate to form strands ~10nm in diameter. The extracellular domains of claudins in apposing strands bind to each other across the junctional space homotypically and heterotypically, with varying affinities. Variation in claudin-claudin binding affinity is thought to be the principal mechanism for the tissue specificity of barrier and pore functions. Occludin is evidently not required for either of these functions, although it is thought to contribute to both of them in normal epithelial cells (Saitou *et al.*, 1998; Saitou *et al.*, 2000; Balda *et al.*, 2000). Conversely, occludin is thought to be essential for the "fence" function of tight junction strands by a mechanism which is as yet undetermined (Balda *et al.*, 1996). The JAM extracellular domain probably assists in tight junction sealing by mediating homotypic interactions across the junctional space (Liu *et al.*, 2000).

The intracellular face of the tight junction strands is thus densely packed with the protruding carboxy-terminal domains of occludin, claudins and JAM (**Figure 1.03B**), and is rich in docking sites for the GUK and PDZ domains of MAGUK and other PDZ domain-containing scaffold proteins. Clustering of these proteins gives rise to the formation of the intracellular tight junction plaque which tethers the strands to

MAGUK Proteins

Protein	Binding Partners	Possible Functions
ZO-1	JAM, occludin, claudins, actin, ZO2/3, AF-6, cingulin, ZONAB-1, α-catenin, connexin-43	Actin-binding, promiscuous scaffold protein, cross-talk -AJ, transcription factor
ZO-2	occludin, claudins, actin, ZO-1, AF-6, α-catenin, cingulin	Actin-binding scaffold protein, cross-talk -AJ
ZO-3	occludin, claudins, actin, cingulin	Actin-binding scaffold-protein
MAGI-1	Rap-1 GEP	Scaffold protein, non-actin-binding
MAGI-2	PTEN	Scaffold protein, non-actin-binding
MAGI-3	PTEN	Scaffold protein, non-actin-binding

Non-MAGUK, PDZ Domain-Containing Proteins

Protein	Binding Partners	Possible Functions
AF-6	ZO-1, ZO-2, JAM	Ras effector
Par-3 (ASIP)	JAM, PKCζ/λ	Establishment of polarity? Evidence mainly from *C. elegans*
Par-6 (ASIP)	PKCζ/λ	
MUPP-1	claudins, JAM	Scaffold protein

Proteins Possessing Neither MAGUK nor PDZ Domains

Protein	Binding Partners	Possible Functions
Cingulin	JAM, ZO-1/2/3	Links TJ to the cytoskeleton
Symplekin	?	?
7H6	?	?
$G_{\alpha 0}$, G_{i2}	?	Formation & maintenance of TJ
PKCζ, PKCλ	Par-3, Par-6	Ser/Thr kinases, role in polarisation; occludin phosphorylation and targeting
ZONAB-1	ZO-1	Transcription factor
huASH1	?	Transcription factor
Rab3B, Rab13	?	Directed vesicular transport
Sec6, Sec8	?	Vesicle targeting, evidence from yeast
PTEN	MAGI-2/3	Tumour-suppressor, lipid phosphatase
Pilt	DlgA	?

Table 1.1: Peripheral Membrane Proteins Found at Tight Junctions.

Tight junction strands associate with diverse peripheral membrane proteins. These include scaffold proteins, most of which contain MAGUK and/or PDZ domains, and signal transduction molecules. Adapted from (Tsukita *et al.*, 2001) with additional information from (Hamazaki *et al.*, 2002; Wittchen *et al.*, 2000; Mino *et al.*, 2000; Ebnet *et al.*, 2000; Yamamoto *et al.*, 1997; Andreeva *et al.*, 2001; Clarke *et al.*, 2000; Kawabe *et al.*, 2001). "TJ" stands for "tight junction," and "AJ" for "adherens junction."

actin and constitutes a massive intracellular complex containing additional signalling proteins. Signal transduction molecules known to associate with the cytoplasmic aspect of tight junctions include the atypical PKC isoforms, PKCζ and PKCλ, the Atypical PKC isoform-Specific Interacting Proteins (ASIP) Par-3 and Par-6 (both related to *C. elegans* genes associated with asymmetric cell division), the heterotrimeric G-proteins $G_{\alpha 0}$ and G_{i2}, the vesicular transport associated proteins Rab3B and Rab13, and the mammalian homologues of the yeast proteins SEC6 and SEC8, which are thought to play a role in the establishment of cell polarity. ZO-1 has also been shown to interact directly with the transcription factor ZONAB-1 (ZO-1 associated Nucleic Acid Binding protein 1) and thereby to regulate transcription of the ErbB-2 gene (summarised in **Table 1.1**). Taken together, current evidence suggests a role for tight junctions in intracellular signalling relating to proliferation, membrane targeting and the establishment and maintenance of polarity.

1.1.04 Adherens Junctions: Physiology and Morphology

Normal polarised epithelial and endothelial cells possess a belt-like *Zonula Adherens* (ZA), which exists at the basal aspect of the tight junction network in the lateral membrane (**Figure 1.01**). The ZA encircles the lateral membrane of the cell in a continuous manner and promotes calcium-dependent, cell type-specific, intercellular adhesion. The ZA is a specialised type of adherens junction which is found only in polarised cells. In non-polarised, adherent cells such as mesenchymal cells, adherens junctions have a discrete, spot like morphology and are expressed all over the cell surface. For review, see (Yap *et al.*, 1997).

1.1.05 Adherens Junctions: the Cadherin-Catenin Complex

The cadherin superfamily of proteins (reviewed extensively by Steinberg & McNutt, 1999) is extremely large, containing at least thirty distinct classical cadherins plus a diverse array of non-classical cadherins. Classical cadherins share a common domain structure, comprising an extracellular amino-terminus, followed by five calcium-binding cadherin repeats, a single transmembrane domain and an intracellular carboxy-terminus. Within the non-classical cadherins, individual proteins may possess between five and

thirty-five cadherin repeats. There is at least one example of a glycosylphosphatidylinositol- (GPI-) anchored cadherin and one example of a cadherin which possesses seven transmembrane domains (Angst *et al.*, 2001). The biologic function of many of the cadherin family members is not understood and a general discussion of the cadherin superfamily is beyond the scope of this introduction, which will focus instead on those cadherins which are known to participate in epithelial adhesion (see below for desmosomal cadherins).

The principal adhesion receptor in epithelial adherens junctions is epithelial (E-) cadherin. E-cadherin was the first member of the cadherin superfamily to be cloned and characterised, having been originally identified as a cell surface glycoprotein involved in calcium-dependent "compaction" (cell-cell adhesion between mouse blastomeres or between embryonic carcinoma cells; Hayafil *et al.*, 1981). E-cadherin is a member of the classical cadherin family and therefore exhibits the classical cadherin domain structure described above (**Figure 1.05**).

Placental (P-) cadherin and neuronal (N-) cadherin (both classical cadherins) are also relevant to epithelial cell biology. Normal epithelial adherens junctions contain E-cadherin but may also contain P-cadherin, which is functionally similar to E-cadherin (Jensen *et al.*, 1997). Co-expression of E- and P-cadherin in epithelial cells is tissue-specific and may also vary in stratified layers (Jensen *et al.*, 1997). Expression of N-cadherin in epithelial-derived tumour cells is associated with epithelial-to-mesenchymal transition, increased motility and increased invasiveness (Kim *et al.*, 2000).

Classical cadherins are linked to the cytoskeleton by the catenin complex. The catenins (α-catenin, β-catenin and plakoglobin/γ-catenin) comprise a family of proteins which share a high degree of sequence homology with each other and with the *Drosophila* gene product armadillo. It is now widely accepted that the E-cadherin cytoplasmic domain (as well as that of other classical cadherins) binds directly to β-catenin or, alternatively, plakoglobin, by means of a 25 amino-acid motif at the extreme cadherin carboxy-terminus. β-Catenin and plakoglobin in turn bind directly to α-catenin which binds

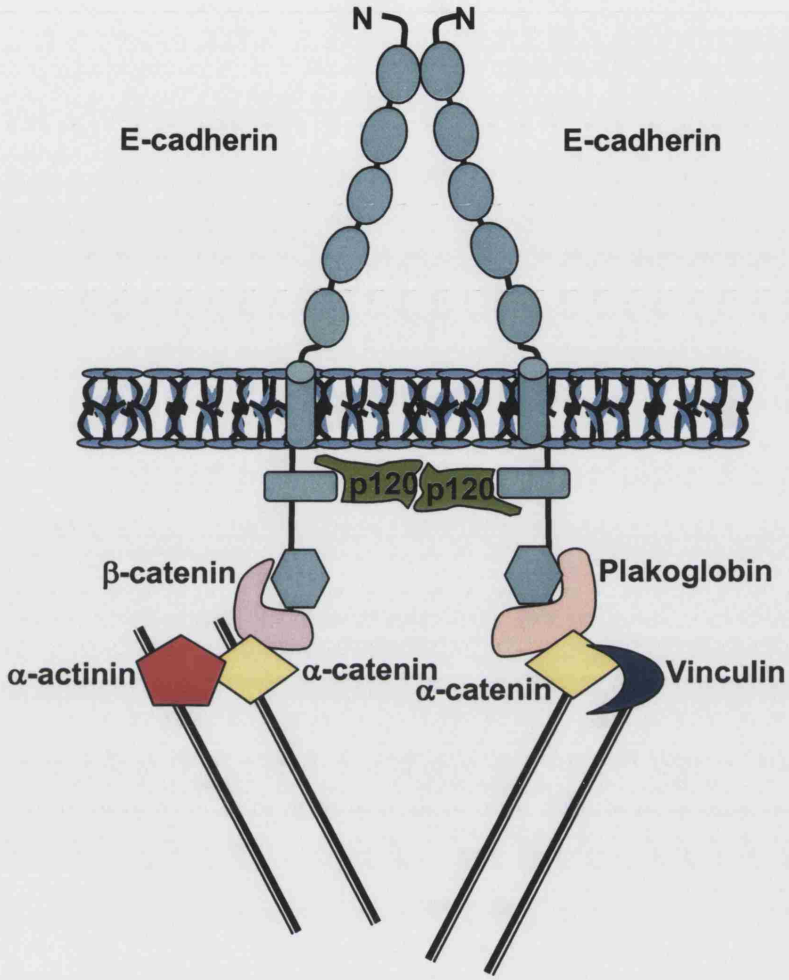

Figure 1.05: The Cadherin-Catenin Complex of Polarised Epithelial Cells.

The principle adhesion receptor in normal, polarised epithelial cells is epithelial (E-) cadherin. This protein possesses five extracellular calcium-binding cadherin repeats, a single transmembrane domain, an intracellular juxtamembrane domain which binds to $p120^{ctn}$ family members, and a membrane-distal catenin-binding motif. Cadherin monomers dimerise laterally (*cis*-interaction) with the assistance of the $p120^{ctn}$ family and also *via* interactions of the cadherin repeats. Once formed, cadherin *cis*-dimers participate in homotypic interactions with *cis*-dimers in the apposing membrane (*trans*-interaction) to form linear zippers and/or cylinder structures which adhere strongly to each other. The catenin-binding motif binds directly to both β-catenin and to plakoglobin. These proteins in turn bind to α-catenin which tethers the complex to actin filaments directly and *via* the actin-binding proteins, α-actinin and vinculin. Formation of the E-cadherin/β-catenin/α-catenin complex is critical for intercellular adhesion.

directly to F-actin, providing a direct link to the cytoskeleton. α-Catenin also binds to a number of actin-binding proteins, including vinculin, α-actinin and ZO-1, which provide further linkage to the cytoskeleton (**Figure 1.05**). The α-catenin/ZO-1 interaction is not thought to be significant in mature, polarised epithelial cells, in which ZO-1 is always localised to tight junctions. Rather, ZO-1 is thought to bind to α-catenin in junctional development, during which process ZO-1 localises to adherens junctions (reviewed by Jensen *et al.*, 1997). Assembly of the cadherin-catenin complex is essential for formation of functional adherens junctions: deletion of the catenin-binding domain of E-cadherin completely abrogates intercellular adhesive capability (Provost & Rimm, 1999).

The juxtamembrane segment of the E-cadherin cytoplasmic domain binds directly to the p120^{ctn} family (**Figure 1.05**), which consists of p120^{ctn}, δ-catenin and p100. Members of the p120^{ctn} family are thought to assist in adherens junction assembly by promoting lateral dimerisation of classical cadherins. Members of the p120^{ctn} family bear a high degree of homology to each other and some homology to the catenins and armadillo. The expression of p120^{ctn} family members is regulated by alternative transcription and is highly cell type-specific (Lu *et al.*, 1999; Staddon *et al.*, 1995; Kiersebilck *et al.*, 1998).

Lateral dimerisation of classical cadherins (*cis*-interaction) is a key step in generating functional adherens junctions. According to the most recent data, cadherin *cis*-dimerisation is promoted by p120^{ctn} family members intracellularly, resulting in a change of conformation in the cadherin extracellular domain (Yap *et al.*, 1998). This conformational change permits co-operative interaction of the extracellular, calcium-binding, cadherin repeats which results in further conformational change. Once formed, mature cadherin *cis*-dimers are able to bind to apposing *cis*-dimers across the junctional space (*trans*-interaction) with high affinity, forming linear zippers and/or cylindrical structures (reviewed by Yap *et al.*, 1997). Classical cadherins mediate cell-type specificity of adhesion due to the fact that they bind homotypically but not heterotypically.

1.1.06 Adherens Junctions: Additional Protein Complexes

Two transmembrane proteins have recently been identified as novel components of adherens junctions (**Figure 1.06**). Vezatin was initially identified as a myosin VIIA-binding protein using the yeast two-hybrid system. This interaction is mediated by the carboxy-terminal tail of vezatin and the FERM (for band four point 1/Ezrin/Radixin/Moesin) domain of myosin VIIA. Vezatin was subsequently found to localise to adherens junctions in a manner dependent on α-catenin. Furthermore, an anti-vezatin antibody successfully co-immunoprecipitated α-catenin, β-catenin and E-cadherin. The functional significance of vezatin in adherens junctions remains to be determined, although there is some speculation that forces generated by myosin VIIA may be involved in supporting cadherin-mediated intercellular adhesion (Küssel-Andermann *et al.*, 2000).

The nectin family consists of at least four independent gene products (nectins 1-4), which are subject to alternate splicing. Nectins are members of the immunoglobulin superfamily and promote calcium-independent intercellular adhesion by homotypic interactions. These proteins possess three extracellular Ig folds, a single transmembrane domain and an intracellular carboxy-terminus. The extreme nectin carboxy-terminus contains an E/A-X-Y-V motif which binds directly to the PDZ domain of large (l-) afadin.

l-Afadin possesses a single, central PDZ domain, followed by three proline-rich sequences and a carboxy-terminal, F-actin-binding domain. l-Afadin interacts functionally with α-catenin and binds directly to the recently-discovered adherens junction protein, ponsin. Ponsin can also bind to vinculin and, like vinculin, is found at both cell-cell and cell-matrix adhesions. Ponsin forms binary complexes with either l-afadin or vinculin but is incapable of forming a ternary complex with them. Thus, the nectin and cadherin systems are thought to be linked *via* l-afadin, α-catenin, ponsin and vinculin (**Figure 1.06**; Takahashi *et al.*, 1999; Yakoyama *et al.*, 2001; reviewed by Nagafuchi *et al.*, 2001).

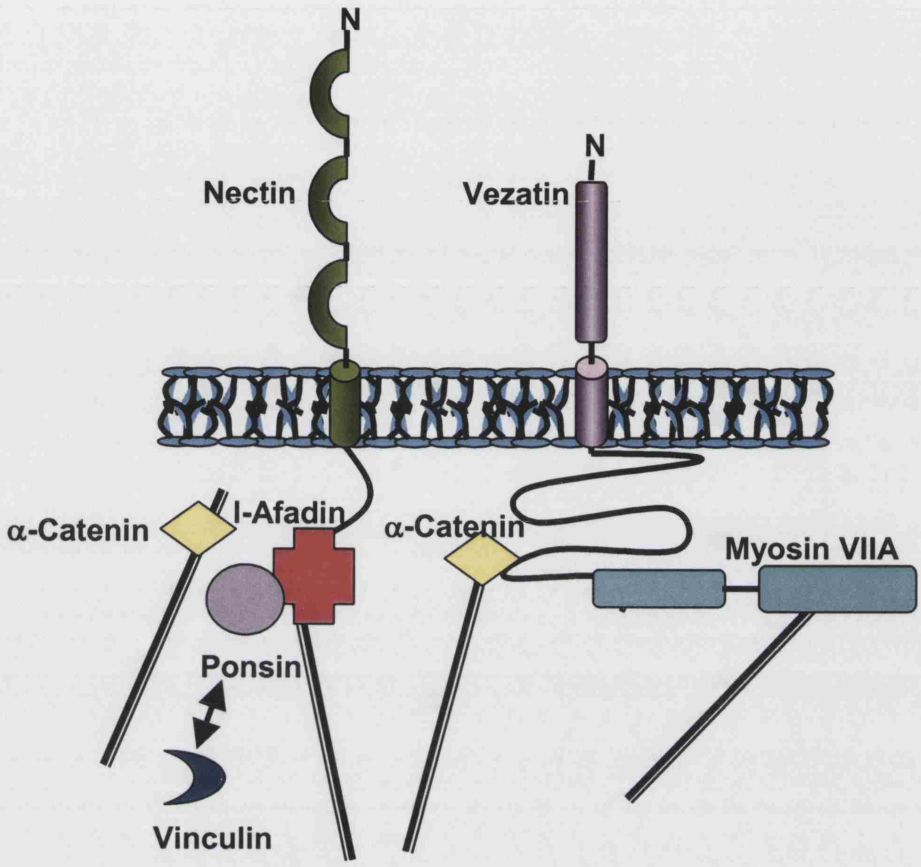

Figure 1.06: Novel Protein Complexes Found in Adherens Junctions.

Vezatin binds directly to the carboxy-terminal FERM domain of myosin VIIA and localises at adherens junctions. Vezatin forms an immunoprecipitable complex with the cadherin-catenin system, possibly mediated by α-catenin. The functional significance of the vezatin-myosin VIIA system in adherens junctions is not understood. Nectin family proteins each possess three extracellular Ig folds, a single transmembrane domain and a carboxy-terminal tail which binds directly to the PDZ domain-containing protein, large (l-) afadin. l-Afadin is an F-actin-binding protein which interacts functionally with α-catenin and may provide a direct link between the nectin-afadin system and the cadherin-catenin system. Nectins mediate calcium-independent, homotypic adhesion by binding to other nectins across the junctional space.

1.1.07 Adherens Junctions: Signal Transduction

Adherens junctions are thought to be largely responsible for contact inhibition of cell proliferation. Loss of E-cadherin expression in epithelial cells, whether occurring

naturally or experimentally induced, is associated with a loss of contact inhibition and can lead to epithelial-mesenchymal transition (Kim *et al.*, 2000). The precise mechanism by which adherens junctions inhibit cell growth is not understood. However, recent work has identified the cyclin-dependent kinase inhibitor, p27(KIP1) as a potential downstream effector of contact inhibition mediated by both E-cadherin and N-cadherin (Levenberg *et al.*, 1999; St Croix *et al.*, 1998).

In addition to its role in the assembly of the cadherin-catenin complex, β-catenin has a well-characterised function in the transduction of Wnt and **W**ing**l**ess (Wg) signals. Briefly, binding of Wnt and Wg to their receptors (e.g. Frizzled) results in phosphorylation of **D**i**s**hevelled (Dsh) which is then recruited to the membrane, where it down-regulates the activity of **G**lycogen **S**ynthase **K**inase 3β (GSK3β). GSK3β normally phosphorylates β-catenin, thus targeting it for degradation in the proteasomes, such that its down-regulation by Dsh results in an accumulation of extrajunctional, cytosolic β-catenin. Elevation of cytosolic β-catenin results in its transport into the nucleus where it forms heterodimers with members of the LEF/TCF (**L**ymphoid **E**nhancer **F**actor/**T**-**C**ell **F**actor) family of transcription factors and thereby promotes transcription of Wg/Wnt responsive genes (reviewed by Novak & Dedhar, 1999).

Although this type of signalling has been most thoroughly described for β-catenin, plakoglobin is also known to participate in Wnt/Wg signalling in a way which is mechanistically very similar. However, whereas β-catenin-mediated gene transcription is thought to be tumourigenic, plakoglobin-mediated gene transcription may be tumour-suppressive (Pfeiffer, 1997; Simcha *et al.*, 1996). The mechanism by which these two proteins exert such distinct effects on cellular function whilst participating in near-identical signalling pathways is not well understood. $p120^{ctn}$ family members have also been shown to localise to the nucleus and interact with transcription factors (Prokhortchouk *et al.*, 2001), and the direct contribution of adherens junctions proteins to transcriptional regulation may well be more complex than previously thought.

Some work has been carried out on the regulation of the cadherin-catenin complex by phosphorylation. As described above, serine/threonine phosphorylation of β-catenin and plakoglobin by GSK3β leads to their degradation in proteasomes. E-cadherin phosphorylation on serine/threonine residues by casein kinase II (CKII) increases the stability of the cadherin/catenin complex, leading to stronger intercellular adhesion (Lickert *et al.*, 2000). E-cadherin, the catenins and the $p120^{ctn}$ family are also susceptible to phosphorylation on tyrosine residues. In general, tyrosine phosphorylation is thought to destabilise the cadherin-catenin complex, leading to a decrease in intercellular adhesive strength. Accordingly, transformation of cells with oncogenic tyrosine kinases such as v-Src tends to lead to a decrease in intercellular adhesion. The search for tyrosine kinases that directly phosphorylate members of the cadherin/catenin system has, however, so far been an unfruitful one.

A number of receptor protein tyrosine phosphatases (RPTP), including protein tyrosine phosphatase 1B (PTP1B) and PTPμ, as well as the PTPμ-binding protein, RACK 1 (Receptor for Activated C-Kinase 1) are known to co-localise with the cadherin-catenin complex. In general, RPTP-mediated dephosphorylation of the cadherin/catenin complex increases its stability and results in an increase in adhesive strength (reviewed by Provost & Rimm, 1999; Steinberg & McNutt, 1999).

Finally, the Ras effector IQGAP-1 is known to interact with E-cadherin, α-catenin, β-catenin and actin (Kuroda *et al.*, 1998), and may negatively regulate cell-cell adhesion by competing for binding sites in the cadherin/catenin complex.

1.1.08 Desmosomes: Physiology and Morphology

Desmosomes are expressed primarily in cells which experience mechanical strain, such as epithelial cells (especially in the epidermis) and the myocardium. Like adherens junctions, desmosomes mediate calcium-dependent intercellular adhesion. Unlike

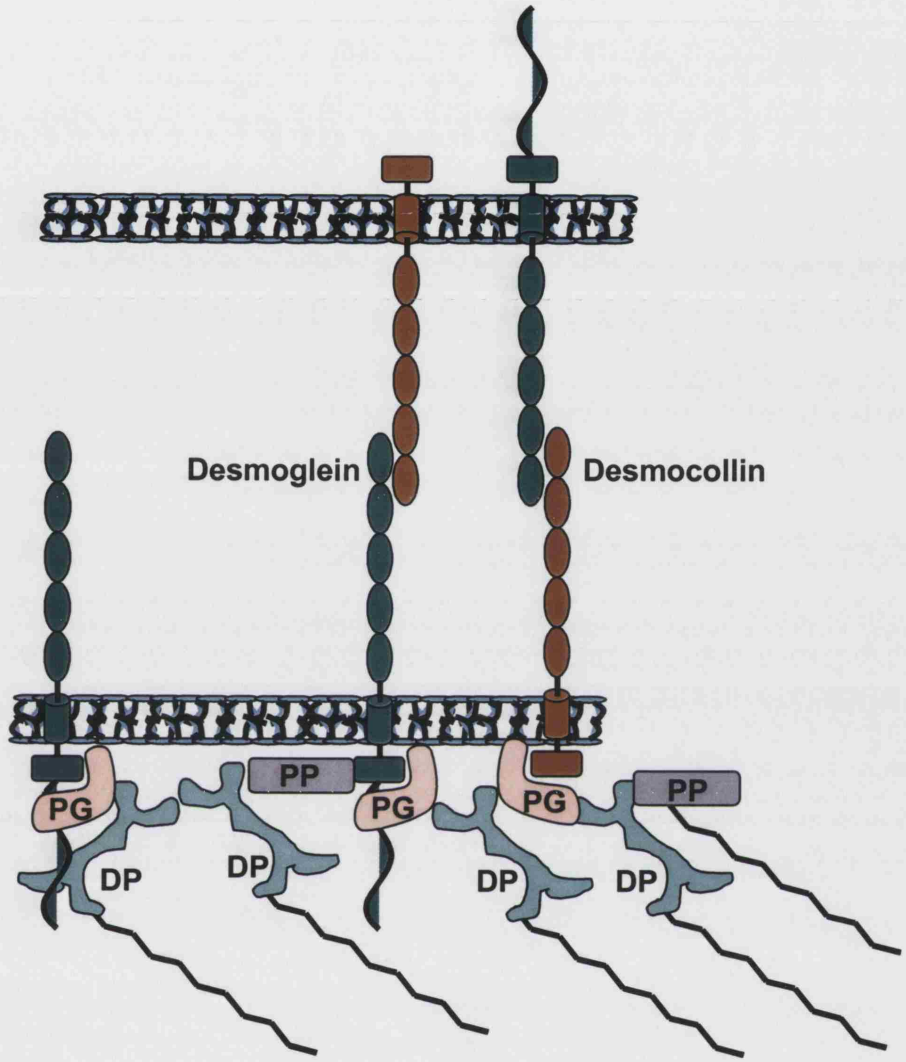

Figure 1.07: Molecular Composition of Desmosomes.

Desmosomes mediate calcium-dependent intercellular adhesion by means of desmosomal cadherins, which can be sub-divided into two groups: desmocollins DC (1-3) and desmogleins DG (1-3). DC and DG each possess five extracellular calcium-binding cadherin repeats through which they bind to each other heterotypically, both laterally and in *trans*. Desmosomal cadherins possess a membrane-proximal catenin-binding sequence which binds directly to plakoglobin (PG), although each DC may be expressed as a splice variant which lacks the catenin-binding domain. PG in turn binds directly to desmoplakin (DP), which binds to acidic and basic cytokeratins. The linear complex is extended laterally by the promiscuity of plakophilins (PP) 1-3, which are related to p120ctn, and desmoplakin (DP). PP1 can bind directly to the catenin-binding domains of DG, to cytokeratins and to DP. DP can also bind directly to itself and to the cytoplasmic tails of both DC and DG. Expression of the various desmocollin, desmoglein and plakophilin isoforms is both tissue-specific and subject to developmental regulation.

adherens junctions, however, which in epithelial cells form the continuous, belt-like ZA, desmosomes are always expressed as discrete, spot-like structures. Furthermore, whereas adherens junctions are anchored to the actin-based, microfilament cytoskeleton, desmosomes are linked to intermediate filaments, whose content varies according to cell type. In epithelial cells, desmosomes are connected exclusively to intermediate filaments composed of acidic and basic cytokeratins.

1.1.09 Desmosomes: Molecular Composition

The desmosomal adhesion receptors are non-classical, desmosomal cadherins. These can be divided into two sub-families, desmocollins and desmogleins, each of which has three members (desmocollin 1-3 and desmoglein 1-3). Like classical cadherins, desmosomal cadherins are glycoproteins which each possess an extracellular amino-terminus, five extracellular calcium-binding cadherin repeats, a single transmembrane domain and an intracellular carboxy-terminus which contains a catenin-binding domain (**Figure 1.07**). Each desmocollin may be expressed as a carboxy-terminal-truncated splice variant which lacks the catenin binding domain. The biologic significance of these truncated desmocollin variants is not understood. Desmogleins are not known to undergo alternative transcription, but possess an extended intracellular domain which is unique to them and the function of which is not understood (**Figure 1.07**; reviewed by Angst *et al.*, 2001).

Desmosomal cadherins are linked to the intermediate filament network by two groups of desmosomal plaque proteins: the armadillo-related proteins and the plakins. The armadillo-related proteins include plakoglobin, which is the most closely related to β-catenin, and plakophilins 1-3, which are more closely related to $p120^{ctn}$. The plakins (desmoplakin, plectin and the envelope proteins envoplakin and periplakin) share a common domain structure in which two globular, amino- and carboxy-terminal head domains are separated by a central, rod-like, coiled-coil domain. In a model which is analogous to the classical cadherin/β-catenin/α-catenin system, desmosomal cadherins

bind directly to plakoglobin, which in turn binds to desmoplakin, which in turn binds directly to intermediate filaments (**Figure 1.07**; reviewed by Green & Gaudry, 2000)

1.1.10 Desmosomes: Complex Assembly

Expression of desmosomal proteins is both tissue-specific and developmentally regulated. Furthermore, in stratified epithelia such as the epidermis, desmosomal components are differentially expressed in the stratified layers, such that the most superficial layers tend to express desmocollin 1, desmoglein 1 and plakophilin 1; whereas the basal layers tend to express more desmocollin 3, desmoglein 3 and plakophilin 2. The structure of any given desmosome is thus likely to differ significantly from that of a cell in another tissue, or even in a different stratified layer (Schmidt *et al.*, 1994; Borrmann *et al.*, 2000).

The structure of the desmosomal complex differs significantly from that of cadherin-based adherens junctions. Firstly, it seems likely that desmosomal cadherins engage in heterotypic interactions, both laterally and in *trans* through their extracellular domains (**Figure 1.07**). Studies seem to indicate that a desmocollin and a desmoglein, plus plakoglobin, must be present in a cell in order to achieve efficient adhesion. Furthermore, current data suggest that not all desmocollin/desmoglein pairs are productive, and that even with productive pairings, the correct stoichiometry of desmocollin: desmoglein is critical (Chitaev & Troyanovsky, 1997; Kowalczyk *et al.*, 1996).

Secondly, it seems that the linear zipper and cylinder models of adherens junction assembly do not pertain to desmosome assembly. Whereas plakoglobin mediates formation of a linear complex of desmosomal cadherin/plakoglobin/desmoplakin, plakophilins are more promiscuous. Plakophillin-1 can bind directly to desmogleins, desmoplakins and keratins through its head domain (**Figure 1.07**) and may thus serve as a focal point for recruiting plaque proteins to the cell membrane (North *et al.*, 1999; Hatzfeld *et al.*, 2000). Furthermore, desmosomal cadherin tails may bind directly to desmoplakin, which can also bind to itself (**Figure 1.07**; Smith & Fuchs, 1998; Hofmann *et al.*, 2000). It is currently thought that, whereas plakoglobin links desmoplakin to cadherin tails through linear interactions, plakophilin extends the plaque laterally through

diverse interactions, generating a mesh-like structure that is rich in binding sites for cytokeratins.

1.1.11 Desmosomes: Regulation and Signal Transduction

Like adherens junctions, desmosomes are sensitive to calcium, such that raised extracellular calcium promotes their assembly (Watt *et al.*, 1984), and perturbation of intracellular calcium stores antagonises it (Stuart *et al.*, 1996). Desmosomal assembly relies on cross-talk with adherens junctions, which is mediated by plakoglobin. In cells transfected with dominant negative E-cadherin, or in cells which lack plakoglobin, true desmosomes do not form, and adherens junction and desmosomal components become mixed (Lewis *et al.*, 1997; Ruiz *et al.*, 1996).

The role of plakoglobin in signal transduction has been fairly well characterised and is both described above and reviewed in (Ben Ze'ev & Greiger, 1998). Plakophilins also localise to the nucleus in addition to their desmosomal localisation, raising the possibility that they might also regulate gene transcription (Mertens *et al.*, 1996). This potential role of plakophilins has not been investigated; however, there is some evidence that plakophilin 1 may regulate cell shape and organisation of the actin cytoskeleton (Hatzfeld *et al.*, 2000).

The vast majority of desmosomal proteins so far identified are subject to phosphorylation. In one cell culture model, the activation of the tyrosine kinase receptor FGFR2c (Fibroblast Growth Factor Receptor 2c) by Fibroblast Growth Factor-1 (FGF-1) resulted in junctional dissolution, however it is not known whether this was mediated directly or by downstream effectors (Savagner *et al.*, 1997). Plakoglobin has been identified as a tyrosine kinase substrate, in which tyrosine phosphorylation leads to weakening of its interaction with desmoplakin and an inhibition of cytoskeletal association (Shibamoto *et al.*, 1994). Similarly, phosphorylation of serine residues in desmoplakin is thought to inhibit its association with intermediate filaments (Stappenbeck *et al.*, 1994).

Finally, in the same way that classical cadherins can drive divergent differentiation pathways (e.g. E-cadherin promotes epithelial differentiation whereas N-cadherin drives mesenchymal differentiation), it is tempting to speculate that desmosomal cadherins may participate actively in differentiation signalling.

1.1.12 Hemidesmosomes: Physiology and Morphology

Hemidesmosomes (reviewed in Borradori & Arnoud, 1999; Nievers *et al.*, 1998) are expressed on the basolateral membrane surface of stratified and other complex epithelia where they promote adhesion to the basement membrane. Hemidesmosomes generally appear as small, semi-circular electron-dense microdomains, possessing a tripartite cytoplasmic plaque to which intermediate filaments are anchored. The term "hemidesmosome" arose due to the fact that, at the electron microscopic level, they appear to be "half desmosomes": desmosomes are circular (comprised of a semicircular plaque from each apposing cell) whereas hemidesmosomes are semicircular.

1.1.13 Hemidesmosomes: Molecular Composition

Two distinct transmembrane receptors participate in hemidesmosome adhesion to matrix: the Bullous Pemphigus (BP) antigen, BP180, and $\alpha_6\beta_4$ integrin (bullous pemphigoid being an autoimmune disease of the skin). Both of these receptors bind primarily to extracellular laminin 5, which is in turn linked *via* collagen VII anchoring filaments to the basement membrane.

BP180 is a type II transmembrane protein, and as such has an extracellular carboxy-terminus and a single transmembrane domain. The extracellular domain is large, consisting of a series of collagen repeats which are thought to form collagen-like triple helices. *In vitro* binding data suggest that the BP180 extracellular domain binds directly to the β3 chain of laminin5. Interestingly, the majority of the collagenous extracellular domain of BP180 can be cleaved by proteases to release a large, soluble, matrix-binding heavy chain (Marinkovich *et al.*, 1996). The biologic significance of this proteolytic cleavage product is not yet understood, but it may well serve to modulate stromal adhesion.

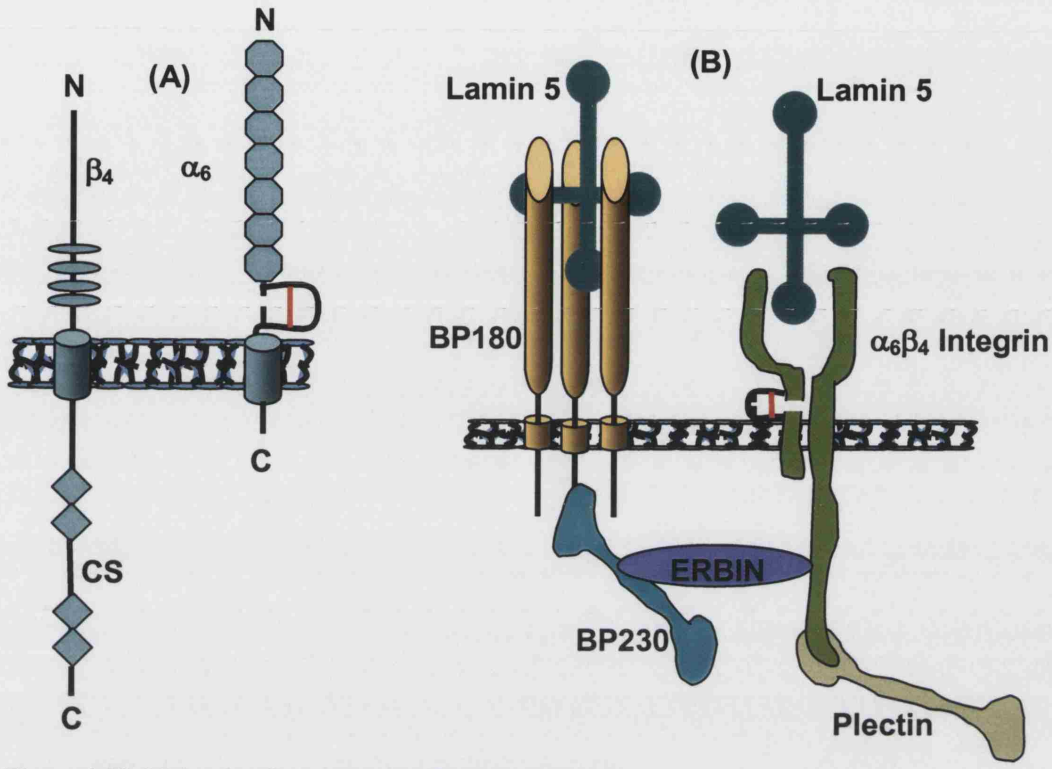

Figure 1.08: Molecular Composition of Hemidesmosomes.

The α6β4 integrin (**A**) is a key component of hemidesmosomes. The β4 chain possesses four extracellular cysteine-rich domains (ovals), a single transmembrane domain and an unusually large cytoplasmic domain, which contains four **F**ibro**n**ectin type III (FNIII) domains (diamonds). The FNIII domains are clustered in pairs which are separated by a **C**onnecting **S**egment (CS). The CS contains several tyrosines which are phosphorylated upon ligation of α6β4 integrin by laminins, and which are referred to as the tyrosine activation motif. The α6 chain possesses seven extracellular homologous repeats, the last three of which are predicted to bind divalent metal cations. (**B**) α6β4 Integrin and BP180 are the adhesion receptors of hemidesmosomes. BP180 is a type II transmembrane protein (i.e. with an intracellular carboxy-terminus) with a large, collagenous extracellular domain. Both BP180 and α6β4 integrin bind to extracellular laminin 5, which in turn binds to collagen VII anchoring filaments. On the cytoplasmic side, the β4 integrin carboxy-terminal tail binds directly to the amino-terminal globular domain of plectin, which in turn binds to cytokeratins *via* its carboxy-terminal domain. Similarly, the intracellular carboxy-terminus of BP180 binds directly to the amino-terminal domain of BP230, which binds to keratins via its carboxy-terminal domain. ERBIN (ERBB2-Interacting protein) binds to both BP230 and α6β4 integrin. BP180/plectin and α6β4 integrin/BP230 interactions are also likely to occur, and BP180 is known to bind to β4 integrin. Some of these protein-protein interactions are co-operative, such that, for example, the binding of plectin to β4 integrin releases the carboxy-terminus of β4 from an intramolecular interaction, thus facilitating binding of BP180 to the β4 chain.

$\alpha_6\beta_4$ integrin (**Figure 1.08A**) is unusual among the integrin receptor family (discussed more generally below) in that the β_4 subunit possesses an extremely large cytoplasmic domain (approximately 1000 amino acids in length). The β_4 cytoplasmic domain contains four fibronectin type III (FNIII) repeats, which are arranged in pairs separated by a connecting segment (CS). The α_6 chain possesses seven extracellular, homologous repeats of which the last three are presumed to bind divalent metal cations. The α_6 subunit is cleaved close to the transmembrane domain, creating a heavy chain and a light chain which are joined by a disulphide bond (**Figure 1.08A**). Both the α_6 and the β_4 subunit may be expressed as cytoplasmic splice variants. Although $\alpha_6\beta_4$ integrin can act as a receptor for various laminins, its principal ligand is laminin 5 (Niessen *et al.*, 1994). The tight binding of monomeric laminin 5 to both $\alpha_6\beta_4$ integrin and collagen VIIA is thought to supply primary resistance to friction forces (Rousselle *et al.*, 1997).

Connection of hemidesmosomal adhesion receptors to the intermediate filament network is mediated by two members of the plakin family: plectin and another bullous pemphigoid antigen, BP230. The amino-terminal globular head of plectin binds directly to the β_4 integrin cytoplasmic tail through its second FNIII repeat and part of the connecting segment (Rezniczek *et al.*, 1998). The carboxy-terminal globular domain of plectin binds to a diverse array of intermediate filaments including vimentin, glial fibrillary acidic protein, cytokeratins, lamin B and all three neurofilament proteins (Green *et al.*, 1992), and may also bind to the microtubule network and to actin. Similarly, the amino-terminal, globular domain of BP230 binds to the intracellular amino-terminus of BP180 and associates with keratins *via* its carboxy-terminal domain (**Figure 1.08B**; Yang *et al.*, 1996).

There is some evidence that the BP180 can bind directly to $\alpha_6\beta_4$ integrin (Schaapveld *et al.*, 1998). This interaction may be facilitated by binding of plectin to $\alpha_6\beta_4$ integrin, which is thought to release the carboxy-terminal tail of β_4 integrin from an intramolecular interaction and thus liberate a binding site for BP180 (reviewed in Nievers *et al.*, 1998). Recently, a protein has been identified which may further serve to cross-link the $\alpha_6\beta_4$

integrin/plectin system with the BP180/BP230 system. ERBIN (**ERB**B2 **In**teracting Protein) is a LAP (**L**RR **A**nd **P**DZ) protein which possesses sixteen amino-terminal **L**eucine **R**ich **R**epeats (LRR) and a carboxy-terminal PDZ domain which has recently been shown to form a complex with both β_4 integrin and BP230 (**Figure 1.08B**; Favre *et al.*, 2001).

The above description applies to Type I hemidesmosomes. Type II hemidesmosomes have also been described, which contain $\alpha_6\beta_4$ integrin and plectin but not the BP antigens. Type II hemidesmosomes are expressed in epithelial cells, such as those of the intestine, which do not express the BP antigens at all. For review, see (Nievers *et al.*, 1998)

CD151 (a member of the tetraspanin family of proteins) has recently been shown to associate with $\alpha_6\beta_4$ integrin, both within hemidesmosomes and in the focal contacts that form at the periphery of hemidesmosomes (Sterk *et al.*, 2000). Tetraspanins comprise a large family of proteins, each of which is predicted to possess four transmembrane domains and two extracellular loops. Interactions between tetraspanins and integrins have been previously reported in the context of focal adhesions (e.g Berditchevski & Odintsova, 1999); however, Sterk *et al.* suggest a role for CD151 in the formation and stabilisation of hemidesmosomes.

1.1.14 Hemidesmosomes: Regulation and Signal Transduction

$\alpha_6\beta_4$ Integrin has been implicated in diverse signal transduction events, ranging from pro-apoptotic to pro-migratory and pro-survival signalling, depending on the specific cell type investigated (reviewed in Nievers *et al.*, 1998). However, the issue of signal transduction from the hemidesmosome *per se* has been complicated by the fact that $\alpha_6\beta_4$ integrin expression is not restricted to these structures in cells that express them. Overall, it appears that hemidesmosomal plaques do not contain the kind of adaptor molecules, kinases and phosphatases that are associated with signalling centres, and that hemidesmosomes are probably not involved in initiating or propagating signal transduction cascades. Hemidesmosomes are, however, thought to be subject to

regulation by signal transduction events incident on $\alpha_6\beta_4$ integrin. In particular, the β4 chain has been shown to be susceptible to phosphorylation on both tyrosine and serine/threonine residues.

The epidermal growth factor receptor (EGFR) tyrosine kinase, ErbB2 has been shown to associate with $\alpha_6\beta_4$ integrin. Furthermore, tyrosine phosphorylation of the β_4 chain in response to epidermal growth factor (EGF) stimulation (together with ligation of $\alpha_6\beta_4$ integrin by laminin 5) promotes hemidesmosomal disassembly and increases the migratory response to laminins (Mainiero *et al.*, 1996). The physical and/or functional association of ErbB2 with $\alpha_6\beta_4$ integrin (Favre *et al.*, 2001) in hemidesmosomes may be facilitated by ERBIN, which was originally identified as an ErbB2 binding protein (Borg *et al.*, 2000).

Conflicting data have been accumulated regarding a proposed Tyrosine Activation Motif (TAM) within the connecting segment of the β_4 integrin cytoplasmic tail. Tyrosine residues within this motif are rapidly phosphorylated in response to ligation of $\alpha_6\beta_4$ integrin (Mainiero *et al.*, 1995). Originally, site-directed mutagenesis studies involving tyrosine-to-phenylalanine mutations confirmed the hypothesis that phosphorylation of TAM tyrosines was essential for localisation of $\alpha_6\beta_4$ integrin to hemidesmosomes (Mainiero *et al.*, 1995). More recently, however a β_4 deletion mutant which lacked the entire TAM sequence localised efficiently into hemidesmosomes (Niessen *et al.*, 1997; Schaapveld *et al.*, 1998) and the role of the TAM sequence has been called into question.

$\alpha_6\beta_4$ integrin has recently been identified as a substrate for PKC-mediated serine phosphorylation. In primary keratinocytes in culture, as well as in some carcinoma cell lines, PKC-mediated phosphorylation of the β_4 chain of $\alpha_6\beta_4$ led to redistribution of $\alpha_6\beta_4$ integrin from hemidesmosomes into either a cytosolic fraction, or into actin-rich cellular protrusions (Alt *et al.*, 2001; Rabinovitz *et al.*, 1999). In both cases, the cells exhibited decreased attachment to matrix and an augmented migratory phenotype.

Taken together, these data suggest that hemidesmosome structure and function may be modulated by pro-migratory signalling. Such signalling, resulting in phosphorylation of hemidesmosomal β_4 integrin, may cause a shift from the stable interaction of $\alpha_6\beta_4$ with cytokeratins to a more dynamic interaction with the actin cytoskeleton, thereby facilitating epithelial cell migration.

1.1.15 Focal Contacts: Physiology and Morphology

Focal contacts were initially described in the early 1970s in experiments using imaging techniques such as interference-reflection microscopy and electron microscopy. These experiments demonstrated the existence of fairly large (a few μm^2), elongated regions of the basolateral plasma membrane which were in close contact (gap ~15 nm) with the substrate (Abercrombie *et al.*, 1971; Abercrombie *et al.*, 1975; Izzard & Lochner, 1976). These structures are now known to provide a link between the extracellular matrix and the actin cytoskeleton, influencing such fundamental cellular functions as adhesion, spreading, migration and morphogenesis, all in association with extracellular matrix.

1.1.16 Focal Contacts: Molecular Composition

The number of proteins known to associate with focal contacts currently stands at over fifty (**Table 1.2**). The picture is further complicated by the fact that many of these proteins are subject to post-translational modification, proteolytic processing and/or alternative transcription. Expression of individual focal contact components, or of specific splice variants, may display both tissue-specificity and developmental regulation. A full and comprehensive description of focal contacts is thus beyond the scope of this introduction but is well reviewed in (Zammer & Geiger, 2001). However, key concepts pertaining to focal contact structure and function can be discussed by adopting a modular approach to both content and signalling.

Adhesion to matrix provided by focal contacts is primarily mediated by integrins, which are heterodimeric matrix receptors consisting of one α and one β subunit (**Figure 1.09**). There are at least eighteen different α subunits and eight β subunits, which can combine to produce around twenty-five different integrin receptors. Ligand binding specificity is

conferred by the large extracellular domains and is defined by the precise combination of α and β subunits. Most integrins are capable of binding to more than one extracellular matrix ligand, and most matrix components can be bound by more than one integrin. Consequently, there is a considerable degree of apparent redundancy in the system from the point of view of receptor-ligand interactions. Notwithstanding, it seems that certain individual integrin subunits may perform unique and necessary functions which, when lost, cannot be compensated by the cell. For example, targeted deletion of the β_4 subunit in mice results in post-natal mortality (van der Neut *et al.*, 1996).

Integrin α and β subunits generally possess short cytoplasmic tails which are nevertheless capable of binding to cytoplasmic plaque proteins. For example, α integrin cytoplasmic domains are thought to bind directly to calreticulin, calnexin (α_6), actin (α_1 and α_2) and paxillin (α_4); and β subunit cytoplasmic domains are capable of binding both structural proteins (e.g. talin and α-actinin) and signalling molecules (e.g. PKCα). Reviewed in (Petit & Thiery, 2000)

$\alpha_6\beta_4$ and $\alpha_3\beta_1$ integrins are of particular interest in epithelial cells. $\alpha_6\beta_4$ integrin (described in detail above) is a component of hemidesmosomes which, under the influence of pro-migratory signalling, is transferred from these (relatively static) structures into (more dynamic) focal contacts, where it participates positively in migration (Rabinovitz *et al.*, 1999). $\alpha_3\beta_1$ integrin is also expressed in several epithelia but, in contrast to $\alpha_6\beta_4$ integrin, is associated with focal contacts and not hemidesmosomes. Both $\alpha_6\beta_4$ and $\alpha_3\beta_1$ are receptors for laminin 5; however, whereas $\alpha_6\beta_4$ integrin ligation in focal contacts is pro-migratory, $\alpha_3\beta_1$ integrin is thought to negatively regulate migration (Hodivala-Dilke *et al.*, 1998). Accordingly, up-regulation of $\alpha_6\beta_4$ integrin in epithelial tumours is associated with increased migration and invasiveness (Mercurio *et al.*, 1994), whereas $\alpha_3\beta_1$ integrin expression is generally down-regulated in tumours of epithelial origin (Pignatelli *et al.*, 1994).

Tyrosine Kinases	*Ser/Thr Kinases*	*Phosphatases*	*GTPase Modulators*	*Other Enzymes*	*Actin-Binding Scaffold Proteins*	*Non Actin-Binding Scaffold Proteins*	*Trans-Membrane proteins*
Abl	ILK	LAR-PTP	ASAP1	Calpain II	α-Actinin	CAS	Integrin
Csk	PAK	PTP1B	GRAF	PI3K	Actopaxin/ Parvin	Caveolin-1	CD98
FAK	PKC	SHP-2	PKL	PLC-γ	EAST	CH-ILKBP	IAP
Pyk2			PSGAP		ERM	Crp1	LAR-PTP
Src					Filamin	DOCK180	Layilin
					Fimbrin	Grb-7	Polycystin
					Nexilin	Hic-5	Syndecan-4
					Profilin	LIP.1	SHPS-1
					Talin	Palladin	Tetraspanins
					Tensin	Paxillin	uPAR
					VASP/ENA	Ponsin	
					Vinculin	Syndesmos	
					Zyxin	Syntenin	
						Vinexin	
						PINCH	

Table 1.2: A List of Proteins Found in Focal Contacts.

The focal contact constitutes a massive structural and signalling complex which contains transmembrane proteins, protein serine/threonine kinases, protein tyrosine kinases, protein phosphatases, modulators of small GTPases, lipid modifying enzymes, the protease calpain II, actin-binding scaffold proteins and non actin-binding scaffold proteins. Adapted from (Zammer & Geiger, 2001).

Tetraspanins are commonly associated with integrins at focal contacts, and appear to assist in integrin-mediated signalling. The mechanism for this remains unclear, although

the tetraspanins are known to bind to phosphatidylinositol-4-kinase (PI4K) (Berditchevski *et al.*, 1997) and may thus link integrins to the phosphoinositide signalling system. Additional transmembrane components of focal contacts include the proteoglycan, syndecan 4 (Woods & Couchman, 1994); the hyaluronan receptor, layilin (Bono *et al.*, 2001); the integrin-associated protein, IAP, which may function as a calcium-channel (Schwartz *et al.*, 1999); the amino acid transporter, CD98 (Fenczik *et al.*, 1997); and several growth factor receptors (e.g. Bartfield *et al.*, 1993). The significance of the association of these proteins with focal contacts is not generally understood, with the exception of the growth factor receptors which are known to participate directly in integrin signalling events. Reviewed in (Eliceiri, 2001).

The cytoplasmic plaque of focal contacts contains both structural and signalling elements. Structural components include actin-binding scaffold proteins such as vinculin, α-actinin, talin, tensin, and profilin; as well as non actin-binding scaffold proteins such as paxillin and Hic-5. Signalling elements of focal contacts include tyrosine kinases, such as focal adhesion kinase (FAK), the FAK-related kinase, PYK2 and Src; serine/threonine kinases, such as the integrin-linked kinase (ILK) and PKCα; protein tyrosine phosphatases (PTP), such as PTP1B; proteins which function in small GTPase signalling, including GRAF (GTPase Regulator Associated with FAK); at least one protease (calpain II); and the lipid-modifying enzymes phosphatidlyinositol-3-kinase (PI3K) and phospholipase Cγ (PLCγ). See **Table 1.02**, reviewed in (Zammer & Geiger, 2001).

Assembly of focal contacts is initiated by ligation of integrin receptors with their extracellular matrix ligands, which results in integrin clustering. Clustering of integrins into focal contacts is apparently dependent on the β subunit cytoplasmic tail (Hayashi *et al.*, 1990; LaFlamme *et al.*, 1992). Conversely, the α subunit cytoplasmic domain does not appear to play a direct role in matrix-mediated integrin clustering, although it may be involved in dictating ligand specificity of insertion into focal contacts (Brieswitz *et al.*, 1993). According to one model, the earliest event following integrin clustering is the recruitment of FAK and tensin to the integrin cytoplasmic domain. This is in turn followed by the phosphorylation-dependent recruitment of α-actinin, talin and vinculin,

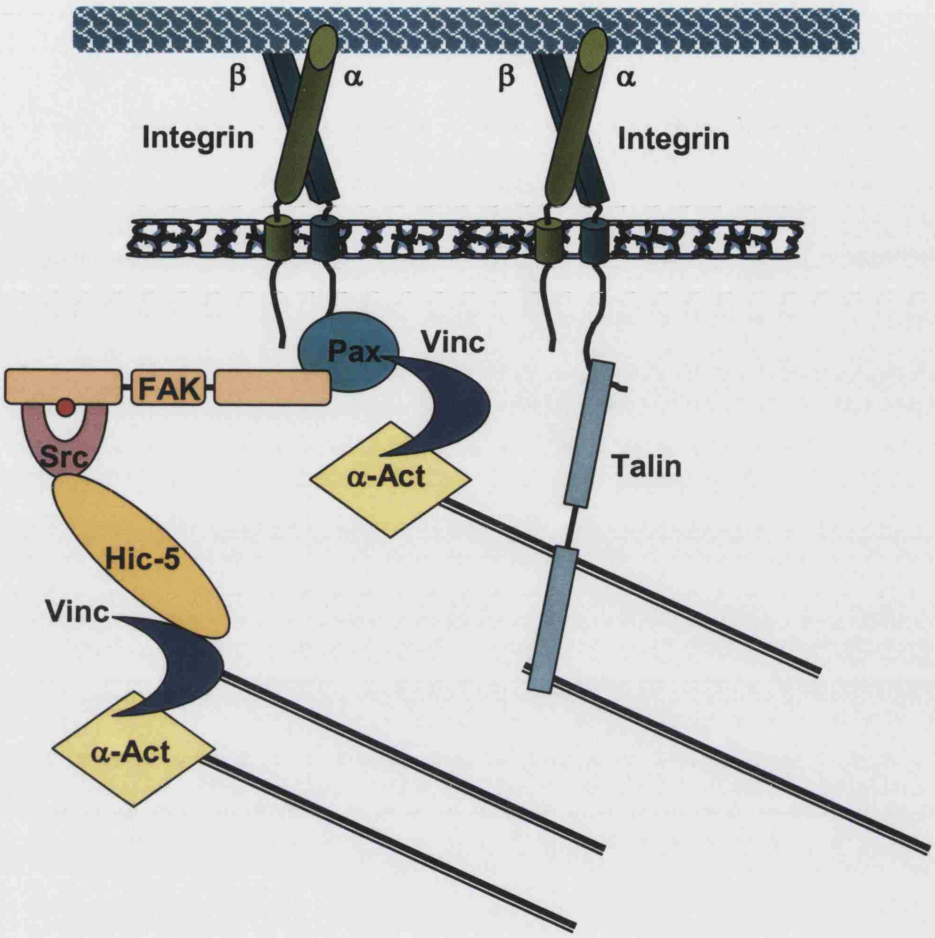

Figure 1.09: Examples of Protein Interactions in Focal Contacts.

α/β Integrin heterodimers bind to extracellular matrix ligands, which results in integrin clustering. The cytoplasmic tails of β integrin subunits bind to a wide variety of cytoplasmic proteins, including paxillin (Pax) and talin, which are in turn capable of binding several other focal contact proteins. Some of these, such as talin, α-actinin (α-Act) and vinculin (Vinc) are actin-binding proteins; others, such as Src and FAK, are signal-transducing enzymes; and still others, such as paxillin and Hic-5 are non actin-binding scaffold proteins. Assembly of the complex is dependent on both structural and signalling elements. Each protein in the complex is capable of binding to other molecules, and over fifty proteins have already been found to participate in focal contacts (see **Table 1.2**).

which permits the recruitment of other focal contact proteins and the association of actin filaments with the focal contact (Miyamoto *et al.*, 1995; reviewed in Petit & Thiery, 2000).

It is probably not possible to accurately describe focal adhesion assembly beyond this sort of level due the redundancy of protein-protein interactions (**Figure 1.09, Figure 1.11**). Most components of focal contacts possess binding sites for several other focal contact proteins. It is not uncommon, for example, for an individual focal contact protein to possess a number of Src-homology 2 (SH2) domains for binding to phosphotyrosines, a number of SH3 domains for binding to proline-rich sequences, and an actin-binding domain. A corollary of this is that a given focal contact containing exactly the same components could, theoretically, assemble in many different ways. Interestingly, there appears to be no single focal contact protein whose presence is critical for focal contact formation, such that cells from vinculin knockouts, paxillin knockouts and FAK knockouts are all able to generate structures resembling focal contacts, albeit with altered morphology and distribution (Ilic *et al.*, 1995; Hagel *et al.*, 2002; Xu *et al.*, 1998). This apparent redundancy is reflected in the relatively late-stage embryonic lethality of these knockout phenotypes. For example, FAK knockout mice survive until the eighth day of embryonic development (E 8), which suggests that until this point FAK function is being compensated for by other focal contact components.

1.1.17 Focal Contacts: Signal Transduction

The assembled focal contact is not only a massive sub-cellular structure, but is also a complex signalling entity (**Table 1.2**). Transduction of extracellular matrix signals by integrin receptors has been implicated in several fundamental cellular processes, including cell cycle regulation, mitogenesis, migration, survival, apoptosis and anoikis (apoptosis due to suspension of adherent cells), see (Giancotti, 1997; Cox & Huttenlocher, 1998; Miao *et al.*, 1997).

Much work has been carried out regarding the activation of the Mitogen-Activated Protein Kinase (MAPK or MAP kinase), ERK (for Extracellular signal Regulated Kinase), by integrin signalling. According to the classic model, recruitment of FAK to the cytoplasmic domains of clustered integrin receptors permits autophosphorylation of FAK on tyrosine 397, creating a high-affinity binding site for Src family kinases. Once bound, Src phosphorylates FAK on additional tyrosine residues (discussed in more detail

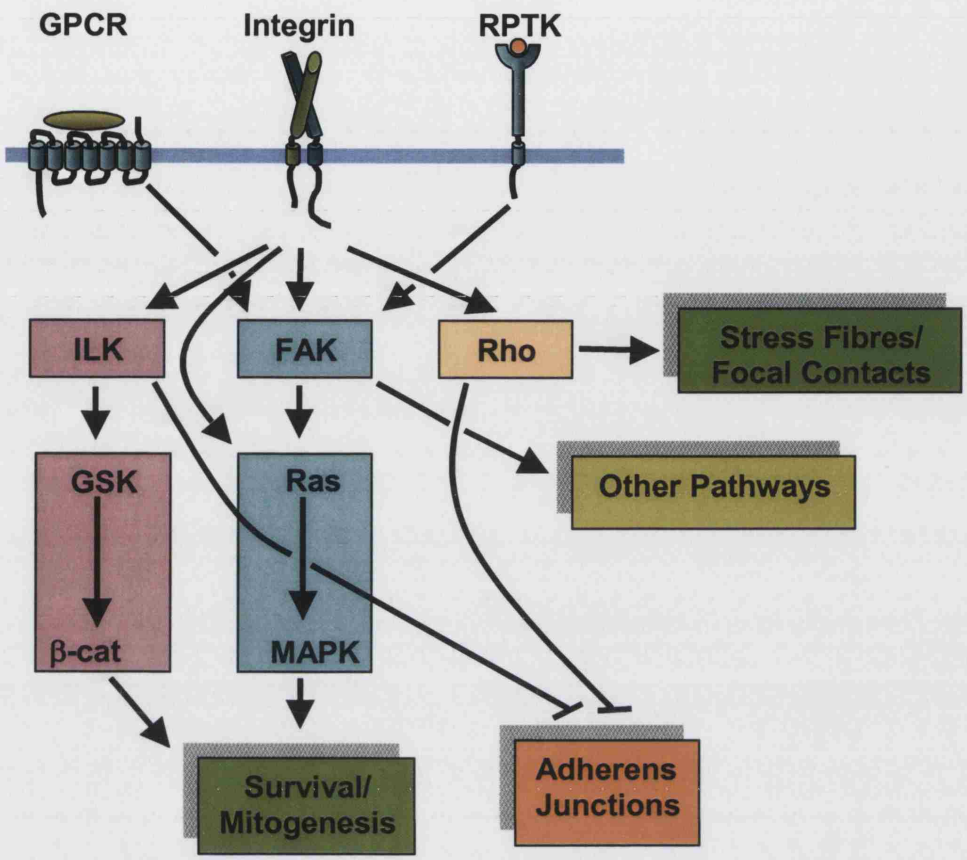

Figure 1.10: Focal Contacts: Divergence and Convergence of Signalling Pathways.

Integrin clustering leads to the recruitment and activation of diverse signal transduction molecules, including FAK, ILK and Rho. Rho activation instigates a cascade which results in the formation of stress fibres and focal contacts, directly modulating adhesion and migration. Active ILK phosphorylates GSK3β, leading to β-catenin stabilisation, transcription of Wg/Wnt responsive genes and, amongst other things, cell survival and proliferation responses. Recruitment of FAK results in its autophosphorylation and subsequent activation of the Ras/MAP kinase pathway leading to survival/proliferation. Integrin signalling can also activate the Ras/MAP kinase pathway independently of FAK, and FAK activation can result in stimulation of other signalling pathways (discussed in detail below). FAK is a point of signal convergence since it can be activated not only by integrins, but also by signalling from receptor protein tyrosine kinases (RPTK) and G-protein coupled receptors (GPCR). Focal contact signalling modulates other adhesive structures. For example, both active Rho and active ILK are known to decrease stability of the cadherin/catenin complex, leading to decreased intercellular adhesion mediated by adherens junctions.

below) and creates a binding site for the adaptor protein, Grb2, which is thus recruited to the complex along with its binding partner, Sos (Son Of Sevenless). Sos is a Guanine

nucleotide Exchange Factor (GEF) for the small GTPase, Ras, promoting the release of GDP and the binding of GTP, and thereby activating Ras. Activated Ras in turn activates the kinase, Raf-1, which activates MEK, which in turn activates ERK, leading to migration and survival/proliferation responses (see **Figure 1.10**). For examples see (Schlaepfer *et al.*, 1997; Clark *et al.*, 1996; Schlaepfer *et al.*, 1994). Other models of integrin-mediated ERK activation have arisen in order to account for experiments in which integrins were able to stimulate ERK activity in the absence of FAK (Lin *et al.*, 1997). In one of these models, caveolin (bound to the inactive form of Fyn) binds directly to integrin cytoplasmic domains. In a subset of integrins, this results in Fyn activation (Wary *et al.*, 1998, Wei *et al.*, 1999). Fyn then phosphorylates the adaptor molecule, Shc, on tyrosine (Wei *et al.*, 1999; Chapman *et al.*, 1999), causing Shc to recruit and activate the Grb2/Sos complex, leading ultimately to activation of the Ras pathway as above. See **Figure 1.10**, reviewed in (Chapman *et al.*, 1999).

Such signalling pathways are not necessarily mutually exclusive. Furthermore, the linear events described above, while useful for the purpose of illustration, are simplified: each point of the pathway can be considered to be a node with potential to receive input from and feed directly into other signalling pathways. For example, several growth factor receptors signal to MAPK using the same signal transduction modules (Zachary *et al.*, 1993; Canbay *et al.*, 1997; Zachary & Rozengurt, 1992), and full activation of MAPK appears to require engagement of both integrins and growth factor receptors (Renshaw *et al.*, 1999). Signalling from the point of FAK alone can activate several other signal-transducing molecules which could potentially lead to MAPK activation, as discussed in detail below. In addition to this there are questions of expression levels and stoichiometry which will add further complexity to this picture as they are characterised. The above pathways are therefore given merely as simplified examples of some of the ways in which ligation of integrin receptors may propagate intracellular signals through focal contacts.

1.2 Focal Adhesion Kinase

1.2.01 An Overview of FAK Function

The non-receptor tyrosine kinase (NRTK), FAK, was originally identified as a hyperphosphorylated protein in v-Src transformed cells (Schaller *et al.*, 1992) and is now known to be a key mediator of both integrin- and growth factor-mediated signalling. Full activation of FAK signalling pathways appears to require input from both integrin receptors and receptors for soluble growth factors. The growth factor receptor component of FAK activation can come from receptor tyrosine kinases and/or G protein-coupled receptors (GPCR). FAK is involved in adhesion-dependent signalling from a host of receptors including the receptors for insulin, insulin-like growth factor (Pillay *et al.*, 1995), platelet-derived growth factor (Cospadel *et al.*, 1999), nerve growth factor (Park *et al.*, 2000), vascular endothelial growth factor (Abedi & Zachary, 1997), bombesin, vasopressin (Zachary *et al.*, 1993), prolactin (Canbay *et al.*, 1997) and the muscarinic m3 receptor for acetylcholine (Slack, 1998).

FAK signalling has been implicated in the regulation of a truly diverse range of cellular events, including cell spreading (Owen *et al.*, 1999), migration (Hauck *et al.*, 2000; Cary *et al.*, 1996), survival signalling from insoluble matrix components (Ilic *et al.*, 1998; Frisch *et al.*, 1996; Valentinis *et al.*, 1998), survival signalling from soluble growth factors (Oktay *et al.*, 1999), transition from G_1 to S phase of the cell cycle (Oktay *et al.*, 1999), T-cell co-stimulation (Berg & Ostergaard, 1997), neurite outgrowth (Ivankovic-Dikic *et al.*, 2000; Anneren *et al.*, 2000), and developmental morphogenesis (Hens & DeSimone, 1995). A role for FAK has also been described in some pathophysiological processes, including cellular invasion by bacterial pathogens such as *E. coli* (Reddy *et al.*, 2000) and *Yersinia pseudotuberculosis* (Alrutz & Isberg, 1998), hypertrophy of cardiac myocytes (Laser *et al.*, 2000) and the acquisition of an invasive phenotype in certain tumours (Maung *et al.*, 1999; Kornberg, 1998).

The critical role of FAK in regulating cell biology is highlighted by the remarkable degree of FAK sequence conservation between species. Human FAK bears

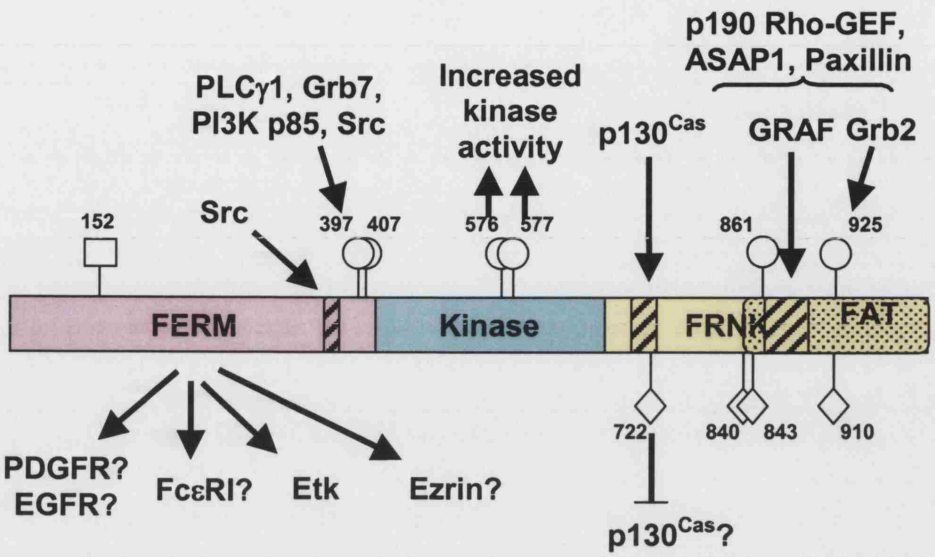

Figure 1.11: Molecular Anatomy of Focal Adhesion Kinase.

FAK is a member of a small family of non-receptor tyrosine kinases which share a common domain structure: the central kinase domain (blue) is flanked by large, non-catalytic, amino-terminal (pink) and carboxy-terminal (yellow) domains. The carboxy-terminal domain of FAK may be expressed as an alternative transcript (FRNK, for **F**AK **R**elated **N**on-**K**inase) which inhibits FAK function by competing for binding sites in focal contacts. FRNK contains the **F**ocal **A**dhesion **T**argeting domain (FAT, stippled) which is critical for localisation of FAK to focal contacts and which contains all of the FAK-inhibitory function of FRNK. The amino-terminal domain has recently been identified as a FERM domain (for band **f**our point one/**E**zrin/**R**adixin/**M**oesin) and is thought to promote interactions with proteins such as the PDGF receptor, the EGF receptor, FcεRI (high-affinity IgE receptor), Etk (**E**pithelial and endothelial **T**yrosine **K**inase) and ezrin, although it is not yet clear whether all of these interactions are directly mediated by the FERM domain. FAK is phosphorylated on several tyrosines (open circles) by itself (Y397) and by Src family kinases (all others), creating high affinity binding sites for the SH2 domains of several signalling molecules, as indicated. FAK also possesses three proline rich sequences (diagonal stripes) which are binding sites for at least three distinct SH3 domain-containing proteins, as indicated. FAK is now known to be susceptible to cell cycle-dependent phosphorylation (open diamonds) on four different serine residues. Although the function of these serine phosphorylation events remains largely uninvestigated, it is thought that phosphorylation of S722 (which is within the p130Cas-binding domain) might disrupt the FAK/p130Cas complex, and that this may somehow be necessary for progression through interphase of the cell cycle. FAK is also subject to sumoylation on lysine 152 (open square), creating a signal for active nuclear import.

approximately 90% sequence identity with *Xenopus laevis* FAK (Zhang *et al.*, 1995) and 93% identity with chicken FAK at the amino acid level (Schaller *et al.*, 1992). The fact that FAK is expressed in nearly all cell types tested, and in organisms ranging from

Drosophila to human, further argues its fundamental role in basic cellular processes. Finally, the embryonic lethality of FAK knockout mice, and the fact that cells from these mice cannot be cultured on a normal p53 background (Ilic *et al.*, 1995) indicates that FAK is essential for both normal organismal development and for normal cell division.

1.2.02 FAK Domain Structure

FAK is a member of a small family of NRTK that includes the proline-rich tyrosine kinase 2 (Pyk2) (Avraham *et al.*, 1995) and a single *Drosophila* gene product, Dfak56, which bears homology to both FAK and Pyk2 (Fujimoto *et al.*, 1999). FAK family members exhibit a characteristic domain structure (**Figure 1.11**) in which a central kinase domain (amino acids 421-680 in murine FAK) is flanked by large, non-catalytic, amino-terminal (amino acids 1-420) and carboxy-terminal (amino acids 681-1052) domains.

The carboxy-terminal domain of FAK is expressed in some tissues as an alternative transcript known as FRNK or p41/42^{FRNK} (for FAK-Related Non-Kinase; Nolan *et al.*, 1999). FRNK antagonises FAK signalling by competing for binding sites in focal contacts (Richardson & Parsons, 1996). FRNK contains a Focal Adhesion Targeting (FAT) domain (840-1052, see **Figure 1.11**) which binds directly to paxillin (Hildebrand *et al.*, 1995; Hayashi *et al.*, 2002), is the sole determinant in targeting FAK to focal contacts, and mediates all of the FAK-inhibitory properties of FRNK (Mortier *et al.*, 2001).

The amino-terminal domain of FAK bears distant homology to FERM proteins. This homology was discovered by an investigator who used the amino-terminal domain of murine FAK as a query for the BLAST2 gapped search algorithm at the NCBI non-redundant database. This search retrieved distant homology between the amino terminus of FAK and the FERM domain of talin. Comparisons were further refined using Hydrophobicity Cluster Analysis (HCA) and secondary structure prediction. Statistically significant homology was identified between the amino-terminal domain of FAK and classical FERM domains (Girault *et al.*, 1998; Girault *et al.*, 1999).

The archetypal FERM domain is that of the band 4.1 protein of the erythrocyte membrane. Band 4.1 acts as an adaptor between the spectrin- and actin-based cytoskeletons and integral membrane proteins. The cytoskeletal binding function is mediated by a short, carboxy-terminal motif whereas binding to the cytoplasmic tails of transmembrane proteins (e.g. glycophorin C) is performed by the FERM domain, which is expressed at varying lengths from the amino-terminus. This overall domain structure is shared with the Ezrin/Radixin/Moesin (ERM) family of proteins but the highest degree of sequence preservation is in the membrane-binding domain, which is thus referred to as a FERM domain (for review see Mangeat *et al.*, 1999; Bretscher *et al.*, 1999).

FERM domains are commonly subject to intramolecular inhibition by carboxy-terminal regions ("head-to-tail" inhibition) and can be activated by signalling events. For example, the head-to-tail inhibition of ezrin can be relieved by PKCα-mediated phosphorylation (Ng *et al.*, 2001).

The FAK FERM domain contains Y397, the role of which in FAK signalling is described below. The size of this domain, however, argues in favour of biologic function(s) beyond those mediated by this single tyrosine residue, and investigators have recently attempted to define further functional roles for it. One paper suggests that the FAK FERM domain may bind directly to EGF receptors and/or the Platelet-Derived Growth Factor Receptor (PDGFR) whilst the FAT domain is bound to integrins at focal contacts. The authors argue that this could be a mechanism for the integration of growth factor and integrin receptor signals regulating migration in murine fibroblasts (Sieg *et al.*, 2000). In another paper, Vial *et al.* demonstrated that the FERM domain could reconstitute the signalling cascade of the high-affinity IgE receptor (FcεRI) in a mast cell line that is deficient in FcεRI-mediated histamine secretion. FAK levels in these cells are low and, since exogenously expressed full-length FAK is able to reconstitute the same signalling cascade, the authors argue that FAK interacts functionally with FcεRI *via* its FERM domain (Vial *et al.*, 2001). Another group has identified the tyrosine kinase Etk (for Epithelial and endothelial Tyrosine Kinase) as a mediator of integrin signalling, claiming that the Pleckstrin Homology (PH) domain of Etk interacts directly with the FAK FERM

domain (Chen *et al.*, 2001). Finally, an additional group has shown that recombinant ezrin which lacks the carboxy-terminal domain can form an immunoprecipitable complex with the FAK FERM domain (Poullet *et al.*, 2001). These authors claim that their truncated construct corresponds to activated ezrin and that its proposed interaction with the FAK FERM domain could be of physiological relevance, although they demonstrate no interaction of endogenous FAK and endogenous ezrin.

The FAK FERM domain is still poorly understood. It is interesting to note, for example, that of the papers published to date on the FAK FERM domain, none of them have reported immunocytochemical co-localisation of the FERM domain with the proposed interacting partners. Overall, the FAK FERM domain is currently thought to be involved in cross-linking growth factor receptors to integrin-containing focal contacts. Evidence showing that overexpression of the FAK FERM domain can inhibit cell migration has been interpreted as evidence that this domain might bind directly to the FAK FAT domain, a form of head-to-tail inhibition that is commonly observed for FERM-domain-containing proteins. Additionally, several authors have reported nuclear localisation of putative FAK FERM domain proteolytic fragments (Lobo & Zachary, 2000, Stewart *et al.*, 2000, Jones & Stewart, 2004). Nuclear FAK leptomycin B (Jones & Stewart, 2004), implying that nuclear FAK fragments are subject to active import by the nuclear import machinery. The mechanism for nuclear targeting of FAK fragments appears to involve the recently discovered SUMOylation (SUMO stands for small ubiquitin-like modifier) of FAK at lysine 152 (Kadare *et al.*, 2003). The biologic function of nuclear FAK remains, however, a mystery and one can only speculate concerning a possible role in cross-linking nuclear envelope proteins to the nuclear lamin matrix or, potentially, more radical roles such as the direct modulation of gene transcription events.

1.2.03 FAK is Regulated by Phosphorylation

FAK molecular function is regulated by multiple phosphorylation events and protein-protein interactions in each of its domains. To date, six tyrosine phosphoacceptor sites have been identified within FAK: Y397, Y407, Y576, Y577, Y861 and Y925. FAK autophosphorylation at Y397 generates a high-affinity binding site for the SH2 domains

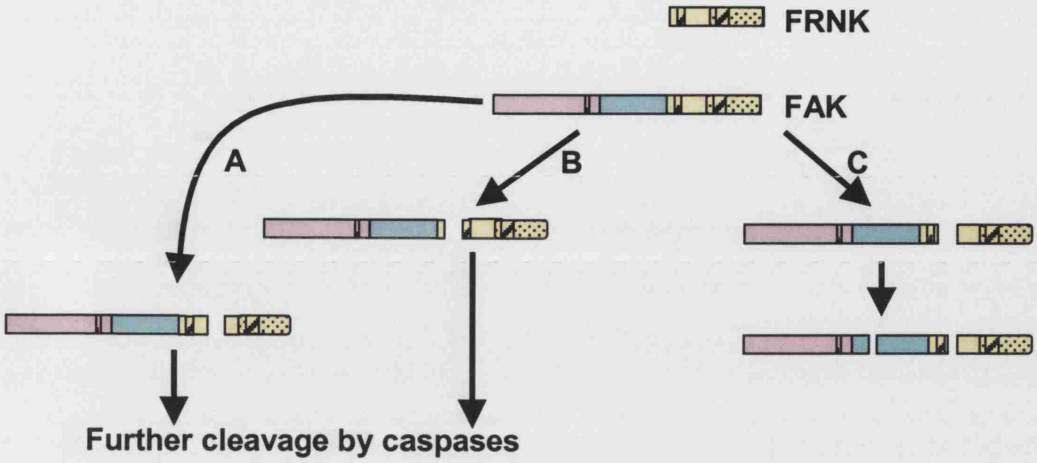

Figure 1.12: Regulation of FAK by Proteolytic Cleavage.

Cleavage of FAK during the early phase of apoptosis by caspase 3 (**A**); and caspase 6 (**B**) generates carboxy-terminal fragments containing the FAT domain (yellow stipples) plus amino-terminal fragments containing the FERM (pink) and kinase (blue) domains. Calpain-mediated FAK cleavage (**C**) liberates similar carboxy-terminal fragments but also generates a fragment roughly corresponding to the FAK FERM domain plus a third fragment consisting of the majority of the kinase domain. The carboxy-terminal fragments liberated by these processes presumably compete with full-length FAK for binding sites at focal contacts, thereby destabilising focal contacts and antagonising FAK signalling.

of Src family kinases (Schaller *et al.*, 1999), the regulatory subunit (p85) of PI3K (Chen *et al.*, 1996), the adaptor molecule Grb7 (Han *et al.*, 2000; Han *et al.*, 1999), PLCγ1 (Zhang *et al.*, 1999) and the protein phosphatase PTEN (Tamura *et al.*, 1999). Src binding to pY397 is thought to result in Src-mediated phosphorylation of all remaining FAK tyrosine phosphoacceptor sites. Src-mediated FAK phosphorylation events result in increased FAK catalytic activity (Y576 and Y577; Calalb *et al.*, 1995) and the creation of an SH2 domain ligand for Grb2 (Y925; Schlaepfer & Hunter, 1996). The functional significance of the phosphorylation events at Y407 and at Y861 has yet to be established. (See **Figure 1.11**).

Recent work on FAK serine phosphorylation has identified residues S722, S840, S843 and S910 as phosphoacceptors (Ma *et al.*, 2001; Yamakita *et al.*, 1999), although the

impact of serine phosphorylation on FAK function is not well understood. Serine phosphorylation of FAK residues S843 and S910 takes place at interphase, coincident with dephosphorylation of FAK tyrosines. One group has provided evidence that phosphorylation of S722 (which is within the $p130^{Cas}$ binding site) disrupts the FAK/Cas complex (**Figure 1.11**; Yamakita *et al.*, 1999) and may thus attenuate FAK signalling.

FAK possesses three proline-rich, SH3 domain ligands (P1-P3). P1 (368-378) may be a ligand for the SH3 domains of Src family kinases (Thomas *et al.*, 1998), augmenting the principal interaction between Src and Y397. P2 (712-733) is a ligand for the SH3 domain of $p130^{Cas}$ (Cary *et al.*, 1998; Harte *et al.*, 1996); and P3 (863-913) provides a docking site for the Rho-GAP, GRAF (see **Figure 1.11**; Hildebrand *et al.*, 1996).

1.2.04 Does FAK Interact Directly with β Integrin Subunits?

A direct interaction between FAK and β integrin cytoplasmic tails has been assumed since FAK was first cloned (Schaller *et al.*, 1992), and the FAK FERM domain was proposed to be responsible for this presumed interaction. Evidence supporting this hypothesis came from *in vitro* binding data using recombinant FAK domain constructs and synthetic β_1 integrin cytoplasmic tails. FERM domain-containing constructs were shown to bind to immobilised synthetic β_1 and β_3 integrin subunit cytoplasmic tails with approximately 10% greater efficiency than those constructs that did not contain the FERM domain (Schaller *et al.*, 1995). However, work on the interaction between FAK and β-integrins in whole-cell systems has argued against the validity of these early experiments. For example, FAK FERM domain constructs are incapable of targeting to β-integrin-containing focal contacts unless a FAT domain is present (Cooley *et al.*, 2000). Conversely, FAT is sufficient to direct the localisation of reporter proteins to β integrin-containing focal contacts; and FAK constructs which lack FAT cannot localise to focal contacts. Since FAT can bind directly to paxillin (Hayashi *et al.*, 2002; Mortier *et al.*, 2001), which can in turn bind directly to β integrin cytoplasmic tails (Schaller *et al.*, 1995), it is conceivable that the FAT/paxillin interaction is responsible for targeting of FAK to focal contacts. However, one group has succeeded in generating FAT constructs that are able to localise to focal contacts whilst being unable to bind to paxillin (Cooley *et*

al., 2000). These and other authors thus claim that FAT localises to focal contacts by means of a direct interaction with β integrin subunits, however they do not present any direct evidence to support this notion. It is not inconceivable, for example, that FAT could mediate focal contact localisation independently of paxillin binding by interacting with some other integrin-binding protein. Hard evidence in favour of a direct interaction between FAK and β integrins has so far been lacking.

1.2.05 FAK is Subject to Proteolytic Processing

In addition to regulation by phosphorylation, FAK activity can be regulated by proteolysis. It has been conclusively demonstrated that FAK is cleaved by caspases during the early stages of several models of apoptosis (Ilic *et al.*, 1998; Cicala *et al.*, 2000; van de Water *et al.*, 1999; Gervais *et al.*, 1998; Wen *et al.*, 1997). Early cleavage of FAK by caspases 3 and 6 liberates proteolytic fragments corresponding roughly to FRNK. Studies have shown that overexpression of FRNK or FRNK-like constructs constitutes a pro-apoptotic signal (Valentinis *et al.*, 1998; Frisch *et al.*, 1996). This pro-apoptotic role is not surprising since FRNK directly inhibits FAK signalling. However, it is interesting to note that by cleaving FAK and thereby producing FRNK-like peptides, caspases not only abrogate the pro-survival signalling capability of FAK, but may also actively generate a pro-apoptotic signal in the form of FRNK. Caspase-mediated cleavage of FAK also results in the liberation of amino-terminal FAK fragments which are thought to contain both the FERM and kinase domains (Ilic *et al.*, 1998; Cicala *et al.*, 2000; van de Water *et al.*, 1999; Gervais *et al.*, 1998; Wen *et al.*, 1997). The potential function of these amino-terminal FAK fragments in apoptosis has not been investigated.

It is also now well established that FAK is also subject to cleavage by calpain in non-apoptotic, viable cells (Carragher *et al.*, 2001; Carragher *et al.*, 1999; Cooray *et al.*, 1996), which is consistent with localisation of calpain II in focal contacts (Beckerle *et al.*, 1987). Calpain-mediated cleavage of FAK results in the generation of three FAK fragments, corresponding roughly to its FERM, kinase and FRNK-like domains (**Figure 1.12**). It is interesting to note that in both caspase- and calpain-mediated cleavage systems, cells adopt a rounded phenotype consistent with inhibition of FAK function by

FRNK-like proteolytic cleavage products and consequent disassembly of focal contacts. The functional significance of calpain-derived FAK cleavage products has, however, not been thoroughly investigated.

Cleavage of FAK by both caspases and calpain results in the early generation of fragments containing an intact FAK FERM domain (**Figure 1.12**). Furthermore, two recent papers, including work from this laboratory, have described a presumptive proteolytic cleavage product of FAK corresponding to the FERM domain which is constitutively expressed and which is subject to distinct regulation and cellular compartmentalisation (Lobo & Zachary, 200; Jones *et al.*, 2001). It is tempting to consider that such a fragment may possess biologic functions which are distinct form those of full-length FAK, although this hypothesis has not been tested to date.

1.2.06 FAK and Epithelial Cell Biology

The role of FAK in epithelial cell biology is poorly understood, especially when compared with the level of understanding that has been achieved regarding FAK function in fibroblasts. Much of the work on FAK in epithelial cells has focussed on its expression and apparent up-regulation in motile tumours, such as ovarian and prostatic carcinomas (McLean *et al.*, 2001; Judson *et al.*, 1999; Agochiya *et al.*, 1999; McCormack *et al.*, 1997; Owens *et al.*, 1995). The idea that FAK signalling is pro-migratory in normal epithelial cells has been supported by migration data from the intestinal epithelial cell line, Caco-2, and from HGF-induced scatter assays using Madin-Darby Canine Kidney (MDCK) cells (Liu *et al.*, 1998; Lai *et al.*, 200). FAK also appears to play an anti-apoptotic role in epithelial cells, suppressing programmed cell death in carcinoma cells as well as in MDCK cells (van de Water *et al.*, 1999; van de Water *et al.*, 2001; Xu *et al.*, 2000; Grossman *et al.*, 2001; Niu & Nachmias, 2000).

FAK signal transduction remains a relative mystery in epithelial cells, although the data so far accrued do not differ greatly from the picture that has emerged in fibroblasts. Tumour Necrosis Factor α (TNFα), EGF and the Insulin-like Growth Factor-1 (IGF-1) are all thought to signal through FAK in various epithelial cells (Guvakova & Surmacz,

1999; Koukouritaki *et al.*, 1999; Kuwada *et al.*, 1998), and FAK is thought to mediate the activation of MAP kinases in response to extracellular matrix signals in Caco 2 cells (Liu *et al.*, 1998; Sanders & Basson, 2000; Yu *et al.*, 2000a).

1.3 Epithelial Cell Dynamics

1.3.01 Epithelial-Mesenchymal Transitions

Under a variety of different circumstances, mature epithelia may undergo a change in phenotype in response to morphogenic pressures. Epithelial-mesenchymal transition (EMT) is an example of such phenotypic change, in which epithelial cells undergo phenotypic conversion to a fibroblastic phenotype. EMT is seen in physiological processes including vertebrate embryogenesis, fibroblast formation in injured tissues and the initiation of metastasis in epithelial cancer (Hey, 1995, Strutz *et al.*, 1995, Iwano *et al.*, 2002, Kiemer *et al.*, 2001, Janda *et al.*, 2002, Vincent-Salomon & Thiery, 2003 and Xue *et al.*, 2003). If there can be said to be a point to cell biological processes, the point of EMT appears to be the generation of motile or migratory cells from the static mature epithelial population. This concept is most concisely illustrated in epithelial tumour metastasis, in which epithelial cells undergo EMT prior to intravasation (entering the systemic circulation), extravasation (exiting systemic circulation), and the establishment of secondary tumours in distant tissue environments (e.g. Kiemer *et al.*, 2003).

Accordingly, the process of EMT involves the breakdown of epithelial cell-cell contacts and loss of intercellular adhesion. The intermediate filament and cortical actin-based cytoskeletons lose their tethering positions at epithelial lateral membranes, and F-actin is rearranged into stress fibres. Cells undergoing EMT lose their cobbled epithelial architecture, adopt a spindled morphology and express mesenchymal markers (for review see Savagner, 2001). The signal transduction pathways that co-ordinate these complex cellular events have been the subject of a great deal of investigation, generally in cultured cells and cell lines. A broad overview of the current understanding of these molecular processes is described below.

TGFβ is perhaps considered to be the archetypal inducer of EMT (Miettinen *et al.*, 1994, Okada *et al.*, 1997, Derynck *et al.*, 2003, Yao *et al.*, 2004); however, EMT can be induced or facilitated by a number of different cytokines that promote proteolytic degradation of the basement membrane by metalloptoteinases or membrane assembly inhibitors (Cittero & Gallard, 1994, Okada *et al.*, 1997, Morali *et al.*, 2001, Strutz *et al.*, 2002). EMT induction by TGFβ appears to require signalling from β integrin subunits Bhowmick *et al.*, 2001); Smad3-dependent transcription or Smad3-independent activation of MAP kinase (Yu *et al.*, 2002); and signalling through small GTPases (Boyer *et al.*, 1999, Janda *et al.*, 2002). In addition to TGFβ, it has been shown that EGF receptors are concentrated in the EMT microenvironment and that EGF signalling can contribute to the completion of EMT (Cittero & Gallard, 1994, Okada *et al.*, 1997); signalling from IGF-II can contribute to EMT by promoting the disassembly of E-cadherin-containing adherens junctions and promoting β-catenin signalling in the nucleus (Morali *et al.*, 2001); and FGF-2 signalling can contribute to the induction of MMP-2 and MMP-9 (Bhowmick *et al.*, 2001), which assist in degradation of the basement membrane.

The signal transduction pathways that result in EMT bare similarities across many of the epithelia that have been experimentally studied with respect to this phenomenon. Occupancy of receptor tyrosine kinases results in the downstream activation of Ras-family GTPases, and this in turn leads to the activation of the Raf/MAP kinase pathway, leading to the transcription of EMT-associated genes and re-organisation of the actin cytoskeleton (Etienne-Manneville & Hall, 2002, Bar-Segi & Hall, 2000, Ward *et al.*, 2004, Janda *et al.*, 2002). Activation of ILK, either by TGFβ-Smad (Li *et al.*, 2003) or integrin receptor signalling (Novak *et al.*, 1998), promotes the nuclear import of β-catenin, increased β-catenin/LEF-mediated transcription and suppression of E-cadherin. Src kinases are also frequently activated downstream of RPTKs and their activity, via PI3K, promotes stabilisation of β-catenin, survival from apoptosis, and removal of E-cadherin from adherens junctions (Janda *et al.*, 2002, Bhowmick *et al.*, 2001, Behrens *et al.*, 1993, Smith *et al.*, 1992, Avizienyte *et al.*, 2004).

The LEF-Smad3/β-catenin system is emerging as a key element of EMT signalling (Kim *et al.*, 2002, Attisano & Wrana, 2002). Wnt-1, IGF-II, Ras and ILK signalling all result in the accumulation of cytosolic β-catenin (Morali *et al.*, 2001, Novak *et al.*, 1998, Espada *et al.*, 1999, He, 2003), perhaps through the inhibition of GSK3β activity. GSK3β-mediated β-catenin phosphorylation enables β-catenin to form a complex with the adenomatous polyposis coli (APC) inhibitor and Axin, resulting in a loss of free β-catenin through ubiquitination and consequent degradation in the proteasomes (Aberle *et al.*, 1997, He *et al.*, 2003, Ha *et al.*, 2004). Conversely, inhibition of GSK3β results in the stabilisation of cytosolic β-catenin, its nuclear import and formation of the transcriptionally active β-catenin-LEF complex (reviewed by Novak & Dedar, 1999). Interestingly, TGFβ-mediated Smad3 activation can promote LEF-dependent transcription in the absence of β-catenin (Attisano *et al.*, 2002), indicating the synergistic regulation of LEF by more than one EMT signalling pathway.

At the level of transcription factors, much work has been carried out with respect to those that regulate E-cadherin levels, since E-cadherin has been used as a classical marker of EMT. Three zinc-finger proteins, Slug, Snail are thought to bind to the E-cadherin promoter at inhibitory E-box sites, in competition with the basic helix-loop-helix protein SIP-1, and thereby directly downregulate E-cadherin levels (reviewed in Thiery & Chopin, 1999, Come *et al.*, 2004). In addition, this pathway downregulates cytokeratins, muc-1 and desmoplakin, whereas fibroblast-specific protein-1 (FSP1), fibronectin, vitronectin and Rho are increased (Nieto, 2002, Peinado *et al.*, 2003) More recently, Twist has also emerged as an additional transcription factor capable of inducing EMT. Unlike Slug, Snail and SIP-1, Twist is thought to act by binding to and inactivating positive regulators of E-cadherin transcription (Yang *et al.*, 2004, Kareth & Tuveson, 2004). Forced expression of Slug, Snail, SIP-1 or Twist is sufficient to cause EMT in epithelial cell lines. Strikingly, Slug, Snail and SIP-1 are particularly well known for their role in gastrulation and neural crest development in early embryogenesis (Hemvathy *et al.*, 2000, Nakagawara *et al.*, 2004) but are also very well characterised as repression of E-cadherin in epithelial tumours (e.g. Blanco *et al.*, 2002). This relationship underlines the biochemical proximity of physiological and pathological EMTs.

A recent paper has shed interesting light on the mechanism by which EMT signals promote the dissolution of tight junctions (Ozdamar *et al.*, 2005). These authors have demonstrated that the tight junction molecule, Par-6 (a known regulator of epithelial polarity and tight junction assembly) is actually a substrate for the type II TGFβ receptor, TβRII. Using a mammary epithelial model of EMT, they further demonstrated that Par-6 phosphorylation promotes its interaction with Smurf1, leading to Smurf1-mediated ubiquitin ligation of RhoA, degradation of RhoA in the proteasomes and, ultimately, a loss off tight junctions. This work therefore provides an elegant delineation of sequential signalling from an EMT inducer to an effector of tight junction dissolution.

A body or work is beginning to emerge relating to the transcriptomic (Higgins *et al.*, 2003, Zavadil *et al.*, 2001, and proteomic (Demir *et al.*, 2004, Lenferink *et al.*, 2004) responses associated with EMT. Although the number of genes and gene-products known to be either up- or down- regulated during EMT is now fairly large, the functional contribution of most of these is not well understood. Further characterisation of the biologic significance of these downstream gene regulatory events, together with an integrated understanding of the complex interplay between Slug, Snail, SIP-1, Twist, β-catenin/LEF, β-catenin-independent LEF and (more recently) NFkB (Huber *et al.*, 2004) in regulating the EMT transcriptomic response will add a new level of sophistication to our understanding of the EMT process, and provide opportunities for clinical fields relating to cancer therapy and tissue engineering.

1.3.02 The Transepithelial Migration of Polymorphonuclear Cells

Transepithelial migration is observed in mucosal infections, in which large numbers of leukocytes (particularly polymorphonuclear leukocytes, PMN) accumulate at sites of inflammation (Kumar *et al.*, 1982, Nostrant *et al.*, 1987, Surawicz *et al.*, 1994). To emigrate from the blood, PMN require chemotactic stimulation that promote adherence to and migration across the endothelium (transendothelial migration), followed by migration across the inflamed epithelium (transepithelial migration). PMN migrate across simple epithelia by migrating across the normally closed paracellular pathway, involving co-

ordinated opening and closing of epithelial intercellular junctions. Transepithelial migration of PMN consists of three principal stages: adhesion of the PMN to the epithelial basolateral membrane; migration of PMN between apposing epithelial cells; and post-migration adhesion of PMN to the apical epithelial surface. (For review see Dunon *et al.*, 1996, Parkos, CA, 1997 and Huber *et al.*, 1998-9).

Adhesion of PMN to the basolateral surface of epithelial cells is mediated exclusively by the leukocyte β2 integrin, CD11b/CD18 (Parkos *et al.*, 1991), in contrast to transendothelial migration (for review see Worthylake *et al.*, 2001) which appears to involve both CD11b/CD18 and CD11a/CD18. The search for epithelial counter-receptors involved in this initial adhesion step has so far proven fruitless: no basolateral receptor for CD11b/CD18 has yet been shown to modulate PMN transepithelial migration in the basolateral-apical direction. Evidence in favour of an epithelial protein counter-receptor for CD11b/CD18 comes largely from studies showing that CD11b/CD18-mediated adhesion is both sensitive to proteinase K (Miyata *et al.*, 1999) and upregulated under inflammatory conditions (Miyata *et al.*, 1999, Colgan *et al.*, 1996). The most attractive candidate epithelial ligand for CD11b/CD18 might be ICAM-1; however, ICAM-1 is not normally expressed on intestinal epithelia except during inflammation, and then its expression is localised to the apical and not the basolateral surface (Parkos, CA *et al.*, 1996). In airway epithelia, ICAM-1 is expressed at apical cellular boundaries; however, airway epithelia have a more flattened morphology than intestinal epithelia, and it is possible that migrating PMN at the basolateral surface might have access to apical ICAM-1 as a counter-receptor in these cells (Taguchi *et al.* 1998, Jahn *et al.*, 2000).

Interesting preliminary data have been gathered concerning the role of carbohydrate determinants in the initial binding of PMN to epithelial cells. Heparin and heparan sulphate have been shown to bind CD11b/CD18 (Diamond *et al.*, 1995) and to block the initial adhesion of PMN to epithelial cells (Colgan *et al.*, 1995). Unfortunately, however, the search for heparan sulphate proteoglycans that might actually function as epithelial receptors for CD11b/CD18 has yet to yield positive results. For example, Syndecans 1 and 3 are expressed abundantly on epithelial cells but neither of these heparan sulphate

proteoglycans is capable of inhibiting the binding of epithelial cells to purified CD11b/CD18 (CA Parkos, unpublished). One group has published data supporting the notion that fucosylated proteoglycans may be involved in initial PMN-epithelial adhesion, demonstrating that probing of western blots of fucosylated proteins from epithelial cells with purified CD11b/CD18 reveals several candidate proteins (Zen *et al.*, 2002). Unfortunately, at the time of writing this introduction, these candidate proteins remain uncharacterised.

During the migration stage, interactions between epithelial CD47 and PMN Signal Regulatory Protein-α (SRP-α) are thought to enhance the rate of transepithelial migration (Parkos *et al.*, 1996). Accordingly, stable exogenous expression of CD47 in CD47-deficient epithelial cells significantly enhances the rate of PMN transepithelial migration (Liu *et al.*, 2001). This picture is complicated by the fact that CD47 is also expressed on PMN and that *cis*-interactions between PMN CD47 and SRP-α are likely to occur (Han *et al.*, 2000). Evidence supporting the importance of PMN-CD47 in this process comes from studies demonstrating that anti-CD47 antibodies are capable of inhibiting PMN migration on filters (Liu *et al.*, 2001). These antibodies are thought to be activating rather than blocking antibodies, and their effects can be reversed by genestin, a known tyrosine kinase inhibitor (Liu *et al.*, 2001). Conversely, almost nothing is known relating to the initiation of signal transduction events by epithelial CD47. Taken together, these data suggest a pro-migratory role for epithelial CD47 and an anti-migratory role for PMN CD47 in transepithelial migration and raise the possibility that CD47 may function as an initiator of signal transduction events in the migration process, although the field is still in relative infancy and a great many questions remain to be answered.

The SIRP-α counter-receptor for CD47 (Jiang *et al.*, 1999) is a member of a large family of transmembrane glycoproteins that are variously expressed in granulocytes, subsets of other leukocytes, cells of the central nervous system and endothelial cells (for review see Brown *et al.*, 2001). SIRP-α possesses an amino-terminal extracellular domain containing three Ig folds, a single transmembrane domain and an intracellular carboxy-terminal domain. The carboxy-terminal domain of SIRP-α contains tyrosine

phosphorylation motifs, and SIRP-α has been shown to interact with tyrosine phosphatases SHP-1 and SHP-2 in response to ligand (Stofega *et al.*, 2000). Functionally, there is some evidence that CD47 ligation of SIRP-α in whole-cell systems may downregulate the PMN phagocytic response (Oldenborg *et al.*, 2000), and it is thought that this may provide a mechanism for the enhanced transepithelial migration of PMN in response to epithelial CD47. This model implies a positive role for signals emanating from PMN SIRP-α in response to ligation of epithelial CD47. Thus, the SIRP-α-CD47 interaction may result in bidirectional signalling in epithelial and PMN cells.

More recently, the number of Ig superfamily proteins found at epithelial intercellular junctions has grown rapidly, and includes JAMs, nectins, Coxsackie Adenovirus Receptor (CAR) and related proteins. These proteins are generally associated with tight junctions and adherens junctions, and are therefore interesting candidate post-adhesion receptors for transmigrating neutrophils. With respect to transmigration, the largest body of evidence has been gathered on the JAM family of proteins. Initially, studies by Dejana and co-workers demonstrated that monoclonal antibodies against JAM-A inhibited transendothelial migration of both monocytes and neutrophils both *in vitro* and *in vivo* (Martin-Padura *et al.*, 1998, Del Maschio *et al.*, 1999). Further investigation of JAM-A in the transendothelial model demonstrated that it might serve as a receptor for CD11a/CD18, mediating T-cell endothelial adhesion (Ostermann *et al.*, 2002). However, CD11a/CD18 is unlikely to be important for transepithelial migration since blocking antibodies against this integrin receptor fail to inhibit transepithelial migration (Parkos *et al.*, 1991). According to more recent evidence, however, JAM-C is likely to be involved in transepithelial migration. Initially, platelets expressing JAM-C were shown to bind to CD11b/CD18 on PMN, and this binding was inhibited by anti-JAM-C antibodies (Santoso *et al.*, 2002). Work by Zen *et al.* subsequently demonstrated expression of JAM-C in epithelial desmosomes and showed inhibition of PMN transepithelial migration by anti-JAM-C as well as by soluble JAM-C/Fc chimeric molecules. The question of whether or not JAM-C might be acting as a receptor for CD11b/CD18 in initial adhesion rather than paracellular transmigration was addressed using JAM-C blocking antibodies,

which were shown to cause no decrease in the number of basolaterally adhering neutrophils.

After migration across the epithelial tight junction network, PMN employ activation of the NADPH oxidase and release of granules to eliminate pathogens. Anti-pathogenic activity by granulocytes is often a "double-edged sword" causing damage to the surrounding tissue as well as clearing pathogens: tissue damage is a well-known consequence of PMN activation and is prevalent in several mucosal diseases characterised by abundant PMN transepithelial migration. Strangely, in many inflammatory conditions, PMN are retained at the epithelial apical surface. In some instances, such as in ulcerative colitis, this may be caused by the deposition of autoimmune anti-epithelial antibodies, promoting retention of activated PMN (Halstensen *et al.*, 1990, Reaves *et al.*, 2001). Additionally, increased ICAM-1 expression on the apical epithelial membrane during inflammation may serve to tether PMN and macrophages to the luminal surface.

1.4 Thesis Goals

The amino terminus of focal adhesion kinase (FAK) is expressed in several cell types as a proteolytic cleavage product, the biologic function of which is not understood. The hypothesis for this study is that the FERM-domain containing amino terminus of FAK acts as a membrane-cytoskeletal linker, tethering cell-cell contact proteins to the F-actin cytoskeleton in epithelial cells. Furthermore, it is hypothesised that Y397, an autophosphorylation site on FAK, is necessary for amino terminal FAK localisation to cell-cell junctions.

In order to test this hypothesis, the following aims were established:

- To generate (or obtain through collaboration) epitope-tagged and fluorescently-labelled full-length and amino-terminal FAK constructs, including constructs which possess, lack and are mutated in the Y397 site
- To overexpress FAK constructs in two different epithelial cell lines (MDCK and HEK 293) and assess expression by biochemical methods and confocal microscopy
- To precisely define the subcellular localisation of amino-terminal FAK constructs, especially with respect to cell-cell contact structures
- To test for association of amino-terminal FAK with actin, and with cell-cell contact proteins

2 Materials and Methods

2.1 Antibodies and Reagents

Primary antibodies used in this study for immunocytochemistry and western blotting are listed in **Table 2.1** below. HRP-conjugated anti-mouse, anti-rabbit and anti-goat were all obtained from Santa Cruz and were used at a dilution of 1:3000. Protein A/G+ agarose was also obtained from Santa Cruz. Rhodamine Red-X- and FITC-conjugated donkey anti-mouse, anti-rabbit and anti-goat secondary antibodies were all obtained from Jackson Immunoresearch Laboratories. Fluorescently labelled secondary antibodies were resuspended to a final concentration of 0.75 mg/ml and used at a dilution of 1:100. OptiMEM and foetal calf serum were obtained from GIBCO. *Pfu* Turbo was purchased from Stratagene, deoxynucleotide triphosphates (dNTP) were purchased from GIBCO and T4 DNA ligase was purchased from New England Biolabs. All other DNA modifying enzymes and restriction enzymes were obtained from Roche Molecular Biochemicals.

All other media and reagents were obtained from Sigma unless specifically stated otherwise.

2.2 Molecular Biology

A series of FAK-derived constructs were generated to investigate novel functions of the Amino-Terminal domain of FAK (ATF). ATF-386 constructs encode residues 1 to 386 of murine FAK tagged with either a Carboxy-terminal GFP (ATF-386-GFP) or an amino-terminal Flag tag (Flag-ATF-386). ATF-420-GFP encodes residues 1-420 of FAK tagged with Carboxy-terminal GFP. ATF-420F-GFP encodes residues 1-420 of FAK with a Y397F mutation and tagged with carboxy-terminal GFP. In addition, two full-length FAK constructs (Flag-FAK and FAK-GFP), two FAT domain (amino acids 840-1052) constructs (Flag-FAT and GFP-FAT), and a construct encoding amino acid residues 387-839 tagged with a Carboxy-terminal GFP tag (CentFAK-GFP, for "the Central region of FAK"-GFP) were used. See **Figure 2.01** for summary.

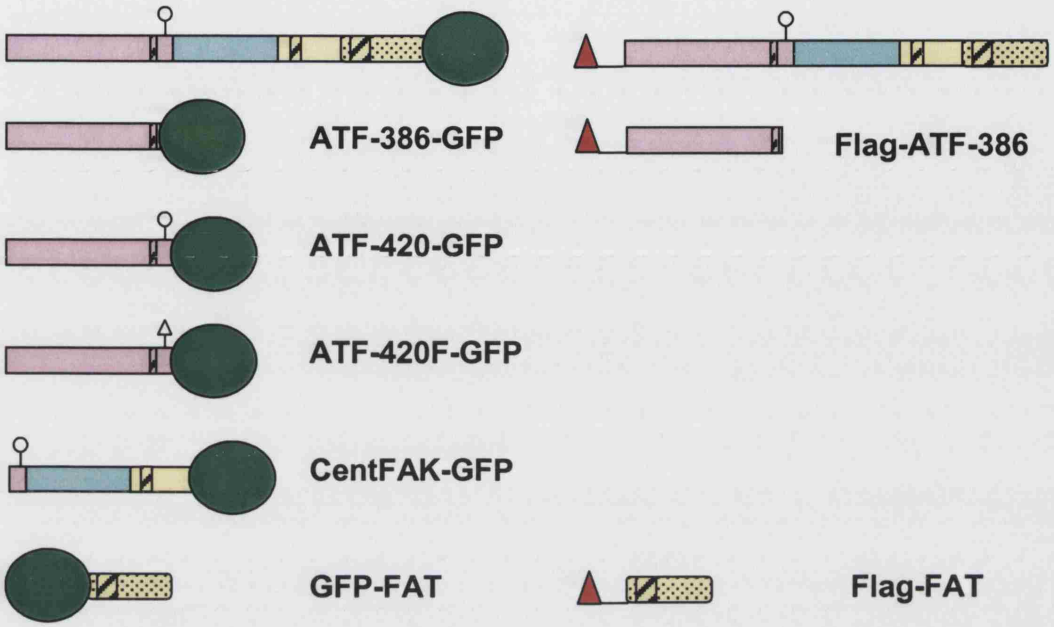

Figure 2.01: Constructs Used in this Thesis.

A series of FAK-derived constructs were used in this thesis. Some of these were "tagged" with the *Aqueoria victoria* Green Fluorescent Protein (GFP, large green oval) and others with the Flag octamer (red triangles). FAK-GFP and Flag-FAK are full-length, wild-type FAK constructs with a carboxy-terminal GFP tag and an amino-terminal Flag tag, respectively. Amino-Terminal FAK (ATF) constructs encode FAK residues 1-386 (ATF-386-GFP and Flag-ATF-386), 1-420 (ATF-420-GFP) and 1-420 with a Y397F mutation (ATF-420F-GFP), possessing the Flag and GFP tags as indicated. CentFAK-GFP contains the Central region of FAK from T387 until the limit of the FAT domain and possesses a carboxy-terminal GFP tag. GFP-FAT and Flag-FAT correspond to the Focal Adhesion Targeting domain with amino-terminal GFP and Flag tags, respectively. The colour scheme of the various FAK regions is the same as that in **Figure 1.11**. Y397 is represented as an open circle and the Y397F mutation is represented as an open triangle.

The full-length murine FAK cDNA cloned into pBluescript (pBS/FAK) was generously provided by Dr JT Parsons (Department of Microbiology and Cancer Center, University of Virginia). ATF-420-GFP and ATF-420F-GFP were the kind gift of Dr Lucia Beviglia and Dr Bill Cance (Department of Surgery and Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill School of Medicine). Full-length

Flag-FAK and full-length FAK-GFP were kindly provided by Dr Barbara Herren (Department of Medicine, University College London). All other constructs were generated by PCR subcloning using pBS/FAK as template, or by restriction enzyme subcloning, as described below.

2.2.01 PCR Primers and Restriction Enzyme Subcloning

Flag-ATF-386 was generated using the 5′ primer 5-TAG CGG CCG CCA CCA TGG ACT ACA AGG ACG ACG ATGACA AGG CAG CTG CTT-3′ and the 3′ primer 5′-AGT GAA AAG CAA GGC ATG CGG ACA TAG TGA GGA TCC GTG TCA-3′ and cloned into the Not I and BamH I sites of pcDNA 3.1 (-).

Flag-FAT was generated using the 5′ primer 5′-TAG CGG CCG CCA CCA TGG ACT ACA AGG ACG ACG ATG ACA AGC TCT CTC GAG GGC AGC ATC-3′ and the 3′ primer ACG CGG ATC CTT ATC AGT GTG GCC GTG TCT GCC C-3′ and was cloned into the Not I and BamH I sites of pcDNA 3.1 (-).

ATF-386-GFP was generated using the 5′ primer 5′-ACT GCT CGA GCG CCA CCA TGG CAG CTG CTT ATC TTG AC-3′ and the 3′ primer 5′-TGA CAC GGA TCC TGT CCG CAT GCC TTG CTT TTC ACT-3′ and was cloned into the Xho I and BamH I sites of pEGFP-N3.

CentFAK-GFP was generated using the 5′ primer 5′-GCA TCT CGA GCA CCA TGG CAG TCT CTG TGT CAG AGA CA-3′ and the 3′ primer 5′-GAT GGA TCC GAG CCT CAC ATC AGG TTT CAG GAA-3′ and was cloned into the Xho I and BamH I sites of pEGFP-N3.

FAT-GFP was generated by excising the Xho I - BamH I fragment from Flag FAT and cloning it directly into pEGFP-C1.

2.2.02 Polymerase Chain Reaction

PCR was performed using *Pfu* Turbo DNA polymerase. This enzyme was chosen for its proof-reading capability, which reduces the error incorporation rate by approximately seven-fold as compared with normal *Taq* polymerase. PCR reactions were set up in 250 µl thin-walled tubes according to the following general formula:

Distilled water	76.0 µl
10 x Buffer	10.0 µl
dNTP (10 mM each)	2.0 µl
Template DNA (100 ng/µl)	1.0 µl
5′ Primer (10 µM)	5.0 µl
3′ Primer (10 µM)	5.0 µl
Pfu Turbo (2.5 U/µl)	2.5 µl

The *Pfu* Turbo reaction buffer at 1 x concentration (20 mM Tris-HCl pH 8.8, 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton-X-100, 0.1 mg/ml nuclease-free BSA) contained Mg^{2+} ions at a concentration that was suitable for all PCR reactions carried out. PCR reaction mixtures were subjected to thermocycling in a PTC-100™ programmable thermocycler (MJ Research, Inc.) with the heated lid enabled as follows:

Initial Melting			98°C 1 minute
↓		melting	98°C 1 minute
Amplification	29 x ↺	annealing	60°C 1 minute
↓		extension	72°C 1minute/kb product
Final Extension			72°C 10 minutes

Although PCR product yield and specificity are generally enhanced by determining an optimal annealing temperature for each individual template/primer combination, an annealing temperature of 60°C allowed for sufficient and specific amplification of all target sequences presented in this thesis.

PCR products were subjected to agarose gel electrophoresis and the relevant bands were excised and purified from the agarose using the QIAquick Gel Extraction Kit from Qiagen.

2.2.03 Restriction Enzyme Digestion and Vector Dephosphorylation

PCR products and their corresponding vectors were subjected to digestion with the appropriate restriction enzymes in succession. In general, DNA was digested at a concentration not exceeding 0.1 μg DNA/μl and using 10 U restriction enzyme per 1 μg DNA. Digestions were allowed to proceed for 2 hours at 37°C. Following the first restriction enzyme digestion, DNA was subjected to agarose gel electrophoresis and the digested band was excised from the gel and purified using the QIAquick Gel Extraction kit. After the second restriction enzyme digest, DNA was purified using the QIAquick PCR Purification Kit (Qiagen). DNA purification kits were used in exact accordance with the manufacturer's instructions.

Following digestion with both appropriate restriction enzymes, vector DNA was dephosphorylated using calf intestinal alkaline phosphatase (CIAP). 10 x CIAP reaction buffer was added to the DNA to a final buffer dilution of 1 x buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1mM dithiothreitol, pH 7.9). CIAP was then added at a ratio of 0.01 U CIAP: 1 pmol free ends. The amount of free ends was calculated using the rule of thumb that 1 μg of a 1 kb fragment of DNA is equivalent to ~1.52 pmol DNA and thus possesses approximately 3 pmol ends, as recommended by the supplier. The DNA/CIAP mixture was incubated for 15 minutes at 37°C, after which a further 0.01 U CIAP/pmol ends was added, and the reaction was allowed to proceed for a further 15 minutes. The dephosphorylation reaction was arrested by the addition of 2 μl 0.5M EDTA and heating for 20 minutes at 65°C. The dephosphorylated vector was then purified from enzyme and buffer salts using the QIAquick PCR Purification Kit.

2.2.04 Ligation Reactions

DNA vector and insert were ligated using T4 DNA ligase. Ligation reactions were set up containing 100 ng of vector and various amounts of insert DNA such that the molar ratios

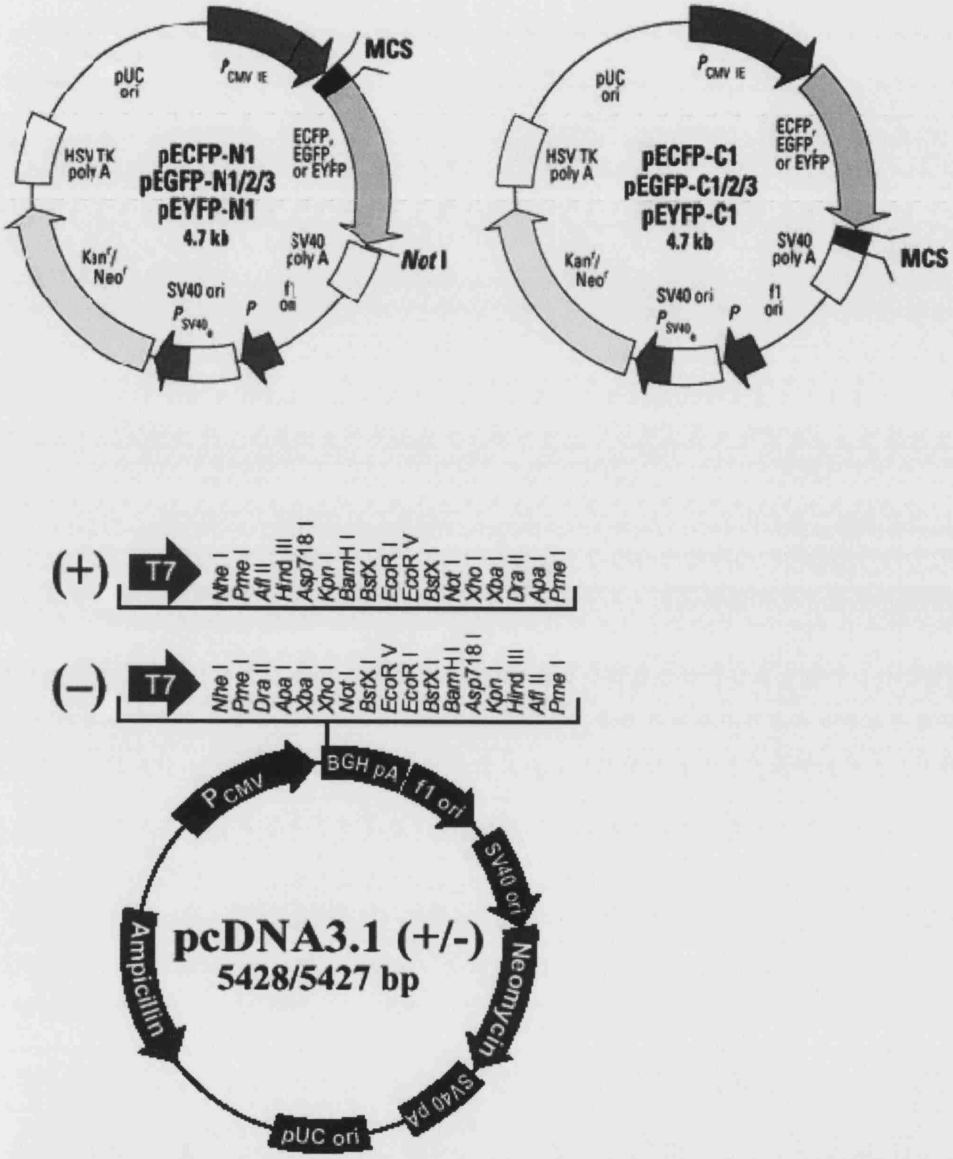

Figure 2.02: Eukaryotic Expression Vectors Used in this Study.

Above are shown vector maps for the vectors used in this study: pEGFP-N1, pEGFP-N3 and pEGFP-C1 from Clontech; and pcDNA3.1 (+) and pcDNA3.1 (-) from Invitrogen. Vector maps were obtained from the suppliers.

of vector: insert of 1:1, 1:3 and 1:5, along with a negative control containing vector but no insert, were prepared for each vector/insert pair. Ligation reaction mixtures contained 1 x ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM ATP, 10 mM dithiothreitol, 25 µg/ml BSA, pH 7.5) and 400 U (1 µl) ligase in a total volume of 20 µl. Ligations were allowed to proceed overnight at 16°C after which time they were stored at 4°C.

2.2.05 Transformation of Competent Cells

Ligated products were used to transform competent *E. coli* strain DH5α, which were prepared using the rubidium chloride method. The bacteria were first grown to mid-log phase in LB medium containing 20 mM $MgSO_4$. Absorbance at 600 nm was used to monitor bacterial growth, an absorbance of 0.4-0.6 indicating mid-log phase. Bacterial cells were then pelleted by centrifugation at 4,500 x *g* for 5 minutes at 4°C. Cells were then gently resuspended in 0.4 x (original culture volume) ice cold Buffer 1 containing:

30 mM potassium acetate
10 mM $CaCl_2$
50 mM $MnCl_2$
100 mM RbCl
15% v/v glycerol

and adjusted to pH 5.8 using acetic acid. Cells were incubated for 5 minutes in Buffer 1 on ice and then pelleted by centrifugation as above. Cells were then resuspended in 0.04 x (original culture volume) in ice-cold Buffer 2 containing:

10 mM PIPES at pH 6.5
75 mM $CaCl_2$
10 mM RbCl
15% v/v glycerol

and adjusted to pH 6.5 using KOH. Cells were incubated in Buffer 2 for 60 minutes on ice before being aliquoted and snap-frozen.

Approximately 10 ng DNA (2 µl) from each ligation mixture was incubated with 50 µl Rb-competent DH5α for 30 minutes on ice. The bacteria were then heat-shocked by incubating at 42°C for 90 seconds and allowed to recover on ice for 2 minutes. Bacterial

cells were then diluted in 450 µl room temperature SOC medium (GIBCO) and incubated at 37°C horizontally on an orbital shaker at ~150 rpm for 45 minutes to allow expression of the antibiotic resistance gene. Finally, 200 µl of transformed bacteria were plated on LB agar plates containing the appropriate antibiotic for selection.

2.2.06 Selection of Positive Clones and Insert Verification

Bacterial plates were incubated overnight at 37°C after which 10 colonies from each set of transformations were used to inoculate 2.5 ml overnight cultures. Plasmid DNA was purified from the overnight cultures using the Plasmid Mini Kit (Qiagen) in exact accordance with the manufacturer's instructions. Purified plasmids were then screened for the presence of insert by restriction enzyme digestion and agarose gel electrophoresis. All insert-positive clones were stored as glycerol stocks and insert identity was confirmed by double-stranded DNA sequencing (Cambridge BioScience).

2.3 Cell Culture and Transfections

HEK 293 cells were the kind gift of Dr Andrew Tinker (Department of Medicine, University College London) and MDCK cells were generously provided by Professor Mike Horton (Department of Medicine, University College London).

HEK 293 cells were maintained in Minimal Essential Medium containing Earle's salts and supplemented with 10% FCS, L-glutamine, non-essential amino acids and penicillin/streptomycin. MDCK cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine and supplemented with 10% FCS, non-essential amino acids and penicillin/streptomycin.

Cells were cultured at 37°C in a humidified atmosphere containing 5% v/v carbon dioxide. Cells were passaged at 90% confluence. At this time, cells were washed twice with phosphate buffered saline lacking in divalent metal cations (Sigma), and then digested with 10 x trypsin-EDTA solution diluted 1:10 in dPBS. Trypsinisation was allowed to proceed in the cell culture incubator for 10 minutes, after which time the digestion was arrested by the addition of 0.1 volume normal cell culture medium. At this

stage, cells were counted using a Neubauer improved haemocytometer and seeded at various cell densities according to specific experimental requirements.

Optimal protocols for transfecting MDCK and HEK 293 cells using the LipofectAMINE range of transfection reagents (GIBO) were obtained from the manufacturer. HEK 293 cells were transfected with LipofectAMINE 2000 for protein analysis experiments, and with LipfectAMINE for imaging experiments. MDCK cells were transfected with LipofectAMINE PLUS. Specific details of transfection protocols are given below under the appropriate experimental headings.

2.4 Immunocytochemistry and Confocal Microscopy

2.4.01 Transfection of HEK 293 Cells for Imaging

For fluorescence microscopy experiments, HEK 293 cells were seeded on glass coverslips of diameter 13 mm in 12-well plates at a density of 1 x 10^5 cells per well. Cells were seeded in normal growth medium containing no antibiotics. Transfections were performed 24 hours after seeding, or once the cells had reached 60% confluence, using LipofectAMINE. Briefly, 400 ng plasmid DNA was diluted into 50 µl OptiMEM per well. In a separate tube, 2.5 µl LipofectAMINE was diluted in 50 µl OptiMEM per well and mixed thoroughly. The diluted DNA and diluted LipofectAMINE were allowed to stand at room temperature for 15 minutes, after which they were combined and allowed to stand for a further 30 minutes to allow transfection complexes to form. During the second incubation the seeding medium was replaced with an OptiMEM wash. After the 30-minute incubation, 400 µl OptiMEM per well to be transfected was added to the transfection complexes. The OptiMEM wash was then removed from the cells, and 500 µl transfection complex was added to each well. Cells were incubated with transfection complexes for 6 hours, after which time the transfection complexes were removed and replaced with medium containing serum but no antibiotics. Cells were fixed and stained 24-48 hours post-transfection.

For imaging analysis of untransfected HEK 293, cells were seeded on glass coverslips as above and fixed for staining at approximately 60% confluence.

2.4.02 Transfection of MDCK Cells for Imaging

MDCK cells were transfected using LipofectAMINE PLUS (LF+). Cells were seeded on glass coverslips of diameter 13 mm in 12-well plates at a density of 1.5 x 10^5 per well in normal growth medium lacking antibiotics. Cells were transfected 24 hours after seeding or at 60% confluence. For transfection, 1 μg DNA was diluted in 50 μl OptiMEM per well to be transfected. 2.5 μl PLUS reagent per well was added to the diluted DNA and the resulting mixture incubated at room temperature for 15 minutes. During this incubation, 4.5 μl LipofectAMINE was diluted in 50 μl OptiMEM per well in a separate tube. After the 15 minute incubation, the DNA/PLUS reagent mixture was added to the diluted LipofectAMINE, mixed well and allowed to stand for a further 15 minutes at room temperature. During the second incubation, MDCK cells were washed with 1 ml OptiMEM and the wash replaced with 400 μl OptiMEM per well. Finally, 100 μl transfection complex was added to each well. MDCK cells were incubated in transfection complex for 3 hours after which time the transfection was stopped by the addition of 1 ml DMEM containing 20% FCS, non-essential amino acids and no antibiotics. Cells were fixed and stained 24-48 hours post-transfection.

For imaging analysis of untransfected MDCK, cells were seeded on glass coverslips as above and fixed for staining at approximately 60% confluence.

2.4.03 Methanol Fixation

Glass coverslips with attached cells were washed 3 times in phosphate buffered saline (PBS), fixed by immersion in 100% anhydrous methanol at -20°C for 2 minutes and washed a further three times in PBS. Non-specific binding was then blocked by incubation with 5% normal donkey serum (NDS) in PBS for 30 minutes at 37°C in a humidified chamber. Coverslips were then incubated in primary antibody diluted in 5% NDS/PBS. Antibody dilutions for immunocytochemistry are shown in **Table 2.1.** Primary antibody staining proceeded for 1 hour at 37°C in a humidified chamber, after

which coverslips were washed three times in PBS. The coverslips were then transferred to secondary antibody diluted in 5% NDS/PBS, and incubated for 30 minutes at room temperature. Coverslips were then washed three times in water before being mounted on Superfrost Plus-coated slides (BDH). The Pro-Long Anti-FADE Kit (Molecular Probes) was used as mounting medium, anti-fade reagent and sealant.

The nuclear dye DAPI (4',6-diaminidino-2-phenylindole) was often used as a counterstain. In these instances, DAPI was added directly to the Pro-Long Anti-FADE reagent to a final concentration of 1 μg/ml.

2.4.04 Paraformaldehyde Fixation

Cell-coated coverslips were washed three times in PBS and then immersed in 4% PFA/PBS for 10 minutes at room temperature. Coverslips were then washed three times in PBS before being permeabilised by immersion in 0.2% Triton X-100 in PBS for 2 minutes at room temperature. Cells were washed three times in PBS and then subjected either to antibody staining (described above) or to phalloidin staining. For phalloidin staining, the coverslips were incubated with 1 μg/ml TRITC-conjugated phalloidin for 30 minutes at room temperature before being washed three times with water and mounted as above.

2.4.05 Imaging Equipment

Fluorescent cell analysis was performed using a Zeiss Axiovert 100M microscope connected to a Hammatsu C4742-95 digital camera, a Hamamatsu camera controller and a Ludl filter turret. Equipment and data were managed using the Openlab 2.2.4 software from Improvision.

2.4.06 Imaging Software: Openlab 2.2.4

The Openlab 2.2.4 software from Improvision is a modular software suite specifically designed for scientific imaging. The software consists of a set of core features (the

Antibody Specificity	Mr of Antigen	Supplier	Host	Dilution for WB	Dilution for ICC
Actin	42k	Sigma	Mouse	1:500	N/A
E-Cadherin	120k	Santa Cruz	Rabbit	1:250	1:20
pan-Cadherin		Zymed	Rabbit	1:500	1:50
β-Catenin	92k	Transduction Laboratories	Mouse	1:500	1:100
γ-Catenin	82k	Transduction Laboratories	Mouse	1:250	1:25
Ezrin	80k	Upstate Biotechnology	Rabbit	1:1000	1:100
FAK A	125k	Santa Cruz	Rabbit	1:400	1:400
FAK C	125k	Santa Cruz	Rabbit	1:400	1:400
FAK pY^{397}	125k	Biosource International	Rabbit	1:200	N/A
Flag M2	N/A	Sigma	Mouse	1:500	N/A
Flag M5	N/A	Sigma	Mouse	N/A	1:500
GFP	25k	BD Clontech	Rabbit	1:200	N/A
β_1 Integrin	130k	Chemicon	Mouse	1:1000	1:100
β_1 Integrin	130k	Transduction Laboratories	Mouse	1:250	1:25
Occludin	65k	Zymed	Rabbit	1:250	1:50
Vinculin	117k	Chemicon	Mouse	1:300	1:30
ZO-1	220k	Zymed	Rabbit	1:250	1:25
ZO-2	160k	Santa Cruz	Goat	1:400	1:20

Table 2.1: Summary of Primary Antibodies Used in this Study

Above are listed all of the primary antibodies used in this study, the suppliers from which they were purchased, the hosts in which the antibodies were raised, and the dilutions used in Western blotting (WB) and immunocytochemistry (ICC).

Openlab Core) to which additional modules can be added to allow for customisation of the suite for specific experimental needs. The Openlab Core comprises a comprehensive series of functions for image handling and processing. Specifically, the core allows opening of images in all standard imaging formats (as well as in some non-standard formats), employs an image layering and caching system that allows large numbers of images the be handled in a single, multi-layered file, facilitates multi-channel imaging for rapid creation of merges, superimpositions and transparencies, contains editing and image annotation functions, possesses spatial calibration and calibrated image measurement tools and contains image enhancement and colour table editing facilities. Further core modules are primarily concerned with image processing and the creation of presentations, video documents and slide shows.

For the work contained in this thesis, additional Openlab modules were used to control the microscope and other equipment remotely. The Microscope module allowed for control of functions on the Zeiss microscope, including the electronic control of the focus drive to 0.1 μm precision. Other modules were used to control the filter wheels, shutters and digital camera using the computer. The Automator module was used to take large series of images through the vertical (z-) axis of the samples at given z-increments, using defined exposure times, incident wavelengths and filter wheel settings. In addition to these, the Confocal Imaging module was used to perform volume deconvolution, which removes out-of-focus haze.

2.2.07 Optical Parameters for Visualisation of Fluorophores

There are three key optical parameters affecting the nature of the data produced in fluorescent cellular imaging: the magnification afforded by the objectives and eyepieces, the wavelength(s) of light incident on the sample and the filter sets that control perception of the emitted light spectrum by the eyepieces and/or by the camera.

Fluorescently labelled cells were viewed using 40x (numerical aperture 1.2) and 63x (numerical aperture 1.4) magnification objectives and eyepieces of 10 x magnification.

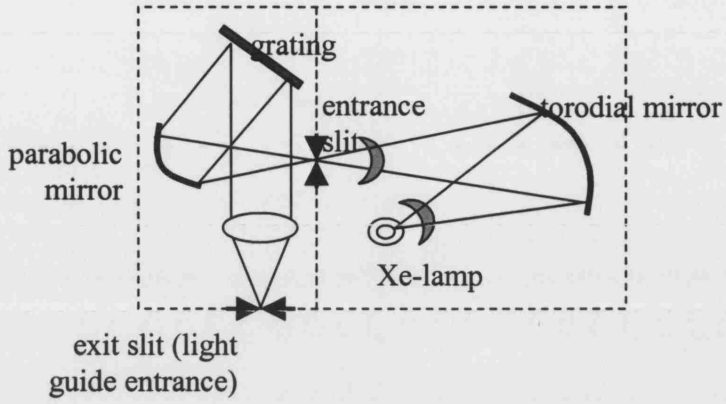

Figure 2.03: The Polychrome II Monochromator.

White light from the xenon lamp is collected through two lenses and a torodial mirror and focussed on an entrance slit. This slit is located in the wall that separates the light source from the monochromator. The beam is deflected *via* the parabolic mirror onto the grating, which is fixed to the galvonometric scanner. The grating generates a spectrum which is focussed onto the light guide. The wavelength of the monochromator is selected by turning the scanner.

Immersion oil with a refractive index of 1.515 (Immersol, Zeiss) was used in conjunction with the 63 x objective.

Most classical fluorescent and confocal microscopes emit a broad spectrum of light onto the sample, so that all fluorophores present are activated. This leaves the question of signal specificity entirely up to the filter sets controlling the spectrum of perceived light. However, for these experiments the Polychrome II (Photonics) was used to control the wavelength of incident light to within 0.5 nm, allowing the incident wavelength to be set at the precise excitation maximum of the relevant fluorophores. The Polychrome II is a monochromatising device with an integrated light source in the form of a 75 Watt xenon lamp. The function of the monochromator and the method employed to control the emission wavelength are summarised in **Figure 2.03**. In practice, the incident wavelength was controlled using the Polychrome module in the Openlab software. The wavelengths selected were 360 nm for DAPI, 490 nm for EGFP and 570 nm for Rhodamine-Red-X.

Filter Set 40 from Zeiss permitted independent detection of DAPI, FITC and Rhodamine-Red-X in double-stained cells without optical spillover.

2.2.08 Image Capture and Volume Deconvolution

Images were captured using the Automator module. Optimal exposure times were determined individually for each fluorophore in each experiment. Contrast enhancement and camera binning functions were frequently used to diminish the minimum exposure time required to gain quality images. The microscope was then focussed on the most apical point at which the fluorescent signal was in focus and this position was registered by the software. The microscope was then focussed to the basolateral-most position at which the fluorescent signal was in focus and this position was also registered. After focussing, the correct filters were selected, and the shutters set so that all of the light emitted from the sample was sent to the digital camera. The z-spacing of the sample slices was then set. In general, a sample z-spacing of 0.1 μm was used, although in some cases a z-spacing of 0.2 μm was used. The computer then proceeded to capture images from the apical surface to the basal-lateral surface at the specified z-increments. Volume deconvolution was performed using the Confocal Imaging module and the intensity of the images was optimised using the Contrast Enhancement function. Superimpositions were performed only on deconvolved images of different fluorophores from the same z-plane of a given sample.

2.5 Western Blotting and Immunoprecipitations

2.5.01Transfection for Western Blotting and Immunoprecipitations

For protein analysis experiments, HEK 293 cells were transfected using LipofectAMINE 2000. The specific format varied largely according to the requirements of the experiment; however, cells were routinely seeded at a density of 1.05×10^5 cells/cm^2 of growth area in normal growth medium containing no antibiotics. The exact numbers of cells seeded in the various culture formats are shown in **Table 2.2**, which also shows the amount of plasmid DNA, the cell seeding volume and the volume of LipofectAMINE 2000 used per dish or per well for each transfection.

Cell Culture Format	Cells seeded per dish/well	Cell seeding volume (ml)	DNA used per dish/well (µg)	Volume LF2K per dish/well (µl)
12-well plate	0.5×10^6	1	1.6	4
6-well plate	1×10^6	2	3.2	8
60 mm dish	2×10^6	4	6.4	16
90 mm dish	6×10^6	10	16	40
150 mm dish	16×10^6	20	32	80

Table 2.2: Variable Parameters for Transfection Using LipofectAMINE 2000.
Above are shown cell number, cell seeding volume, amount of DNA and volume of transfection reagent used to transfect HEK 293 cells with LipofectAMINE 2000 in various formats. The values given are per well (6- and 12-well plates) or per dish (all other formats).

Transfection was performed 24 hours after seeding. The appropriate amount of DNA was diluted into 0.1 x (cell seeding volume) OptiMEM. The corresponding volume of LipofectAMINE 2000 was also diluted in 0.1 x (cell seeding volume) OptiMEM in a separate tube and allowed to stand at room temperature for 5 minutes. The diluted DNA and the diluted LipofectAMINE 2000 were then combined, to a total volume of 0.2 x (cell seeding volume), mixed thoroughly and allowed to stand at room temperature for 30 minutes to allow transfection complexes to form. After this second incubation, the transfection complexes were added directly to the cell seeding medium and mixed by gently rocking the culture dish backwards and forwards.

LipofectAMINE transfection of HEK 293 cells was performed in a 6-well plate format. In this case, 2.0×10^5 cells per well were seeded in 2 ml normal growth medium containing no antibiotic and cultured for 24 hours prior to transfection. 800 ng DNA was diluted in 100 µl OptiMEM. 5 µl LipofectAMINE was then diluted in 100 µl OptiMEM per well and allowed to stand at room temperature for 15 minutes. After this time, the diluted DNA and diluted LipofectAMINE were combined and allowed to stand for 30 minutes at room temperature to allow transfection complexes to form. During this second incubation, the seeding medium was removed from the HEK 293 cells and replaced with

an OptiMEM wash. After the 30-minute incubation, 800 µl OptiMEM was added to the DNA/LipofectAMINE mixture. The OptiMEM wash was then removed from the cells and replaced with 1 ml transfection complex. Cells were incubated in the presence of transfection complexes for 6 hours, after which time the complexes were removed and replaced with normal growth medium containing no antibiotics.

2.5.02 Western Blotting

For Western blotting experiments, cells were seeded either in 6-well plates or in 60 mm dishes and transfected using LipofectAMINE 2000 as described above. Transfected cells were harvested 24 hours after the addition of transfection complexes whereas untransfected cells were harvested at confluence. Cells were scraped into ice-cold Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 8.0, 150 mM NaCl) and washed twice with ice-cold TBS. Cells were then lysed in ice-cold lysis buffer, containing 1 x COMPLETE protease inhibitor cocktail (Roche Molecular Biochemicals), 1% Triton X-100, 0.5% NP-40 and 0.2 mM Na_3VO_4 in TBS. Cells were lysed with gentle agitation at 4°C for 30 minutes. Cell lysates were then centrifuged at high speed for 15 minutes at 4°C and the insoluble pellets discarded. Protein estimation was performed using the ***DC*** Protein Assay Kit (BioRAD) in exact accordance with the manufacturer's instructions. For Western blots, 20-50 µg protein in 2 x SDS PAGE sample buffer was subjected to SDS-PAGE in gels ranging from 8-12% acrylamide. Proteins were transferred to PVDF membranes (Immobilon-P, Millipore) and blocked in 5% non-fat milk in TBS for 1 hour at 37°C, or overnight at 4°C. Membranes were then blotted with primary antibodies diluted in TBS according to **Table 2.1**, with the exception of the anti-vinculin antibody which was diluted in 1% bovine serum albumin (BSA) in TBS, as recommended by the supplier. Membranes were then washed six times with TBS and incubated with HRP-conjugated secondary antibodies (also diluted in TBS). After further washing, antibody binding was visualised by chemiluminescence using the ECL+ kit (Amersham).

2.5.03 Protocol for Immunoprecipitation

For experiments involving immunoprecipitation, cells were seeded and transfected in 90 mm or 150 mm dishes, as described above. Transfections were allowed to proceed for 24

hours, after which time cells were harvested and lysed as described above for Western blotting. Protein estimation was performed as above and concentration of lysates was adjusted to 1 mg protein/ml with lysis buffer; a total of 1 mg protein was used for each immunoprecipitation. The adjusted lysates were pre-cleared by incubation with 25 µl protein A/G+ agarose for 30 minutes at 4°C on a daisy wheel. The agarose beads were then pelleted by high-speed centrifugation at 4°C for 30 minutes. The supernatants were kept and the pellets discarded. The supernatants were then incubated in the presence of 5 µg antibody for 1 hour at 4°C on a daisy wheel. 25 µl protein A/G+ agarose was then added to the lysate/antibody mixture and incubated for 1 hour at 4°C on a daisy wheel. Finally, the agarose/antibody complexes were spun down at high speed and washed three times with lysis buffer (all at 4°C). After the final wash, the agarose beads were spun down, the supernatant discarded and the immunoprecipitates eluted from the beads by boiling in 200 µl 2 x SDS PAGE sample buffer. The eluted immunoprecipitated proteins were then subjected to SDS-PAGE as described above, running either 25 µl or 50 µl sample per lane, followed by Western blotting.

2.5.04 Cytoskeleton Binding Assay

Cells were seeded in 90 mm dishes and transfected with LipofectAMINE 2000 as described above. 24 hours post-transfection, cells were incubated with 1 µg/ml cytochalasin D in normal growth medium (or with a corresponding dilution of DMSO as a control) for 1 hour at 37°C. Cells were then scraped in ice-cold TBS and washed twice in TBS. Cells were then lysed in ice-cold lysis buffer containing 1% Triton X-100, 0.2 mM Na_3VO_4 and 1 x COMPLETE in TBS. Lysis was allowed to proceed for 30 minutes on a daisy wheel at 4°C. After lysis, the Triton-insoluble fraction was spun down at 9,000 x g and the supernatant removed to a fresh tube ("triton soluble fraction"). The triton-insoluble pellet was washed three times in ice-cold lysis buffer before being extracted with RIPA buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM HEPES [pH 7.4], 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 1 x COMPLETE. The pellet was incubated in RIPA buffer at 4°C on a daisy wheel for 1 hour after which time insoluble material was spun down at high speed and discarded, and the supernatant kept

("triton insoluble fraction"). Both the triton soluble and the triton insoluble fractions were assayed for protein concentration using the ***DC*** Protein Assay Kit from BioRAD. 20 μg of protein from each sample was subjected to SDS-PAGE and Western blotting as described above. This method was adapted from (Schlaepfer *et al.*, 1998).

3. Expression of Endogenous Cellular Contact Proteins in HEK 293 Cells

Introduction

The immortalised cell line HEK 293 is highly susceptible to transfection and is thus a commonly used vehicle for heterologous expression studies. The expression of endogenous cellular contact proteins had not been extensively investigated in HEK 293 cells prior to commencing this thesis. It was therefore important that the expression and subcellular distribution of key cellular adhesion proteins should be characterised in this cell line prior to commencing studies using exogenous FAK constructs.

3.1 Focal Contact Proteins

HEK 293 cells were first of all assayed for expression of the focal contact proteins FAK, β_1 integrin and vinculin (**Fig 3.01** and **3.02**). FAK expression was assessed using one antibody against the amino-terminal domain (N-FAK antibody) and another antibody raised against the carboxy-terminal domain (C-FAK antibody). Immunocytochemistry using these antibodies gave rise to strikingly different patterns of subcellular distribution. C-FAK immunoreactive protein was expressed in the cytosol and was negligible in the nucleus and at areas of cell-cell contact (**Figure 3.01A**). Conversely, N-FAK immunoreactive protein was expressed in the nucleus as well as in the cytosol, and appeared to be concentrated in discontinuous, elongated structures at areas of cell-cell contact (**Figure 3.01B**).

Western blot analysis of HEK 293 lysates with N- and C- FAK antibodies revealed a band at ~125k, corresponding to the expected relative molecular weight for FAK. In addition, blotting with the C-FAK antibody revealed a band at ~52k (**Figure 3.01C**) and blotting with the N-FAK antibody revealed a band at ~42k (**Figure 3.01D**).

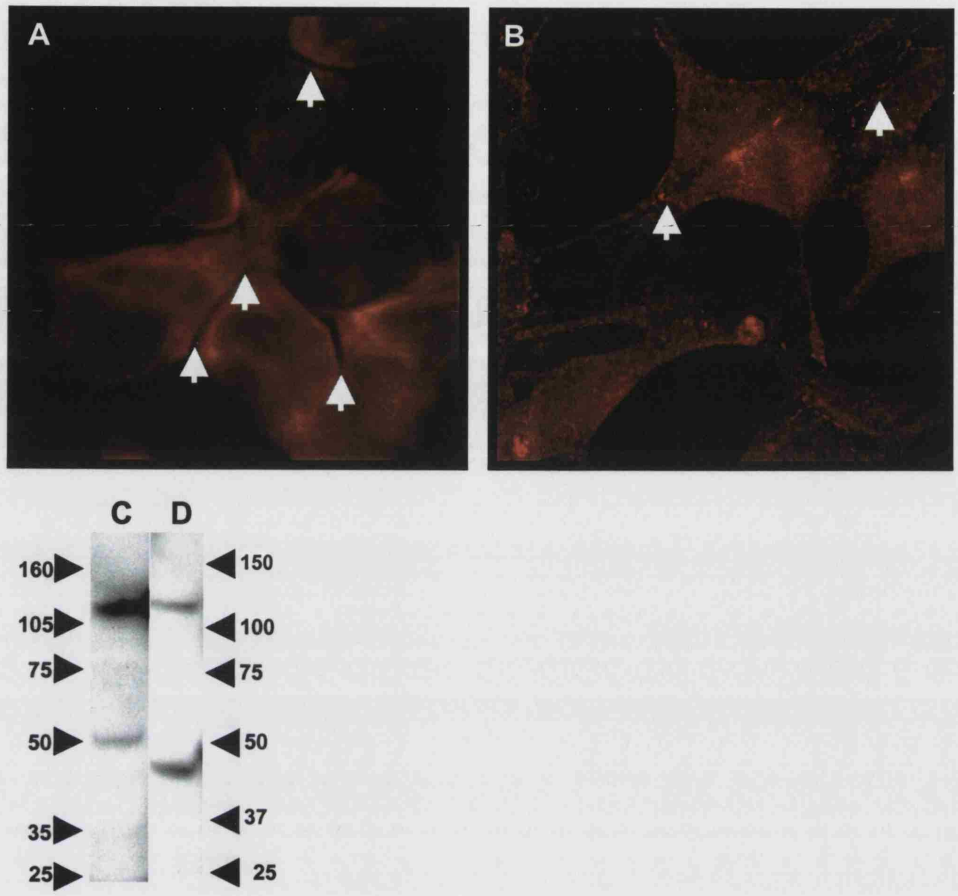

Figure 3.01: FAK Expression in HEK 293 Cells.

Expression of endogenous FAK in HEK 293 cells was analysed by immunocytochemistry (**A** and **B**) and by Western blotting (**C** and **D**) with antibodies raised against the carboxy-terminal domain (**A** and **C**) or against the amino-terminal domain (**B** and **D**) of FAK and visualised using Rhodamine-Red-X-conjugated goat anti-rabbit. Carboxy-terminal immunoreactivity (**A**) is largely cytoplasmic and perinuclear, and there is a clear avoidance of areas of cell-cell contact (arrows). Amino-terminal immunoreactivity (**B**) is also largely cytoplasmic but there is marked focal intensity at areas of cell-cell contact (arrows). Western blotting reveals that in addition to expressing full-length FAK, HEK cells express a carboxy-terminal fragment of approximately 55k (**C**) and an amino-terminal fragment of around 42k (**D**). The carboxy-terminal fragment probably corresponds to a FRNK-like species, and the amino-terminal fragment may correspond to a constitutive proteolytic cleavage product, which has been previously identified in other cell types (Carragher *et al.*, 2001; Carragher *et al.*, 1999; Cooray *et al.*, 1996; Ilic *et al.*, 1998; Cicala *et al.*, 2000; van de Water *et al.*, 1999; Gervais *et al.*, 1998; Wen *et al.*, 1997; Lobo & Zachary, 2000; Jones *et al.*, 2001). Each image is the representative of three experiments.

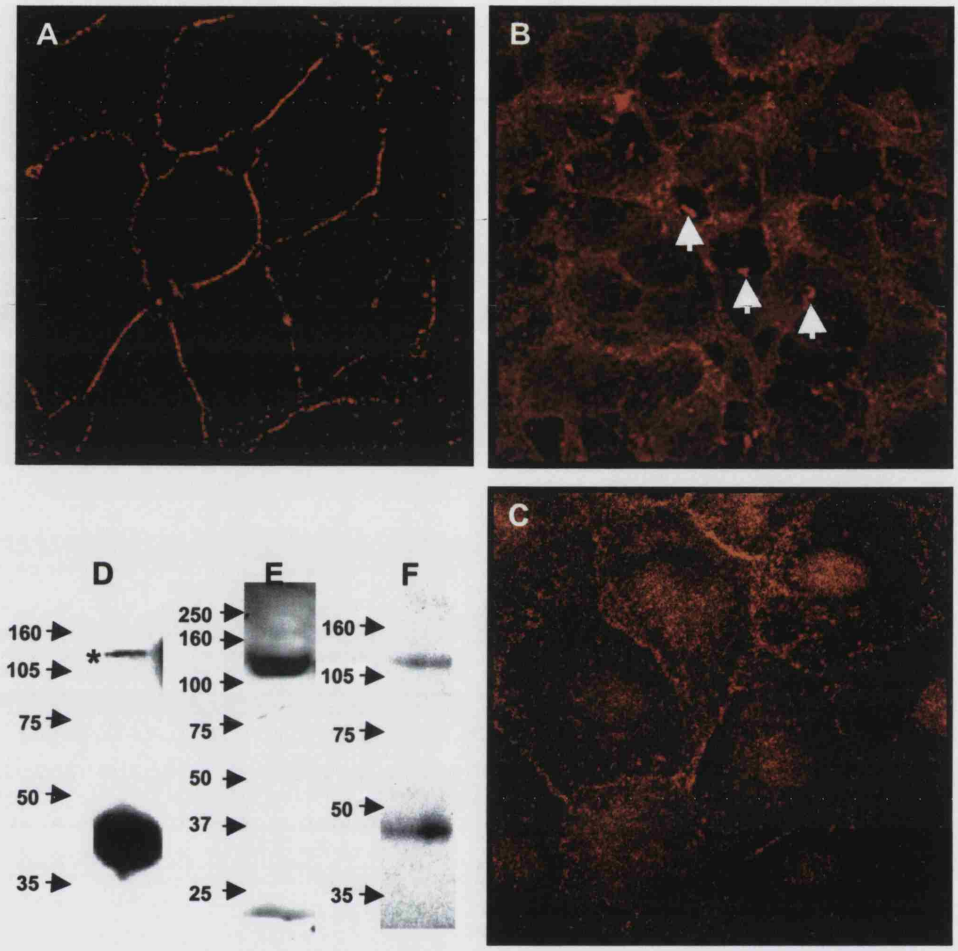

Figure 3.02: β_1 Integrin and Vinculin Expression in HEK 293 and MDCK Cells

Expression of endogenous β_1 integrin subunits (**A** and **D**) and vinculin (**B** and **E**) in HEK 293 cells was determined by immunocytochemistry and Western blot analysis. β_1 integrin subunit immunoreactivity (apical plane of the cell) is found primarily and consistently at areas of cell-cell contact (**A**); whereas vinculin expression (**B**) appears to be largely cytosolic, with some concentration in punctate structures in the basal plane of the cell (arrows) which could represent a form of focal contact. Western blotting with anti-β_1 integrin showed a band corresponding to the expected molecular weight of 130k for β_1 integrin (**D**, *) along with a much more intense band at <50k. Vinculin (**E**) ran as a single band at the expected molecular weight of ~117k. In MDCK cells, β_1 integrin subunits (apical plane) were found in the membrane, at areas of cell-cell contact (**C** and **F**) and in the nucleus. MDCK cells express full-length immunoreactive β_1 (**F**, *) plus a smaller band running at <50k. Anti-β_1 integrin was obtained from Chemicon. Each image is the representative of three experiments.

β_1 integrin was seen to localise primarily to areas of cell-cell contact in HEK 293 cells using a monoclonal anti-β_1 integrin antibody (**Figure 3.02A**). This sub-cellular distribution was confirmed by staining HEK 293 cells with an alternative monoclonal anti-β_1 (**Figure 4.09**), as well as by staining MDCK cells anti-β_1 (**Figure 3.02B**). Staining of HEK 293 cells with both anti-β_1 monoclonals revealed similar localisation at cell-cell contacts. Staining of MDCK cells with anti-β_1 also revealed cell-cell contact localisation (**Figure 3.02C**). Western blotting of HEK 293 cell lysates consistently resulted in a band corresponding to the predicted β_1 relative molecular weight (130k) (**Figure 3.02D**) as well as a much more intense band at just over 40k. Blotting of MDCK lysates with anti-β_1 revealed the expected 130k band plus a lower molecular weight band (<50k) of approximately equal intensity (**Fig 3.02F**).

Vinculin immunoreactive protein was seen to localise to the cytosol of HEK 293 cells, with some concentration in punctate structures (basal plane) which may represent a form of focal contact (**Fig 3.02B**). Vinculin was expressed as a single band corresponding to the predicted relative molecular weight of 117k in Western blot analysis (**Fig 3.02E**).

3.2 Tight Junction Proteins

HEK 293 cells were then assayed for expression of the tight junction proteins occludin, ZO-1 and ZO-2 (**Figure 3.03**). In these cells, occludin was seen to be expressed as a series of bands, the largest of which corresponded to the predicted molecular weight of 65k (**Figure 3.03D**). Occludin was located primarily at areas of cell-cell contact in the apical plane of the cell, with little or no cytosolic expression (**Figure 3.03A**). ZO-1 was expressed as two distinct bands, one at >250k and another corresponding to the expected ZO-1 molecular weight of 220k (**Figure 3.03E**). Immunocytochemical staining with ZO-1 antibody revealed membrane staining with some cytosolic and nuclear localisation (**Figure 3.03B**). ZO-2 was expressed as a single band corresponding to the predicted molecular weight of 160k (**Figure 3.03F**). ZO-2 expression was cytosolic, with some

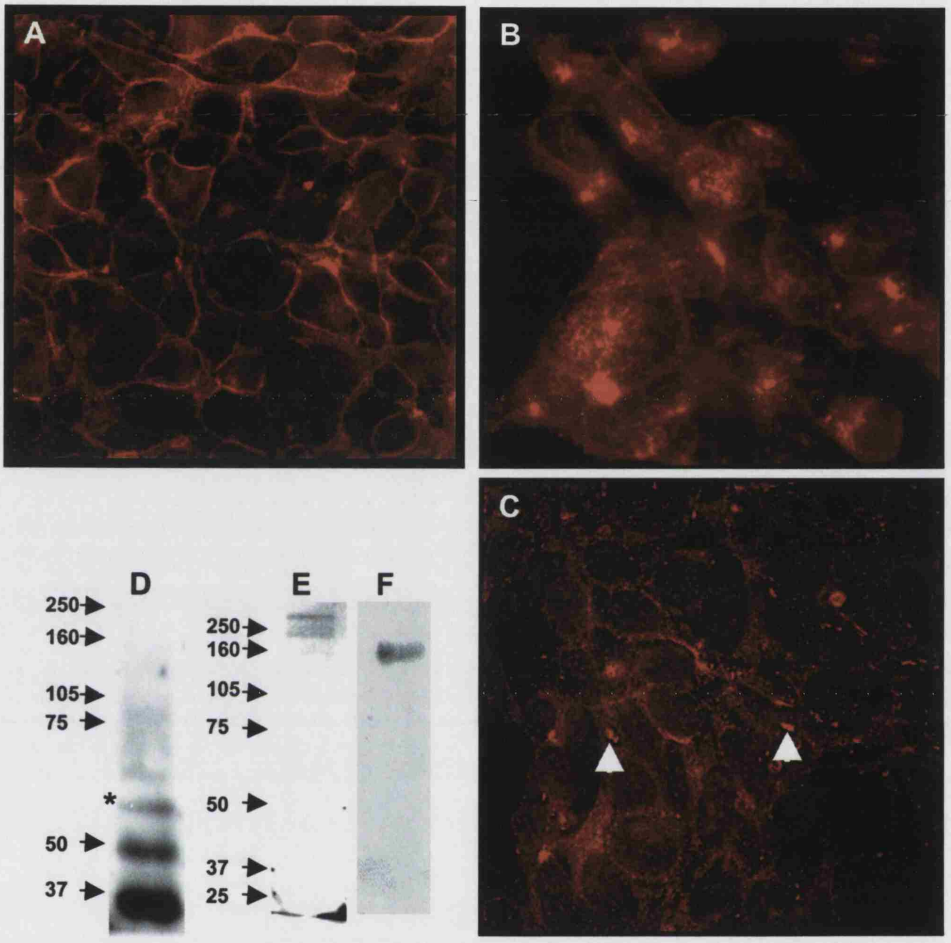

Figure 3.03: Expression of Tight Junction Proteins in HEK 293 Cells.

HEK 293 cells were analysed for the expression of the tight junction proteins occludin (**A** and **D**), ZO-1 (**B** and **E**) and ZO-2 (**C** and **F**) by immunocytochemistry (**A-C**) and Western blotting (**D-F**). Occludin is expressed mainly at areas of cell-cell contact (**A**), ZO-1 exhibits diffuse membrane staining (**B**), and ZO-2 expression appears to be largely cytosolic with some punctate concentration (arrows). ZO-1 is expressed as a doublet at ~220k (expected molecular weight) and >260k. ZO-2 is expressed as a single band as expected. Occludin appears to be expressed in three major bands, the heaviest of which (*) corresponds to the predicted M*r* of ~65k. Each image is the representative of three experiments.

apparent concentration in punctate structures in the basal aspect of the cell (**Figure 3.03C**), a pattern of expression which was similar to that of vinculin in these cells (**Figure 3.02B**).

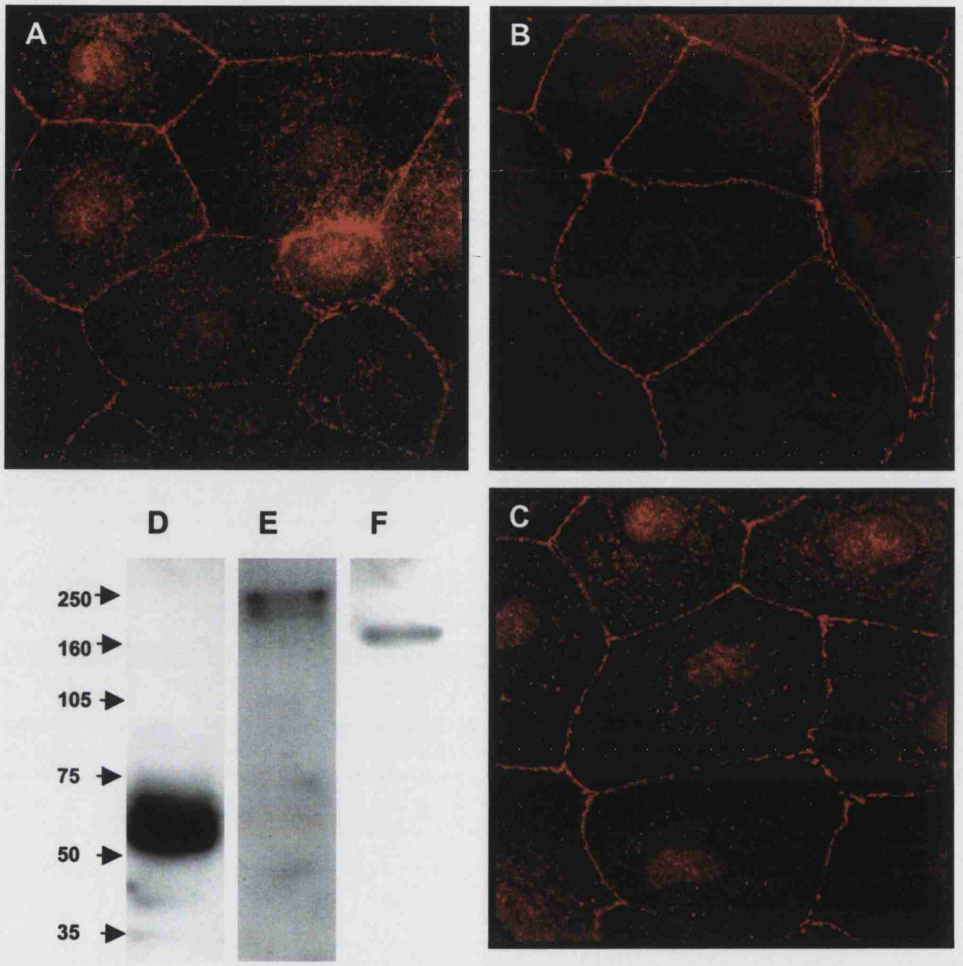

Figure 3.04: Validation of Tight Junction Antibodies in MDCK Cells.

The tight junction antibodies used to assess expression of occludin, ZO-1 and ZO-2 in HEK 293 cells were validated by immunocytochemistry (**A-C**) and Western blotting (**D-F**) using MDCK cells. Striking junctional localisation was observed for occludin (**A**), ZO-1 (**B**) and ZO-2 (**C**) and some nuclear immunoreactivity was also observed for each protein. In MDCK cells, occludin appears to migrate as one band (**D**). ZO-1 (**F**) is present as a doublet, presumably corresponding to splice variation as previously reported (Gonzalez-Mariscal *et al.*, 1999). ZO-2 migrates as a single band (**F**). Each image is the representative of three experiments.

Since the occludin, ZO-1 and ZO-2 antibodies were all originally raised against canine antigens, and since some of the immunostaining observed in HEK 293 cells differed from

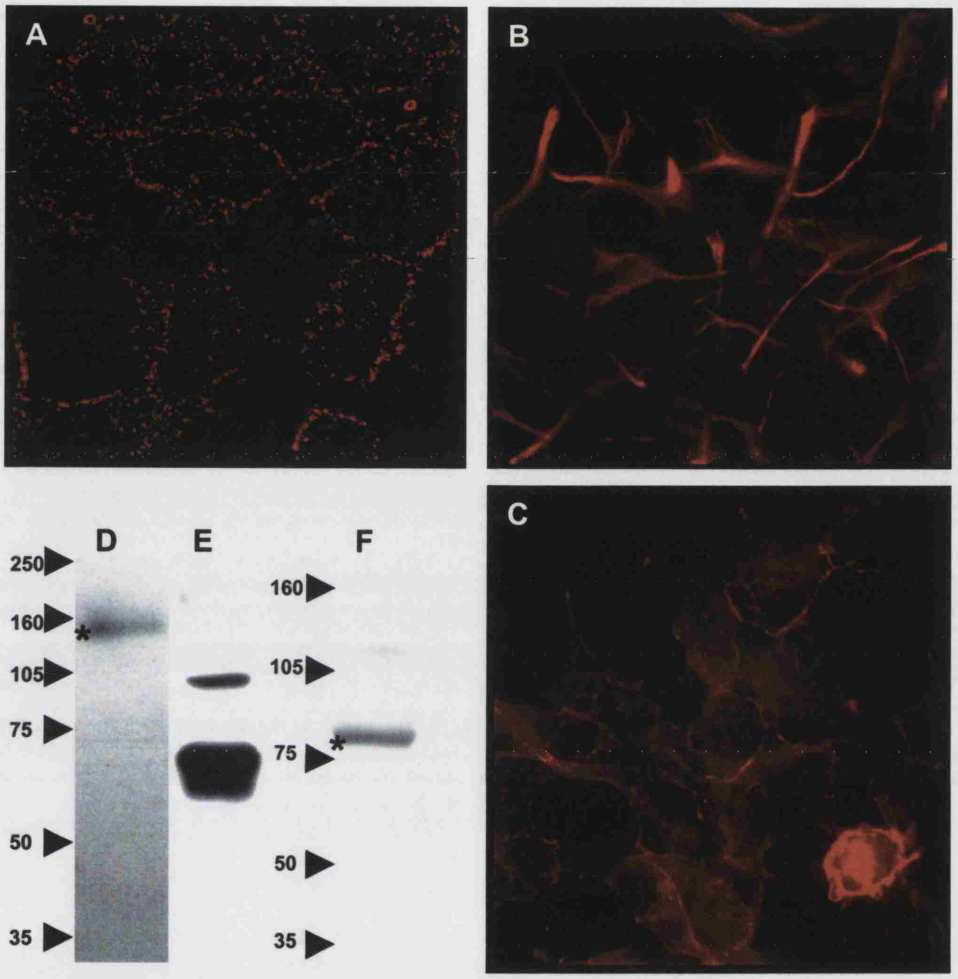

Figure 3.05: Expression of Adherens Junction Proteins in HEK 293 Cells.

HEK 293 cells were analysed by immunocytochemistry (**A-C**) and Western blotting (**D-F**) for expression of the adherens junction proteins E-cadherin (**A** and **D**), β-catenin (**B** and **E**) and plakoglobin (**C** and **F**). E-cadherin is discontinuously expressed at cell-cell contacts (**A**) and as a single band (**D**) corresponding to the expected molecular weight of ~92k (*). β-catenin subcellular distribution staining is highly unusual (**B**) and the protein is expressed in two major bands (**E**), one corresponding to the expected molecular weight (*) and a lower band which may represent a proteolytic cleavage product. Plakoglobin exhibits cytosolic and membrane staining with some concentration at areas of cell-cell contact (**C**), and the protein is expressed as a single band (**F**) corresponding to the expected molecular weight (*). Each image is the representative of three experiments.

expected, these antibodies were validated using MDCK cells (**Figure 3.04**). Immunocytochmical staining of MDCK cells with all three antibodies revealed classic

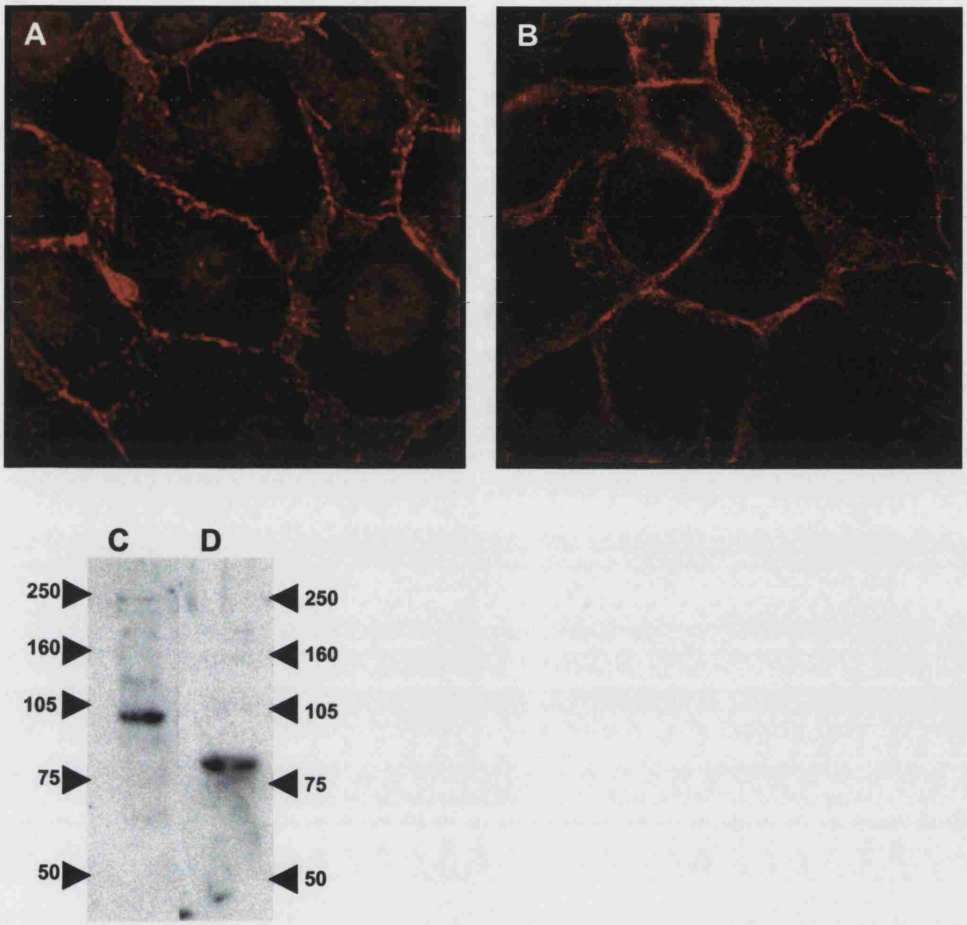

Figure 3.06: Validation of Catenin Antibodies in MDCK Cells.

MDCK cells were used to validate the antibodies used to assess catenin expression in HEK 293 cells. MDCK cells were analysed by immunocytochemistry (**A** and **B**) and Western blotting (**C** and **D**) with antibodies raised against β-catenin (**A** and **C**) and plakoglobin (**B** and **D**). β-Catenin (**A**) and plakoglobin (**B**) both exhibit membrane and junctional staining in the apical plane of the cell. Both β-catenin (**C**) and plakoglobin (**D**) are expressed as single protein bands corresponding to their expected relative molecular weights. Each image is the representative of three experiments.

junctional localisation (apical), with additional nuclear and perinuclear staining (**Figure 3.04A-C**). Western blot analysis with these antibodies revealed a single occludin band at the predicted molecular weight of 65k (**Figure 3.04D**); two ZO-1 bands consistent with the ZO-1 alternative transcripts reported for MDCK cells (**Figure 3.04E**; Gonzalez-

Mariscal *et al.*, 1999), and a single ZO-2 band at the predicted relative molecular weight of 160k (**Figure 3.04F**).

3.3 Adherens Junctions Proteins

E-cadherin, β-catenin and plakoglobin were used as examples of adherens junction proteins in epithelial cells. Western blotting of HEK 293 cells with anti-E-cadherin revealed a single band at the predicted relative molecular weight of 130k (**Fig 3.05D**), and confocal microscopy revealed discontinuous membrane staining (**Figure 3.05A**). β-Catenin was expressed as two distinct bands – a heavier band corresponding to the predicted molecular weight of 92k, and a much more intense band at <75k (**Figure 3.05E**). β-Catenin expression in HEK 293 cells was highly abnormal, being concentrated in cellular protrusions and being negligible in the cell body and nucleus (**Figure 3.05B**). Plakoglobin was expressed as a single band corresponding to the predicted molecular weight of 82k (**Figure 3.05F**) and its subcellular distribution was cytosolic with some apparent concentration at the plasma membrane and cell-cell contacts (**Figure 3.05C**).

The β-catenin and plakoglobin antibodies were also validated in MDCK cells (**Fig 3.06**). Immunocytochemistry and confocal microscopy with both of these antibodies revealed membrane and cell-cell contact localisation in the apical plane with some β-catenin staining in the nucleus (**Figure 3.06A-B**). Both revealed a single band corresponding to the predicted relative molecular weight for their antigens by Western blot analysis (**Figure 3.06 C-D**).

3.4 Negative Control Data for Antibody Staining

Pre-immune rabbit, mouse and goat sera were used in place of primary antibodies in both Western blotting and immunocytochemistry experiments to address the issue of non-specific staining (**Figure 3.07**). In order to mirror experimental imaging as closely as possible, negative control images were subjected to volume deconvolution and contrast enhancement. For each serum tested, contrast enhancement revealed even, diffuse

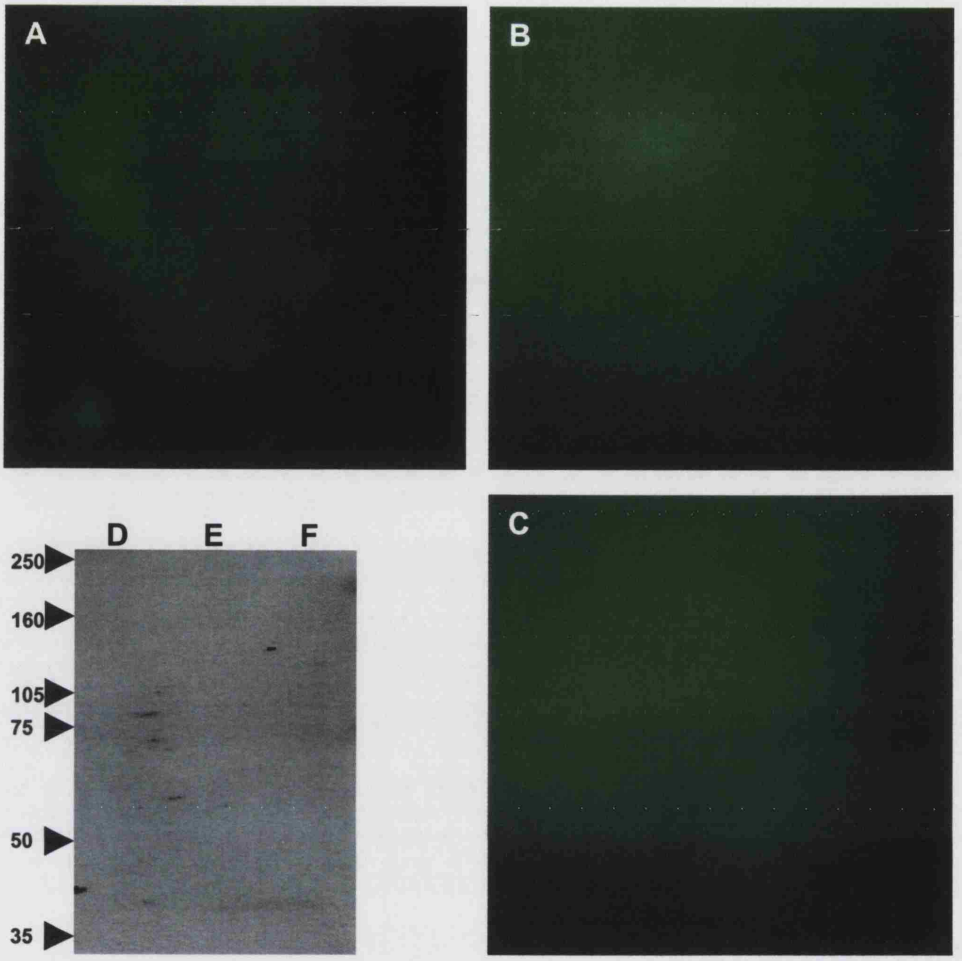

Figure 3.07: Negative Control Data for Immunostaining.

Pre-immune beast sera were used to probe HEK 293 cells as negative controls for immunocytochemistry (**A-C**) and Western blotting (**E-F**). No specific signals were detectable by confocal microscopy. Western blotting failed to reveal any bands even after prolonged exposure (1hour). Each image is the representative of three experiments.

illumination (Figure 3.07 A-C) and Western blots showed no detectable bands, even after prolonged exposure (Figure 3.07 E-F).

4 Distinctive Localisation of Amino-Terminal FAK Constructs in the Nucleus and at Areas of Cell-to-Cell Contact

Introduction

Transient heterologous expression of FAK-derived constructs in HEK 293 cells was used to determine the subcellular distribution of ATF constructs and to attempt to reproduce the distinctive subcellular distribution of endogenous N-FAK immunoreactivity observed in this cell line (**Figure 3.01B**). It was hoped that co-localisation studies would lead to the identification of potentially novel protein-protein interactions, and lend insight into the function of the FAK amino-terminal domain in epithelial cells.

4.1 Optimisation of Transfection and Initial Characterisation of FAK-Derived Constructs by Western Blotting

Optimisation of HEK 293 cell transfection was performed using the Flag-FAK, Flag-ATF-386 and Flag-FAT constructs (**Figure 4.01**). LipofectAMINE (LF) and LipofectAMINE 2000 (LF2K) cationic lipid transfection reagents were tested according to the manufacturer's instructions. Briefly, HEK 293 cells were transfected with each construct using either LF or LF2K and were harvested either 24 or 48 hours post-transfection. The cells were lysed and the lysates subjected to protein quantitation. For each lysate, 20 μg total cellular protein was loaded per lane in an SDS gel and subjected to Western blotting using either anti-Flag M2 (**Figure 4.01A**), anti-N-FAK (**Figure 4.01B**) or anti-C-FAK (**Figure 4.01C**). For Flag-FAK and Flag-ATF constructs, LF2K transfection resulted in consistently greater exogenous protein expression than did LF. For Flag-FAT, no qualitative difference was observed between LF- and LF2K-mediated transfection. In all cases, there was no apparent difference in the level of exogenous protein expression between those cells harvested 24 hours post-transfection and those harvested 48 hours post-transfection (**Figure 4.01A**). For all subsequent Western blotting and immunoprecipitation experiments, cells were transfected using LF2K for 24 hours.

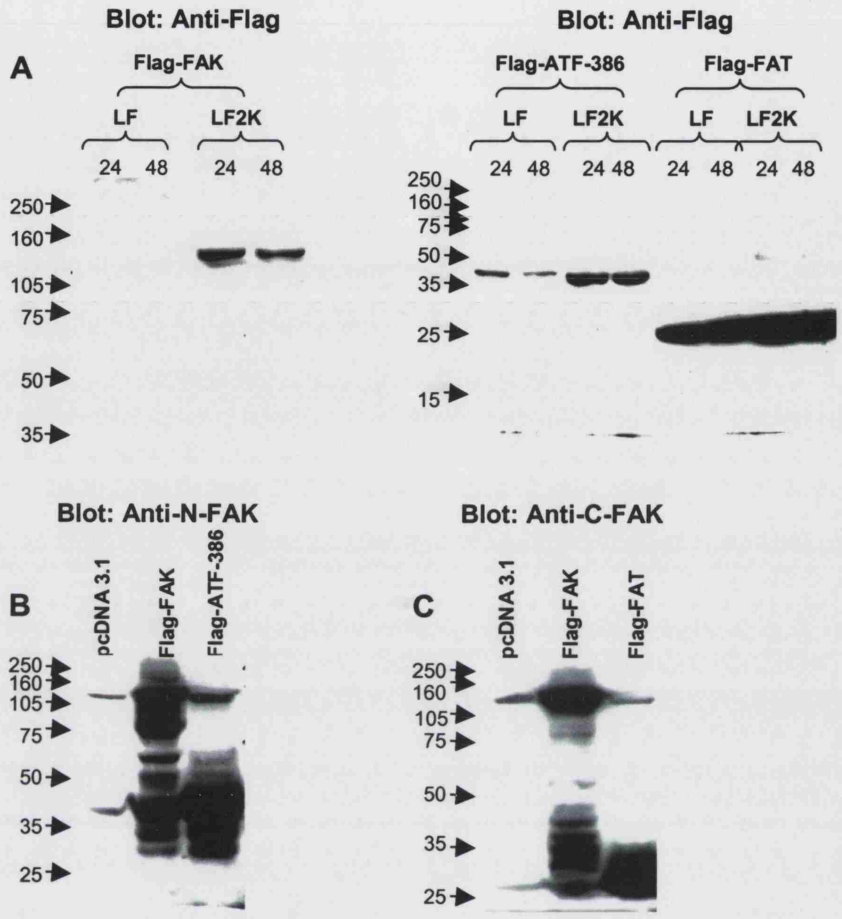

Figure 4.01: Optimisation of HEK 293 Transfection Using Lipofectamine Reagents and Flag-Tagged Constructs.

(**A**) HEK 293 cells were transfected with Flag-FAK, Flag-ATF-386 and Flag-FAT using either Lipofectamine (LF) or Lipofectamine 2000 (LF2K), as indicated. Protein was extracted from transfected cell cultures harvested 24 and 48 hours post-transfection, as indicated. Protein extracts were subjected to a protein assay, and 20 µg protein was loaded per lane in 8% (Flag-FAK) and 12% (Flag-ATF-386 and Flag-FAT) SDS gels. The gels were transferred to PVDF membranes which were probed with anti-Flag M2 (**A**). (**B** and **C**) Expression of transfected Flag constructs was compared with that of endogenous FAK protein. HEK 293 cells were transfected with empty vector, Flag-FAK, Flag-ATF-386 and Flag-FAT constructs, as indicated, using LF2k. Cells were harvested after 24 hours and 20 µg protein extract was loaded per well and subjected to SDS PAGE (10% gels) and Western blotting using anti-N-FAK (**B**) and anti-C-FAK (**C**). Each image is the representative of three experiments.

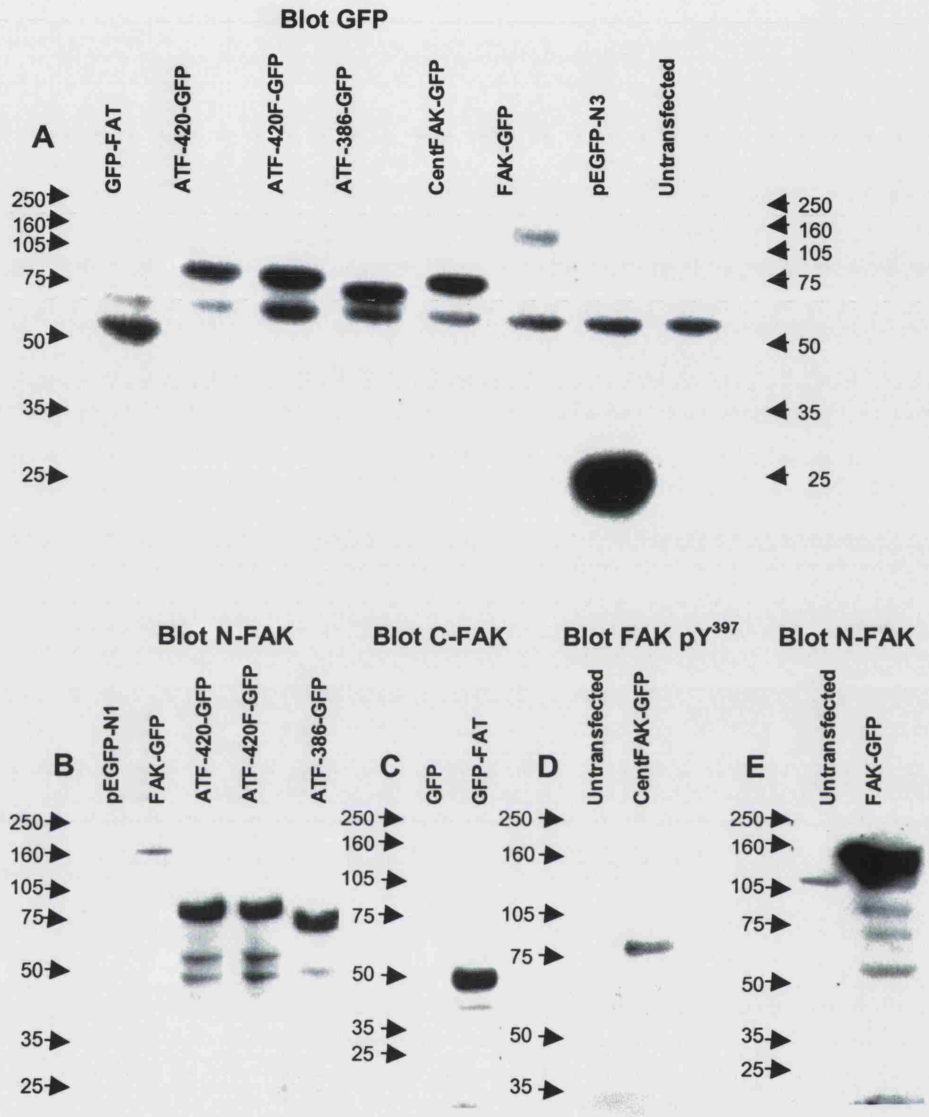

Figure 4.02: Western Blot Analysis of GFP-Tagged Construct Expression.

GFP-tagged FAK constructs were transiently transfected into HEK 293 cells using LF2K. Cells were harvested 24 hours post-transfection and 20 µg of protein extract from untransfected, vector-only transfected or FAK construct-transfected cells was loaded per lane, as indicated, in SDS-PAGE gels which were run, transferred to PVDF membrane and blotted with anti-GFP (**A**), anti-N-FAK (**B** and **E**), anti-C-FAK (**C**) or anti-FAKpY397 (**D**). Bands corresponding to the expected molecular mass were observed for each protein, and the anti-GFP antibody also recognised a non-specific band (*). Each blot is the representative of three experiments.

In order to compare LF2K-mediated expression with endogenous protein levels, HEK 293 cells were transfected with either empty vector (pcDNA 3.1) or Flag-tagged constructs. Lysates were prepared and in each case 20 μg total protein was run on an SDS gel and subjected to Western blotting using either anti-N-FAK (**Fig 4.01B,E**) or anti-C-FAK. In each case, FAK immunoreactivity was much stronger for the exogenously-expressed construct than for endogenous FAK. In all cases, a major band corresponding to the predicted molecular weight of the construct (125k for Flag-FAK, ~40k for Flag-ATF-386 and ~27k for Flag-ATF) was observed.

GFP-tagged construct expression was then characterised (**Figure 4.02**). pEGFP-N1, FAK-GFP, ATF-386-GFP, ATF-420-GFP, ATF-420F-GFP, CentFAK-GFP and GFP-FAT were transfected into HEK 293 cells. Cells were harvested after transfection and 20 μg total protein was loaded per land in an SDS gel and subjected to Western blotting analysis using either anti-GFP (**Figure 4.02A**), anti-N-FAK (**Figure 4.02B**), an antibody raised against phospho-tyrosine 397 of FAK (anti-FAK pY397, **Figure 4.02C**), or anti-C-FAK (**Figure 4.02D**). In some blots using anti-FAK antibodies (**Figure 4.02 B-C**), endogenous FAK was not detected as exposure was minimised to allow for sufficient resolution of the transfected construct. However, endogenous p125 FAK is shown in **Figure 4.02E**.

Using anti-GFP (**Figure 4.02A**), major bands corresponding to the predicted molecular weights of ~25k for GFP; ~50k for FAT-GFP; ~60k for ATF-386-GFP; ~75k for ATF-420-GFP, ATF-420F-GFP and CentFAK-GFP; and ~160 for FAK-GFP were observed. A non-specific band between 50k and 75k was observed in all lanes, including the untransfected lane.

Using anti-FAK antibodies, major bands were also observed corresponding to the expected molecular weights of the transfected constructs (**Figure 4.02B-E**). In addition, minor N-FAK-reactive bands were seen in ATF-386-GFP, ATF-420-GFP, ATF-420F-GFP and FAK-GFP transfected lysates (**Figure 4.02B and E**); and a single minor C-FAK reactive band was seen in FAT-GFP transfected lysates (**Figure 4.02C**). Comparing 20

μg total protein from untransfected and FAK-GFP-transfected cells, N-FAK immunoreactivity was much more intense in transfected than untransfected cells (**Figure 4.02E**). Anti-pY397 immunoreactivity was observed in CentFAK-GFP transfected cells but not in untransfected lysates (**Figure 4.02D**) even after prolonged exposure.

4.2 Characterisation by Confocal Microscopy Reveals Distinctive Subcellular Distribution of ATF Constructs in HEK 293 and MDCK Cells

For imaging analysis of construct expression, HEK 293 cells were transfected with LF rather than LF2K. This was due to the fact that LF transfection was performed at <50% confluence (compared to ~90% confluence for LF2K), and imaging of cells from sub-confluent cultures facilitated better resolution of neighbouring cells and more precise definition of cell-cell contacts.

In general, methanol fixation prior to antibody staining produced clearer images than did paraformaldehyde fixation. However, methanol fixation of GFP-transfected cells seemed to result in decreased GFP signal compared to paraformaldehyde fixation. Therefore, for imaging experiments, untransfected cells and cells expressing Flag-tagged constructs were fixed in methanol, whereas cells expressing GFP constructs were fixed in paraformaldehyde.

As a preliminary investigation into subcellular distribution, all ATF constructs (ATF-386-GFP, ATF-420-GFP, ATF-420F-GFP and Flag-ATF) were transfected into HEK 293 cells. Cells were fixed and Flag-ATF-386-transfected cells were stained with anti-Flag M5 antibody and visualised using a FITC-conjugated anti-mouse antibody (**Figure 4.03**). The subcellular distribution of all four constructs was similar, with increased intensity in the nucleus and at areas of cell-to-cell contact, although cytosolic and membrane expression was also observed.

HEK-293 cells were transfected with Flag-FAK, FAK-GFP, CentFAK-GFP, Flag-FAT and GFP-FAT for comparison (**Figure 4.04**). For each of these constructs, expression

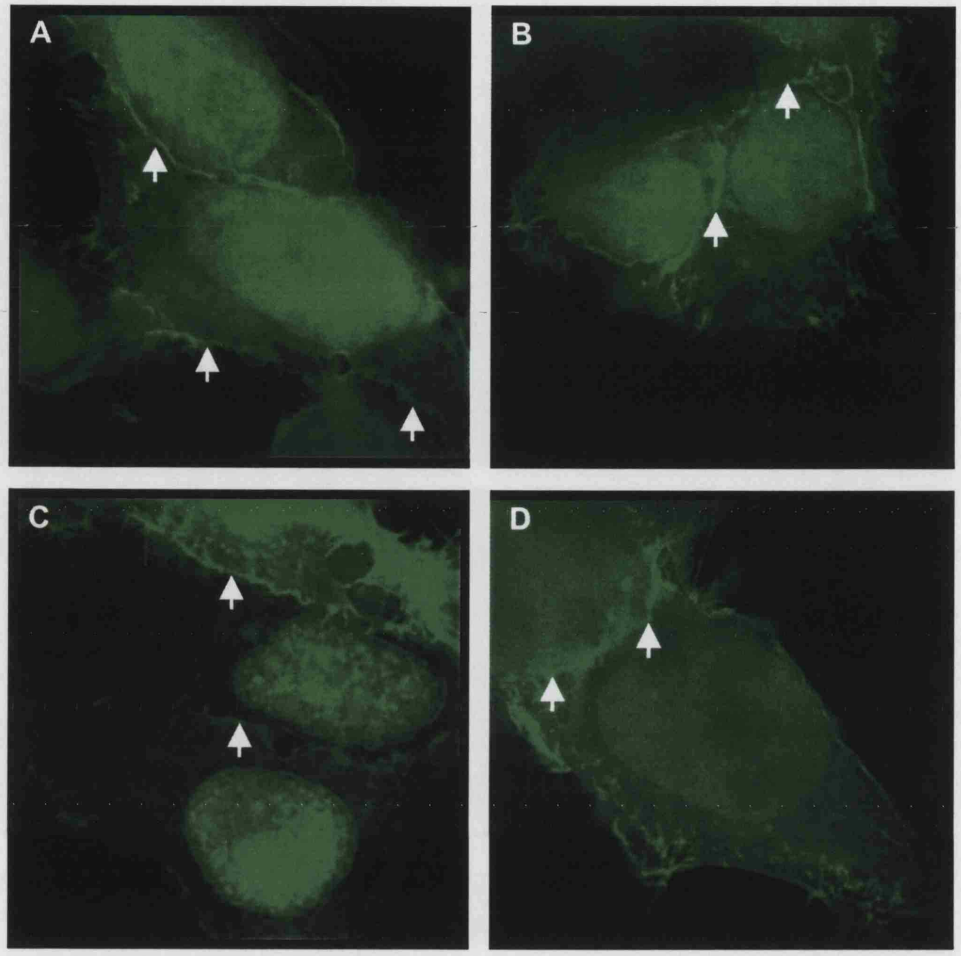

Figure 4.03: Subcellular Localisation of Amino-Terminal FAK Constructs in HEK 293 Cells.
HEK 293 cells grown on glass coverslips were transfected with ATF-420-GFP (**A**), ATF-420F-GFP (**B**), ATF-386-GFP (**C**) and Flag-ATF-386 (**D**). Approximately 24 hours post-transfection, coverslips were washed and either fixed with paraformaldehyde (**A-C**); or fixed in methanol, stained with anti-Flag M5 antibody and visualised using FITC-conjugated anti-mouse (**D**). Fixed and/or stained cells were mounted as described and viewed under the fluorescent microscope. Fluorescence intensity is increased at areas of cell-to-cell contact in the apical plane of the cells (arrows) and in the nuclei. Each image is the representative of four experiments.

appeared to be largely perinuclear and diffusely cytoplasmic, with low signal intensity in the nucleus and at areas of cell-to-cell contact.

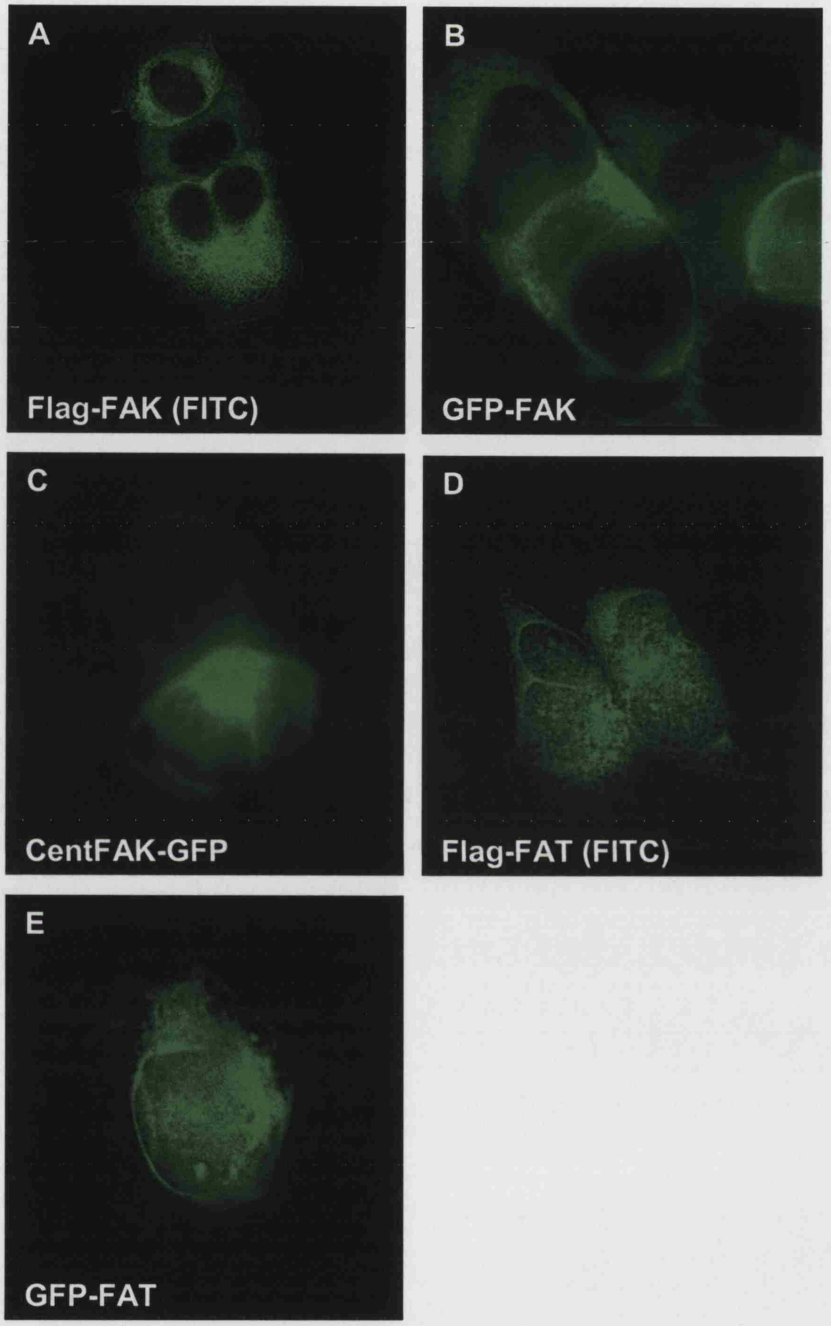

Figure 4.04: Subcellular Distribution of FAK, FAT and CentFAK Constructs in HEK 293 Cells.
HEK 293 cells were seeded on glass coverslips and transfected using LF with Flag-FAK (**A**), FAK-GFP (**B**), CentFAK-GFP (**C**), Flag-FAT (**D**) or GFP-FAT (**E**). Cells were either fixed in paraformaldehyde and viewed directly under the microscope (**B,C, E**) or else were fixed in methanol and stained with anti-Flag M5 antibody prior to examination (**A, D**). Fluorescence is largely cytosolic and perinuclear, with little signal in the nucleus and cell-cell contacts. Each image is the representative of three experiments.

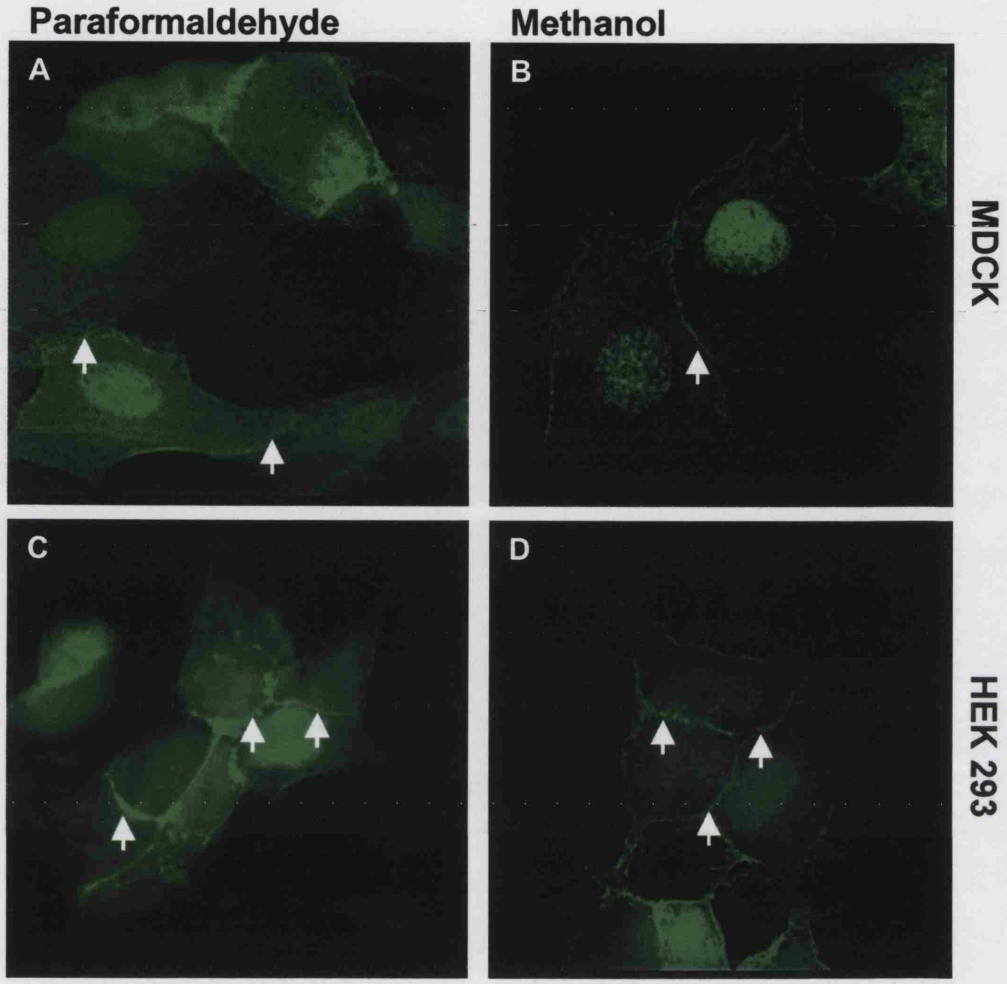

Figure 4.05: Flag-ATF-386 Localise to Areas of Cell-Cell Contact in HEK 293 Cells and in MDCK Cells.

MDCK cells (**A** and **B**) and HEK 293 cells (**C** and **D**) were seeded on glass coverslips and transfected with Flag-ATF-386 using LF+ (**A** and **B**) or LF (**C** and **D**). Cells were subsequently fixed either with paraformaldehyde (**A** and **C**) or with methanol (**B** and **D**) prior to staining with the anti-Flag M5 antibody. Fluorescence intensity is particularly marked at areas of cell-cell contact in the apical plane of the cells. Each image is the representative of four experiments.

In order to control for any effect that might be produced due to some peculiarity of HEK 293 cells or of the fixation process, Flag-ATF-386 was transfected into MDCK and HEK 293 cells in parallel experiments, and cells were fixed in either paraformaldehyde or methanol (**Figure 4.05**). Subcellular distribution of Flag-ATF-386 in MDCK cells

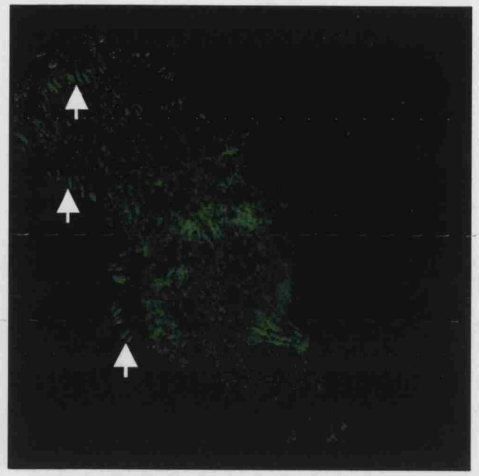

Figure 4.06: Flag-FAK is Expressed in Structures Resembling Focal Contacts in MDCK Cells.
MDCK cells were transfected with Flag-FAK using LF+ as described. The following day, cell-coated coverslips with fixed with methanol and stained with the anti-Flag M5 antibody, as described. Flag immunoreactivity is concentrated in discrete, elongated structures that resemble focal contacts (arrows) in the basolateral aspect of the cell. This image is the representative of four experiments.

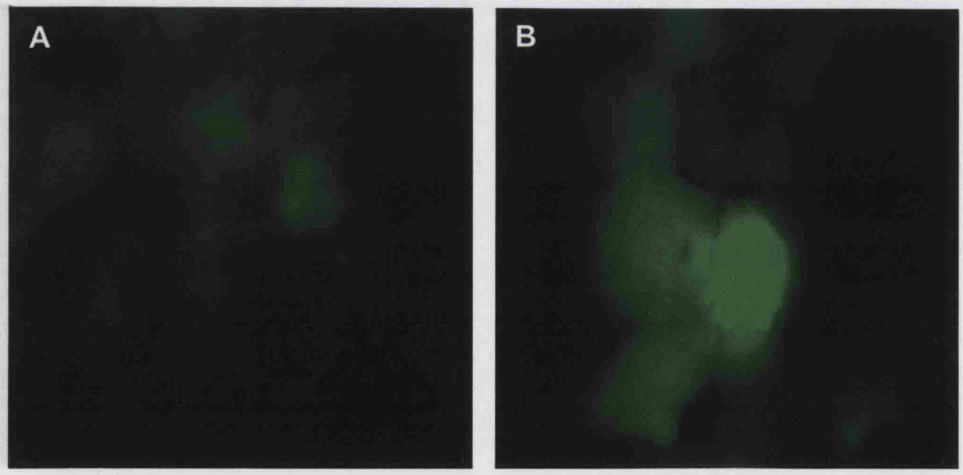

Figure 4.07: Negative Control Data for Fluorescence Microscopy Experiments using Flag- and GFP-Tagged Constructs.
HEK 293 cells were transfected with either pcDNA 3.1 (**A**) or pEGFP-N3 (**B**) using LF. Transfected cells were either fixed in methanol and stained with anti-Flag M5 antibody (**A**) or fixed in paraformaldehyde and visualised directly (**B**). Images were subjected to volume deconvolution and contrast enhancement as for experimental images. Each image is the representative of three experiments.

(**Figure 4.05A-B**) was similar to that observed in HEK 293 cells (**Figure 4.05C-D**), being particularly marked in the nucleus and at areas of cell-cell contact. No consistent difference was observed in Flag-ATF-386 distribution in paraformaldehyde (**Figure 4.05A** and **C**) *versus* methanol fixed cells (**Figure 4.05B** and **D**).

In contrast to results obtained using the Flag-ATF construct, confocal microscopy of MDCK cells transfected with Flag-FAK revealed anti-Flag immunoreactivity in focal contacts (**Figure 4.06**).

In order to determine whether the expression tags and detection systems for the exogenous constructs may have given rise to any of the observed results, HEK 293 cells were transfected with either empty pcDNA 3.1 (**Figure 4.07A**) or empty pEGFP-N1 (**Figure 4.07B**). Cells were fixed and pcDNA transfected cells were stained using anti-Flag M5 and FITC-conjugated anti-mouse prior to image analysis. Anti-Flag staining was extremely dim, however contrast enhancement revealed diffuse staining (**Figure 4.07A**), and GFP expression was diffuse throughout the cell (**Figure 4.07B**).

4.3 ATF Constructs Co-Localise with Filamentous Actin, β1 Integrin, Occludin and the Nuclear Dye DAPI

ATF constructs consistently displayed increased fluorescence intensity at areas of cell-to-cell contact (**Figure 4.03** and **Figure 4.05**). Having established that localisation of ATF constructs to areas of cell-cell contact is not dependent on Y397, ATF-386 constructs were used to further define subcellular localisation. It was hoped that in using ATF-386 constructs, identification of Y397-mediated protein-protein interactions might be avoided in co-localisation studies as well as in subsequent immunoprecipitation experiments. Therefore, ATF-386 construct localisation was assessed with respect to endogenous proteins shown to localise to cell-cell contacts in HEK 293 cells. The candidate proteins chosen for this investigation, based on their observed localisation in untransfected cells, were β_1 integrin and occludin. In addition, co-localisation of ATF constructs with F-actin was investigated.

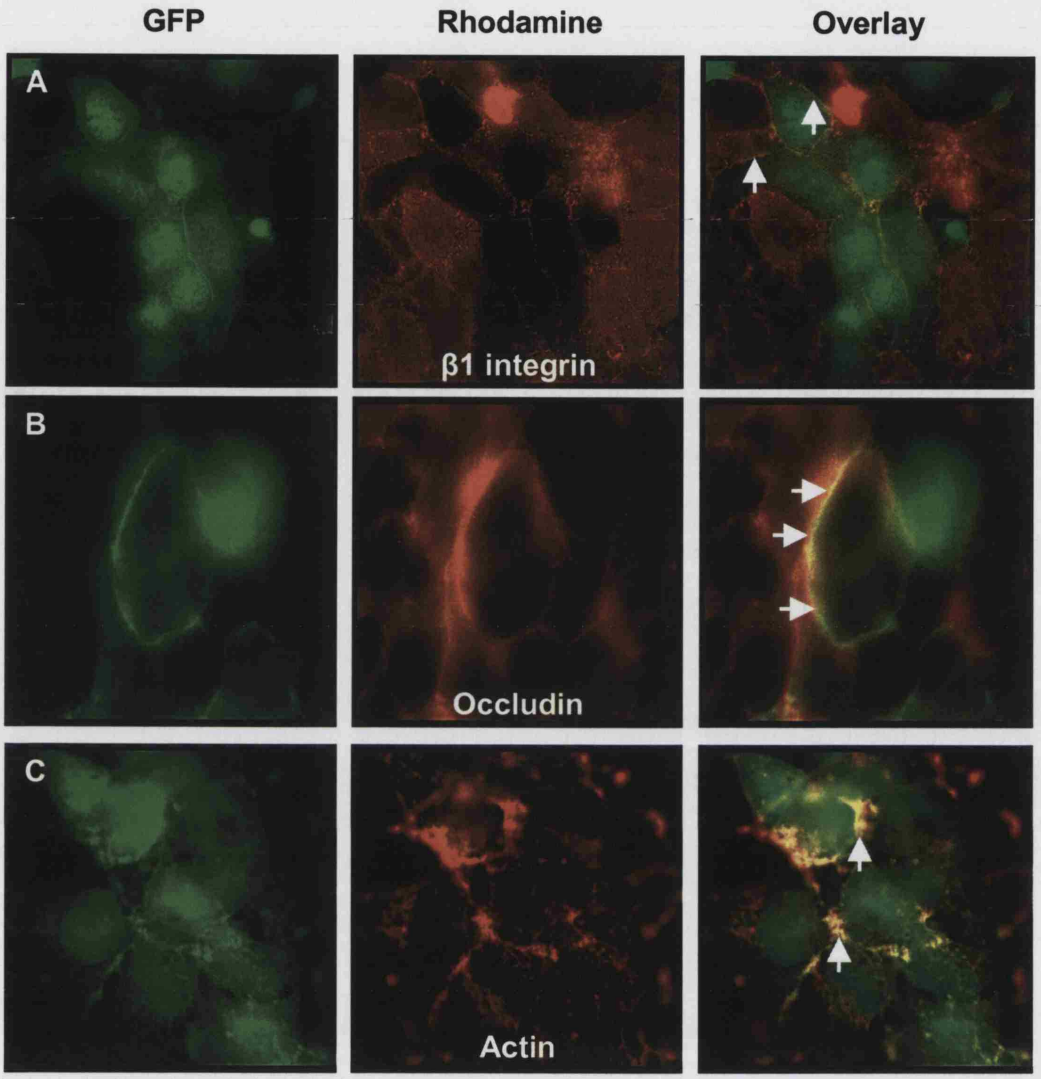

Figure 4.08: Exogenously Expressed Flag-ATF-386 Co-Localises with Endogenous β_1 Integrin, Occludin and Actin.

HEK 293 cells were transiently transfected with ATF-386-GFP fixed with paraformaldehyde and stained with anti-β_1 integrin (**A**), anti-occludin (**B**) or rhodamine-phalloidin (**C**). Staining of β_1 integrin and occludin were visualised using Rhodamine-Red-X conjugated secondary antibodies. Images from identical focal planes were taken of GFP and rhodamine fluorescence, as indicated, and overlaid. Areas of overlapping fluorescence intensity are seen as yellow in the overlaid images (arrows). Co-localisation of ATF-386-GFP is observed with actin, β_1 integrin and occludin in the apical plane of the cell. Each image is the representative of four experiments.

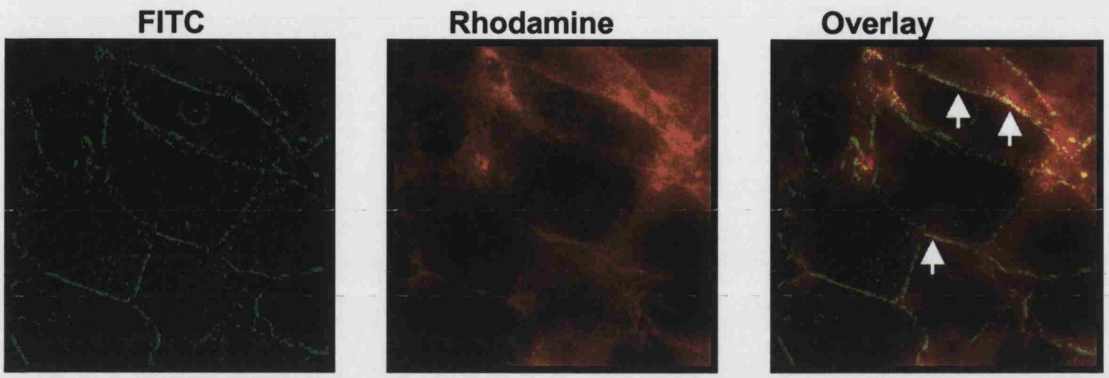

Figure 4.09: Co-Localisation of Endogenous β_1 Integrin and Occludin in HEK 293 Cells.
Untransfected HEK 293 cells were fixed in methanol and stained with polyclonal anti-occludin and monoclonal anti-β_1 integrin from Transduction Laboratories. Primary antibody staining was visualised using anti-mouse FITC (β_1 integrin) and anti-rabbit Rhodamine-Red-X (occludin). FITC and rhodamine images from the apical plane were subjected to volume deconvolution and overlaid. Overlapping fluorescence intensity, which appears yellow in the overlaid image (arrows), is particularly marked at areas of cell-cell contact. Each image is the representative of three experiments.

HEK 293 cells were transfected with ATF-386-GFP and stained with anti-occludin, β_1 integrin and rhodamine-conjugated phalloidin (**Figure 4.08**). Localisation of occludin and β_1 integrin subunits was visualised using Rhodamine-Red-X conjugated anti-rabbit and anti-mouse secondary antibodies, respectively. β_1 integrin subunit fluorescence appeared to be diminished in ATF-386-GFP transfected cells compared to neighbouring cells that did not express green fluorescence; however, co-localisation of β_1 with ATF-386-GFP was observed at cell-cell contacts (**Figure 4.08A**). ATF-386-GFP also co-localised consistently with occludin (**Figure 4.08B**) and F-actin (**Figure 4.08C**) at areas of cell-to-cell contact.

The observed distribution of β_1 integrin subunits at areas of cell-cell contact prompted investigation of the co-localisation of β_1 with occludin. Co-localisation of β_1 integrin and occludin staining was strong, consistent and almost exclusively observed at areas of cell-to-cell contact (**Figure 4.09**).

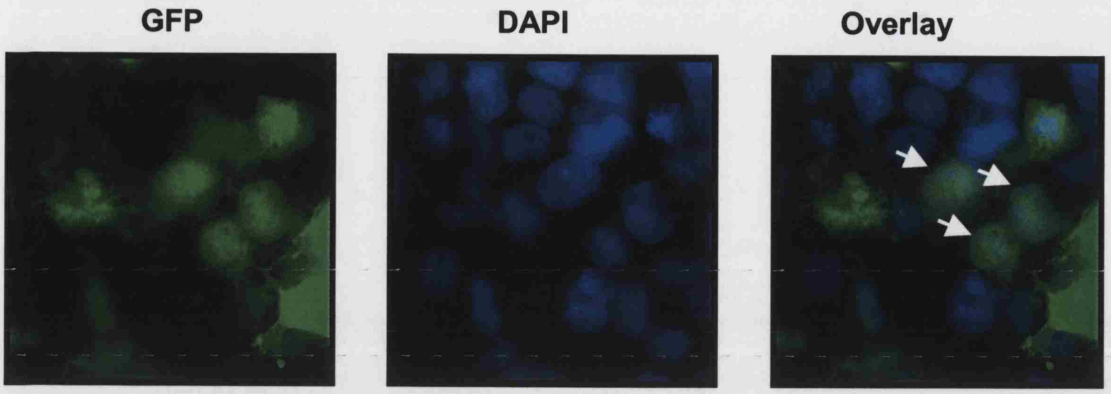

Figure 4.10: ATF-386-GFP Exhibits Nuclear Localisation.

HEK 293 cells transfected with ATF-386-GFP were fixed with paraformaldehyde and stained with the nuclear dye DAPI. Images of GFP and DAPI fluorescence from the basal plane were taken and overlaid such that overlapping fluorescence intensity appears cyan. Co-localisation of GFP and DAPI fluorescence is seen in GFP expressing cells (arrows). Each image is the representative of three experiments.

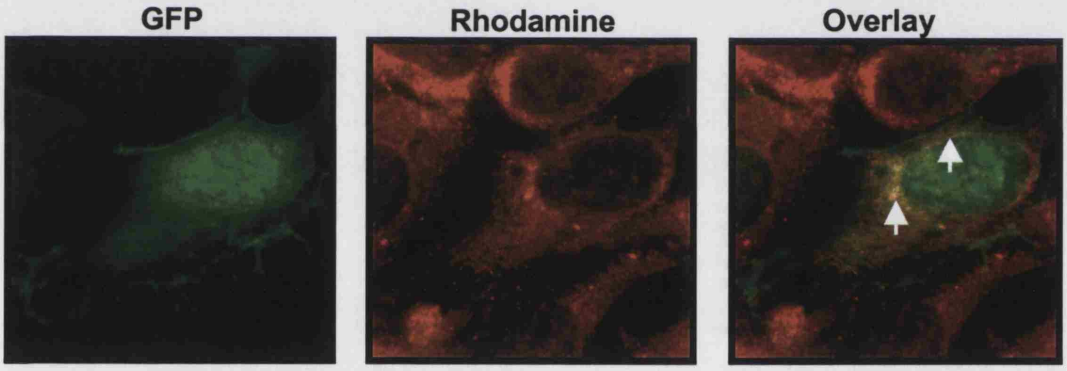

Figure 4.11: Differential Localisation of Exogenously-Expressed ATF-386-GFP and Endogenous C-FAK Immunoreactivity.

ATF-386-GFP transfected HEK 293 cells were fixed in paraformaldehyde and stained with anti-C-FAK antibody, which was visualised using anti-rabbit Rhodamine-Red-X. Images of FITC and rhodamine staining were of the same focal plane and overlaid such that overlapping signal intensity appears yellow. A degree of perinuclear and cytosolic co-localisation is seen (arrows). However, the striking nuclear and cell-cell contact localisation of ATF-386-GFP is not mirrored by C-FAK immunoreactivity. Each image is the representative of four experiments.

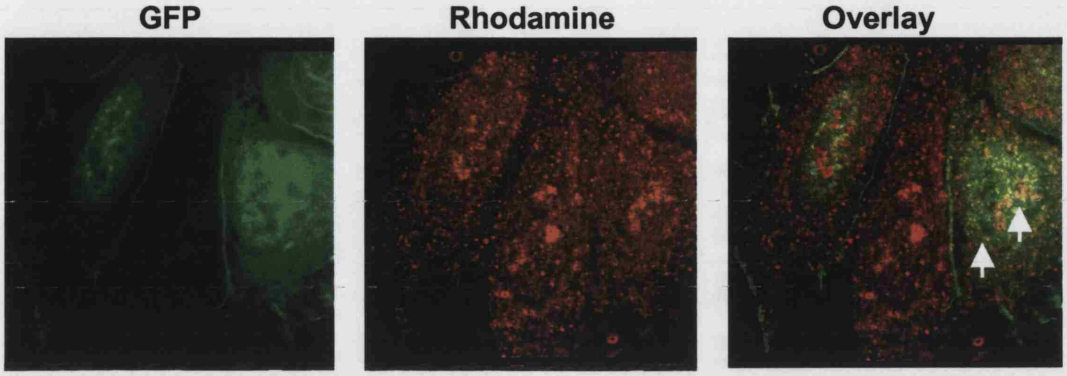

Figure 4.12: Differential Subcellular Distribution of ATF-386-GFP and Epidermal Growth Factor Receptors.

ATF-386-GFP transfected HEK 293 cells were fixed with paraformaldehyde and stained with anti-EGF receptor (EGFR) antibody, which was visualised using a Rhodamine-Red-X conjugated anti-rabbit. Images of GFP and rhodamine fluorescence from the same focal plane were overlaid such that the overlapping signals produce a yellow colour. EGFR immunoreactivity is seen to be evenly-dispersed through all compartments of the cell. In some cells, marked nuclear co-localisation of ATF-386-GFP and EGFR signals was observed (arrows).

Finally, the apparent nuclear localisation of ATF-386-GFP was investigated using the nuclear dye DAPI. HEK 293 cells transfected with ATF-386-GFP were fixed in paraformaldehyde and counterstained with DAPI as described (**Figure 4.10**). Co-localisation of ATF-386-GFP with DAPI was observed in all cells that expressed nuclear green fluorescence.

4.4 ATF-386-GFP Localisation is Distinct from that of Endogenous FAK and of Endogenous EGF Receptors.

Subcellular localisation of ATF-386-GFP with respect to endogenous FAK was investigated using HEK 293 cells transfected with ATF-386-GFP, and anti-C-FAK antibody, as described. Although a degree of perinuclear co-localisation was observed, C-FAK immunoreactivity was negligible in both the nucleus and at areas of cell-to-cell contact, the brightest areas of ATF-386-GFP fluorescence (**Figure 4.11**).

During the progress of this thesis, data were published that provided evidence of an interaction between FAK and EGF receptors mediated by the FAK amino-terminal domain (Sieg *et al.*, 2004). Since EGF receptors can be expressed at cell-cell contacts (Kwiatkowska *et al.*, 1991), the co-localisation of ATF-386-GFP with endogenous EGFR at areas of cell-cell contact was assessed. In HEK 293 cells transfected with ATF-386-GFP, EGF receptors were expressed throughout the nucleus and cytoplasm of the cell (**Figure 4.12**). Although nuclear co-localisation of ATF-386-GFP with EGF receptors was observed (**Figure 4.12**), EGF receptors did not co-localise with ATF-386-GFP at areas of cell-cell contact (**Figure 4.12**).

5 Investigation of a Mechanism for the Localisation of Amino-Terminal FAK Fragments

Introduction

The localisation of endogenous amino-terminal FAK fragments at cell-cell contacts in HEK 293 cells (**Figure 3.01B**) was reproduced using epitope-tagged ATF constructs (**Figure 4.03**). Furthermore, ATF constructs were observed to co-localise with actin, β_1 integrin subunits and occludin (**Figure 4.08**). Transient heterologous expression and immunoprecipitation were used to determine whether ATF constructs form complexes with these and other proteins.

5.1 Characterisation of Anti-Flag and Anti-FAK Antibodies in Immunoprecipitation

Immunoprecipitation experiments were performed to determine whether ATF constructs form complexes with those proteins with which they co-localised at areas of cell-cell contacts in intact HEK 293 cells, namely β_1 integrin, occludin and actin (**Figure 4.08**). Flag-tagged FAK constructs were transfected into HEK 293 cells which were harvested 24 hours post-transfection. Protein lysates were prepared as described and subjected to immunoprecipitation using anti-Flag M2 antibody. Lysates of untransfected cells and cells transfected with empty vector were also prepared as controls. The resulting immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with anti-occludin, anti-β_1 integrin and anti-actin (**Figure 5.01**). Bands corresponding to the expected molecular weights for β_1 integrin (**Figure 5.01A**), occludin (**Figure 5.01B**) and actin (**Fig 5.01C**) were seen in all lanes, including those that had not been transfected with Flag-tagged constructs.

Immunoprecipitations were subsequently performed using anti-FAK antibodies. HEK 293 cells transfected with Flag-tagged FAK constructs or empty vector pcDNA3.1 were subjected to immunoprecipitation using anti-N-FAK or anti-C-FAK. The resulting

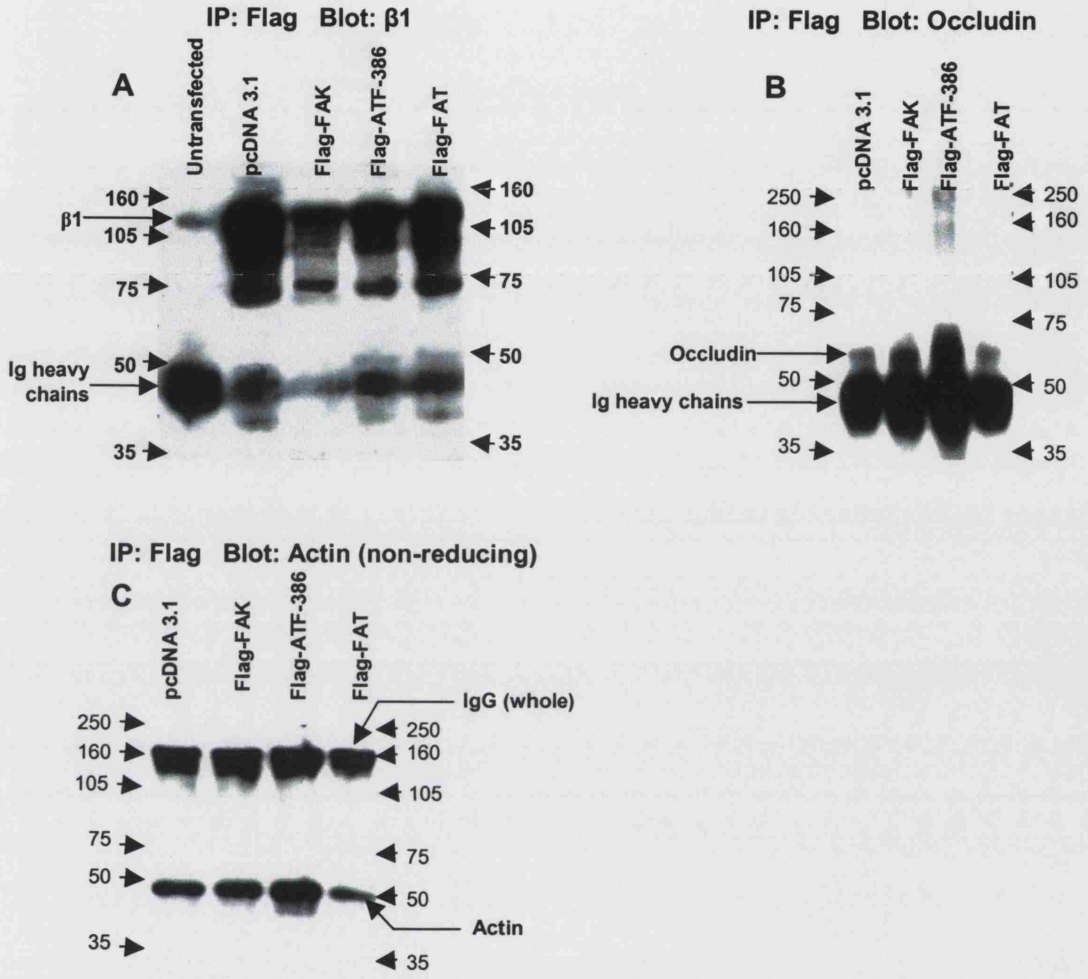

Figure 5.01: Anti-Flag Antibody Immunopreciptates β_1 Integrin, Actin and Occludin Independently of the Presence of Flag-Tagged Constructs.

HEK 293 cells were transfected with empty pcDNA 3.1, Flag-FAK, Flag-ATF-386 or Flag-FAT, as indicated using LF2K. Cells were harvested and lysates prepared for immunoprecipitation as described. Each lysate was then subjected to immunoprecipitation using 5 μg anti-Flag M2 antibody, followed by SDS-PAGE in reducing conditions (**A** and **B**) or non-reducing conditions (**C**). The protein gels were transferred to PVDF membranes and subjected to Western blotting using anti-β_1 integrin (**A**), anti-occludin (**B**) or anti-actin (**C**). Bands are seen in each lane corresponding to the expected molecular weights of β_1 integrin, occludin and actin (arrows). Antibody heavy chains (**A** and **B**) and whole IgG (**C**) are indicated. Each image is the representative of five experiments.

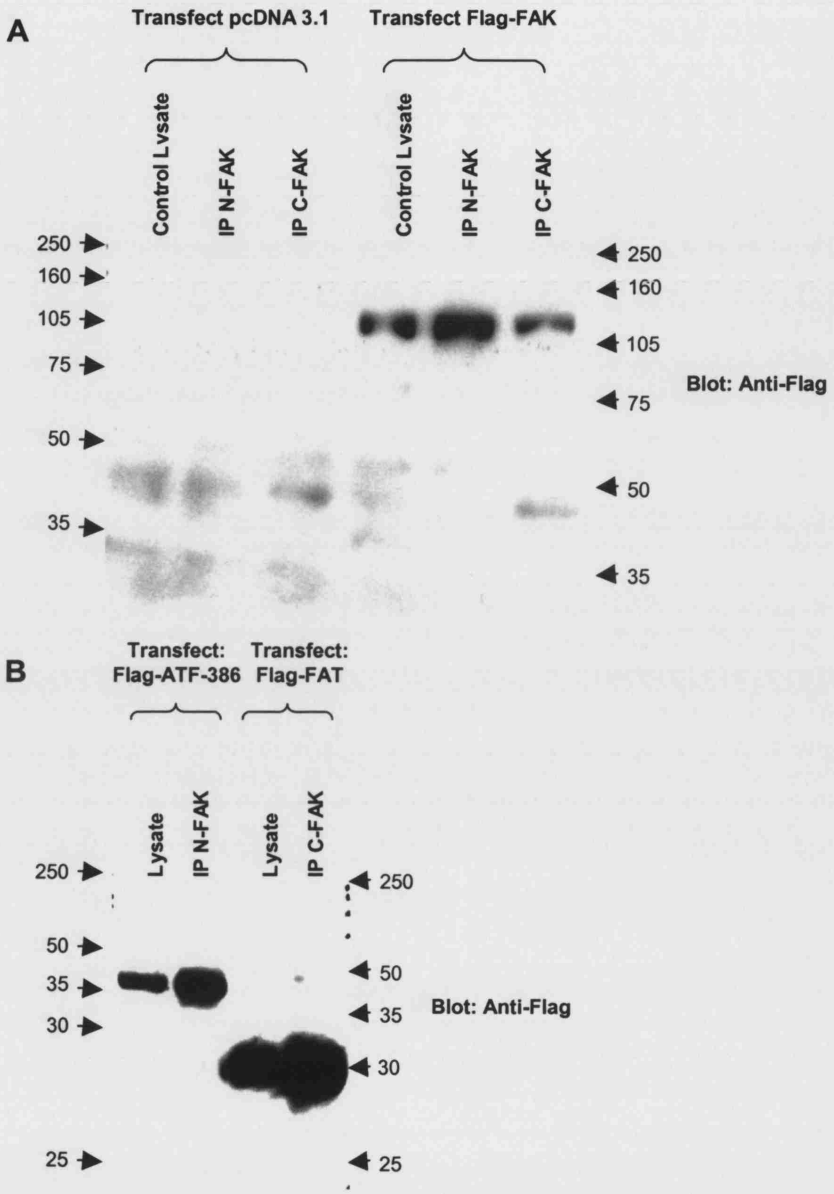

Figure 5.02: Immunoprecipitation of Flag-Tagged FAK Constructs Using Anti-FAK Antibodies.
HEK 293 cells transfected with either empty pcDNA 3.1, Flag-FAK, Flag-ATF-386 or Flag-FAT, as indicated. Lysates were prepared as described and immunoprecipitated using 5 µg anti-N-FAK or 5 µg anti-C-FAK, as indicated. The resulting immunoprecipitates were run on 8% (**A**) or 12% (**B**) SDS-PAGE gels, transferred onto PVDF membranes and blotted with anti-Flag M2 antibody. In each case, 20 µg of transfected whole cell lysate was run alongside the immunoprecipitate for comparison. In each case, the anti-FAK antibodies are seen to successfully precipitate the Flag-tagged constructs, which are not seen in the empty vector-transfected lysates (**A**). Each image is the representative of four experiments.

immunoprecipitates were subjected to SDS-PAGE and Western blotting using anti-Flag M2 antibody (**Figure 5.02**). Anti-Flag-reactive bands corresponding to those expected for Flag-FAK (**Figure 5.02A**), Flag-ATF-386 (**Figure 5.02B**) and Flag-FAT (**Fig 5.02B**) were observed in both immunoprecipitated and non-immunoprecipitated transfected lysates, however no bands were observed in empty vector pcDNA3.1-transfected lysates (**Figure 5.02A**).

5.2 β1 Integrin and Occludin do not Co-Immunoprecipitate with FAK-Derived Constructs

Flag-ATF-386, ATF-386-GFP, Flag-FAK and Flag-FAT were transfected into HEK 293 cells together with the corresponding empty-vector controls. Lysates were subjected to immunoprecipitation with N-FAK and C-FAK antibodies. Immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with anti-β_1 integrin (**Figure 5.03**) or anti-occludin (**Figure 5.04**). Immunoprecipitation of Flag-tagged constructs (**Figure 5.03A** and **B**) and of ATF-386-GFP (**Figure 5.03A**) failed to co-immunoprecipitate any band corresponding to the predicted full length β_1 integrin or occludin (**Figure 5.04**).

5.3 FAK-Derived Constructs do not Form an Immunoprecipitable Complex with Actin, nor do they Display Differential Triton Solubility in the Presence of Cytochalasin D

HEK 293 cells were transfected with Flag-ATF-386, ATF-386-GFP and empty vectors pcDNA 3.1 and pEGFP-N1. Protein lysates were prepared 24 hours post-transfection and subjected to immunoprecipitation using anti-N-FAK (**Figure 5.05**). The resulting immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with anti-actin antibody. No band was seen corresponding to actin in any lane.

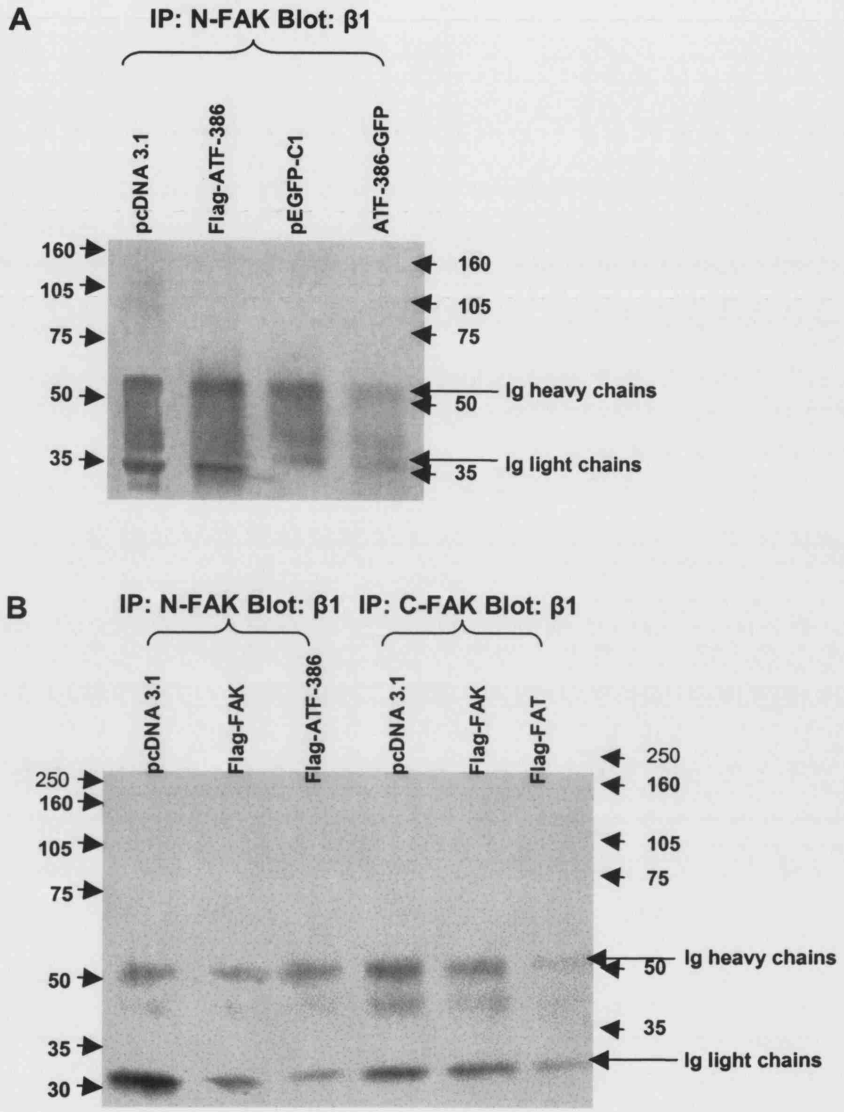

Figure 5.03: Amino-Terminal FAK Constructs do not Form an Immunoprecipitable Complex with β_1 Integrin.

HEK 293 cells were transfected with either Flag-FAK, Flag-ATF-386, Flag-FAT, ATF-386-GFP or the empty vectors pcDNA3.1 or pEGFP-C1, as indicated. Lysates were prepared as described and subjected to immunoprecipitation using either anti-N-FAK antibody or anti-C-FAK antibody, as indicated. The resulting immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with anti-β_1 integrin antibody. No band corresponding to the predicted molecular weight for β_1 integrin is seen in any lane. Ig heavy and light chains are indicated. Each image is the representative of two experiments.

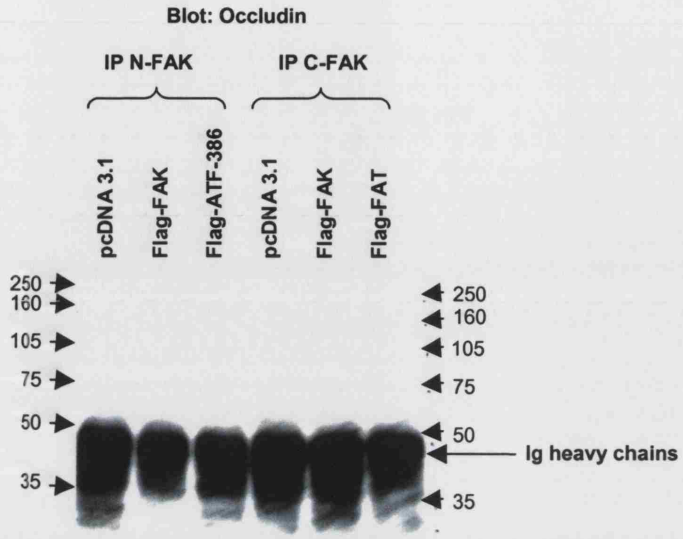

Figure 5.04: FAK Constructs do not Form an Immunoprecipitable Complex with Occludin.
HEK 293 cells were transfected with empty pcDNA 3.1, Flag-FAK, Flag-ATF-386 or Flag-FAT, as indicated. Lysates were prepared as described and subjected to immunoprecipitation with either anti-N-FAK or anti-C-FAK, as indicated, followed by SDS PAGE and Western blotting. No band is seen corresponding to the predicted relative molecular weight of 65k for occludin. Antibody heavy chains are indicated. Each image is the representative of three experiments.

A cytoskeleton-binding assay that exploits the insolubility of the actin-based cytoskeleton in Triton-X-100 has been used to investigate in the interaction of FAK with the F-acton cytoskeleton (Schlaepfer *et al.*, 1998). This assay was used to determine whether ATF constructs might bind directly or indirectly to F-actin in a manner which was not detectable by immunoprecipitation. HEK 293 cells were transfected with Flag-FAK, Flag-ATF-386, Flag-FAT, FAK-GFP, ATF-386-GFP, ATF-420-GFP, ATF-420F-GFP and GFP-FAT. 24 hours post-transfection, cells were treated either with the known cytoskeletal inhibitor cytochalasin D or with a comparable volume of the solvent DMSO (**Figure 5.06**) for one hour. Cells were harvested and Triton-soluble and Triton-insoluble fractions were prepared as described. The resulting lysates were subjected to SDS-PAGE followed by Western blotting using either anti-Flag (**Figure 5.06A** and **B**) or anti-GFP (**Figure 5.06C**).

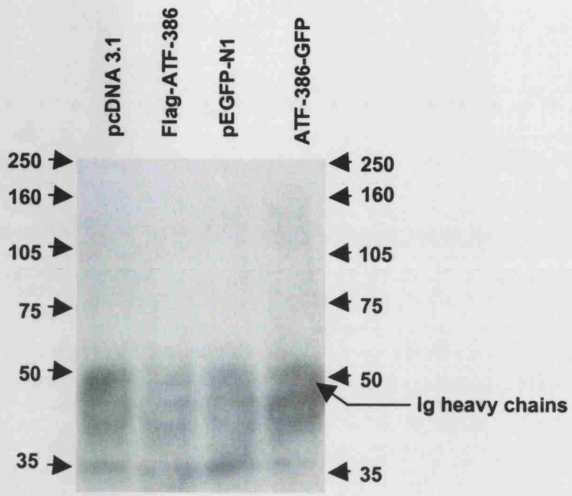

Figure 5.05: Amino-Terminal FAK Constructs do not Form an Immunoprecipitable Complex with Actin.

HEK 293 cells were transfected with pcDNA 3.1, Flag-ATF-386, p-EGFP-N1 or ATF-386-GFP, as indicated. 24 hours post transfection, protein lysates were prepared and these were immunoprecipitated using anti-N-FAK antibody. The resulting immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with anti-Actin. No band was seen in any lane corresponding to the ~60k band expected for actin. Antibody heavy chains are indicated. This image is the representative of two experiments.

Flag-FAK, Flag-ATF-386 and Flag-FAT proteins did not display any consistent differential distribution between the Triton-soluble (**Figure 5.06A**) and Triton-insoluble (**Figure 5.06B**) fractions in a manner affected by the presence or absence of cytochalasin D. Flag-FAK was observed in the Triton-soluble but not the Triton-insoluble fraction, however the presence of Flag-FAK in the Triton-soluble fraction was not affected by treatment with cytochalasin D (**Figure 5.06A** and **B**). The GFP-tagged constructs FAK-GFP, ATF-386-GFP, ATF-420-GFP, ATF-420F-GFP and GFP-FAT were consistently expressed in the Triton-soluble fraction in the presence or absence of cytochalasin D (**Figure 5.06C**).

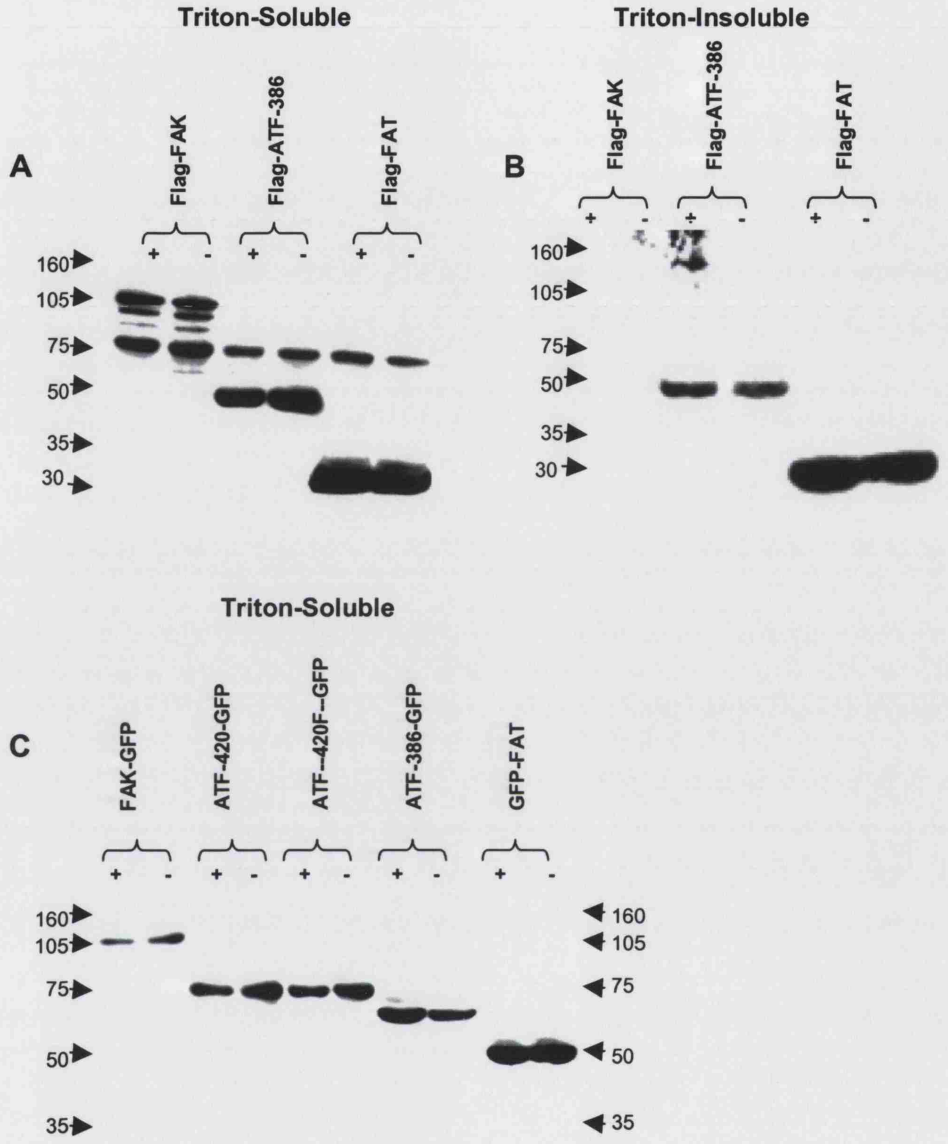

Figure 5.06: Cytochalasin D does not Affect the Partitioning of FAK-Derived Constructs into the Triton-Soluble Cell Fraction.

HEK 293 cells were transfected with Flag-tagged (**A** and **B**) or GFP-tagged (**C**) FAK constructs as indicated. 24-hours post-transfection, cells were treated with either cytochalasin D (+) or DMSO (-) as described. Triton-soluble (**A** and **C**) and Triton-insoluble (**B**) fractions were prepared and 5-20 μg total protein per sample was subjected to SDS-PAGE and blotted using either anti-Flag M2 (**A** and **B**) or anti-GFP (**C**). Autographs were subjected to varying degrees of exposure; however, differential Triton-solubility in the presence of cytochalasin D was not observed for any of the constructs tested. Each image is the representative of five experiments.

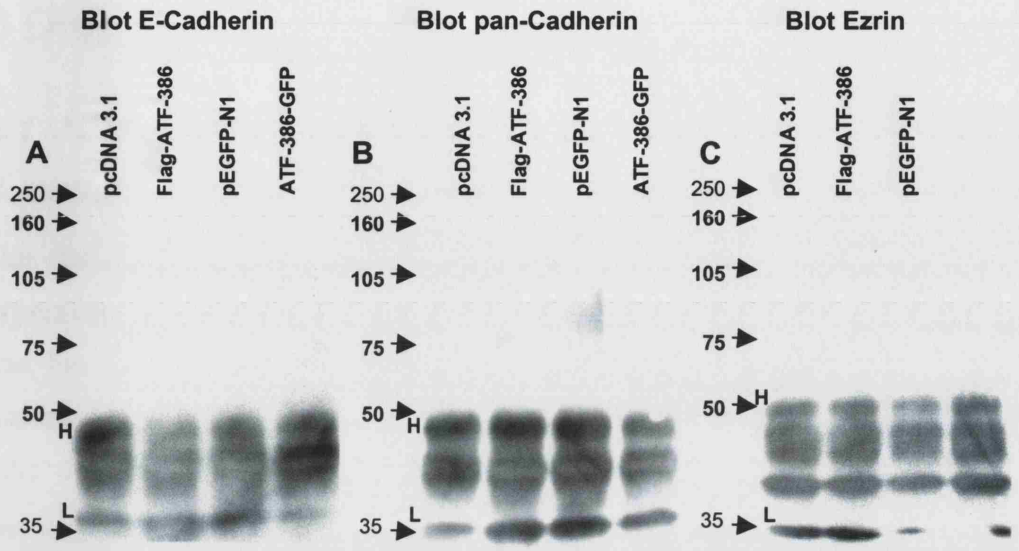

Figure 5.07 Amino-Terminal FAK Constructs do not form Immunopreciptable Complexes with E-Cadherin, pan-Cadherin or Ezrin.

HEK 293 cells were transfected with pcDNA3.1, Flag-ATF, pEGFP-N1 or ATF-386-GFP, as indicated. 24 hours post-transfection, lysates were prepared and subjected to immunoprecipitation as described using anti-E-cadherin (**A**), anti-pan-cadherin (**B**) or anti-ezrin (**C**). The resulting immunoprecipitates were subjected to SDS-PAGE followed by Western blotting using the anti-N-FAK antibody. No bands are seen corresponding to those expected for E-cadherin, pan-cadherin or ezrin. Antibody heavy chains (**H**) and light chains (**L**) are indicated. Each image is the representative of two experiments.

5.4 Amino-Terminal FAK Fragments Do Not Form Immunopreciptable Complexes with Cadherins or Ezrin

HEK 293 cells transfected with Flag-ATF-386, ATF-386-GFP or the empty vectors pcDNA3.1 and pEGFP-N1 were harvested 24 hours post-transfection and the resulting lysates were immunoprecipitated using anti-N-FAK antibody. Immunoprecipitates were subjected to SDS-PAGE followed by Western blotting using anti-E-cadherin (**Figure 5.07A**), anti-pan-cadherin (**Figure 5.07B**) or anti-ezrin (**Figure5.07C**). Immunoprecipitation of exogenous ATF proteins failed to co-immunoprecipitate E-cadherin, pan-cadherin or ezrin immunoreactivity.

6 Discussion of Results and Conclusions

6.1 HEK 293 Cells Express Proteins Associated with Tight Junctions, Adherens Junctions and Focal Contacts

The transformed epithelial cell line, HEK 293 is frequently used in heterologous expression studies due to its susceptibility to transfection. In spite of the fact that this cell line has been used previously to investigate aspects of integrin-mediated signalling relating to epithelial migration (e.g. Rodriguez-Frade *et al.*, 1999; Simon *et al.*, 1997), little work describing the expression of endogenous cellular contact proteins in this cell line had been published prior to commencing this thesis. Therefore, in order to establish this cell line as a useful model system in which to characterise the amino-terminal domain of FAK, it was assayed for expression of key focal contact (**Figure 3.01-3.02**), tight junction (**Figure 3.03**) and adherens junction (**Figure 3.05**) proteins using both confocal microscopy and Western blotting. HEK 293 cells were found to express focal contact proteins FAK, β_1 integrin and vinculin; the adherens junction proteins E-cadherin and β-catenin; the adherens junction and desmosomal protein plakoglobin; and the tight junction proteins occludin, ZO-1 and ZO-2 (**Figures 3.01-3.03, 3.05**). However, both confocal microscopy and Western blotting revealed unusual expression of these proteins in HEK 293 cells.

6.1.01 Subcellular Distribution of Focal Contact Proteins

Although HEK 293 cells were found to express proteins classically associated with focal contacts, they were not found to express focal contacts *per se*. Analysis of FAK expression using N-FAK and C-FAK antibodies revealed amino-terminal FAK staining throughout the cell, including the nucleus, with increased intensity at areas of cell-cell contact in the apical plane of the cell (**Figure 3.01B**). C-FAK staining, on the other hand, was primarily cytosolic, with increased perinuclear signal intensity, minimal staining of the nucleus and an absence of staining at cell-cell contacts (**Figure 3.01A**).

β_1 integrin subunit expression was largely found at areas of cell-cell contact in the apical plane of HEK 293 cells (**Figure 3.02A**). Junctional localisation of β_1 integrin staining was confirmed using an independent β_1 antibody and an occludin counterstain. (**Figure 4.09**) as well as by staining MDCK cells with anti-β_1 (**Figure 3.02C**). Cell-cell contact localisation of β_1 has previously been reported in both epithelial and endothelial cells (Tafazoli *et al.*, 2000; Yanez-Mo *et al.*, 1998). Furthermore, investigators have long assumed a direct interaction between FAK and β_1 cytoplasmic domains, proposing that this interaction is mediated by the FAK amino-terminal domain (Schaller *et al.*, 1992). The subcellular localisation of β_1 integrin in HEK 293 cells was therefore consistent both with published literature and with a potential interaction between the ~42k amino-terminal FAK fragment and β_1 at cell-cell contacts.

Vinculin expression in HEK 293 was found to be largely cytosolic with some concentration in basal punctate structures that could potentially represent a form of primitive focal contact (**Figure 3.02B**).

In summary, of those proteins examined that are normally thought to associate with focal contacts, none displayed the type of subcellular distribution consistent with unambiguous focal contact localisation. It can therefore reasonably be concluded that HEK 293 cells either do not express focal contacts or else express a highly unusual form of focal contact that may contain vinculin but does not contain FAK or β_1 integrin, in spite of the fact that both of these proteins are expressed in the cell.

6.1.02 Subcellular Distribution of Tight Junction Proteins

Confocal microscopy of HEK 293 cells with anti-occludin showed distinct cell-cell contact staining with a lesser degree of diffuse cytoplasmic staining (**Figure 3.03A**). ZO-1, on the other hand, exhibited what appeared to be diffuse membrane staining with perhaps some nuclear concentration (**Figure 3.03B**). ZO-2 displayed nuclear and perhaps membrane staining, with some expression in discrete basal punctate structures (**Figure 3.03C**) similar to those revealed by staining with anti-vinculin (**Figure 3.02B**). Therefore, of the tight junction proteins examined, only occludin exhibited the type of clear,

continuous, circumferential subcellular distribution associated with tight junction localisation (**Figure 3.03A**). Furthermore, the cellular distributions of ZO-1 and ZO-2 appeared to be quite distinct from each other (**Figure 3.03B-C**) and did not seem to correspond to either occludin (**Figure 3.03A**) or E-cadherin (**Figure 3.05A**) staining in HEK 293 cells. The subcellular distributions observed for ZO-1 and ZO-2 being both unexpected and inconsistent with tight junction localisation, all three tight junction antibodies were validated by staining MDCK cells. Anti-occludin, anti-ZO-1 and anti-ZO-2 antibodies exhibited classical cell-cell contact localisation in the apical aspect of MDCK cells (**Figure 3.04A-C**).

6.1.03 Subcellular Distribution of Adherens Junctions Proteins

In HEK 293 cells, E-cadherin displayed discontinuous membrane distribution, with a degree of cytosolic staining (**Figure 3.05A**). The staining observed for E-cadherin is consistent with loss of E-cadherin from cell-cell contacts, a phenomenon associated with epithelial-mesenchymal transition (Stockinger *et al.*, 2000). Plakoglobin displayed cytosolic and membrane distribution with increased signal intensity at cell-cell contacts (**Figure 3.05C**). Cell-cell contact localisation of plakoglobin in cells that lack strong circumferential E-cadherin staining could be explained by its potential participation in desmosomes, or by interaction with N-cadherin, the expression of which in epithelial cells is associated with epithelial-mesenchymal transition (Kim *et al.*, 2000). β-catenin was expressed in unusual, elongated protrusions (**Figure 3.05B**) that could not be identified. This subcellular localisation has not apparently been reported previously in the published literature. Anti-plakoglobin and anti-β-catenin antibodies were validated on MDCK cells, where they displayed classical cell-cell contact and membrane localisation in the apical plan of the cell (**Figure 3.06A-B**).

6.1.04 Western Blotting of HEK 293 Lysates Reveals High Constitutive Proteolytic Activity

The expression of endogenous cellular contact proteins in HEK 293 cells was confirmed by Western blotting, and bands were seen corresponding to the predicted relative molecular weights for FAK (**Figure 3.01C-D**), β_1 integrin (**Figure 3.02D**), vinculin (**Figure 3.02E**), occludin (**Figure 3.03D**), ZO-1 (**Figure 3.03E**), ZO-2 (**Figure 3.03F**),

E-cadherin (**Figure 3.05D**), β catenin (**Figure 3.05E**) and plakoglobin (**Figure 3.05F**). However, Western analysis of HEK 293 cells revealed the presence of additional bands of lower-than-expected relative molecular weight for a subset of proteins that included FAK (**Figure 3.01C-D**), β_1 integrin (**Figure 3.02D**), occludin (**Figure 3.03D**) and β catenin (**Figure 3.05E**).

Two FAK bands were observed in HEK 293 cells in addition to p125: a C-FAK reactive band migrating at ~55k and presumably corresponding either to FRNK or a FRNK-like proteolytic cleavage product (**Figure 3.01C**); and an N-FAK reactive band migrating at ~42k (**Figure 3.01D**) that has been previously reported (Carragher *et al.*, 2001; Carragher *et al.*, 1999; Cooray *et al.*, 1996; Ilic *et al.*, 1998; Cicala *et al.*, 2000; van de Water *et al.*, 1999; Gervais *et al.*, 1998; Wen *et al.*, 1997; Lobo & Zachary, 2000; Jones *et al.*, 2001). The distinctive subcellular distribution of N-FAK antibody staining observed in untransfected HEK 293 cells (**Figure 3.01B**; discussed above) could therefore be explained by cell-cell contact and nuclear targeting of the ~42k amino-terminal FAK fragment, consistent with the original hypothesis of this thesis.

Comparing Western blotting data from HEK 293 and MDCK cells revealed that the two additional occludin bands (**Figure 3.03D**) and the single additional β-catenin band (**Figure 3.05E**) found in HEK 293 lysates were not present in MDCK lysates (**Figures 3.04D, 3.06C**). Furthermore, although both HEK 293 and MDCK cells express a <50k β_1 integrin band in addition to the ~130k band expected for this protein, the relative signal intensity of the <50k band is greater in HEK 293 cells than it is in MDCK cells. The relative abundance of these lower-than-expected molecular weight bands in HEK 293 cells may be indicative of a high degree of constitutive proteolytic activity in this cell line. Elevated calpain proteolytic activity is frequently associated with cellular transformation (Carragher *et al.*, 2004) and since HEK 293 is a transformed cell line, it possible that calpain contributes to the increased proteolytic activity seen in these cells. Such a notion would be consistent with the observed degradation of exogenously expressed Flag-FAK (**Figure 4.01B-C**; discussed in more detail below) as FAK is a known substrate for calpain-mediated proteolysis (Cooray *et al.*, 1996; Carragher *et al.*,

2001; Carragher *et al.*, 1999). This hypothesis could be experimentally proven using calpain inhibitors such as ACCM. Alternatively, the abundance of these smaller protein fragments in HEK 293 cells could be the result of the activity of other proteases, or due to gene duplication/truncation events in this aneuploid cell line.

6.1.05 HEK 293 Cells as a Model for Studying the Amino-Terminal Domain of FAK

Although it cannot be said that HEK 293 cells express focal contact, adherens junctions or tight junction proteins in a way that exactly matches the classical models for these structures, they clearly do express protein components pertaining to each of them. The expression of the epithelial-specific proteins occludin (**Figure 3.03A,D**) and E-cadherin (**Figure 3.05A,D**) in these cells implies that they retain a degree of epithelial differentiation. Furthermore, the expression of occludin (**Figure 3.03A**) and β_1 integrin (**Figure 3.02A**) at areas of cell-cell contact in HEK 293 cells provides a strong indication that elements of their junctional adhesion complexes remain intact. Finally, the apparent lack of focal contacts in HEK 293 cells may have facilitated the identification of cell-cell contact localised amino-terminal FAK: it may be that amino-terminal FAK fragments localise at cell-cell contacts in epithelial cells but lie below a threshold of detection when focal contacts are present, or that previous investigators have simply passed over them, focussing their attention on more intense focal contact localisation of p125 FAK and p55 FRNK.

6.2 Cell-Cell Contact and Nuclear Localisation of ATF Constructs in Epithelial Cells: Independence of Tyrosine 397

Imaging and Western blot analysis of endogenous FAK in HEK 293 cells supported the original hypothesis that FAK FERM domain might participate in cell-cell contact structures (discussed above) and confirmed earlier reports that amino-terminal FAK fragments display nuclear localisation (e.g. Lobo & Zachary, 2000; Jones *et al.*, 2001). A series of GFP-labelled and epitope-tagged FAK-derived constructs (see **Figure 2.01** for summary) was used to determine whether the FAK amino-terminal domain might be capable of targeting to cell-cell contacts and to the nucleus in the absence of the FAT and

kinase domains. Furthermore, the hypothesis that cell-cell contact and nuclear localisation of amino-terminal FAK fragments is mediated by protein-protein interactions involving Y397 was tested using constructs in which this residue was present, absent or mutated to phenylalanine. Prior to embarking on imaging experiments, the transfection of HEK 293 cells was optimised (**Figure 4.01**), and construct expression was characterised by Western blotting using anti-Flag (**Figure 4.01A**), anti-FAK (**Figures 4.01B-C, 4.02B-E**) and anti-GFP antibodies (**Figure 4.02A**).

6.2.01 Localisation of ATF Constructs in HEK 293 Cells

Of the constructs used in this thesis, ATF-420-GFP probably corresponds most closely to the ~42k amino-terminal proteolytic fragment reported both in this thesis (**Figure 3.01D**) and by others (Carragher *et al.*, 2001; Carragher *et al.*, 1999; Cooray *et al.*, 1996; Ilic *et al.*, 1998; Cicala *et al.*, 2000; van de Water *et al.*, 1999; Gervais *et al.*, 1998; Wen *et al.*, 1997; Lobo & Zachary, 2000; Jones *et al.*, 2001). When transfected with ATF-420-GFP, HEK 293 cells exhibited green fluorescent signal that was particularly intense at cell-cell contacts and in the nucleus (**Figure 4.03A**). Compared to staining of endogenous FAK using the anti-N-FAK antibody (**Figure 3.01B**), the cell-cell contact signal from ATF-420-GFP was both stronger relative to cytosolic staining and more continuous.

ATF-420-GFP contains Y397, a site of autophosphorylation that is a known binding site for a several SH2 domain-containing proteins, particularly (but not exclusively) of the Src family (Schaller *et al.*, 1999; Chen *et al.*, 1996; Han *et al.*, 2000; Han *et al.*, 1999; Zhang *et al.*, 1999; Tamura *et al.*, 1999). It was therefore postulated that the cell-cell contact distribution of ATF-420-GFP might be mediated by as yet undetermined SH2 domain interactions involving Y397. However, transfection of HEK 293 cells with ATF-420F-GFP, in which Y397 is mutated to phenylalanine, resulted in a subcellular distribution that was indistinguishable from that of ATF-420-GFP (**Figure 4.03B**). Furthermore, ATF-386-GFP, which lacks the Y397 site entirely, also displayed a subcellular distribution that was identical to that of ATF-420-GFP (**Figure 4.03C**).

In order to determine whether the carboxy-terminal GFP expression tag used in these constructs in any way affected their observed subcellular distribution, the construct Flag-ATF-386 (possessing an amino-terminal Flag expression tag and lacking the Y397 region) was transfected into HEK 293 cells. Detection of Flag-ATF-386 with anti-Flag M5 monoclonal antibody again reproduced the subcellular distribution observed for ATF-420-GFP (**Figure 4.03D**). Furthermore, CentFAK-GFP, in which Y397 is both present and phosphorylated (**Figure 4.02D**) and therefore capable of interacting with SH2 domains, localised neither at cell-cell contacts nor in the nucleus of HEK 293 cells (**Figure 4.04C**). In fact, exogenously expressed Flag-FAK, FAK-GFP, CentFAK-GFP, Flag-FAT and GFP-FAT all exhibited similar patterns of subcellular distribution, being largely expressed in the cytosol and with diminished expression in the nucleus and absence of staining at cell-cell contacts (**Figure 4.04**).

These data therefore demonstrate that the amino-terminal domain of FAK is both required and sufficient for cell-cell contact localisation, and that such localisation is independent of Y397.

6.2.02 Confirmation of Cell-Cell Contact and Nuclear Localisation of ATF Constructs in MDCK Cells

HEK 293 cells display phenotypic characteristics of epithelial-mesenchymal transition: stellate morphology (**Figure 3.01B**), partial loss of E-cadherin from cell-cell contacts (**Figure 3.05A**) and loss of contact inhibition (Mayor *et al.*, 1977). Furthermore, HEK 293 cells express unusual cellular contact structures (**Figures 3.01-3.03, 3.05**, discussed above). MDCK cells, on the other hand, are used as a standard model cell line for epithelial function and cell-cell contact structures, and much of our current understanding of tight junction function has been obtained through studying this cell line. Furthermore, MDCK cells have been used for a number of studies pertaining to FAK function in epithelial cells (e.g. Rakish *et al.*, 1995; Prahalad *et al.*, 2004; Frisch *et al.*, 1996; Wang *et al.*, 2001) and express endogenous FAK in focal contacts (Stewart *et al.*, 2002). The subcellular distribution of ATF constructs was therefore investigated in MDCK cells in order to control for any artefact that might have been produced as a result of using HEK

293 cells. Paraformaldehyde and methanol fixation were also compared to control for fixation artefacts.

In a parallel experiment, HEK 293 and MDCK cells were transfected with Flag-ATF-386 and stained with the anti-Flag M5 antibody (**Figure 4.05**). In both cell types, Flag-ATF-386 was observed to localise primarily at areas of cell-cell contact and in the nucleus, independently of the method of fixation. When transfected with the Flag-FAK construct and stained with the anti-Flag M5 antibody, MDCK cells exhibited staining typical of focal contacts (**Figure 4.06**). Data from MDCK cells therefore confirmed earlier findings from HEK 293 cells, namely that ATF constructs localise at cell-cell contacts in a manner that is independent of Y397.

6.2.03 Co-localisation Studies Further Define the Subcellular Distribution of ATF in HEK 293 Cells

The subcellular distribution observed for the ATF group of constructs was further defined using ATF-386-GFP-transfected HEK 293 cells and a number of different counterstains. First of all, the co-localisation of ATF-386-GFP with endogenous FAK was determined using the anti-C-FAK antibody (**Figure 4.11**). Although a degree of perinuclear and cytosolic co-localisation was observed, the C-FAK antibody did not stain those parts of the cell expressing the brightest green fluorescence, namely the nucleus and cell-cell contacts.

Two groups have suggested that that the FAK FERM domain binds directly to the carboxy-terminal domain of FAK (Dunty *et al.*, 2004; Cooper *et al.*, 2003), with supporting evidence from *in vitro* studies. If that were the case, one might expect a FAK FERM domain construct such as ATF-386-GFP to localise closely with endogenous C-FAK immunoreactivity and/or to affect its subcellular distribution. On the contrary, C-terminal FAK immunoreactivity was not changed in ATF-386-GFP transfected cells (**Figure 4.11**) as compared to untransfected cells (**Figure 3.01A**), exhibiting predominantly perinuclear and cytosolic localisation, and signals from ATF-386-GFP and C-FAK were largely non-overlapping, as judged by overlay of confocal images (**Figure**

4.11). These data could still be consistent with head-to-tail inhibition of endogenous FAK mediated by ATF-FAT interactions if that inhibition were strong enough to entirely block binding of exogenous ATF to p125 FAK. If that were the case, then the degree of perinuclear and cytosolic co-localisation observed between ATF and C-FAK antibody staining would be attributable to ATF-p55 FRNK interactions.

The cell-cell contact localisation of ATF constructs was investigated using anti-occludin, anti-β_1 integrin and rhodamine-phalloidin (**Figure 4.08**). Occludin and β_1 integrin were selected as counterstains due to the fact that both antibodies exhibited consistent cell-cell contact localisation (**Figures 3.02A, 3.03A, 4.09**), whereas co-localisation of ATF with the actin-based cytoskeleton was assessed due to the well-characterised role of FERM domains in cross-linking transmembrane receptors with the cytoskeleton (Girault *et al.*, 1998). ATF-386-GFP signal co-localised consistently with actin, β_1 integrin and occludin signals at areas of cell-cell contact (**Figure 4.08**) However, the type of clear and continuous cell-cell contact staining observed for β_1 integrin in untransfected cells (**Figures 3.02A, 4.09**) was not found in cells transfected with ATF-386-GFP. Rather, when compared directly with β_1 staining in untransfected neighbouring cells, β_1 exhibited discontinuous cell-cell contact staining and diminished cytosolic staining in ATF-transfected cells (**Figure 4.08A**). Hence, co-localisation of β_1 integrin and ATF-386-GFP was discontinuous and restricted to areas of cell-cell contact. One hypothesis that could be used to explain these data is that circumferential β_1 integrin is expressed in a distinct focal plane as compared to ATF-386-GFP. However, the fact that ATF-386-GFP co-localises more or less continuously with occludin (**Figure 4.08B**), and that endogenous occludin co-localises consistently with β_1 integrin (**Figure 4.09**), argues in favour of ATF-386-GFP expression in close proximity to circumferential β_1 integrin. It may therefore be that ATF-386-GFP exerts an epitope-masking effect on β_1 integrin or that ATF-386-GFP somehow alters the subcellular distribution of β_1 subunits.

Some evidence has been published concerning a potential interaction between FAK and EGF receptors mediated by the FAK amino-terminal domain (Sieg *et al.*, 2000). Furthermore, EGF receptors are capable of localising at cell-cell contacts (e.g. Yu *et al.*,

2000b). The possibility was therefore considered that ATF might interact with EGF receptors at cell-cell contacts. Although ATF and EGF receptors did co-localise in the nucleus of HEK 293 cells (**Figure 4.12**), EGFR signal was largely absent from cell-cell contacts. Since EGF receptors were not found to associate with ATF at cell-cell contacts, the relationship between these proteins was not further investigated.

Nuclear localisation of ATF constructs was confirmed using the nuclear dye DAPI as a counterstain (**Figure 4.10**). In all cells examined, ATF-386-GFP co-localised with DAPI fluorescence. Although nuclear localisation of amino-terminal FAK fragments has been previously published (e.g. Lobo & Zachary, 2000; Jones *et al.*, 2001; Yi *et al.*, 2003), these studies have relied on antibody staining for and/or biochemical separations of endogenous proteins. Since nuclear distribution is thought to be a relatively common "artefact" in immunofluorescence microscopy, the data contained herein provide important corroboration of earlier findings, and also provide the first evidence that Y397 is not required for nuclear localisation of amino-terminal FAK fragments. Although a nuclear targeting sequence has yet to be clearly defined for the FAK amino-terminal domain, one group has recently provided important evidence demonstrating that export of these fragments from the nucleus is mediated by the nuclear import-export machinery, reporting that amino-terminal FAK fragments accumulate in the nucleus in the presence of a nuclear export inhibitor (Jones & Stewart, 2004). Current data therefore provide strong indication that amino-terminal FAK fragments may be subject to active nuclear translocation. However, the mechanism of nuclear targeting and the biologic function of amino-terminal FAK in the nucleus have yet to be thoroughly investigated and, unfortunately, it was not possible to pursue these avenues of investigation in this thesis due to time constraints.

6.2.04 Exogenous FAK is Subject to Proteolysis in HEK 293 Cells

One of the hypotheses on which this work is based is that proteolytic cleavage of FAK liberates an amino-terminal fragment roughly corresponding to the FAK FERM domain. Furthermore, this presumptive proteolytic cleavage product is seen in HEK 293 cell lysates analysed by Western blotting with the N-FAK antibody (**Figure 3.01D**).

Therefore, since Flag-FAK contains an amino-terminal Flag epitope tag, it might reasonably have been expected that expression of the Flag-FAK construct in HEK 293 cells might reproduce, at least to a degree, the cell-cell contact and nuclear distribution of the ATF set of constructs. The fact that the Flag-FAK construct did not exhibit any apparent cell-cell contact or nuclear localisation (**Figure 4.04A**) might therefore seem to contradict this assumption. However, Western blotting of Flag-FAK transfected HEK 293 lysates detected a single band corresponding to the predicted relative molecular weight of 125k for FAK (**Figure 4.01A**). On the other hand, Western blotting of Flag-FAK-transfected HEK 293 cell lysates with anti-N-FAK detected several bands, including major products at ~42k and ~77k (**Figure 4.01B**), in addition to the full-length 125k FAK band. These data indicate that Flag-FAK undergoes extensive proteolytic processing in HEK 293 cells to produce amino-terminal fragments, and that the Flag epitope is not present on these breakdown products. The data therefore support the argument that the ~42k amino-terminal FAK fragment is a product of FAK proteolysis and explain the absence of cell-cell contact localisation of Flag-FAK in HEK 293 cells. The fact that amino-terminal FAK-GFP proteolytic cleavage products were of relatively low abundance in transfected HEK 293 cells (**Figure 4.02E**) may be indicative of differential susceptibility of FAK-GFP and Flag-FAK to proteolysis.

6.3 Investigation of the Mechanism of ATF Localisation at Cell-Cell Contacts

Based on the co-localisation results discussed above, the hypothesis was formulated that amino-terminal FAK fragments bind directly to occludin and/or β_1 integrin and/or circumferential actin, fulfilling the type of anchoring function between transmembrane proteins and the cytoskeleton that is well characterised for other FERM domain-containing proteins (Chishti *et al.*, 1998). This hypothesis was tested using co-immunoprecipitation. It was thought that the Flag expression tag might be particularly suitable for determination of protein-protein interactions since it is a relatively small (8 amino acids) epitope tag, and owing to the availability of a monoclonal anti-Flag antibody that was recommended by its manufacturers for co-immunoprecipitation studies. For these experiments, it was considered important to use a construct that did not contain

Y397 since this residue mediates several well-characterised protein-protein interactions (Schaller *et al.*, 1999; Chen *et al.*, 1996; Han *et al.*, 2000; Han *et al.*, 1999; Zhang *et al.*, 1999; Tamura *et al.*, 1999) which are apparently not involved in the cell-cell contact localisation of amino-terminal FAK fragments (**Figure 4.03**). Hence, Flag-ATF-386 was considered a suitable construct for co-immunoprecipitation studies, with Flag-FAK and Flag-FAT constructs being included as suitable controls.

6.3.01 ATF Does Not form Physical Associations with β1 Integrin, Occludin or Actin

Unfortunately, immunoprecipitation using the anti-Flag M2 antibody resulted in the co-immunoprecipitation of β_1 integrin, occludin and actin in cells transfected with Flag-ATF-386, Flag-FAK, Flag-FAT or with empty vector pcDNA3.1 (**Figure 5.01**). This unlooked-for promiscuity of the anti-Flag antibody in immunoprecipitation experiments compelled the adoption of a different experimental approach.

Immunoprecipitation of Flag-FAK, Flag-ATF-386, Flag-FAT or empty vector pcDNA3.1 using either anti-N-FAK or anti-C-FAK successfully co-immunoprecipitated Flag-tagged proteins of the correct molecular weight in those lysates containing FAK constructs but not in empty vector-transfected cells (**Figure 5.02**). Anti-FAK antibodies were therefore used for all subsequent immunoprecipitation experiments. However, immunoprecipitation of transfected cell lysates using anti-FAK antibodies failed to co-immunoprecipitate full-length β_1 integrin (**Figure 5.03**), occludin (**Figure 5.04**) or actin (**Figure 5.05**).

The interaction of FAK with the actin-based cytoskeleton has previously been investigated using an assay that exploits the fact that F-actin is not soluble in lysis buffers containing Triton as the sole detergent (e.g. Schlaepfer *et al.*, 1998). The principle of the assay is that F-actin and associated proteins display differential expression in Triton soluble *versus* Triton-insoluble fractions, such that when FAK associates with the cytoskeleton it shifts from the Triton-soluble to the Triton-insoluble cell fraction. Furthermore, disruption of the F-actin-based cytoskeleton with the pharmacological agent cytochalasin D should bring proteins associated with the F-actin cytoskeleton from the

Triton-insoluble fraction to the Triton-soluble fraction. Given that immunoprecipitation studies had failed to demonstrate any interaction between Flag-tagged FAK constructs and actin, this assay was used to determine whether Flag-ATF-386 might participate in an interaction with the F-actin cytoskeleton that was not detectable by immunoprecipitation.

Flag-FAK, Flag-ATF-386 and Flag-FAT all displayed approximately equivalent expression in the Triton-soluble fraction of HEK 293 cells in the presence or absence of cytochalasin D (**Figure 5.06A**). Flag-FAK was not detectable in the Triton-insoluble fraction at all (**Figure 5.06B**); however, Flag-ATF-386 and Flag-FAT were both expressed in the Triton-insoluble fraction in a manner that was independent of cytochalasin D (**Figure 5.06B**). Furthermore, the GFP-tagged constructs FAK-GFP, ATF-420-GFP, ATF-420F-GFP, ATF-386-GFP and FAT-GFP were all expressed in the Triton-soluble fraction independently of cytochalasin D (**Figure 5.06C**). It was therefore concluded that none of the constructs tested formed a physical association with the F-actin cytoskeleton in HEK 293 cells.

6.3.02 ATF Does Not form Physical Associations with Cadherins or Ezrin

Finally, immunoprecipitation was used to determine whether or not Flag-ATF-386 or ATF-386-GFP might interact directly with ezrin, as had been recently published (Poullet *et al.*, 2001), with E-cadherin or with some other member of the cadherin superfamily. The pan-cadherin antibody used in these experiments is recommended by the manufacturers for detection of cadherin family members including P-cadherin, N-cadherin, E-cadherin, K-cadherin, M-cadherin and R-cadherin of mouse, rat, human and *Xenopus* origin. Immunoprecipitation of HEK 293 cells transfected with Flag-ATF-386, ATF-386-GFP or control empty vectors failed to co-immunoprecipitate immunoreactive ezrin, E-cadherin or pan-cadherin (**Figure 5.07**).

6.3.03 Activation Status of FAK in HEK 293 Cells

Several lines of evidence presented in this thesis provide strong indication that full-length FAK is inactive in HEK 293 cells: both endogenous and exogenously-expressed full-length FAK failed to localise to focal contacts (**Figures 3.01A-B, 4.04A**); phosphorylation of Y397 was not detectable in endogenous FAK (although it is

detectable in exogenous CentFAK-GFP; **Figure 4.02D**); and exogenous FAK did not bind the actin-based cytoskeleton, as determined by a Triton-solubility assay (**Figure 5.06B**). These findings suggest that endogenous FAK is inactive in HEK 293 cells: it may be that the apparent absence of focal contacts leaves endogenous FAK incapable alleviating the auto-inhibitory head-to-tail interaction discussed above. The question of whether or not FAK activation can be induced in this cell line, for example by plating on matrix, has not apparently been investigated and would have some impact on published work (e.g. Rodriguez-Frade *et al.*, 1999). Pyk-2 expression has been noted in epithelial cells (e.g. Keely *et al.*, 2000) and it is possible that HEK-293 cells express Pyk-2 in focal contacts rather than FAK; unfortunately, due to time constraints, it was not possible to investigate this hypothesis.

6.4 Difficulties Experienced in the Execution of Practical Work in this Thesis

Several difficulties were experienced in the execution of the practical work in this thesis. These difficulties can be grouped into three main categories: problems associated with protein biochemistry with respect to the identification of ATF-interacting partners; problems associated with improper procedures for preparation of cells for fluorescence microscopy; and problems associated with attempted functional assays. These difficulties resulted in one chapter of negative data, inconclusive fluorescence microscopy data, and an absence of any functional data at all in the final thesis.

With respect to biochemistry, the selection of the Flag epitope tag was most ill advised as, in this student's hands, the anti-Flag antibody itself participated in promiscuous interactions in the IP-Western blot approach that was adopted to identify potential ATF interacting partners. As discussed elsewhere in this thesis, the c-Myc tag has been more commonly reported in FAK publications employing IP-Western blots, and this tag would probably have been the better choice. As also discussed elsewhere, however, the adoption of the IP-Western methodology for determining interacting partners was also almost certainly a mistake. A better biochemical method to accomplish this goal would have been to generate FAK construct-GST (for Glutathione-S-Transferase) fusion proteins. In

this method, the protein of interest is cloned in-frame with GST and expressed in the target cells, in this instance HEK 293. The cells are then lysed in a non-reducing buffer and loaded on to a glutathione-sepharose reducing column. The column is washed several times in a non-reducing buffer, the stringency of which can be varied, with the aim of retaining specific protein-protein interactions and washing off any non-specifically bound molecules. The protein complexes can then be eluted using a reducing buffer. SDS PAGE followed by Western blotting with anti-phospho-tyrosine might then provide clues as to which signalling proteins might be bound to the protein of interest. Although similar to the IP-western approach in terms of work flow, the GST fusion system has the advantage in that all recombinant protein expressed in frame with GST (and not having undergone any proteolytic cleavage) would bind to the column, guaranteeing greater yields of the protein of interest and thereby increasing the likelihood of a positive result. GST fusion proteins have been used in several publications to identify focal contact associated proteins, and have also been used to investigate FRNK-interacting partners (e.g. Feruse *et al.*, 2001, Bellis *et al.*, 2005, Turner *et al.*, 1999).

Difficulties with fluorescence microscopy arose due to the lack of experience of this student and a lack of experience generally within the group. Accordingly, many of the images presented in this thesis do not show any conclusive subcellular distribution of the relevant proteins. The assembly of both adherens junctions and tight junctions is dependent on the availability of extracellular calcium ions. Therefore, in order to maintain correct junctional morphology, cells for fluorescence microscopy should have been washed in Dulbecco's PBS containing divalent metal cations at 37°C. The cells were instead washed in normally PBS, lacking in divalent metal cations, at room temperature, and these conditions almost certain contributed to the disarray in cellular contact proteins observed in **Figures 3.1-3.4**. Another problem with imaging data presented in this these is that, rather than showing transfected cells surrounded by a larger number of cells, I made the mistake of focusing on a small number of transfected cells, often without the context of neighbouring untransfected cells for comparison. This makes it difficult to exclude the possibility that some of the peripheral cellular fluorescence intensity observed for ATF constructs (e.g. **Figure 3.1D**) is truly cell-cell contact

associated rather than membrane-associated. The use of phase contrast comparisons or a suitable counterstain would have given more credibility to the claims of cell-cell contact localisation of ATF constructs presented herein. Finally, try though I might to force HEK 293 cells to express FAK-containing focal contacts, I did not succeed. To this end, I tried plating HEK 293s on a number of different surfaces including poly-L-lysine, collagen I, fibronectin, vitronectin, laminin and gelatin, but to no avail.

Concerning functional assays for the ATF constructs, several were attempted in both HEK 293 cells and MDCK cells but no reproducible data were obtained. To my eye, cells transfected with ATF constructs looked slightly healthier and more numerous than did untransfected cells; however, proliferation assays proved unreproducible and I put this down to variation in transient transfection efficiency. One way in which this could have been overcome would have been to sort the transfected cells prior to plating. I then attempted to perform wound-healing assays with HEK 293 cells, in order to determine whether ATF-transfected cells might migrate at a different rate (as compared to untransfected or other FAK construct transfected cells) into a scar formed by moving a pipette tip across the coverslip. Unfortunately, HEK 293 cells do not adhere strongly, and in creating the scar, I never failed to cause the entire layer of cells to lift off the cover slip. Again, I attempted to plate the cells on the range of surfaces listed above but did not succeed in making the cells adhere strongly enough to permit the assay. I then attempted to perform these assays in MDCK cells; however, I did not succeed in obtaining an MDCK transfection efficiency (which remained stubbornly at about 5%) to obtain data in either the proliferation assay or the wound-healing assay. If I had transfected a very large plate of MDCK cells, I could perhaps have sorted a suitable number of cells by flow cytometry to perform both the proliferation and wound healing assays with a pure transfected population.

A different way altogether in which the question of functional assays could have been addressed would have been to use microinjection in conjunction with video time-lapse microscopy, through collaboration with an imaging centre. Microinjection is a method of delivering plasmid DNA to individual cells whose expression of exogenous construct can

then be monitored using video time-lapse microscopy. For example, in the wound healing assay, MDCK cells could be wounded and then cells at the leading edge of the wound could be transfected with the ATF construct. The subcellular distribution of ATF at the leading edge could then be seen in real time, and the rate of migration of those cells into the wound could be directly compared with that of vector-only transfected neighbours. Another interesting way in which this technique could have been employed would be to microinject MDCK cells at different levels of confluence and, for underconfluent cells, to microinject both isolated cells and cells in colonies. The subcellular distribution of the ATF construct, as well as its impact on MDCK colony formation could then have been investigated.

6.5 Future Directions

6.5.01 Application of Proteomic Technologies in Defining Protein-Protein Interactions Mediated by ATF

The data presented in this thesis support the hypothesis that the FAK FERM domain interacts with intercellular adhesive structures: the cell-cell contact localisation of endogenous amino-terminal FAK fragments observed in HEK 293 cells (**Figure 3.01B**) was reproduced by transfection of epitope-tagged ATF constructs in both HEK 293 (**Figure 4.03**) and MDCK cells (**Figure 4.05**). Furthermore, targeting of ATF to areas of cell-cell contact was found to be independent of the presence, absence or mutation to phenylalanine of Y397 (**Figure 4.03**), demonstrating that the amino-terminal domain contains other sites capable of mediating protein-protein interactions. Although not published previously, this concept is consistent with the well-established role of FERM domain-containing proteins in mediating protein-protein interactions that serve to cross-link transmembrane receptors with cortical actin structures (Chishti *et al.*, 1998).

However, the ability to draw firm conclusions regarding the molecular basis for the observations presented herein is hampered by the fact that co-immunoprecipitation and Triton solubility assays failed to yield positive results concerning potential protein-protein interactions (**Figures 5.03-5.07**). In this respect, the use of the Flag epitope

expression tag was highly problematic and hindered the search for ATF-interacting partners through the promiscuous interactions of the anti-Flag M2 antibody in immunoprecipitation (**Figure 5.01**). The myc epitope tag could perhaps have provided a more suitable system of co-immunoprecipitation.

Intercellular adhesive structures are extremely complex entities. Assuming that ATF constructs participate in a direct physical interaction with one or more proteins from tight junctions, adherens junctions or desmosomes, the number of proteins that would have to be tested in order to exhaust all currently known possibilities is vast, including the claudins, JAM, ZO-3, MUPP1, N-cadherin, desmocollins, desmogleins, plakophilins, desmoplakins and elements of the nectin-afadin system, to name but a few. In hindsight, the adoption of a one-by-one approach for identifying these protein-protein interactions was short-sighted in two respects: first of all, such an approach would be too time-consuming to allow all possibilities to be thoroughly investigated; and secondly, the scope of the search would be limited by the understanding and/or imagination of the investigator. Instead, more open-ended proteomic methodologies would provide better means of carrying out such a search.

In outline, and alternative approach would have involved transient expression of myc-tagged ATF, FAK and FAT constructs into HEK 293 cells. Transfected lysates would be subjected immunoprecipitation with anti-myc antibodies, followed by 2D-gel electrophoresis and mass spectrometry to identify spots associated with the amino-terminal domain. Alternatively, immunoprecipitates could have been subjected to PowerBlot screening (BD Pharmingen). PowerBlot is a proteomic screening service based on Western blotting technology, in which test/treated and control samples are screened in triplicate with over 1000 monoclonal antibodies and semi-quantitative analysis of expression levels is performed using the PD Quest software from Bio-Rad.

MDCK cells were not used more frequently in this thesis due to the fact that they were simply not as transfectable as HEK 293 cells. However, with sufficient time available, MDCK clones could be generated that stably express ATF, FAK and FAT constructs.

Such stable cell lines would presumably require an inducible expression system, such as the Tet-on system, due to the fact that FAT constructs are known to induce apoptosis in several cells. Once such cell lines were generated, construct expression could be induced, immunoprecipitations with anti-myc antibody performed, and these could be subjected to proteomic screening as above and compared to the HEK 293 data.

Finally, protein-protein interactions mediated by the FAK FERM domain could be identified by screening HEK 293 and/or MDCK and/or other epithelial cell cDNA libraries using an ATF-386 type construct as bait in a yeast 2-hybrid screen.

Open-ended proteomic screening approaches such as these are frequently subject to the criticism of being insufficiently hypothesis-driven. In this case, however, the hypothesis is quite clearly defined, namely that ATF constructs interact with cell-cell adhesion proteins. Such an unambiguous hypothesis would provide a clear framework for analysing the potentially large amounts of data that might be gathered in these systems. Any interactions uncovered would then be confirmed by immunofluorescence with confocal microscopy prior to developing rational functional assays to determine the biological significance of those interactions.

Another interesting avenue for further investigation might be to perform site-directed alanine-scanning mutagenesis to determine which residues in the FAK FERM domain are involved in the localisation of amino-terminal FAK fragments to areas of cell-cell contact and/or in its physical interaction with binding partners that would be uncovered using the proteomic methodologies described above. The advent of siRNA technology has already been used to investigate FAK function (e.g. Walsh *et al.*, 2003) and, combined with transfection of ATF constructs both capable and incapable of localising at cell-cell contacts would no doubt provide useful insights into the biologic function of amino-terminal FAK fragments. Data collected from such functional studies could ultimately be confirmed in primary epithelial and even endothelial cells using adenoviral or retroviral technology.

6.5.02 Emerging Role of FAK in Adherens Junctions and Epithelial Cell Migration

In switching from non-migratory to migratory phenotype, epithelial cells co-ordinate a complex series of events. Focal contacts, which are static in non-migrating cells, become dynamic, entering into a cycle of assembly and disassembly that permits movement along extracellular matrix. At the same time, intercellular adhesive structures weaken and disassemble in order to permit movement. For review, see (Schlaepfer *et al.*, 2004). Therefore, since epithelial migration requires co-ordinated assembly and disassembly of various cellular contact structures, it is not surprising that systems should exist to facilitate cross-regulation of tight junctions, adherens junctions, desmosomes and hemidesmosomes.

Several lines of recently published data support the notion of a functional link between FAK and cadherin-based adherens junctions. Phosphorylation of FAK by Src has recently been shown to be required for Src-induced de-regulation of E-cadherin Furthermore, FAK signalling appears to modulate N-cadherin-mediated adhesion (Yano *et al.*, 2004; Schaller, 2004); N-cadherin has been shown to be capable of regulating FAK phosphorylation in retinal pigment epithelial cells (van Aken *et al.*, 2003); and FAK may be required for maintenance of cell-cell adhesion in collective cell migration (Yano *et al.*, 2004). One group has recently published data showing that FAK and paxillin localise at epithelial cell-cell contacts in the developing zebra fish, that junctional FAK is phosphorylated on Y397 in this model system and that FAK may associate with actin in cadherin-containing epithelial junctions (Crawford *et al.*, 2003). Taken together, these data suggest a functional role for FAK in mediating cross-talk between focal contacts and adherens junctions, associated with deregulation of E-cadherin-based adherens junctions and with signalling to and/or from the more dynamic N-cadherin system. Based on data presented in this thesis, it seems likely that targeting of FAK to cell-cell contacts is mediated by the amino-terminal domain, and that Y397 phosphorylation is not necessary for junctional localisation. It is tempting to consider that amino-terminal FAK fragments, liberated by, for example, calpain-mediated proteolysis, might contribute to this cross-communication, since data arising from this thesis support such a notion. As questions

Molecule	*Tight Junctions*	*Adherens Junctions*	*Desmosomes*	*Hemidesmosomes*	*Focal Contacts*
ZO-1	+	+	-	-	-
ZO-2	+	+	-	-	-
LIN-7	+	+	-	-	-
Plakoglobin	-	+	+	-	-
Polycystin	-	+	-	-	+
Ponsin	-	+	-	-	+
Vinculin	-	+	-	-	+
α-Actinin	-	+	-	-	+
Ponsin	-	+	-	-	+
Zyxin	-	+	-	-	+
Erbin	-	-	+	+	-
Plectin	-	-	-	+	+
$\alpha_6\beta_4$ integrin	-	-	-	+	+
β_1 integrin	?	-	-	+	+
CD151	-	-	-	+	+
FAK	?	+	?		+

Table 7.1: Examples of Shared Components of Cellular Contact Structures in Epithelial Cells. Although normally investigated in isolation, cellular contact structures seem to include an ever-increasing number of shared components, and no single cellular contact structure is entirely composed of molecules that are found exclusively in that structure. Data presented herein indicate that FAK may participate in intercellular junctions, and recently published data describe communication between focal contacts and adherens junctions mediated by FAK. (Nagafuchi, A, 2001; Mitic & Anderson, 1998; Roh *et al.*, 2002; Stewart *et al.*, 2002; Tiwari-Woodruff *et al.*, 2001; Borradorri & Arnoud, 1999; Nievers *et al.*, 1998; Zammer & Geiger, 2001; Jaulin-Bastard *et al.*, 2002).

relating to cross-regulation of cellular contact structures are investigated more thoroughly, it may come to light that FAK interacts functionally with tight junctions, desmosomes or junctional β_1 integrin, in addition to its recently discovered interaction with adherens junctions.

The ability of intracellular proteins to perform such "multitasking" functions is becoming increasingly evident, and is highlighted by the fact that the classical cellular contact structures are not wholly distinct entities: each type of cellular contact contains components that are shared with other contact structures (**Table 7.1**). Moreover, since the sequencing of the human genome identified only around 30,000 genes where around

300,000 proteins are thought to be required for human biology, it is evident that the number of different biologic functions attributable to each gene is likely to be larger than initially expected. In this respect, post-translational modification, including events such as protein phosphorylation and proteolytic cleavage, is likely to be an important element in generating functional diversity in the proteome. Accordingly, previously overlooked proteolytic cleavage products, such as the ~42k amino-terminal FAK fragment discussed in this thesis, will have to be re-examined with an open eye and an open mind.

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Appendix: Publications Arising from this Thesis

Conference Abstracts

Stewart, A and Zachary, I (2000). "The amino-terminal domain of FAK localises with β_1 integrin at lateral junctions in HEK 293 cells." *The American Society for Cell Biology.*

Stewart, A and Zachary, I (2001). "Localisation of the amino-terminal domain of FAK at epithelial cell-cell contacts is independent of tyrosine 397." *The British Societies for Cell & Developmental Biology.*

Published Articles

Stewart, A, Ham, C and Zachary, I (2002). "The focal adhesion kinase amino-terminal domain localises to nuclei and intercellular junctions in HEK 293 and MDCK cells independently of tyrosine 397 and the carboxy-terminal domain." *Biochem Biophys Res Commun* **299**: 62-73