TNF-α Signalling to the Cell-Cell Junctions
And the Cytoskeleton in Endothelial cells

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Abstract

Tumour necrosis factor-α (TNF-α) is known to induce changes in endothelial cell morphology and paracellular permeability, but the mechanisms have not been extensively characterised. The purpose of this study was to establish the effects of TNF-α on human umbilical vein endothelial cell (HUVECs) paracellular permeability and tight junctions and relate these responses to changes in the actin cytoskeleton.

TNF-α caused progressive changes to HUVECs over 24 h. TNF-α induced RhoA activation, myosin light chain phosphorylation, cortical F-actin thickening and the formation of tiny inter-cellular gaps within 10 min. A small increase in permeability accompanied these changes. By 24 h, TNF-α caused stress fibre formation, cell elongation and extensive gap formation. Occludin and JAM-A were lost from the tight junctions, ZO-1 was partially redistributed and permeability was increased. RhoA and ROCK inhibition prevented TNF-α-induced changes in F-actin and cell morphology, but ROCK inhibition did not re-establish the junctional localisation of ZO-1, nor did it prevent increased permeability. Myosin light chain kinase inhibition had no impact on TNF-α-induced stress fibres, cell elongation or permeability at 24 h. These results indicate that the TNF-α-induced morphological and cytoskeletal changes are not solely responsible for increased permeability and that signalling to the tight junction proteins may be more important for TNF-α regulation of barrier function.

To identify potential interacting partners of occludin, a yeast two-hybrid experiment was performed using the C-terminus of occludin. The transcriptional co-activator protein, Ski-interacting protein (SKIP) and the Ser/Thr protein kinases, casein kinase Iε (CKIε) and UNC-51 like kinase-1 (ULK-1) were identified in this screen. These novel interactions may be important for occludin regulation and function.

During the course of these studies, adenoviruses were used to introduce genes into HUVECs. An inhibitory effect of control adenoviruses on TNF-α-induced cytoskeletal changes and permeability was observed, suggesting that adenovirus binding and/or entry could modulate endothelial cell behaviour and responses.
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<td>β-galactosidase-carrying adenoviruses</td>
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<tr>
<td>Ad-GFP</td>
<td>GFP-carrying adenoviruses</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CAR</td>
<td>Coxsackie and adenovirus receptor</td>
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<tr>
<td>CRIB</td>
<td>Cdc42/Rac interactive binding</td>
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<td>Dulbecco’s Modified Eagle’s Medium</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
<td>EDTA</td>
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<td>FITC</td>
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<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
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<td>Green fluorescent protein</td>
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<td>Mitogen activated protein kinase</td>
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<tr>
<td>MEM</td>
<td>Eagle's Minimum Essential Medium</td>
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1 Introduction

1.1 Endothelial cell function

Endothelial cells (ECs) line blood vessels and form a physical barrier that separates the vascular lumen from the vessel wall. ECs maintain blood circulation and fluidity, and regulate vascular tone and coagulation (Cines et al., 1998). Furthermore, ECs control tissue homeostasis by regulating the exchange of solutes, macromolecules and cells between the blood and the surrounding tissues.

Transport across the endothelium is achieved through either paracellular or transcellular routes. Transcellular routes are formed by cell surface plasmalemmal vesicles, particularly caveolae, which can shuttle across the endothelium and/or fuse to form channels for the delivery of plasma proteins to subadjacent cells and tissues (transcytosis) (Simionescu et al., 2002). Caveolae comprise 95% of endothelial cell-surface vesicles and are defined as cholesterol- and glycosphingolipid-rich membrane microdomains (Predescu et al., 1993; Minshall et al., 2003). Caveolin is a major protein constituent of caveolae, that coats the cytoplasmic surface of the microdomain, binds to cholesterol, and associates with a number of signalling molecules, including G-proteins and kinases (Kurzchalia et al., 1992; Rothberg et al., 1992; Minshall et al., 2003). Caveolae are therefore platforms for signalling, trafficking and protein organisation. Albumin, low-density lipoproteins (LDL), metalloproteases, transferrin and insulin are transported by transcytosis (Simionescu et al., 2002). Most transcytosis occurs nonselectively in the fluid phase or by adsorption to the vesicle membrane (Tuma and Hubbard, 2003).

However, there are some reports of receptor-mediated transcytosis. For instance, albumin, which binds to important hydrophobic and sparingly soluble molecules such as free fatty acids, thyroid, steroid hormones, bile acids and drugs, engages a protein expressed on the luminal endothelial cell surface called gp60. Gp60 also binds to caveolin-1. Albumin binding to gp60 results in tyrosine phosphorylation of gp60 and caveolin-1 and albumin transcytosis (Tiruppathi et al., 1996; Tuma and Hubbard, 2003).
Chapter 1

Introduction

Paracellular permeability is regulated by endothelial tight junctions (discussed below) and is utilised by ions, water and small molecules, as well as immune cells such as leukocytes (Van Buul, 2004).

Endothelial dysfunction contributes to pathological conditions such as chronic inflammation and atherosclerosis (Gonzalez and Selwyn, 2003). In a normal inflammatory response, pathogenic invasion stimulates the release of cytokines, such as tumour necrosis factor (TNF-α) and interleukin-1 (IL-1) from activated macrophages and other immune cells, which activate the endothelium to express cell adhesion molecules such as intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin. Blood leukocytes adhere to cell adhesion molecules expressed on the surface of the endothelium, which facilitates their transmigration through the endothelium to infected tissues (Liu et al., 2004). Such inflammatory responses are required for an effective defence against infection, however, persistent or chronic inflammation is central to diseases such as rheumatoid arthritis, inflammatory bowel disease and atherosclerosis (Gonzalez and Selwyn, 2003; Ulbrich et al., 2003; Glass and Witztum, 2001).

Atherosclerosis arises from an accumulation of fatty streaks that underlie the endothelium of large arteries. Fatty streaks form when macrophages take up oxidised low density lipoprotein (LDL)-cholesterol, progressively accumulate in the subendothelial space and develop into foam cells, which contain massive amounts of cholesterol ester. Smooth muscle cells also migrate to this region and synthesize extracellular matrix proteins, forming a fibrous cap. Localized chronic inflammation ensues, including the production of TNF-α, interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1), and apoptosis and necrosis of macrophages and smooth muscle cells leads to the formation of a necrotic core. Neovascularisation further destabilises the plaque and eventual thrombosis and plaque rupture can cause myocardial infarction and stroke (Glass and Witztum, 2001).

It is therefore of clinical importance to understand the regulation of endothelial function and inflammatory responses.
1.2 Intercellular Junctions

Figure 1A shows the space between two neighbouring epithelial cells. This space contains a number of intercellular junctions including the adherens junctions, tight junctions, desmosomes and gap junctions. Many of these junctional components are also present in endothelial cells (Fig. 1B).

The primary role of the adherens junctions is cell-cell adhesion, which is mediated by classic cadherins and their association with actin via members of the catenin family. Vascular endothelial cadherin (VE-cadherin) is a cell adhesion molecule that is specifically expressed in endothelial cells. Electron micrographs show that although the adherens junctions bring the two neighbouring membranes into close apposition, there is still a gap of about 20 nm between the two cells.

The tight junctions are the most apical of the intercellular junctions and they seal the paracellular space completely. This reflects one of the main roles of the tight junctions: to act as a permeability barrier, restricting the flow of fluid from the vascular lumen through the intercellular space (Gonzalez-Mariscal, 2003). The second role for tight junctions is that of a membrane diffusion barrier, restricting the mixing of apical and basolateral plasma membrane components (fence function) (Dragston et al. 1981; van Meer et al., 1986; Anderson, 1995; Balda et al., 1996). Tight junctions are composed of integral membrane proteins from the occludin, claudin and JAM families, which form homotypic interactions with the same proteins on neighbouring cells (Fig. 1.1B). Membrane associated proteins, such as ZO-1, also reside at the tight junctions, and serve to bridge the transmembrane proteins to the actin cytoskeleton, whilst others have signalling and scaffolding roles. Tight junctions of endothelial cells lack some of the proteins present in epithelial tight junctions, such as 7H6 and symplekin (Wachtel et al., 1999). Tight junctions have been described as membrane microdomains which are rich in cholesterol and caveolin-1 and are therefore, DIG-like in structure (detergent-insoluble glycolipid rafts). Indeed, occludin not only resides in the same fraction as detergent-insoluble lipid rafts in sucrose gradients, but it also colocalizes and coimmunoprecipitates
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A)

B)
Figure 1.1
Ultrathin section of a tight junction (A). Ruthenium red has been added to the apical surface of epithelial monolayers and it is unable to penetrate past the tight junctions (arrow). Scale bar, 10 nm. Taken from González-Mariscal et al, 2003. Schematic diagram showing the intercellular junctions between two neighbouring endothelial cells (B). Tight junctions, adherens junctions, gap junctions and some other proteins reside in this region.
with caveolin-1 in the epithelial cell line, T84. These raft-like domains may play a central role in the spatial organisation of tight junctions, as well as enrichment of signal transduction proteins to this structure (Nusrat et al., 2000).

Platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) is a member of the Immunoglobulin (Ig) superfamily, containing six extracellular Ig domains, and is expressed in endothelial cells as well as platelets, monocytes, neutrophils and some T-cells. PECAM-1 is assembled at the cell-cell borders and forms homotypic interactions in confluent monolayers, but it is not restricted to any particular cell junction. PECAM-1 has been shown to co-precipitate with β-catenin (Matsumura et al., 1997; Ilan and Madri, 2003). Antibodies to either leukocyte, or endothelial PECAM-1 Ig domains have been shown to block neutrophil and monocyte transmigration, indicating an important role for PECAM-1 in this process (Muller et al., 1993).

Gap junctions allow the exchange of ions and small molecules between neighbouring cells and are formed by a channel made from six transmembrane connexin protein monomers. There is some evidence that tight junctions are in relatively tight proximity to the gap junctions. This has been observed in fibroblasts transfected with occludin (Furuse et al., 1998) and in sertoli cells (McGinley et al., 1977). The coiled-coiled region of occludin associates with connexin-26 (Nusrat et al., 2000) and others have shown coprecipitation of connexin-32 and occludin (Kojima et al., 1999). Indeed, connexins may have a role in tight junction assembly and actin organisation (Kojima et al., 2002). Furthermore, in MDCK cells, connexin-45 co-localises and co-precipitates with ZO-1 (Kausalya et al., 2001), and in cardiac myocytes, connexin-43 has been shown to associate with α-spectrin via ZO-1 (Toyofuku et al., 1998). Endothelial cells express connexins -37, -40 and -43 (Vestweber, 2000).

Epithelial cells also contain desmosomes. These junctions stabilise adhesion through their association with keratin filaments. Integral membrane desmosomal cadherins - desmoglein and desmocollin – bind to cytoplasmic proteins desmoplakins I and II and the armadillo-related plakoglobin and plakophilin family proteins, which in turn bind to
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intermediate filaments (Vestweber et al., 2000). However, endothelial cells do not contain classic desmosomes, but do express desmoplakin in a region termed the ‘complexus adhaerentes’ (Schmelz et al., 1993). Endothelial cells also lack keratins.

1.2.1 Adherens junction and tight junction cross-talk

Polarized epithelial cells and endothelial cells that exhibit high electrical resistance, such as those from the blood-brain barrier, form distinct tight and adherens junctions (Rüffer et al., 2004). However in microvascular endothelial cells that are the sites for leukocyte transmigration and are thought to be more ‘leaky’ and in other non-epithelial cells, the intercellular junctions are not discrete, but are intermingled (Vestweber, 2000; Anderson, 1995). For example, the tight junction protein ZO-1 colocalises and co-immunoprecipitates with the adherens junction protein α-catenin when overexpressed in fibroblasts (Itoh et al., 1997). Co-localisation has been observed between occludin, ZO-1, the catenins and VE-cadherin in pulmonary arterial ECs (Drenckhahn and Ness, 1997). Moreover, a recent study has shown that in human microvascular endothelial cells (HMEC), ZO-1 and β-catenin have overlapping localisations in confocal x-z scans, whereas these are clearly separable in MDCK cells (Rüffer et al., 2004). This study also demonstrated coprecipitaion of ZO-1 and ZO-2 with VE-cadherin in HMEC-1 cells. Tight junction assembly depends upon adherens junction assembly (Baida et al., 1993). Exogenous occludin localises correctly in occludin-null fibroblasts, which contain ZO-1 and adheren-like junctions. However, neither occludin nor ZO-1 localize correctly to cell-cell contact regions in L-cells lacking cadherin-adhesion junctions (Van Itallie, 1997). It is possible that the adherens junctions signal for the formation of tight junctions. In support of this, activation of protein kinase C (PKC) can induce tight junction formation in the absence of cadherin-based cell-cell adhesion (Balda et al., 1993). Alternatively, the adherens junctions might form a scaffold onto which tight junctions can form: ZO-1 transiently binds to β-catenin in the early stages of tight junction formation in MDCK cells and in L-cell fibroblasts which lack cadherin-based adherens junctions, neither ZO-1 nor occludin localises to sites of cell contact (Mitic and Anderson, 1998). Other signals are also transmitted between tight and adherens junctions. A synthetic peptide homologous to the second extracellular loop of occludin
induces down regulation of occludin itself, upregulation of β-catenin and the transcription of β-catenin/TCF/LEF target gene c-myc (Vietor et al., 2001). However, in Ras-transformed MDCK cells, the recruitment of occludin to the areas of cell-cell contact precedes the appearance of E-cadherin at cell-cell contacts (Chen et al., 2000). VE-cadherin association with the adherens junction protein, p120, has recently been shown to be important for barrier function (Iyer et al., 2003).

1.3 The structure of tight junctions
Endothelial tight junctions contain integral membrane proteins that directly or indirectly associate with F-actin, essentially bridging F-actin fibres between neighbouring cells. A host of soluble proteins form a 'plaque' at the tight junction, where they fulfill structural, signalling and regulatory roles.

1.3.1 Occludin
The first membrane spanning protein found to localise at the tight junction was occludin (Furuse et al., 1993). Occludin is a ~65 kDa phosphoprotein with a short intracellular N-terminus, four membrane-spanning regions that link two extracellular loops, a short intracellular turn and a long cytoplasmic C-terminus (Fig. 1.2A). Occludin molecules polymerise to form structures called tight junction fibrils (Staehalin et al., 1973) and they also form lateral, homotypic interactions with occludin molecules of opposing cell membranes. The lateral association of opposing occludin proteins is thought to involve the high tyrosine and glycine residue content within the first extracellular loop of occludin (Ando-Akatsuka et al., 1996). The occludin C-terminus is predicted to adopt a coiled-coil conformation from Leucine 440 to Glutamine 469 (Ando-Akatsuka et al., 1996, 1997; Nusrat et al., 2000).

Occludin is expressed in essentially all epithelial and endothelial tissues, as well as neurons, astrocytes and T-cells (Alexander et al., 1998; Bauer et al., 1999). The function of occludin in cell types that do not form tight junctions has not been determined. There are several splice variants of occludin (Fig. 1.2B). Occludin 1B contains a 193-base pair insertion, encoding a longer form of occludin with a unique N-terminal sequence of 56
amino acids (Muresan et al., 2000). Occludin 1B has identical localisation to occludin in MDCK cells. There has been no report so far of the expression of occludin 1B in human umbilical vein endothelial cells (HUVECs). Occludin II (occludin TM-4) is a further alternative splice variant of occludin containing a 162-base pair deletion (Ghassemifar et al., 2002, Mankertz et al., 2002). This represents a deletion of exon 4, resulting in a protein of ~58 kDa that lacks the fourth transmembrane (TM) domain and immediate C-terminal flanking region. In contrast to occludin and occludin 1B, occludin II is therefore predicted to have an extracellular localisation of its C-terminus. This has been shown in an HT-29/B6 intestinal cell line (Mankertz et al., 2002). Occludin II does not localise to the tight junctions of HT-29/B6 cells and is instead diffuse across these cells (Mankertz et al., 2002). Occludin II is also absent from the tight junctions of confluent Caco-2 cells, but is present at the periphery of islands of confluent cells and cells bordering a wounded edge (Ghassemifar et al., 2002). Occludin II mRNA has been detected in HUVECs (Ghassemifar et al., 2002). Occludin III is a further occludin splice variant that lacks its fourth transmembrane domain, and also has an extracellular C-terminus (Mankertz et al., 2002). Occludin III does not localise to the tight junctions in HT-29/B6 cells but instead exhibits diffuse staining similarly to occludin II (Mankertz et al., 2002). Occludin IV contains a small deletion (19 amino acids) within its C-terminus and localises to the tight junction in HT-29/B6 cells. How these splicing events are regulated has not yet been determined.
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A) Extracellular Space
   Plasma Membrane
   Cytoplasm

B) Occludin I
   (65 kDa)

Occludin TM-4
   (Occludin II)
   (58 kDa)

Occludin III

Occludin IV
   (~58-60 kDa)

Occludin IB
   (70 kDa)
Figure 1.2
Schematic diagram of occludin (A). Occludin contains an intracellular N-terminus, followed by two large extracellular loops that are connected by a short intracellular region, and a long intracellular C-terminal region. Acidic (solid circle), basic (cross-hatch) and uncharged (open circle) residues at neutral pH are indicated. Glycines are represented as (triangle) and tyrosines as (inverted triangle). Taken from Mitic and Anderson, 1998. The splice variants of occludin (B). The solid boxes are the transmembrane (TM) regions of occludin. Arrows indicate regions of occludin I that are lacking in each of the splice variants; lines indicate where these sites lie in the variants. The size of each protein is indicated where known.
1.3.1.1 Occludin binding partners

Occludin has been shown to bind to several tight junction proteins and signalling molecules (Fig. 1.3). Occludin directly associates with cingulin (Cordenonsi et al., 1999), and the membrane associated guanylate kinase homologues (MAGUK) proteins ZO-1 (Furuse et al., 1994), -2 and -3 and also to F-actin through the occludin C-terminus (Nusrat et al. 2000; Wittchen et al., 1999). Occludin also binds to the ubiquitin protein ligase, Itch, via the occludin N-terminus (Traweger et al., 2002). A novel bait peptide method revealed an association between the coiled-coiled domain of occludin and ZO-1, PKC-ζ, c-Yes, p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase (PI 3-kinase), connexin-26 and other occludin molecules (Nusrat et al., 2000).

1.3.1.2 Occludin function

Occludin has roles in maintaining tight junction barrier and fence function. There are several lines of evidence for this. Brain endothelial cells, which have much higher expression of occludin than non-neuronal tissue, also have much lower permeability (Hirase et al., 1997). Occludin protein and mRNA is down regulated in some inflammatory bowel diseases, which correlates with enhanced paracellular permeability (Kucharzik et al., 2001). Measurement of electrical resistance across cultured monolayers is used extensively for determining barrier function, and is particularly useful for epithelial cells as these exhibit high transepithelial resistance (TER) values of up to several thousand Ωcm² (Wong et al., 1997). However, cultured endothelial cells such as HUVECs or even brain microvessel endothelial cells lose their high electrical resistance in culture, which makes it difficult to use this as a measure of permeability (Wachtel et al., 1999). Expression of full length occludin in MDCK cells enhances TER and increases the number and width of tight junction strands (McCarthy et al., 1996) and a synthetic peptide corresponding to either the first or second extracellular loop of occludin decreases TER (Wong et al., 1997; Lacaz-Vieira et al., 1999). The second extracellular loop may also be required for stable assembly of occludin at the tight
Figure 1.3
Ocludin-interacting proteins
Schematic diagram showing the known ocludin-interacting proteins. Proteins shown to bind to the N-terminus are boxed in blue. Those known to associate with the C-terminus are boxed in red. Those that have specifically been shown to associate with the coiled-coiled region of ocludin are boxed in green. Green coil indicates the coiled-coil region of ocludin. p85 is the regulatory subunit of PI 3-kinase. N.B. CK2 and p34cdc2/cyclin B have also been shown to phosphorylate ocludin in vitro.
junction (Medina et al., 2000). Experiments in *Xenopus* embryos have shown that expression of C-terminally truncated occludin increases paracellular permeability (Chen et al., 1997). Truncations of the C-terminus have also been reported to increase macromolecular permeability in MDCK cells, but to simultaneously increase TER, indicating two separable responses. The C-terminus, therefore has roles in regulating both electrical resistance and paracellular permeability. C-terminal truncations have also been reported to abolish fence function in MDCK cells (Balda et al., 1996). Finally, truncation of the N-terminus of occludin increases paracellular permeability, decreases TER and disrupts strand morphology in murine epithelial cells (Bamforth et al., 1999). In all these truncation experiments, occludin appeared to be correctly targeted to the tight junction, most likely through oligomerization with endogenous occludin (Chen et al., 1997) or association with ZO-1 (Medina et al., 2000). Occludin also contributes to cell-cell adhesion via the first extracellular loop (Van Itallie et al., 1997) and is implicated in modulation of transepithelial migration of neutrophils (Huber et al., 2000) and T-cells (Alexander et al., 1998). Mutation of some residues within the extracellular loops of occludin results in relocalisation of occludin along the basolateral membrane, as well as decreased paracellular permeability (Balda and Matter, 2000). This indicates that the loops are important for the correct localisation of occludin to the tight junctions and for the formation of aqueous pores. Surprisingly, occludin is not essential for the formation of tight junction fibrils as shown by occludin-deficient embryonic stem cells, which form tight junction fibrils (Saitou et al., 1998). This led to the search for other proteins that might compensate for the lack of occludin, or that might themselves be more important for tight junction integrity. The claudins are a second membrane spanning family of proteins that are located at the tight junction and polymerise along tight junction fibrils (Furuse et al., 1998) (see below).

### 1.3.1.3 Occludin regulation
Occludin is extensively phosphorylated and migrates as a smear of 62-82 kDa on SDS gels (Sakakibara et al., 1997). This phosphorylation is important for occludin localisation to the tight junction and for permeability. There are no reports of glycosylation of occludin so far.
1.3.1.3.1 Occludin tyrosine phosphorylation

In epithelial cells, heavily phosphorylated occludin localises to the junctions, and less-phosphorylated occludin is seen in the cytoplasm and/or basolateral membrane (Sakakibara et al., 1997, Tsukamoto et al., 1999, Andreeva et al., 2001). As such, loss of occludin tyrosine phosphorylation correlates with decreased TER (Chen et al., 2002; Tsukamoto et al., 1999). Tight junction reassembly and TER recovery in MDCK cells after ATP repletion requires tyrosine kinase activity and induces occludin, ZO-2 and ZO-3 tyrosine phosphorylation (Tsukamoto et al., 1999, Chen et al., 2002). So occludin phosphorylation may therefore regulate its association with the junction, which in turn affects permeability. However in *Xenopus* embryos, dephosphorylated occludin is found preferentially at the junctions (Cordenonsi et al., 1997), and others have correlated tyrosine phosphorylation with increased permeability (Gloor et al., 1997; Esser et al., 1998). So regulation of occludin through phosphorylation may have different roles depending on cell type, and the site and type of phosphorylation (Tyr or Ser/Thr). c-Yes has been shown to bind to the occludin C-terminus and its activity is required for occludin tyrosine phosphorylation during calcium switch assays, so it is likely that this kinase directly phosphorylates occludin, but this has not yet been determined *in vitro* (Chen et al., 2002; Nusrat et al., 2000, Gonzales-Mariscal, 2003).

1.3.1.3.2 Occludin and Ser/Thr phosphorylation

Occludin has been shown to be a target for phosphorylation of serine and threonine residues by PKC (Andreeva et al., 2001), CK2 (Cordenonsi et al., 1999) and p34^cd^ cyclin B, which is a protein involved in cell cycle progression (Cordenonsi et al., 1997). In *Xenopus laevis* occludin C-terminal Ser^{379} and Thr^{375} have been shown as *in vitro* targets for CK2 (Cordenonsi et al., 1999). PKC phosphorylates occludin Ser^{338} *in vitro* and activation of PKC with phorbol esters induces occludin phosphorylation and recruitment to sites of cell-cell contact in MDCK cells in low calcium medium (Andreeva et al., 2001). However, others have reported occludin Thr dephosphorylation in response to PKC activation, which correlated with increased paracellular permeability in LLP-CK1 epithelial cells (Clarke et al., 2000).
1.3.1.4 Occludin degradation

Occludin and tight junction barrier function may also be regulated through protein degradation, both extracellularly and intracellularly. Occludin proteolysis and increased permeability, induced by protein tyrosine phosphatase inhibitors, can be blocked by a metalloproteinase inhibitor, PHEN (Wachtel et al., 1999). Some membrane-bound metalloproteases, such as members of the ADAM (a disintegrin and metalloproteinase) family, are responsible for proteolytic cleavage of integral membrane proteins in a process called 'shedding'. One example of an ADAM family member is TACE (tumour necrosis factor-alpha-converting enzyme), which cleaves a precursor of TNF-α, releasing the soluble, active cytokine extracellularly (Black et al., 2002). The large occludin extracellular loops make attractive potential targets for metalloprotease activity and could also be cleaved in this way. The E3 ubiquitin ligase, Itch (Perry et al., 1998), binds to the occludin N-terminus, ubiquinates it and induces occludin internalisation and proteolysis (Traweger et al., 2002).

1.3.1.5 Occludin transcriptional regulation

Two promotors for control of occludin mRNA expression have been identified, one of which contains potential binding sites for transcription factors myc, NFIL6, TCF1 and AP2, and the other contains sites for AP2, c-myb, and a GC-box (a CG-rich region that is recognised by several transcription factors such as Sp1) (Fig 1.4). The pro-inflammatory cytokine, TNF-α, has been reported to reduce reporter gene expression in HT-29/B6 intestinal cells transfected with either the occludin exon 1 or the occludin exon 1a promoter region (Mankertz et al., 2000 and 2002). In astrocytes, TNF-α downregulates occludin, although these cells do not form a functional permeability barrier and in epithelial and brain microcapillary cells occludin levels were unaffected by TNF-α (Wachtel et al., 2001).
Fig 1.4

Ocludin transcription occurs from two promoters

E1, E1A, E2, E2B and E3 are ocludin exons. Dotted lines indicate regions that are spliced out. P = promoter regions. Blue arrows are the transcription start sites, red arrows are the translation start sites. Black arrows show where the indicated transcription factors bind to the two promoter regions.
1.3.2 Claudins

Claudins-1 and -2 belong to the second family of integral membrane proteins to be shown to integrate into tight junction strands and to be able to reconstitute them when transfected into fibroblasts that lack tight junctions (Furuse et al., 1998a and b). Currently there are 24 known members of the claudin family. These 22-27 kDa tight junction proteins contain an intracellular N-terminus, four transmembrane regions, two extracellular loops; with the first being much larger than the second, and a short intracellular C-terminus (Fig. 1.5). Claudins associate via their extracellular loops to claudin molecules present on neighbouring cells at the tight junction. This association is specific, as claudin-3 associates with claudin-1 or -2, but claudin-1 will not interact with claudin-2 (Furuse et al., 1999).

1.3.2.1 Claudin tissue distribution

Claudins exhibit a diverse tissue distribution and have been detected, for example in mouse lung, liver, kidneys, brain, spleen and testis (Tsukita et al., 2000). Some claudins are tissue-specific, for example claudin-5, which is found only in endothelial cells. Other examples are claudin-1 and -2, which are expressed at high levels in the liver and kidney or claudin-3, which is detected mainly in the lung and liver. Usually two or more claudins are expressed together in one cell type (Tsukita et al., 2000; Furuse et al., 1998).

1.3.2.2 Claudin binding partners

Most claudins associate with several PDZ (PSD95/DLG/ZO-1) -containing proteins at the tight junction through conserved PDZ-binding motifs within the C-terminus. PDZ domains are protein-binding modules that mediate protein-protein interactions. The most well-characterised PDZ domains are the type I PDZ domains that recognise the motif S/T-X-V (where X is any amino acid) and type II PDZ domains that recognise the motif $-X - $ (where $ is a hydrophobic residue) (Nourry et al., 2003; Bezprozvanny et al., 2001). PDZ domains can also bind to other PDZ domains. Some claudins, such as claudins 1-5 contain a type I PDZ-binding motif, whilst others, such as claudin-16 contain a type II-PDZ motif. Claudins-11 and -13 lack PDZ-binding motifs altogether. The tight junction proteins ZO-1, ZO-2, ZO-3 (Itoh et al., 1999), PATJ (Roh et al., 2002)
Figure 1.5

The structure of claudins

Claudins span the plasma membrane four times and contain a large extracellular loop, followed by a short intracellular region, a smaller second extracellular loop and a cytoplasmic C-terminus. Adapted from Tsukita et al., 2000.
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and MUPP1 (Hamazaki et al., 2002; Jeanson et al., 2003) each have been shown to associate with claudin PDZ C-terminal motifs. Claudins also associate indirectly with AF-6 and cingulin (Heiskala et al., 2001).

1.3.2.3 Claudin functions

Claudins are involved in tight junction barrier function. For example, overexpression of claudin-5 in mouse L-fibroblasts induces the formation of tight junction fibrils (Morita et al., 1999), indicating their role in maintaining the tight junction structure. Expression of claudins-1, -3 or -5 in mouse fibroblasts induces the formation of tight junction strands observed in freeze frame replicas (Morita et al., 1999; Furuse et al., 1998). Individual claudins or combinations of different claudins can produce tight junction fibrils of differing morphology (Furuse et al., 1999, Morita et al., 1999a and b) and they can confer high or low TER and permeability values. For example claudin-1 overexpression results in increased TER and decreased paracellular permeability in MDCK cells (Inai et al., 1999). Similarly, co-expression of claudin-1 and -3 in NIH/3T3 cells decreases solute permeability and increases electrical resistance (Coyne et al., 2003). However, there is some evidence that claudin-5 may confer a leaky phenotype in some cells. Claudin-5 overexpression in NIH/3T3 cells results in tight junction formation, but increased solute permeability compared with untransfected cells. The untransfected NIH/3T3 cells expressed ZO-1, but did not contain any organised tight junction strands, and only exhibited baseline electrical resistance (Coyne et al., 2003). Furthermore, co-transfection of claudin-3 and claudin-1 into NIH/3T3 cells increases TER and reduces paracellular permeability, which is reversed by further transfection of claudin-5. These data indicate that a mix-and-match of claudins can determine the level of tightness of the junctions, and that the endothelial-specific claudin-5 probably contributes to more leaky junctions (Coyne et al., 2003; Wang et al., 2003). How exactly claudin-5 achieves this is yet to be determined (Coyne et al., 2003).

There are also other lines of evidence for claudin involvement in barrier function. Removal of claudin-4 induces a decrease in TER in MDCK cells (Sonoda et al., 1999) and removal of claudin-2 changes high resistance MDCK I cells to low resistance cells.
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(Furuse et al., 2001). Claudin-1 expression has been shown to be lacking in human breast cancer cells, and re-introducing this claudin decreases the paracellular flux of tracers (Hoevel et al., 2002). Claudin-1-deficient mice die within 1 day of birth and despite containing occludin and claudin-4-positive tight junction fibrils, exhibit a tracer-permeable epidermis (Furuse et al., 2002).

Claudins are involved in determining which ions pass through the tight junction. Claudins are thought to create paracellular, charge selective pores, whose selectivity depends upon the claudin protein expressed. For example, claudin-4 expression in epithelial cells decreases Na\(^+\) permeability, but leaves Cl\(^-\) permeability unaffected (Van Itallie et al., 2000). Furthermore, claudin-2 is thought to encode a leaky cation-permeable channel, whereas claudin-8 acts more as a cation barrier (Yu et al., 2003; Amasheh et al., 2002). The extracellular loops of individual claudin molecules exhibit big differences in their isoelectric points and charged residues, which could account for such selectivity. Claudin-16 is expected to act as a cation pore, and claudins-4, -11 and -17 are probably anionic channels (Mitic and Van Itallie et al., 2001). Substitution of some negative charged residues with positive charges on the first extracellular loop of claudin-15 reverses the paracellular charge selectivity preference from Na\(^+\) to Cl\(^-\) (Colegio et al., 2002).

1.3.2.4 Regulation of claudins
Claudins have phosphorylation sites within their C-terminus including a consensus motif for PKC, casein kinase II and cAMP-dependent protein kinase (PKA) (Heiskala et al., 2001). Cyclic AMP (cAMP) is a member of the cyclic nucleotide family of second messengers, which is generated by conversion of ATP to cAMP by adenylyl cyclases (Heldin and Purton, 1997). Recently, phosphorylation of claudin-5 immunoprecipitates by PKA has been reported and is associated with the promotion of tight-junction function in endothelial cells (Ishizaki et al., 2003). PKC might also regulate claudins, as PKC\(\varepsilon\) has been shown to shift claudin-1 from the Triton-X-100 soluble fraction to the insoluble fraction and enhance TER (Yoo et al., 2003). Some compensatory crosstalk may occur
between the claudins as overexpression studies involving one claudin have resulted in decreased expression of an endogenous claudin (Yu et al., 2003).

1.3.2.5 Claudins and human disease
Several human diseases have provided clues as to the claudin family function. Patients with hypomagnesemia with hypercalciuria and nephrocalcinosis (HHN) have a mutation in the gene coding for claudin-16 and exhibit defective Mg\(^{2+}\) and Ca\(^{2+}\) reabsorption in the thick ascending limb of Henle in the kidney, whereas NaCl reabsorption is unaffected (Simon et al., 1999). Mutations in claudin-14 result in profound deafness, probably due to improper ionic composition of fluid surrounding the basolateral surface of outer hair cells as a result of loss of a cationic barrier (Ben-Yosef et al., 2003). Claudins may also have a role in cancer. Claudin-4 is overexpressed in pancreatic cancer and gastrointestinal tumours, and is a receptor for *Clostridium perfringens* enterotoxin (CPE), which targets claudin-4 expressing cells, induces tumour cell necrosis and is of potential therapeutic value (Michl et al., 2001). Interestingly, claudin-4 is associated with well-differentiated, less invasive tumours and overexpression studies have demonstrated its ability to reduce tumour cell line invasiveness and growth by increasing the number of tight junctions (Michl et al., 2003). Claudins-3 and -4 are also frequently overexpressed in ovarian tumours (Rangel et al., 2003). However, claudin-1 expression is lost in many breast cancers (Hoovel et al., 2002; Kramer et al., 2000), and there is lower expression of claudin 7 in head and neck squamous cell carcinomas (Al Moustafa et al., 2002). Increased permeability precedes the onset of colon carcinoma, and several isoforms of PKC are implicated in intestinal tumorigenesis via their effect on the tight junction (Heiskala et al., 2001).

1.3.3 JAMs

1.3.3.1 JAM family and structure
Junctional adhesion molecules (JAMs) are a single membrane spanning protein, which belongs to the immunoglobulin superfamily (IgSF), and localises to the tight junction (Martin-Padura et al., 1998). The JAMs have confusing nomenclature due to the human and mouse JAMs being independently named. This has been resolved by the renaming of
the JAM proteins by Muller et al. (2003) and this convention is followed here (Table 1.1). JAMs have been further categorized as members of the cortical thymocyte xenopus (CTX) gene family (Aurrand-Lions et al., 2001b), which interestingly, also encorporates the coxsackie and adenovirus receptor (CAR) (see below) and endothelial-selective adhesion molecule (ESAM) that are also present at tight junctions. JAMs contain a 215 amino acid extracellular region containing two Ig domains, a membrane-spanning region and a short intracellular tail of 45 amino acids. The JAM proteins have conserved length and composition of their C-termini. This tail contains a type II PDZ-binding motif in all three JAM proteins (Martin-Padura et al., 1998), which differs from ESAM and CAR, in that the latter proteins contain type I PDZ-binding motifs in this region (Bergelson et al., 1997; Hirata et al., 2001) and neither of these proteins bind to PAR3, whereas the JAMs do (Ebnet et al., 2003). Human JAM-A migrates as a doublet on SDS-PAGE, which represents two glycosylated forms of approximately 40 and 37 kDa (Liang et al., 2000). The X-ray structure of the extracellular domain of JAM-A has been determined, and suggests that JAM molecules dimerise via their first variable Ig loop (Kostrewa et al., 2001). JAMs form homotypic interactions with JAMs on neighbouring cells. JAM-B has also been shown to form heterotypic interactions with JAM-C, but not with JAM-A (Cunningham et al., 2000 and 2002, Arrate et al., 2001; Liang et al., 2002).
Chapter 1  

*Introduction*

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**Table 1.1**

Current nomenclature for the JAMs

JAMs have been re-named JAM-A, -B or -C respectively. The original authors and their JAM designations are shown.
1.3.3.2 Expression and localisation

Human JAM-A is expressed in epithelial and endothelial cells (Liu et al., 2000, Williams et al., 1999) as well as peripheral blood leukocytes such as monocytes, neutrophils, platelets, T-lymphocytes and B-lymphocytes, and red blood cells (Williams et al., 1999, Sobocka et al., 2000, Liu et al., 2000). Human JAM-B is largely confined to endothelial cells (Ebnet et al., 2003). Human JAM-B is expressed mainly in the heart, placenta and lymph nodes, where its expression is particularly high in high endothelial venules (HEV) and was reported to be lacking in peripheral blood leukocytes and platelets (Palmeri et al., 2000). Indeed, Liang et al. (2002) demonstrated a lack of binding between JAM-B and B-cells, neutrophils or monocytes, which would be consistent with a lack of homotypic interactions. However, JAM-B expressing cells are capable of binding T-cells, natural killer cells and dendritic cells; in the case of T-cells through binding JAM-C (Liang et al., 2002). Human JAM-C was detected in HEVs and HUVECs (Aurrand-Lions 2001, Johnson-Leger 2002). JAM-C expression has been reported in monocytes, B-cells and T-cells (Johnson-Leger 2002, Liang 2002), although two other groups did not detect JAM-C in peripheral blood leukocytes except for in platelets (Santoso et al., 2002; Arrate et al., 2001).

JAMs (JAM-A) localise to cell-cell contact regions and co-localise with tight junction components in epithelial and endothelial cells in mice and humans (Martin-Padura 1998, Williams 1999). However, JAM-A does not incorporate into tight junction strands in the same way as occludin and claudin. JAM-A transfected into L-cells does not form tight junction fibrils, but instead forms small aggregates (compare JAM-A Itoh et al., 2001; claudin-5; Morita et al., 1999b and occludin; Furuse et al., 1998b). However, there does appear to be lateral association between JAM-A and the tight junction strands in freeze fracture replicas (Itoh et al., 2001).

1.3.3.3 Binding partners

JAM-A binds through its PDZ-binding motif to AF6 (Ebnet et al., 2000). JAM-A, -B and -C bind to ASIP/PAR-3 (Itoh et al., 2001; Ebnet et al., 2001, 2003) and ZO-1 (Bazzoni et al., 2000, Ebnet et al., 2003). JAM-A also co-immunoprecipitates with cingulin (Bazzoni
et al., 2000). JAMs are also capable of acting as receptors for several integrin family members (see below).

1.3.3.4 JAM functions

JAMs are implicated in diapedesis (Muller et al., 2003). This is thought to involve both homotypic and heterotypic interactions between JAM molecules on the endothelium and on leukocytes. Monoclonal antibodies directed against JAM-A can block monocyte transmigration (Martin-Padura et al., 1998). JAM-A-blocking antibodies also prevented monocyte and neutrophil infiltration in a mouse meningitis model (Del Maschio et al., 1999). As well as binding other JAMs, JAM-A has been shown to bind to the leukocyte integrin, LFA-1 (αLβ2 / CD11a-CD18) (Ostermann et al., 2002). TNF-α and IFN-γ in combination have been shown to alter JAM-A distribution from tight junctions, but without any change in overall cell-surface expression (Ozaki et al., 1999). This is comparable to the effect of these cytokines on PECAM-1 (Rival et al., 1996). This redistributed JAM-A co-operates with ICAM-1 in arresting memory T-cells on the cell surface. This is achieved by the lymphocyte LFA-1 interaction with both JAM-A and ICAM-1 on the endothelium. The contribution of JAM-A to this arrest is abolished where JAM-A redistribution from the junction has not been induced. JAM-A does not exhibit this arresting-role in neutrophil adhesion assays, but for both T-cells and neutrophils, diapedesis requires JAM-A, as blocking antibodies to JAM-A inhibits this, presumably by inhibiting the JAM-A association with LFA-1. JAM-A binds to LFA-1 via the JAM membrane-proximal Ig-like domain 2 (Ostermann et al., 2002).

JAM-B interacts heterotypically with JAM-C (Arrate et al., 2001, Cunningham et al., 2000) and can also interact with integrin α4β1 (VLA-4, CD 49d-CD29), which is expressed in lymphocytes, monocytes and eosinophils. This occurs only in a JAM-C dependent way (Cunningham et al., 2002). Both interactions are dependent upon the first Ig fold of JAM-B.

Platelet JAM-C has also been described as a counter-receptor to the leukocyte β2 integrin, Mac-1, but not for LFA-1, which may facilitate the accumulation of leukocytes at sites of platelet deposition in regions of vascular damage (Santoso et al., 2002).
JAM-A has also been described as a reovirus attachment protein $\sigma$ – receptor (Barton et al., 2001, Tyler et al., 2001).

### 1.3.3.5 JAMs and the tight junctions

JAMs appear to contribute to tight junction assembly. Tight junction disruption using calcium depletion resulted in JAM-A redistribution on the cell membrane (Liang et al., 2000). Localisation of occludin to the tight junction during recovery from calcium depletion is inhibited by anti-JAM-A antibodies. Moreover, antibodies to JAM prevent TER recovery in calcium depletion assays (Liu et al., 2000).

JAMs may also have a role in paracellular permeability, as CHO cells transfected with JAM-A demonstrate reduced paracellular flux (Martin-Padura et al., 1998). The characteristic increased albumin flux in a mouse meningitis model was inhibited by JAM-A-blocking antibodies (Del Maschio et al., 1999). JAM-A is expressed in brain endothelial cells and epithelial cells, whereas JAM-B is not detected in brain blood vessels, but is in the more leaky lymphatics, which may indicate a differential role for these two JAMs in regulating paracellular permeability (Aurrand-Lions et al., 2001).

### 1.3.6 JAM regulation

It has recently been show that JAM-C is phosphorylated on two serine residues. Mutation of one of these serines ($\text{Ser}^{91}$) in the cytoplasmic tail to an alanine residue results in accumulation of JAM-C at tight junctions, whereas exchange for the phospho-mimicking aspartic acid residue results in loss of it from cell-cell contacts (Ebnet et al., 2003). This phosphorylation may therefore be important for localisation of JAM-C to the tight junctions. It is also interesting to note that JAM-B contains a PKC phosphorylation consensus sequence in its intracellular tail (Cunningham et al., 2000). The physiological relevance of this site has not yet been determined.

### 1.3.4 Coxsackie and adenovirus receptor

The coxsackie B (CVB) and adenovirus (Ad) receptor (CAR) is a member of the Ig family of membrane-spanning proteins shown to localise to cell-cell contacts. CAR was
first identified in 1997 as a receptor for the coxsackie B virus and adenovirus 2 and 5 (Bergelson et al., 1997; Tomko et al., 1997; Carson et al., 1997). CAR is a 46-kDa membrane-spanning protein that contains a 222-amino acid extracellular region of two Ig-like domains, a single transmembrane region and a 107-amino acid intracellular domain (Bergelson et al., 1997). Despite their similarity, JAM and CAR exhibit less that 30% identity in their extracellular regions (Carson et al., 2001). The CAR intracellular C-terminus contains a type I PDZ-binding motif (SXV).

1.3.4.1 CAR tissue distribution and subcellular localisation
CAR is expressed on the basolateral surface of epithelial cells (Walters 1999) and endothelial cells including HUVECs (Carson et al., 1999) and targeting to this region requires multiple regions within the CAR cytoplasmic domain (Cohen et al., 2001). CAR has been shown to co-localise and co-immunoprecipitate with ZO-1 in epithelial cells (Cohen et al., 2001). Despite this, it has not been shown whether CAR encorporates in the tight junction strands or whether it laterally associates to it. It seems unlikely that CAR is an integral part of the tight junction fibrils, as other Ig family members localised at cell-cell junctions (JAM and PECAM) do not encorporate into the strands. Walters et al (2002) published a more basal localisation of CAR and its association with β-catenin. The exact location of CAR is, therefore, unclear at present. In both cases, a low level of infectivity of adenovirus where it is applied apically does indicate that CAR is buried within the cell-cell junctions and is relatively inaccessible in epithelial cells. This has been a major obstacle in the use of adenoviruses for gene therapy for conditions such as cystic fibrosis (Einfeld et al., 2002).

1.3.4.2 CAR function
CAR is most well known for its function as a coxsackie B virus and adenovirus 2 and 5 receptor, and hence for its uses in gene therapy for conditions such as cystic fibrosis (Einfeld et al., 2002). The physiological role for this receptor is less well understood, but is has been reported to have roles in cell-cell adhesion, tight junction formation and barrier function: over-expression of CAR results in homotypic aggregation (Cohen et al., 2001; Honda et al., 2000) and cell adhesion has been shown to be facilitated by the CAR
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CAR has also been implicated in cellular growth control, as CAR protein levels are down-regulated in bladder cancer specimens and in a human prostate cancer line and CAR expression in the former cells results in their growth being inhibited (Okegawa et al., 2000 and 2001). This effect requires both the extracellular and transmembrane domains of CAR and is probably mediated through CAR-facilitated intercellular adhesion, as interrupting intercellular adhesion of CAR by a specific antibody inhibits the growth-inhibitory effect of CAR. CAR may therefore be a mediator of contact inhibition, which is downregulated in cancer (Okegawa et al., 2001).

CAR protein expression is induced by TNF-α in HeLa and ovarian cancer cells but decreased in glioblastoma cells. Phythemagglutinin (PHA) and interleukin-2 (IL-2) activation of T-cells increases adenovirus binding, which could indicate an increase in CAR expression (Mentel et al., 1997). If this is the case, it is possible that, like JAM and PECAM, CAR expressed in endothelial cells could form homotypic interactions with leukocytes and be involved in transmigration during inflammation. This has not been investigated so far.

1.3.5 The MAGUK family of tight junction plaque proteins

ZO-1 was the first molecule identified exclusively at the tight junction (Stevenson et al., 1986). It was subsequently recognised to be a member of MAGUK family of proteins.
1.3.5.1 Structure of ZO proteins

ZO-1, ZO-2 and ZO-3 are characterised by the presence of SH3, PDZ and guanylate kinase-like (GK) domains (Fig. 1.6). The PDZ domains confer clustering and scaffolding properties to the MAGUK proteins, particularly as they contain multiple PDZ regions. For example, the second PDZ domain of ZO-1 associates to the second PDZ domains of ZO-2 and ZO-3 (Haskins et al., 1998; Gonzalez-Mariscal 2000). The GK domain is homologous to the enzyme, guanylate kinase, which catalyses the conversion of GMP to GDP by hydrolysing ATP. The MAGUK protein GK region lacks this catalytic ability, as it cannot bind to GMP or ATP, and instead is used as an adaptor module, which, for example, mediates ZO-1 binding to occludin (see below) (Kim et al., 1997; Fanning et al., 1998; Schmidt et al., 2001). The ZO proteins also contain an acidic region and several proline-rich regions within their C-termini. These polyproline regions are recognised by SH3 domains, which are 70 amino-acid motifs that bind to proline rich regions that are at least seven residues in length and generally contain the sequence PXXP. SH3 and GK domains have also been shown to associate in trans as well as intramolecularly (McGee et al., 1999; 2001), although the GK domain lacks the polyproline region usually recognised by SH3 domains. Instead, both intact domains are required for binding to occur (McGee et al., 2001).

Three alternative splice sites are present in the C-terminus of ZO-1. The $\alpha^+$ isoform of ZO-1 contains an extra 80-amino acid motif and is more abundant than the $\alpha^-$ ZO-1 isoform in epithelial cells, whereas the opposite is the case for endothelial cells. Other splice variants of ZO-1 are $\beta_1$, $\beta_2$ and $\gamma$ encoding 7, 20 and 45-amino acid insertions respectively. Their functions are not yet fully understood. There are two known isoforms of ZO-2, one of which contains an amino-terminal 23-amino acid insert. This results from activation of transcription from different promoters and different start codons (Gonzalez-Mariscal 2000).

1.3.5.2 ZO-protein interacting proteins and functions

As expected for the role of ZO proteins in organising the junctions, ZO proteins bind to many other tight junction proteins (Fig. 1.7). ZO-2 and ZO-3 can each bind to ZO-1 and
all three ZO proteins are capable of binding to the occludin C-terminus. ZO-1 has been shown to bind to the coiled-coil region of occludin (Furuse et al., 1994; Nusrat et al., 2000), which involves the GK domain of ZO-1 (Schmidt et al., 2001). The ZO-1 binding region of occludin is required for targeting occludin to the tight junction, indicating a role for ZO-1 in the recruitment of occludin to the cell junctions (Furuse et al., 1994). The first PDZ domain of ZO-1, -2 and -3 binds to the claudin C-terminal YV sequence, and this interaction is thought to localize ZO-1 to the tight junction (Itoh et al., 1999).

ZO-1 also associates with the JAM-A cytoplasmic tail, which requires the second and third PDZ domains of ZO-1 as well as the interconnecting region between the two and is an interaction which may be important for the recruitment of JAM to cell-cell junctions (Ebnet et al., 2000). ZO-1 has been shown to co-precipitate with CAR, and CAR recruits ZO-1 to areas of cell-cell contact in CHO cells (Cohen et al., 2001). ZO-1 binds directly to AF-6, an interaction that is abolished by activated Ras and may be involved in the regulation of cell-cell contact (Yamamoto et al., 1997). ZO-1 also binds to the gap junction component, connexin-43 (C-terminus) through the second PDZ domain of ZO-1 (Toyofuku et al., 1998; Giepmans et al., 1998). In addition, ZO-1 and ZO-3 also bind to a short PDZ binding motif in the C-terminus of connexin-45 through their PDZ domains (Kausalya et al. 2001). The functional relevance of these associations is unclear, but could serve to organise or recruit proteins to the gap junctions.
Figure 1.6
The ZO family of MAGUKs
Each ZO protein contains three PDZ domains (light blue; 1-3), a basic region (+), an acidic region (-), a proline rich region (+) and an SH3 domain (red). ZO-1 splice sites are labelled α, β and γ. ZO-2 splice sites are labelled n (normal and not cancerous cells), c (canine; where it was first identified) and a (avian; where it was first identified). Black arrows are NLS sites, and red arrow is the NES site. 3' termination sites for human (h), mouse (m), chick (ch) and canine (C) are shown. Adapted from Gonzalez-Mariscal, 2000.
Fig 1.7
ZO-1-interacting proteins
Where known, the precise ZO-1 domain involved in the protein-protein interaction is indicated by an arrow. Otherwise the interactions are limited to N-terminal, C-terminal or unknown. All interactions shown are direct with the exception of CAR, whose interaction with ZO-1 has been shown through co-immunoprecipitation (IP) only (Cohen et al., 2001).
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ZO proteins also bind to some cytoskeletal proteins. ZO-1, -2 and -3 associate directly with F-actin via their C-termini (Itoh et al., 1997; Fanning et al., 1998; Gonzalez-Mariscal, 2000), and to the cingulin N-terminus (Cordenonsi et al., 1999). Cingulin contains a globular head and tail linked by a central α-helical rod domain, through which it forms dimers. Cingulin is an actin-binding protein, which also associates with myosin and may be important for cross-linking the tight junction to the actin cytoskeleton. It may contribute to tight junction biogenesis, as it localises with ZO-1 to adherens junctions in fibroblasts that lack tight junctions (D'Atre et al., 2002). In this context, ZO-1 (N-terminus) and ZO-2 can bind directly to the adherens junction protein α-catenin (Itoh et al., 1997). Erythroid protein 4.1 is a cytoskeletal protein that attaches the spectrin/actin cytoskeleton to the plasma membrane (see below). Two isoforms of non-erythroid protein 4.1 have been shown to bind to ZO-2 (proline-rich region), ZO-1 and occludin (Mattagajasingh et al., 2000). This complex may function to attach the membrane cytoskeleton to the membrane. In support of this, ZO-1 has also been shown to bind to the actin-binding protein, spectrin (Itoh et al., 1991). This is a tetrameric actin cross-linking protein that contains triple α-helical repeats (spectrin repeats) and has a flexible rod-like morphology that confers mechanical support to the plasma membrane (Thomas, 2001).

$G_{o12}$ binds to ZO-1 (SH3 domain) and ZO-2 and co-localises with ZO-1 in MDCK cells. This association is implicated in the regulation of barrier function, as overexpression of dominant active $G_{o12}$ increased paracellular permeability (Meyer et al., 2002).
ZO-1 has been observed in the nuclei of some cells, particularly sparse cells lacking cell-cell contact or in cells along the wounded edge (Gottardi et al., 1996, Gonzalez-Mariscal 2000). Furthermore, ZO-1, ZO-2 and ZO-3 contain both a nuclear export (NES) and import motif (NIS) and the SH3 domain of ZO-1 has been shown to associate with the Y-box transcription factor ZONAB and regulates erbB-2 expression, cell proliferation and cell density (Balda and Matter, 2000; 2003). ZO-2 binds to the transcription factors Fos, Jun and C/EBP (Betanzos et al., 2004) and co-localises with the splicing factor SC35 within the nucleus (Islas et al., 2002). ZO-2, like ZO-1, is observed in the nucleus of sparse cells or cells of a wounded edge, but disappears from the nucleus as the cells become confluent.

These associations indicate that ZO proteins have roles in tight junction biogenesis, recruiting other proteins to the tight junction, cross-linking junctions to the actin cytoskeleton and anchoring of the cytoskeleton to the plasma membrane, as well as signalling to the nucleus for transcription of target genes. ZO proteins are also implicated in the regulation of barrier function (see below).

1.3.5.3 ZO-1 Phosphorylation

ZO proteins are phosphoproteins, but the effect of ZO phosphorylations are controversial (Gonzalez-Mariscal, 2003). Therefore, the outcome of ZO-1 tyrosine and/or Ser/Thr phosphorylation probably depends upon the phosphorylation site, cell type and kinase involved. Total phosphorylation of ZO-1 from low-resistance MDCK cells (leaky) is greater than in high-resistance MDCK cells (tight) (Stevenson et al., 1989). However, low phosphorylated ZO-1 has been detected in cells that lack tight junctions, or where they have been dissassembled by removal of calcium (Howarth et al., 1994)

1.3.5.3.1 ZO protein Ser/Thr phosphorylation

Currently there are two known protein kinases that target ZO-1 directly. ZO-1-associated kinase (ZAK), a serine kinase, binds to the SH3 domain of ZO-1 and phosphorylates it C-terminally to this region (Balda et al., 1996). The effect of this phosphorylation event is unknown. Protein kinase C (PKC) has also been shown to induce phosphorylation of
ZO-1, although evidence for direct phosphorylation of ZO-1 by PKC and the result of such an event is contradictory. PKC is a Ser/Thr protein kinase of which there are at least eleven different isoforms. These are classified into three groups; the conventional (cPKC; α, βI, βII, γ), novel (nPKC; δ, ε, η, θ, μ) and atypical (aPKC; ξ, λ/ι). Conventional PKC isoforms require diacylglycerol (DAG), calcium and phosphatidylserine for their activation, whereas novel PKCs do not need calcium and atypical PKCs only require phosphatidylserine (Harhaj et al., 2004). PKC-ζ and λ colocalize with ZO-1 at the junction (Dodane et al., 1996), and purified PKC phosphorylates immunoprecipitated ZO-1, and possibly ZO-2 and ZO-3 in vitro (Stuart and Nigam, 1995). aPKC has been shown to bind to ASIP/PAR-3, which in turn binds to JAM. JAM also binds to ZO-1, thus a multiprotein complex could form bringing aPKC into close association with ZO-1 (Itoh et al., 2001, Ebnet et al., 2001). Activation of PKC induces the formation of TER and ZO-1 localisation to the junction (Balda et al., 1991, 1993). Moreover, inhibition of PKC during calcium-repletion blocks the development of TER (Balda 1991, Denisenko 1994). On the other hand, a PKC agonist was unable to induce phosphorylation of ZO-1, -2 or a 130 kDa protein (probably ZO-3) in MDCK cells (Balda et al., 1993) and PKC inhibition blocks tight junction disassociation where calcium chelators or cytoskeletal disruptors are added, indicating a role for PKC in tight junction disassembly (Citi et al., 1992).

ZO-2 is Ser/Thr phosphorylated by PKA and PKC β, ε, ζ and λ in vitro and its phosphorylation is observed where tight junctions are absent or disassembled (Avila-Flores et al., 2001). Ser/Thr phosphorylation of ZO-2 is associated with impaired barrier function (Ward et al., 2002).

1.3.5.3.2 ZO protein tyrosine phosphorylation
There is some evidence that tyrosine phosphorylation of ZO-1 increases permeability. Tyrosine phosphatase inhibition using vanadate has been shown to increase paracellular permeability and increase tyrosine phosphorylation of ZO-1 in MDCK cells, indicating that ZO-1 tyrosine phosphorylation weakens barrier function. Similar results were observed with the tyrosine phosphatase inhibitor phenylarsine oxide (Staddon et al.,
1995). Furthermore, VEGF has been shown to increase retinal endothelial permeability
in vivo, which also correlated with increased ZO-1 tyrosine phosphorylation (Antonetti et
al., 1999). In contrast, ATP repletion of epithelial cells results in tyrosine
phosphorylation of ZO-2 and ZO-3 (Tsukamoto et al., 1997) and ZO-1 becomes tyrosine
phosphorylated during tight junction assembly in Ras-transformed MDCK cells
following MEK-1 inhibition (Chen et al., 2000). Furthermore, epidermal growth factor
induces tyrosine phosphorylation of ZO-1 and recruits it to the tight junction (Van Itallie
et al., 1995). Hepatocyte growth factor/scatter factor (HGF/SF) induces ZO-1 tyrosine
phosphorylation, decreased ZO-1 protein levels, and increased permeability of HUVECs
(Martin et al., 2002)

1.3.5.3 ZO protein dephosphorylation
The Ser/Thr protein phosphatase 2A isoform, ABαC, has been shown to co-localise with
tight junction proteins during junction biogenesis induced by Ca^{2+}. Active ABαC
dephosphorylates ZO-1, occludin and claudin-1, increases permeability and blocks tight
junction formation (Nunbhakdi-Craig et al., 2002).

1.3.5.4 ZO protein expression
ZO-1 is also regulated through its protein expression levels. This has been shown to
correlate with changes in tight junction barrier function. For example, IFN-γ induces
increased permeability in T84 intestinal epithelial cells, which correlates with reduced
ZO-1 protein expression and concomitant loss of ZO-2 and occludin from the junctions
(Youakim et al., 1999). ZO-1 protein expression is also down-regulated by HGF/SF in
HUVECs, which also increases permeability (Martin et al., 2002).

1.3.6 Other Tight Junction Proteins
A host of other proteins that localise to the tight junction have been identified and have
been recently reviewed (Gonzalez-Mariscal, 2003). Many of these have not so far been
reported in endothelial cells.
1.4 The Actin Cytoskeleton in Endothelial Cells

Tight junctions are associated with the actin cytoskeleton, which has been shown to be important for the regulation of permeability (Shasby et al., 1982; Alexander et al., 1988; Phillips et al., 1989; Goldblum et al., 1993). Three actin cytoskeletal structures have been described in endothelial cells (Dreckhahn and Ness, 1997): the cortical web (membrane cytoskeleton or spectrin-based membrane skeleton), the junction-associated actin filaments and actin stress fibres (Fig. 1.8).

1.4.1 The cortical web

Although the spectrin-based membrane skeleton has largely been studied in erythrocytes, some protein components of this structure have been identified in endothelial cells. In erythrocytes, the membrane skeleton forms a two-dimensional polygonal network, which undercoats the plasma membrane (Byers and Branton, 1985). Using electron microscopy, the membrane skeleton appears as a lattice-like organisation of 5-6 rod shaped molecules 200 nm long linked to 'vertices', forming an overall sheet of five or six polygons. The legs of the polygon are formed of spectrin, and the vertices consist of actin filaments of 13-15 actin monomers. Spectrin is a tetrameric actin-binding and cross-linking protein that contains two α and two β subunits. Many tetramers can bind to a single actin filament to create a branched network (Thomas et al., 2001). Spectrin anchors the membrane skeleton to the plasma membrane through direct association with membrane-spanning proteins, PH domain (β-subunits)-mediated interaction with phospholipids, and through interactions with ankyrins and protein 4.1. Ankyrins can bind to the cytoplasmic portion of several integral membrane proteins and ion channels and protein 4.1 stabilises the cell membrane by promoting a ternary complex of spectrin, protein 4.1 and F-actin. In endothelial cells, the cortical web is a 50-100 nm thick layer that extends underneath the apical and basal endothelial plasma membrane and contains non-erythroidal spectrin (fodrin, also known as spectrin II) and spectrin-associated proteins (Sun et al., 2003; Dreckhahn and Ness, 1997). Actin filaments of the cortical web are associated with caveolae (Fujimoto et al., 1995). The endothelial cortical web controls scaffolding and remodelling of the endothelial plasma membrane, generating stiffness in response to shear stress, and inducing membrane ruffling and microvillus...
formation. It also immobilises some integral membrane proteins via ankyrin, spectrin and protein 4.1, within the plasma membrane (Heltianu et al., 1986, Leto et al., 1986). Membrane proteins that are non-detergent extractable are thought to be associated with the membrane-cytoskeleton in this way.

1.4.2 Junction-associated actin filaments (JAF)

The junction-associated actin filaments can be considered as specialized portions of the cortical web, which are strategically positioned adjacent to the interendothelial junctions and associate with the plasma membrane by binding to complexes of junctional proteins (Fig. 1.8). The JAF is involved in controlling the barrier properties of the endothelium, as depolymerisation of F-actin with cytochalasins or botulimun C2-toxin breaks down the permeability barrier, whereas stabilisation with phalloidin enhances the endothelial barrier (Dreckhahn and Ness, 1997).

1.4.3 Actin Stress Fibres

Stress fibres are contractile, myofibril-like straight filament bundles composed of actin filaments interspersed with myosin filaments and also contain many of the proteins found in smooth muscle filaments (Ridley et al., 1999; Chrzanowska-Wodnicka et al., 1996). They are located mainly along the basal endothelial cell surface or, in elongated cells, can align along the cellular long axis both below and above the nucleus (Drenckhahn and Ness, 1997). Stress fibres terminate at β1-integrin-containing focal contacts. Actin stress fibres are mostly observed in vivo at regions that are exposed to high levels of shear stress (Drenckhahn and Ness, 1997). This has subsequently been seen in vitro, where there is a correlation between the induction of actin stress fibres and arterial levels of fluid shear stress (Wojciak-Stothard et al., 2003). High levels of fluid shear stress induce cellular elongation, increase in β-actin mRNA and the induction of stress fibres that align with the direction of flow. Stress fibres assist endothelial cells in adapting to mechanical forces exerted by blood flow and disassembly of stress fibres has been shown to reduce shear stress resistance of endothelial cells (Drenckhahn and Ness, 1997).
Figure 1.8
The Actin cytoskeleton in endothelial cells
Schematic diagram to show the cortical web, JAF and stress fibres present in endothelial cells. Adapted from Drenckhahn and Ness, 1997.
1.5 Rho GTPases

The appearance of actin stress fibres is linked with an increase in cell tension and intercellular gap formation, which results in changes to paracellular permeability. The Rho (Ras homologous) family of small GTPases are key regulators of the actin polymerisation and organisation and regulate endothelial permeability by actin rearrangement and contractility (Wojciak-Stothard and Ridley, 2003). Rho is the founding member of the Rho-family GTPases; a subgroup of the Ras superfamily (Kaibuchi et al., 1999). There are currently at least twenty human Rho GTPase family members: Rho (A, B, C), Rac (1, 2, 3/1B), Cdc42 (G25K/Cdc42, TC10, TCL, Chp1,2 (Wrch1/2)), RhoG, Rnd (Rnd 1and 2, RhoE/Rnd3), RhoBTB (RhoBTB 1 and 2), RhoD, Rif and TTF/RhoH, each sharing about 50-55% amino-acid identity (Hall and Nobes, 2000). Furthermore, splice variants are known for Rac1 (Jordan et al., 1999). The most well-characterised of these members are RhoA, Rac1 and Cdc42. Like other Ras-related proteins, Rho GTPases contain a CAAX box within their C-terminus (where C is a cysteine, X is usually a methionine or serine, but for some Rho-proteins is a leucine and A is any aliphatic residue). This cysteine residue undergoes geranylgeranylation (C-20 isoprenoid) or farnesylation (C-15) by geranylgeranyl or farnesyl transferases, followed by cleavage of the AAX residues and carboxyl methylation of the remaining cysteine (Zerial and Huber, 1995). This isoprenoid modification is essential for the correct localisation of the active Rho protein to the plasma membrane, and allows Rho GTPases to cycle on and off the plasma membrane according to their activity (Bokoch et al., 1994; Lian et al., 2000).

1.5.1 RhoA, B and C

There are three known isoforms of Rho; RhoA, RhoB and RhoC and they share 92% amino acid identity (RhoA and RhoC) and 85% identity (RhoA/C and and RhoB), with most diversity at their C-termini (Ridley, 1997). RhoA, B and C are widely expressed and RhoA, RhoC largely localise to the cytosol, but translocate to the plasma membranes upon activation, whereas RhoB is localised to endosomes and lysosomes as well as the plasma membrane (Zerial and Huber, 1995; Ridley, 1997). This RhoB localisation
Chapter 1

Introduction

Fig 1.9
The regulation of Rho GTPase activity

Rho GTPases cycle between active, GTP-bound forms and inactive GDP-bound forms. This is controlled by the regulatory proteins depicted. GEF = Guanine nucleotide exchange factor; GAP = GTPase activating protein; GDI = GDP dissociation inhibitor protein; R = receptor. Dotted arrow indicates indirect activation of GEF or inhibition of GAP.
depends upon the addition of a geranylglyceranyl group as well as a 15-carbon chain farnesyl group, unlike RhoA and RhoC, which only contain the geranylglyceranyl modification (Ridley, 1997; Adamson et al 1992).

1.5.2 Regulation of Rho GTPases

The Rho GTPases act as molecular switches that cycle between the active GTP-bound form and the inactive GDP-bound form (Fig. 1.9), with the exception of Rnd proteins (Rnd1, 2, RhoE/Rnd3) and probably RhoH, which are constitutively GTP-bound (Chardin et al., 1999). The Rho GTPases are maintained in their inactive state by GDP dissociation inhibitor proteins (GDI). These proteins bind preferentially to GDP-bound Rho-proteins and prevent the dissociation of GDP, and also inhibit GDP-bound Rho proteins from translocation and binding to cell membranes (Bokoch et al., 1994). They have also been shown to bind to GTP-Rho and Rac (Sasaki et al., 1993). This may be as a result of a pocket formed between two β-sheets within the immunoglobulin fold of GDI, which is thought to accommodate the C-terminal isoprenyl group of the Rho-protein (Keep et al., 1997; Lian et al., 2000; Scheffzek et al., 2000), thereby sequestering the Rho-protein in the cytosol. A total of three GDI isoforms are now known: Rho GDI-1, Rho GDI-2 (or D4-GDI) and RhoGDI-3 (Olofsson, 1999). RhoGDI-1 is active upon Rho, as well as Cdc42 and Rac (Bokoch et al., 1994; Keep et al., 1997).

GEFs catalyse the exchange of GDP for GTP on Rho proteins, thereby activating them, which requires dissociation of the GDI (Robbe et al., 2003). Dissociation of GDI may be regulated by PKC-induced phosphorylation (Mehta et al., 2001) or local integrin-mediated adhesion (Del Pozo et al., 2002). Activated Rac is able to translocate to the plasma membrane of adherent cells, but this is not the case for non-adherent cells, indicating that cell adhesion regulates the targeting of Rac to the membrane. This is important for localising Rac to sites where it can associate with its effectors (Del Pozo et al., 2000). There are currently two known families of GEFs. The first family share a common Dbl-homology (DH) domain; a region of sequence homology to the Dbl proto-oncogene, which is essential for GEF activity. A PH domain is found adjacent to this DH domain in many of these GEFs and is known to bind to phosphorylated phosphoinositides.
and other proteins (Schmidt and Hall, 2002). Not only is the PH domain important for GEF intracellular localisation, but can also affect the DH domain catalytic activity and has been reported to participate in GTPase binding (Schmidt and Hall, 2002). There are currently 60 known human GEFs of this family (Schmidt and Hall, 2002). The second family of GEFs are called DOCKs. These lack the PH and DH domains found in GEFs, and instead contain a DHR-2 (DOCK Homology Region-2) domain. In DOCK-1 (DOCK180) this region interacts with nucleotide-free Rac in vitro and induces the GTP loading of Rac both in vitro and in vivo (Côté et al., 2002). Eleven human DOCK proteins have been identified so far and they have been shown to target Rac and/or Cdc42 for nucleotide exchange (Côté et al., 2002).

Inactivation of GTP-bound Rho proteins is achieved through the action of GTPase activating proteins (GAPs). These molecules contain a GAP homology domain and they stimulate the intrinsic GTPase activity of Rho proteins, resulting in Rho-GDP-bound status (Kaibuchi et al., 1999).

1.5.3 Rho function and targets
Rho was originally identified as a regulator of the actin cytoskeleton, specifically in the formation of stress fibres and focal adhesions (Ridley et al., 1992). In Swiss 3T3 fibroblasts, activation of RhoA with lysophosphatidic acid (LPA) or microinjection of RhoA results in a strong induction of actin stress fibres which can be inhibited by the Rho-specific toxin, Clostridium botulinum C3 transferase (Ridley and Hall, 1992; Ridley et al., 1992). C3 transferase specifically ADP-ribosylates and inhibits RhoA, B and C (Aktories and Hall, 1989; Balsh, Channing and Hall, 1995).

Several Rho-targets have been identified (Fig. 1.10), many of which mediate Rho-induced cytoskeletal reorganisation. For example, Rho associated-kinase (ROCK) is a Ser/Thr protein kinase that mediates Rho-induced stress fibre contraction (Riento and Ridley, 2003). Similarly, citron-K is a Ser/Thr kinase downstream of Rho that regulates contractility during cytokinesis. Dial is a further target of Rho, which binds to the actin-
Fig 1.10

Rho target proteins

Rho targets many effector proteins, some of which are involved in cytoskeletal rearrangements.
binding protein, profilin and promotes actin polymerisation, and phosphatidylinositol 4-phosphate 5-kinase (PIP 5-K) is activated by Rho and generates the phospholipid, phosphatidylinositol (4,5) bisphosphate (PIP$_2$), which interacts with actin-capping proteins, and induces actin polymerisation. These and other Rho targets regulate many biological functions (Ridley, 1996; Hall, 1998), including neurite retraction (Jalink et al., 1994), smooth muscle contraction (Hirata et al., 1992), cell motility (Takaishi et al., 1993), cell adhesion (Ridley et al., 1992), cytokinesis (Madaule et al., 2000) and cellular contraction (Jalink et al., 1994; Ridley and Hall, 1992), serum response factor activation (Hill et al., 1995), mast cell secretion (Norman et al., 1996), apoptosis (Jimenez et al., 1995) and vesicle trafficking (Ridley, 2001).

1.5.4 Rho function in endothelial cells
Rho activity has been shown to contribute to endothelial barrier dysfunction. Constitutively active RhoA induces the formation of actin stress fibres, gaps and reorganisation of VE-cadherin (Wojciak-Stothard et al., 1998), although another report failed to observe redistribution of VE-cadherin or a loss of cell adhesion in HUVECs (van Wetereing et al., 2002). Dominant negative RhoA inhibits thrombin and histamine-induced decrease in the number of tight junctions in HUVECs, decreases peripheral stress fibres but does not significantly alter VE-cadherin, β-catenin or ZO-1 distribution or basal permeability (Wojciak-Stothard et al., 2001), indicating a role for Rho in modulation of HUVEC morphology and cytoskeleton, but less so for regulation of junctional proteins. However, long-term inactivation of Rho leads to a loss of intercellular junctions and increased permeability (Hordijk et al., 1999). Others have shown that PKA, which prevents increases in permeability induced by inflammatory mediators, does so through inhibition of Rho (Qiao et al., 2003). Rho is able to regulate permeability through a number of mechanisms, most of which depend upon Rho-dependent cytoskeletal reorganisation. These include actin polymerisation, bundling of actin filaments into stress fibres and anchoring them at focal adhesions and by stimulating stress fibre contractility.
1.6 Rho regulates contractility

Rho-induced stress fibres are contractile. This has been shown in fibroblasts plated on a flexible rubber substrate, which wrinkle the substratum in response to Rho activation (Chrzanowska-Wodnicka and Burridge, 1996). Furthermore, Rho has been shown to induce contraction of smooth muscle cells (Hirata et al., 1992; Uehata et al., 1997). Rho regulates contractility mainly through its downstream target, ROCK. ROCK targets the regulatory light chain of myosin II (MLC) for phosphorylation and this leads to actin-myosin interaction and contraction. This is an important mechanism for Rho-dependent increased permeability in endothelial cells and has been extensively studied using thrombin. Thrombin has been shown to induce permeability by activating phosphorylation of MLC, both by Rho-dependent and independent mechanisms. This leads to cellular contraction, the appearance of tiny gaps between neighbouring endothelial cells and increased paracellular permeability (Bogatcheva et al., 2002).

1.6.1 Myosins

Myosins are protein molecular motors that bind to F-actin in an ATP-dependent way and are involved in muscle cell and non-muscle cell contraction (Berg, 2001; Ruegg et al., 2002). There are at least 12 different classes of myosins in humans, generated from 40 myosin genes (Berg, 2001). The most well-characterised of these are the conventional myosins (Class II). Class II myosins are expressed ubiquitously, with non-muscle myosin II sharing several biological properties with smooth muscle myosin II. Three different non-muscle myosin II isoforms have been described, which are the products of three separate genes and differ in their heavy chain polypeptides (Simons et al., 1991; Colomb et al., 2004). These isoforms are therefore called nonmuscle myosin heavy chain (NMHC) II-A, II-B and II-C (also called myosin II-A –II-B and II-C). Furthermore, there are four alternatively spliced forms of NMHC II-B and two splice variants of NMHC II-C (Takahashi et al., 1992; Itoh et al., 1995; Phillips et al., 1995; Golomb et al., 2004).

Class II Myosins consists of a pair of heavy polypeptide chains, a pair of essential light chains (ELC) and a pair of regulatory light chains (RLC or MLC) (Ruegg et al., 2002).
The heavy chain has a globular head domain containing actin and ATP-binding sites, an intermediate region that forms a coiled coil (or rod) and is involved in heavy chain dimerisation, and a non-helical carboxy terminal region. One of each type of light chain associates with the globular N-terminal head. The α-helical region forms a hydrophobic strip promoting the association of another such helix, which results in a left handed parallel coiled-coil. Myosin is able to ‘row’ thin actin filaments past thick myosin filaments, whilst hydrolysing a single ATP molecule with each stroke (Ruegg et al., 2002; Voet and Voet, 1995). The extended α-helix of myosin II forms a neck or regulatory domain, which acts as a lever arm and is important for the production of mechanical force and the working stroke (Fig. 1.11).

Many stimuli including thrombin induce phosphorylation of the MLC, which exposes the ATP binding site of MLC and induces actomyosin interaction and contractility (Morel et al., 1990; Sheldon et al., 1993). Thrombin induces MLC phosphorylation by activating both non-muscle myosin light chain kinase (MLCK, also known as Ca²⁺/CaM-dependent protein kinase I) (Garcia et al., 1997) as well as the Rho effector, ROCK.

1.6.2 Rho-associated Ser/Thr kinases (ROCK)

Rho regulates phosphorylation of myosin II and permeability through its effector, ROCK. Inhibition of Rho with C3 transferase results in reduced MLC phosphorylation (van Nieuw Amerongen et al., 1998; Essler et al., 1998; Garcia et al., 1999) and reduced endothelial permeability (van Nieuw Amerongen et al., 1998; Essler et al., 1998). ROCKs are Ser/Thr kinases of which there are two known isoforms: ROCK I (also known as ROKβ or p160ROCK) and ROCK II (also known as ROKα or Rho-kinase). ROCKs I and II share 65% overall sequence identity, with 93% identity within their kinase domains (Nakagawa et al., 1996; Leung et al., 1996). Expression of ROCK II in HeLa cells induces the formation of focal adhesions and stress fibre-like actin bundles, both of which require ROCK kinase domain (Leung et al., 1996). Similar results are observed with overexpressed ROCK I (Ishizaki et al., 1997). Furthermore, ROCK inhibition using Y-27632, or by using a Rho-binding-defective mutant of ROCK I, blocks Rho-induced focal adhesion and stress fibres (Uehata et al., 1997; Ishizaki et al.,
Figure 1.11
Schematic representation of the actomyosin cross-bridge cycle
Y-26732 is a ROCK inhibitor that has been used to inhibit smooth muscle contraction and is frequently used to study ROCK functions. Y-27632 is a relatively specific inhibitor of ROCK I and II, although it has also been shown to inhibit PRK2 at similar concentrations to those used for ROCK II inhibition (Uehata et al., 1997; Davies et al., 2000). These results demonstrate that ROCK functions downstream of Rho in these biological responses. This is thought to be due primarily to the action of ROCKs on MLC and myosin light chain phosphatase (MLCP). ROCK II phosphorylates MLC on Ser^{19} residue (Amano et al., 1996), which contributes to stress fibre assembly, tension formation and contractility (Katoh et al., 2001). MLC phosphorylation is negatively regulated by MLCP. This protein consists of three subunits: a myosin-binding subunit (MBS), a catalytic subunit containing a protein phosphatase type 1c (PP1c) and a subunit of unknown function. MLCP targets MLC for dephosphorylation, thereby inhibiting contractility. ROCK II can phosphorylate MBS at Thr^{679}, Ser^{854} and Thr^{855}, which results in dissociation of MBS from myosin and MLCP inactivation (Kimura et al., 1996; Feng et al., 1999; Velasco et al., 2002) and an increase in MLC phosphorylation and contractility. Rho is also able to bind to MBS although this does not affect MLCP activity, and the relevance of this interaction is unknown, but it could be to co-localize Rho with ROCK in order to activate it (Kimura et al., 1996) (Fig. 1.12).

ROCK is downstream of Rho in thrombin-induced permeability. Inactivation of ROCK using Y-27632 results in loss of thrombin-induced stress fibres and partially inhibits the thrombin-induced increase in permeability, although ROCK inhibition alone is not sufficient to block intercellular gap formation (Carbajal et al., 2000; Wojciak-Stothard et al., 2001). This is due to thrombin-induced activation of MLCK, which also phosphorylates MLC (Fig 1.12). Y-27632 together with MLCK inhibition using the calcium chelator, BAPTA, have an additive effect on inhibition of thrombin-induced permeability (van Nieuw Amerongen et al., 2000).
Figure 1.12

Model for phosphorylation of MLC by ROCK and MLCK

ROCK is activated by RhoA to phosphorylate MLC on Serine 19, whilst MLCK is activated by Calcium/Calmodulin (Ca/Cam) to phosphorylate MLC on Threonine 18 and Serine 19 residues.
1.6.3 Myosin light chain kinase

Myosin light chain kinase (MLCK) is a second important regulatory protein for myosin II. MLC can be phosphorylated on Thr\(^{18}\) and Ser\(^{19}\) residues by MLCK (Seto et al., 1996), resulting in actomyosin interaction and contraction. Indeed, mimicking MLC phosphorylation by substitution of Thr\(^{18}\) and Ser\(^{19}\) with negatively charged Asp residues results in activation of myosin ATPase and a conformational change of myosin II in vitro (Kamisoyama et al., 1994; Bresnick et al., 1995). HUVECs have been shown to express non-muscle MLCK, but not the related SM MLCK form (Verin et al., 1998). Four further splice variants have been identified for non-muscle MLCK resulting in a total of 5 non-muscle MLCK isoforms: MLCK 1, MLCK 2, MLCK 3a, MLCK 3b and MLCK 4. Each of these has been detected in HUVECs (Lazar and Garcia 1999).

1.6.4 Regulation of MLCK by calcium

Thrombin activation of MLCK is regulated by calcium/calmodulin (CaM) (Fig. 1.12). Thrombin triggers the release of calcium from intracellular stores by activating G protein-coupled proteinase receptor 1 (PAR1) (Bogatcheva et al., 2002) and inducing inositol 1,4,5-trisphosphate (IP\(_3\)) generation. IP\(_3\) binds to its receptor (IP\(_3\)R) on the endoplasmic reticulum (ER) and signals calcium release from ER stores. This store depletion also triggers an influx of extracellular calcium through store-operated channels (SOC) (Birnbauer et al., 2000). In the absence of Ca\(^{2+}\), the MLCK autoinhibitory sequence interacts with MLCK active site, inhibiting activation and substrate binding. Upon elevation of intracellular Ca\(^{2+}\), 4Ca\(^{2+}\)-CaM binds to MLCK and CaM forms a collapsed conformation around the CaM-binding domain of MLCK resulting in the release of MLCK autoinhibitory mechanism (Heller et al., 2003).

RhoA has recently been reported to form a complex with IP\(_3\)R and SOC in endothelial cells (Mehta et al., 2003). C3 transferase blocks IP\(_3\)R and SOC interaction and translocation to the plasma membrane, and the thrombin-induced sustained increase in cytosolic calcium is Rho-dependent (Mehta et al., 2003). Furthermore, inhibition of actin polymerisation inhibits the association between IP\(_3\)R and SOC. These data indicate that
sustained extracellular calcium influx in response to thrombin is dependent upon Rho and actin polymerisation.

1.6.5 Phosphorylation of MLCK

LPA also increases permeability through MLCK activation, as inhibition of MLCK reduces basal and LPA-stimulated permeability (van Nieuw Amerongen et al., 2000). However, LPA is unable to increase cytosolic Ca$^{2+}$. This suggests that other mechanisms for MLCK regulation apart from Ca$^{2+}$/CaM-binding are occurring. Non-muscle MLCK contains several consensus sites for protein kinases that might regulate its activity. Moreover, the tyrosine phosphatase inhibitor, vanadate, increases MLC phosphorylation and endothelial permeability, which correlates with an increase in MLCK phosphotyrosine content. This indicates tyrosine phosphorylation might activate MLCK (Gilbert-McClain, 1998). Furthermore, the tyrosine kinase activator and tyrosine phosphatase inhibitor, diperoxovanadate (DVP, vanadate) have been shown to induce MLCK tyrosine phosphorylation, which also correlates with MLCK activation and MLC phosphorylation (Garcia et al., 1999). This phosphorylation might be due to Src, as tyrosine kinase activation results in increased association between cortactin and MLCK, and cortactin and Src, thus it is possible that a complex of these proteins forms, bringing Src into close proximity to MLCK. Indeed endothelial MLCK contains a Src kinase consensus site (Garcia et al., 1999).

MLCK has also been reported as being a target for Ser/Thr phosphorylation. A PKA phosphorylation consensus sequence is present, adjacent to the CaM-binding domain of MLCK. Increasing intracellular cAMP levels results in enhanced MLCK phosphorylation, but unlike tyrosine phosphorylation, reduces MLCK activity (Garcia et al., 1997; Verin et al., 1998). MLCK also contains multiple MAPK consensus phosphorylation sites (Klemke et al., 1997) and has been shown to be a target for ERK1/2. These MAPKs are capable of phosphorylating MLCK downstream of the epidermal growth factor (EGF) receptor as well as integrin receptors, to increase cell motility. MLCK can be directly phosphorylated by ERK1/2, which results in increased MLCK activity, a decreased requirement for CaM, increased MLC phosphorylation and
increased cell motility (Klemke et al., 1997). Rho regulation of MLCK has been hypothesised (Garcia et al., 1999), but there is no evidence to date for Rho-dependent MLCK phosphorylation.

1.6.6 Other methods for regulation of myosin II

Rac and Cdc42 also regulate MLC phosphorylation. Their downstream effector, PAK, has been shown to phosphorylate MLCK directly and inactivate it in HeLa cells (Chew et al., 1998; Sanders et al., 1999; Goeckeler et al., 2000), but in endothelial cells, PAK can phosphorylate MLC on Ser\(^1\) and activate it (Chew et al., 1998; Kiosses et al., 1999).

Rac can also induce myosin heavy chain (MHC) threonine phosphorylation in neuroblastoma cells (Van Leeuwen et al., 1999). In contrast to cell rounding and contraction observed by Rho-induced MLC phosphorylation, Rac-induced MHC phosphorylation results in cell spreading (Leeuwen et al., 1999). Furthermore, phosphorylation of rabbit MHC prevents filament formation in vitro (Murakami et al., 1998) and MHC phosphorylation in Dictyostelium inhibits assembly or activity of myosin II (Brzeska et al., 1996). These data suggest that MHC phosphorylation results in stress fibre disassembly, and that Rac may serve to reduce tension, disassemble actin stress fibres and rearrange stress fibres into branched filaments in lamellipodia.

PKC also targets MLC for phosphorylation on Ser\(^1\)/Ser\(^2\) and Thr\(^9\) residues which inhibits the ATPase activity of myosin II and decreases the affinity of MLC for MLCK, thus preventing phosphorylation of myosin by MLCK (Bresnick, 1999). Moreover, PKC and casein kinase II (CKII) can phosphorylate the heavy chains of myosin–IIA and myosin–IIB. Heavy chain phosphorylation of myosin-IIIB by CKII or PKC inhibits its assembly into filaments, but has no such effect on myosin-IIA (Bresnick, 1999).

1.6.7 Citron

Citron is a target of activated RhoA and RhoC, which can also phosphorylate MLC (Yamashiro et al., 2003). The citron gene encodes for three splicing isoforms, Citron-N, Citron-K and Citron-short (Madaule et al., 2000). Citron shares some homology with ROCK, including 43% homology within the kinase domain, however citron contains an
SH3 and PDZ-binding domain in the C-terminus, which is lacking in ROCK. Citron-K is the longer variant, containing an N-terminal Ser/Thr kinase domain, whereas citron-N lacks this kinase domain and citron-short contains only the kinase domain. Citron-K is ubiquitously expressed and localises to the cleavage furrow of dividing cells (Maduale et al., 2000) where it is a target for Rho in regulating cytokinesis. Cells expressing truncation mutants of citron-K exhibit multinucleation, indicating a defect in completing cytokinesis. As ROCK and citron-K have similar structures, it is possible that they have similar targets and functions. Like ROCK, citron-K has been shown to phosphorylate and regulate MLC. Citron-K phosphorylates MLC on Thr\(^{18}\) and Ser\(^{19}\) \textit{in vitro}, and overexpression of the kinase domain induces this di-phosphorylation \textit{in vivo}.

Overexpression of this domain also restores stress fibre formation in the presence of the ROCK inhibitor, Y-27632 (Yamashiro et al., 2003). This indicates that citron-K might also be involved in stress fibre formation and correct fibre bundling. Whether citron-K contributes to increased paracellular permeability by inducing contraction of junctionally associated cortical actin has not been investigated. Whether citron-K localises to the tight junction in endothelial cell monolayers remains to be seen.

1.7 Actin polymerisation

Actin monomers (G-actin) are single polypeptides, which fold into bilobal, globular molecules consisting of a total of four subdomains, of which the majority is \(\alpha\)-helical in structure. A cleft deep between the two main lobes in the centre of actin forms a binding site for ATP or ADP-Pi. ATP binds here as a complex with \(\text{Mg}^{2+}\) (Dos Remedios, 2003). There are 6 known isoforms of actin, four of which are specific to muscle, whilst the remaining two are found in all cells. There are three \(\alpha\)-actins (\(\alpha\)-skeletal, \(\alpha\)-cardiac, \(\alpha\)-smooth muscle) one \(\beta\)-actin (\(\beta\)-nonmuscle) and two \(\gamma\)-actins (\(\gamma\)-smooth muscle, and \(\gamma\)-nonmuscle). The actin isoforms share 90% overall sequence homology (Herman et al., 1993; Kreis and Vale, 1993). G-actin polymerises to form filamentous actin (F-actin). The monomers within F-actin are in the same orientation creating a directional, polar filament. Each monomer is able to bind a single myosin S1 head, which gives an arrowhead-like appearance by electron microscopy. From this view, the filament ends were designated ‘barbed’ and ‘pointed’. The barbed end of F-actin exhibits rapid
polymerisation, whereas the pointed end is slow-growing (Voet and Voet, 1995). Actin polymerisation is known to be important for many biological processes including cell motility, cell adhesion, and is a dynamic process tightly controlled by many regulatory and actin-associated proteins. These include monomer-binding proteins, filament capping proteins, proteins that sever F-actin and those that nucleate new actin filaments (Dos remedios, 2003).

There are two main mechanisms for stimulating actin polymerisation: through the Arp2/3 complex, and through the formin family of proteins. The dendritic nucleation model (Pollard et al., 2001) shows how the Arp2/3 complex and other proteins co-operate to induce actin polymerisation and branching at the leading edge (Fig. 1.13). This is regulated by Rac and Cdc42.

1.7.1 Cdc42 and Rac and actin polymerisation

WASP (Wiscott Aldrich Syndrome protein) is the founding member of a family of proteins that regulate the Arp2/3 complex (Pollard et al., 2000; Millard et al., 2004). Currently there are 5 known mammalian WASP-related proteins; WASP, N-WASP (which was originally identified in brain but is widely expressed) and three isoforms of Scar/WAVE (Millard et al., 2004). The Arp2/3 complex is composed of seven subunits: actin-related proteins 2 and 3 (Arp2 and Arp3), p40 (ARPC1), p35 (ARPC2), p19 (ARPC3), p18 (ARPC4) and p14 (ARPC5). This protein complex is responsible for 'nucleation', or the branching of new actin filaments from existing polymers at a 70° angle, whilst Arp2/3 itself is incorporated into the new branch, as such it caps the pointed ends of actin filaments (Pollard et al., 2000) (Fig.1.13). WASP family proteins activate the Arp2/3 to nucleate new actin filaments. WASP and N-WASP have a GTPase binding domain (GBD), which is a consensus site for binding to Cdc42. Scar/WAVE lacks this domain, but nevertheless is an effector for Rac. WASP proteins adopt an autoinhibited conformation, in which their C-terminal Arp2/3-interacting domain is masked by binding to the GBD. This inhibition is relieved by binding either to Cdc42 or to SH3-containing proteins Nck or Grb2. For N-WASP, Cdc42 binds to the GBD region, and Grb or Nck bind to the polyproline region. PIP₂ acts synergistically with these activators and
Fig 1.13
The dendritic nucleation model for actin polymerisation
Activation of WASP family proteins activates Arp2/3 to nucleate new actin filaments, which branch at a 70° angle from existing filaments. Barbed end polymerisation is halted by the binding of capping proteins. ATP hydrolysis within the actin filaments triggers severing and depolymerisation by ADF/cofilin. Profilin binds to G-actin and catalyses the exchange of ADP for ATP. Profilin-bound G-actin is then ready for re-incorporation into new filament barbed ends. Adapted from Pollard et al., 2000.
associates with N-WASP’s WH1 domain (Yin and Janmey, 2003) to activate N-WASP and induce activation of the Arp2/3 complex and filament branching. WASP and Scar/WAVE are therefore key modulators of actin polymerisation downstream of Cdc42 and Rac.

There is an alternative mechanism for the regulation of actin polymerisation by Cdc42 and Rac. The Cdc42 and Rac target, p21-activated kinases (PAKs), as well as the Rho target, ROCK, can phosphorylate and activate LIM kinases. LIM kinases 1 and 2 (LIMK1 and 2) are Ser/Thr kinases that phosphorylate and inactivate the actin severing and depolymerising protein, ADF/cofilin. ADF/cofilin phosphorylation results in a fall in ADF/cofilin affinity for actin (Edwards et al., 1999).

1.7.2 Rac and Cdc42 functions in endothelial cells
There is some evidence that Rac is important for maintenance of the endothelial barrier function. Thrombin has been shown to activate RhoA and inhibit Rac1 resulting in cell rounding and contraction (Vouret-Craviari, 2002; Mehta et al., 2003), whereas sphingosine-1 phosphate, a phospholipid released by blood platelets that increases barrier function, activates Rac1 and only weakly activates RhoA (Vouret-Craviari et al., 2002). Similarly, HGF/SF activates Rac and reduces permeability (Liu et al., 2002; Wojciak-Stothard and Ridley, 2003). Inhibition of Rho, Rac and Cdc42 using Clostridium difficile toxin B (toxin B) disrupts barrier function (Hippenstiel et al., 1997; Adamson 2002).

Toxin B catalyses the monoglucosylation of Rho GTPases, including Rho, Rac and Cdc42, but it does not target other small GTPases such as Ras (Hall, 2000). This disruption of barrier function by toxin B is unlikely to be due to an effect on Rho, as C3 transferase inhibition of RhoA enhances endothelial barrier function in bovine pulmonary artery endothelial cells (Carbajal et al., 1999). This is in contrast with epithelial cells, where C3 transferase has been reported to increase permeability (Nusrat et al., 1995). Rac may be responsible for the effect of Clostridium difficile toxin B. Inhibition of Rac using Clostridium sordellii lethal toxin (LT), which glucosylates and inactivates Rac (Popoff et al., 1996), results in increased permeability of rat microvessels in vivo (Waschke et al., 2004). Furthermore, this study showed that Rac inhibition with this
toxin induces F-actin depolymerisation, disjointed junctional localisation of VE-cadherin and β-catenin and gap formation in cell cultures. However, this toxin also targets cdc42, Ras, Rap and Ral as well as Rac (Hall, 2000; Popoff et al., 1996) and so the effects observed cannot be solely attributed to Rac inhibition. A novel adhesion assay showed that Rac inhibition reduced VE-cadherin-mediated adhesion to VE-cadherin-coated microbeads both through disassembly of actin filaments, and by acting on the junction, as LT reduced adhesion even in the presence of an actin-stabilising compound. On the other hand, Rac activation has been shown to induce tyrosine phosphorylation of α-catenin and decrease VE-cadherin-mediated adhesion, the latter of which depends upon ROS (reactive oxygen species) production (van Wetering et al., 2002).

Others have reported that both dominant active and dominant negative Rac increase HUVEC permeability (Wojciak-Stothard et al., 2001; van Wetering et al., 2002). Activated Rac induces the accumulation of cortical F-actin, the formation of stress fibres, intercellular gaps, and VE-cadherin and β-catenin redistribution. ZO-1 is also fragmented and the number of tight junctions decreases (Wojciak-Stothard et al., 1998 and 2001). Actin stress fibre formation in response to Rac activation is not observed in most other cell types apart from endothelial cells. For instance, constitutively active Rac induces a loss of stress fibres in HeLa cells (Manser et al., 1997), and it inhibits Rho and Rho-induced stress fibres in NIH3T3 fibroblasts (Sander et al., 1999; Zondag et al., 2000; Burridge and Wennerberg, 2004). This difference may be attributed to the action of the Rac and Cdc42 effector, PAK, as in endothelial cells it can phosphorylate MLC, whereas in other cell types PAK has an inhibitory effect upon MLC phosphorylation (Chew et al., 1998; Kioosses et al., 1999; Sanders et al., 1999; Goeckeler et al., 2000).

Dominant negative Rac decreases peripheral stress fibres in unstimulated HUVECs (Wojciak-Stothard et al., 1998 and 2001; Lampugnani et al., 2002) and induces a meshwork-like pattern of VE-cadherin and β-catenin at cell-cell junctions. ZO-1 staining is reduced and fragmented at the tight junction, intercellular gaps are observed and a reduction in the number of tight junctions occurred (Wojciak-Stothard et al., 1998 and 2001). Precise regulation of Rac might thus be required for control of barrier function.
and cell adhesion. Another report showed that constitutively active Rac resulted in accumulation of cortical F-actin and cell rounding of a human vascular endothelial cell line (Vouret-Craviari et al., 1998), but also augmented thrombin-induced cell-rounding. In this study, dominant negative Rac inhibited thrombin-induced cell rounding and retraction, whereas constitutively active or dominant negative Cdc42 had no effect on resting or thrombin-stimulated cell morphology or stress fibres in HUVECs.

In another study, overexpression of constitutively active Cdc42 in resting HUVECs increased cortical F-actin, induced stress fibres and intercellular gaps and dispersed VE-cadherin from the junctions (Wojciak-Stothard et al., 1998). Dominant negative Cdc42 reduced peripheral stress fibres, but did not affect VE-cadherin, β-catenin or ZO-1 distribution, nor did it induce intercellular gaps or affect basal permeability. Dominant negative Cdc42 does not affect thrombin or histamine-induced permeability and thrombin does not rapidly activate Cdc42 (Vouret-Craviari et al., 2002). However, dominant negative Cdc42 did inhibit TNF-α-induced formation of filopodia, lamellipodia, stress fibres and cell contraction, although dispersion of VE-cadherin is not totally inhibited (Wojciak-Stothard et al., 1998 and 2001). It also attenuated thrombin-induced actomyosin contractility and stress fibres (Wojciak-Stothard et al., 2001). This indicates that Cdc42 is capable of regulating cytoskeletal changes and cell morphology, but there is no evidence that it affects tight junctions or permeability. Cdc42 may play a role in maintaining cell-cell adhesion by associating with the Rac/Cdc42 effector protein, IQGAP1. Active Cdc42 has been shown to inhibit the binding of IQGAP1 to the VE-cadherin and β-catenin complex. IQGAP1 association with this complex induces α-catenin dissociation and a loss of cell-cell adhesion (Kuroda et al., 1998). As IQGAP1 is also a Rac effector protein, so it is possible that Rac also regulates cadherin-mediated cell-cell adhesion in a similar way. Interestingly, Cdc42 has recently been implicated in the restoration of adherens junctions and the re-establishment of barrier function after thrombin-induced permeability (Kouklis et al., 2003). In this study, Cdc42 was activated by thrombin, but at a later time of 1 h and dominant negative Cdc42 delayed the reformation of VE-cadherin-containing adheren junctions and barrier recovery.
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1.7.3 Rho and actin polymerisation

1.7.4 Formins

The formation of branched actin filaments by the Arp2/3 complex has been well characterised, whereas the source of unbranched filaments has, until recently, remained elusive. There is now growing evidence that formins have an important role in actin nucleation and unbranched filament formation independently of Arp2/3 (Zigmond, 2004). Formins are a family of conserved cytoskeletal-organising proteins that are regulated by Rho GTPases (Pruyne et al., 2002) and are involved in cytokinesis, polarised growth and stress fibre formation (Wasserman, 1998).

Formins contain a conserved formin homology domain 2 (FH2), which is necessary and sufficient to nucleate actin in vitro (Pruyne et al., 2002; Sagot et al., 2002; Kovar et al., 2003) (Fig. 1.14A). This region is flanked by an FH1 domain, which is proline-rich and binds to profilin. Profilin is an actin-monomer binding protein, that catalyses the exchange of G-actin-bound ADP for ATP. The FH1 region also binds to SH3 domains and WW domains, which bind to proline-containing ligands (Macias et al., 2002; Zigmond et al., 2004). Some formins also contain an FH3 domain. This is the least conserved of the FH domains, and its function is not yet clearly defined, although it may be responsible for targeting the formin to specific sub-cellular compartments, such as to mitotic spindles (Kato et al., 2001) or it may contribute to the binding of Rho GTPases (Wallar and Alberts, 2003).

The yeast formin Bni1p FH1 and FH2 domains are sufficient to induce the assembly of unbranched actin filaments in vitro (Pruyne et al., 2002). This mechanism is thought to involve stabilisation of actin dimers by dimerised FH2 domains (Fig.1.14B). Profilin acts as a cofactor in this process by binding to the FH1 domain and profilin-actin probably acts as a substrate for formin-induced nucleation. Indeed, the yeast formin, Cdc12, inhibits elongation by free G-actin, but not profilin-actin (Zigmond, 2004).
Domains that are present in diaphanous-related formins (Diaphanous, hDia1/mDia1, hDia2, hDia12C/mDia3, mDia2/hDia3, FigA/SepA) are shown in (A). D is the diaphanous autoregulatory domain region. Adapted from Bradley, 2003. Schematic diagram of actin nucleation by formins is shown in (B). G-actin binds sequentially to dimers of FH2. Adapted from Zigmond, 2004.
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Formins also act as a leaky cap at the barbed end of the actin filament (Pollard, 2004). The FH2 domain of Bni1p preferentially associates with the barbed ends of F-actin (Pruyne et al., 2002). FH2 competes with, but differs from other F-actin capping proteins, as it allows polymerisation and depolymerisation to occur whilst it is still associated to the filament. Bni1p inhibits free G-actin elongation at the barbed end by 50% (Pruyne et al., 2002). However, a filament nucleated by FH2 retains the formin for some time, allowing elongation to occur for longer than a free barbed end, which would be rapidly capped by capping protein or gelsolin. Formins therefore contribute to actin polymerisation through nucleation of new filaments and leaky capping at the barbed end of the filament.

Diaphanous-related formins (DRFs) are activated by Rho GTPases, which bind to the N-terminal GTPase binding domain (GBD) (Fig. 1.14). This interaction relieves an inhibitory interaction formed between the GBD and DAD (Diaphanous autoregulatory domain) (Zigmond, 2004; Alberts et al., 2001; Watanabe et al., 1999). The GBD is, however, not well conserved between the DRFs. mDia2 and its human homologue, hDia3, have been shown to contain a partial Cdc42/Rac interactive binding (CRIB) motif (Peng et al., 2003) commonly found in Cdc42 effectors, however Bni1p, which binds to Cdc42p (a yeast homologue of Cdc42) (Evangelista et al., 1997), lacks this motif. The mechanism of activation of formins lacking a GBD or a CRIB domain is not clear (Wallar and Alberts, 2003).

Formins have been shown to act downstream of Rho in the formation of actin stress fibres. Both ROCKs and formins are required for Rho-induced stress fibres (Nakano et al., 1999). Overexpression of the diaphanous-related formin, mDia1, induces an increase in fine F-actin filaments in COS-7 cells, even in the presence of C3 transferase, indicating that mDia1 is downstream of RhoA signaling (Wantanabe et al., 1997). Others have reported that ROCK and mDia1 act co-operatively downstream of Rho in actin cytoskeletal reorganisation. Either dominant negative ROCK or mDia1 inhibit stress fibres in MDCK cells (Nakano et al., 1999) and overexpression of active mDia in HeLa cells modifies thick ROCK-induced stress fibres into stress fibres that are more
reminiscent of Rho-induced fibres and co-expression of different ratios of ROCK and mDia induce stress fibres of varying thickness and densities (Watanabe et al., 1999).

Formins have also been implicated in regulating cytokinesis. For example, Bni1 and Bnr1 are required for actin ring formation and cytokinesis in budding yeast (Tolliday et al., 2002). The FH protein, CYK-1 of nematode Caenorhabditis elegans is required for cortical microfilament formation and cytokinesis (Severson et al., 2002) and mutations in the genes that encode diaphanous and cappuccino in Drosophila melanogaster result in defective cytokinesis (Tanaka, 2000; Castrillon et al., 1994).

There have been no reports so far that link endothelial permeability and formins. However, formin-1 has recently been shown to interact with α-catenin and localizes to adherens junctions in keratinocytes (Kobiela et al., 2004) and may be involved on junction formation. It is possible that formins also contribute to endothelial cortical actin ring formation, similar to that observed during cytokinesis, or to stress fibre formation across the cell body, both of which have been reported to correlate with increased permeability (Fig. 1.15) (Goldblum et al., 1993; Brett et al., 1989).
Formin-dependent actin polymerisation

Formins are involved in actin fibre formation at cytokinesis and this is shown schematically in (A). Similar formin-dependent contractile-ring formation might occur during increased endothelial permeability (B). Alternatively, formins could be involved in forming stress fibres that traverse the cells and increase permeability (C). Red box indicates endothelial tight junctions; dark circles are cortical F-actin rings; dark lines are stress fibres; arrows indicate loss of barrier function.
1.7.5 Other mechanisms for Rho-induced actin polymerisation

Rho has also been shown to regulate phosphatidylinositol 4-phosphate 5-kinase (PIP5-K). GTP-bound Rho stimulates its activity and GTPγS-stimulated PIP5-kinase activity is blocked by botulinum C3 exoenzyme (Chong et al., 1994). The mechanism through which Rho activates PIP5-kinase is not clear, but it could involve phosphatidic acid, the product of phospholipase D activation. Rho has been proposed to activate phospholipase D (PLD) (Malcolm et al., 1994; Singer, Brown and Stemweis, 1997). PLD hydrolyses phosphatidylcholine to phosphatidic acid (PA) and choline. PA activates PIP5-kinase, and in turn, PIP5-kinase has been shown to activate PLD2 (Powner, 2002).

Alternatively, Rho-induced activation of PIP5-kinase may involve ROCK (Oude Weernink et al, 2000). Rac and Cdc42 have also been shown to interact with PIP5-K in vivo and in vitro (Tolias et al., 1995). The PIP5-K product, PIP2, can regulate several actin-binding proteins to induce actin polymerization and it also signals for the establishment of cytoskeleton-plasma membrane linkages.

PIP2 can prevent some proteins from associating with actin, such as gelsolin and capping protein. These are proteins that bind to the barbed ends of actin filaments and prevent their elongation. Gelsolin caps actin filaments and prevents the exchange of actin subunits at the barbed end. Gelsolin can also sever actin filaments and it remains attached to the barbed end after severing. Capping protein also binds tightly to the barbed end of actin filaments, but not the pointed ends, and prevents elongation. Capping protein can also bind to and stabilise actin monomers for elongation, and therefore act as nuclei for new filament formation. Removal and inhibition of gelsolin by PIP and PIP2 from the barbed ends frees actin filaments for rapid polymerisation (Yin and Janmey, 2003). However, there has been no link so far between Rho and gelsolin or capping protein.

The F-actin severing and depolymerising protein, ADF/cofilin, is also inhibited by PIP2, which promotes actin polymerisation. Furthermore, profilin is stimulated by PIP2 to release GTP-actin which also promotes actin polymerisation (Yin and Janmey, 2003).
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Again, however there is no evidence to date for Rho inducing polymerisation via these mechanisms.

1.8 Rho Regulates focal adhesions

Rho also regulates the formation of focal adhesions (Ridley and Hall, 1992). Focal adhesions (FA) form points of contact for the tips of actin stress fibres that are linked via transmembrane receptors to the surrounding extracellular matrix components (ECM). Focal adhesions are found beneath the cell body, whereas the related smaller focal complexes are located at the tip of extending protrusions, such as filopodia and lamellipodia. Such complexes are crucial for cell adhesion and migration, cell morphology, growth and differentiation (Petit and Thiery, 2000).

The major receptors found at FAs are the integrins (Martin et al., 2002). These heterodimeric transmembrane glycoproteins are composed of an α and a β–subunit, each of which spans the membrane once, usually containing a large extracellular region and a short intracellular domain. The β-subunit can pair with multiple α-subunits, which creates multiple receptors that bind to different ECM substrates. So far at least 24 adhesion receptors have been identified, some of which participate in cell-cell adhesion, such as vascular-cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM). FA integrins engage extracellular matrix proteins such as fibronectin (α5β1), laminin (α5β1), collagens (α2β1) and vitronectin (α5β3). FAs consist of a host of intracellular structural and signalling proteins. In order for FAs to form, cells must be activated to contract by Rho-activating agents. Activation of Rho results in ROCK-mediated MLC phosphorylation, myosin II activation and the bundling of actin and myosin filaments, which results in tension formation (Petit and Thiery, 2000). This tension is transmitted to integrins in the plasma membrane, leading to integrin aggregation. Clustering of integrins, together with integrin-ligand engagement are required for complete co-localisation of all FA proteins, and FA formation (Burridge and Chrzanowska-Wodnicka, 1996).
1.9 Endothelial barrier dysfunction and non-cytoskeletal regulation

Although there is a lot of evidence for regulation of barrier function by the cortical actin ring and stress fibres, it is likely that independent signalling occurs directly to the tight junction proteins, which also regulates permeability. Histamine induces actin stress fibres, phosphorylation of occludin and increased permeability in ECV304 cells, but only the formation of actin stress fibres is inhibited by Rho or ROCK inhibition. Occludin phosphorylation occurs independently of this pathway and could result in increased permeability (Hirase et al., 2001). Furthermore, as previously cited, Rac inhibition has been shown to perturb VE-cadherin-mediated adhesion both dependently and independently of Rac-induced cytoskeletal reorganisation (Waschke et al., 2004). C3 transferase induces a loss of stress fibres, but this effect is not sufficient to block thrombin-induced increased permeability, indicating that other non-cytoskeletal pathways are also involved (Carbajal et al., 1999). Phosphorylation, degradation and redistribution of tight junction components have all been associated with increased permeability. Occludin proteolysis and increased permeability can be blocked by a metalloproteinase inhibitor (Wachtel et al., 1999) and down-regulation of occludin protein has been observed in response to VEGF (Kevil et al., 1998; Antonetti et al., 1998).

1.10 TNF-α function and signalling

TNF-α (formally called cachectin) is a pro-inflammatory cytokine produced predominantly by activated macrophages and monocytes, but also by B cells, T cells, NK cells, glial cells, Kupffer cells and adipocytes (Tracey and Cerami, 1993). TNF-α contributes to inflammatory responses to pathogens and can be induced after intravenous administration of lipopolysaccharide (LPS) or other bacterial components (Tracey and Cerami, 1993). TNF-α is also associated with the pathogenesis of several diseases, such as septic shock, cachexia, reperfusion injury, adult respiratory distress syndrome (ARDS) and atherosclerosis.

Shock is defined as inadequate tissue perfusion, usually in the presence of low blood pressure, which causes tissue injury, organ failure and death. Septic shock syndrome develops in association with invasive infections and not only is TNF-α produced during
septic shock, but TNF-α itself causes septic shock syndrome when administered to uninfected animals. TNF-α also triggers a secondary cytokine cascade leading to the appearance of other cytokines, such as IL-1, which increases TNF-α toxicity (Tracey et al., 1986). Cachexia is a state of weight loss, weakness and anaemia that complicates illness caused by infection, inflammation, cancer or injury. Chronic exposure of a host to TNF-α or other cytokines induces the cachectic state (Tracey and Cerami, 1993). ARDS is characterised by hypoxia, increased capillary permeability and pulmonary oedema (Bhatia and Moochhala, 2004). Patients with ARDS have elevated levels of TNF-α in the bronchoalveolar fluid, which induces increased capillary permeability and lung water content (Stephens et al., 1988; Tracey and Cerami, 1993).

TNF-α levels are also elevated in patients with myocardial infarction, congestive heart failure and ischemia/reperfusion injury. Reperfusion injury occurs when blood flow is restored after a period of transient ischaemia and is characterised by neutrophilic infiltrate, oedema and haemorrhage (Meldrum, 1998). TNF-α has been linked with the development of increased pulmonary capillary permeability following ischaemia/reperfusion (Colletti et al., 1990a and b). TNF-α has also been shown to depress heart contractile function and induce apoptosis of cardiac myocytes following ischaemia (Meldrum, 1998; Belosjorow et al., 1999).

TNF-α is expressed as a 26-kDa transmembrane protein on the surface of many immune cells, including macrophages. This polypeptide is cleaved by the metalloprotease, TNF-α converting enzyme (TACE), into a 17-kDa soluble form. Soluble TNF-α can bind as a homotrimer to one of two endothelial cell surface receptors of the TNF receptor family, TNFR1 (p55 TNFR, p60, CD120a) and TNFR2 (p75TNFR, p80, CD120b, TNFRSF1b), which are single transmembrane glycoproteins (Madge and Pober, 2001; Vandenabeele et al., 1995). Most of the TNF-α effects that have been determined are elicited via the TNFR1, as opposed to TNFR2, although it is TNFR2 that predominates on the endothelial cell surface, whereas the majority of TNFR1 is found on the Golgi (Madge and Pober, 2001). A ligand-passing model has been a proposed function for the TNFR2,
TNFR1, as opposed to TNFR2, although it is TNFR2 that predominates on the endothelial cell surface, whereas the majority of TNFR1 is found on the Golgi (Madge and Pober, 2001). A ligand-passing model has been a proposed function for the TNFR2, whereby the TNFR2-bound ligand is passed to the TNFR1 receptor at both the plasma membrane and at the Golgi membrane after TNFR2 internalisation (Tartaglia et al., 1993). It is thought that TNFR2 may also bind to transmembrane-bound TNF-α on macrophage cell surfaces (Grell et al., 1995). A third TNF-α receptor may also exist on the mitochondria (Ledgerwood et al., 1998). Yet to be fully characterised, this 60-kDa TNF-α binding protein has been shown to bind to 20% of internalised TNF-α.

Engagement of TNF-α with its receptors triggers the activation of kinases, lipases, phosphatases, and caspases (Fig. 1.16) (Heller and Kronke, 1994; Madge and Pober, 2001). Unlike growth factor receptors which elicit downstream signalling cascades through autophosphorylation or G-protein activation, TNFR1 and TNFR2 form initial receptor signalling complexes by recruitment of adaptor proteins that contain specific domains, primarily the death domain (DD)-containing proteins (Hsu et al., 1996). TNFR1 itself contains a death domain, which is a region of approximately 80 kDa, located near the cytoplasmic carboxy-terminus. TNFR2 lacks this domain. The recruitment of adaptor proteins to TNFR1 and 2 can lead to apoptotic and/or cell-survival signals. TNF-α-associated death domain protein (TRADD) binds to TNFR1 and recruits Fas-associated death domain (FADD), which in turn associates with and activates caspase 8 (MacEwan et al., 2001). Activation of the caspase cascade leads to cleavage of key house-keeping proteins, resulting in cell-death (Madge and Pober, 2001; MacEwan 2002). A signalling complex of TNF receptor associating proteins (TRAFs), receptor interacting protein (RIP) and FADD is implicated in activation of the transcription factor, NFκB (Wajant, 2003). NFκB is maintained in the cytosol by masking of its nuclear localisation signal by inhibitor of κB (IκB). TRAF2 binds to NFκB-inducing kinase (NIK), a MAPK kinase kinase. NIK phosphorylates IκB kinase, which in turn phosphorylates IκB on Ser\textsuperscript{176}, labelling it for ubiquitination and degradation, exposing the NFκB nuclear localisation signal and therefore allows it to migrate to the nucleus.
TNF-α signalling

TNF-α triggers a diverse range of signalling cascades that result in proliferative, apoptotic and inflammatory responses. Adapted from MacEwan, 2002.
TNF-α also activates acidic and neutral sphingomyelinases (SMase), which are phospholipases that cause the breakdown of sphingomyelin to the second messenger ceramide, as well as phosphorylcholine (Kolesnick and Kronke, 1998; MacEwan, 2002). The ceramide generated can then be converted to other ceramide-containing lipids such as sphingosine and sphingosine-1-phosphate (S-1-P) (MacEwan et al., 2002). Ceramide activates a membrane bound kinase called ceramide-activated protein kinase (CAPK) (Liu et al., 1994), and has also been implicated in the activation of NFκB, Jnk and ERK1/2 (Heller and Kronke, 1994, Madge et al., 2001). However, the generation, and role, of ceramide as a second messenger in endothelial cells in response to TNF-α has been disputed (Madge et al., 2001). Exogenous ceramide does not mimic TNF-α responses such as activation of NFκB or JNK in endothelial cells (Modur et al., 1996; Slowik et al., 1996).

TNF-α also activates the MAPKs, ERK1 and ERK 2, p38 MAPK and JNK in HUVECs (Goebeler et al., 1999; Surapisitchat et al., 2001; Hoefen et al., 2002). The mechanism for MAPK activation has not been fully characterised, but may involve Grb2 recruitment to TNFR1. One of the two SH3 domains of Grb2 associates with the proline-rich region of TNFR1 (Hildt and Oess, 1999). The other SH3 domain of Grb2 is known to bind to the GEF, Sos, and this may also be recruited to the TNFR1 receptor complex (Hildt and Oess, 1999). Both Grb2 and Sos are known to be upstream of MAPK activation in response to growth factors and a similar mechanism may occur in response to TNF-α (Madge and Pober, 2001).

1.10.1 TNF-α and Permeability
TNF-α has been reported to increase endothelial permeability (Ferrero et al., 2001; Nwariaku et al., 2002). There are several possible mechanisms through which this may occur. p38 MAPK has been implicated in the control of actin polymerization because of its ability to phosphorylate MAPK-activated kinase-2 (MAPKAP-2), a protein that regulates the actin filament capping protein, heat shock protein 27 (HSP27) (Landry et al., 1995; Garcia et al., 2002). p38 MAPK inhibition attenuates TNF-α-induced barrier
dysfunction, VE-cadherin redistribution, microtubule reorganization, and actin rearrangement (Petrache et al., 2003; Kiemer et al., 2002; Nwariaku et al., 2002). More recently, Rho inhibition with C3 transferase and ROCK inhibition with Y-27632 have been shown to inhibit TNF-α-induced MAPK activation and increased permeability, implying a role for Rho and ROCK upstream of p38 MAPK activation (Nwariaku et al., 2003). However, *Bordetella pertussis* toxin, known to increase lung permeability and airway oedema, also activates p38 MAPK and increases TER, but this p38 MAPK activation does not require Rho family GTPases (Garcia et al., 2002), therefore Rho involvement in p38 MAPK activation probably depends upon the stimulus applied.

PKC may be involved in TNF-α-induced permeability. PKC is a Ser/Thr protein kinase of which there are eleven different isoforms. These isoforms are classified into 3 groups according to their regulatory domain structure; the classical (cPKC), novel (nPKC) and atypical (aPKC) groups. Of these, the classical PKCs are Ca^{2+} and DAG-dependent. TNF-α has been reported to induce translocation of PKC-α to the membrane fraction by 1 minute, which subsides by 15 min but remains elevated at 3 h (Ross et al., 1997). TNF-α–induced translocation of PKC-α and β isotypes to the plasma membrane has also been reported in pulmonary endothelium in vitro (Ferro et al., 1993). With regard to the permeability however, Yonemaru et al. reported no change in TNF-α-induced permeability across bovine endothelial monolayers in the presence of the protein kinase inhibitor H-7, which blocks PKC. Furthermore, in this system the phorbol ester, phorbol myristate acetate (PMA) was unable to increase permeability. However, this is in conflict with other reports (Yonemaru et al., 1997; Lynch et al., 1990; Verin et al., 2000). On the contrary, others have shown TNF-α-induced permeability can be prevented by inhibitors and antisense oligonucleotides to PKC-α (Ferro et al., 2000). How PKC-α is activated by TNF-α is unknown, as an early release of Ca^{2+} does not occur in endothelial cells in response to TNF-α and PKC-α is a classical, Ca^{2+} and DAG-dependent PKC isoform.
Another way in which PKC might regulate permeability is by PKC-α-dependent phosphorylation of RhoGDI and activation of RhoA (Mehta et al., 2001). Thrombin and PMA are known to utilise this mechanism to increase permeability (Mehta et al., 2001; Van Nieuw Amerongen et al., 2000). Activation of PKC-α in HUVECs does not induce MLC phosphorylation or the formation of actin-stress fibres, but rather disrupts VE-cadherin and increases threonine phosphorylation at intercellular junctions, which correlates with an increase in permeability to albumin (Sandoval et al., 2001). PKC has been reported to phosphorylate occludin and ZO-1, which may regulate endothelial permeability (see sections 1.3.1.3.2 and 1.3.5.3.1).

There is some evidence that activation of PKA inhibits thrombin-induced permeability by inhibiting RhoA, and therefore blocking stress fibre formation. cAMP promotes barrier integrity by activation of PKA (Yuan et al., 2003). Increasing intracellular cAMP and subsequent activation of PKA using forskolin and 3-isobutyl-1-methylxanthine inhibits the thrombin-induced dissociation of RhoA from GDI, as well as inhibiting RhoA activation, and translocation to the plasma membrane (Qiao et al., 2003). In epithelial cells, a decrease in transepithelial resistance in response to TNF-α can be inhibited by the cAMP-non-hydrolysable analogue, dibutyryl-cAMP, or by activation of PKA with forskolin or phosphodiesterase inhibitors (Marano et al., 1995). This implies that TNF-α may employ a mechanism of inhibiting PKA in order to induce permeability increases in these cells.

1.10.2 Regulation of Rho and TNF-α induced permeability

Rho activation by TNF-α has not been extensively studied, and has not been shown directly. However, TNF-α rapidly induces the formation of stress fibres in endothelial cells and dominant negative Rho inhibits TNF-α-induced stress fibres and contraction, and partially prevents VE-cadherin dispersion (Wojciak-Stothard et al., 1998). It is therefore likely that a signal from the TNF-α receptors, TNFR1 or 2, to Rho exists, most likely through a Rho-GEF. There are several mechanisms for activation of GEFs that have recently been reviewed (Schmidt and Hall, 2002) and involve relief of autoinhibition, protein-protein interaction, and/or GEF re-localisation. Some GEFs
contain a regulatory domain, which auto-inhibits the activity of the GEF. For example, Vav is a GEF for Rho, Rac and Cdc42 that is thought to act downstream of the epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and B-cell and T-cell receptors (Bustelo, 2000). Receptor activation results in phosphorylation of Vav by the non-receptor tyrosine kinases Src and Syk, which results in Vav activation (Schmidt and Hall, 2002). The PH domain provides a second, auto-inhibitory constraint to Vav, by interacting with the DH domain (Schmidt and Hall, 2002) and this is relieved by association with the PI 3-kinase product, PIP3. This disruption of the PH-DH interaction also enhances phosphorylation of Vav by Lck (Schmidt and Hall, 2002). For other GEFs, protein-protein interactions are sufficient for activation. Activation of p115RhoGEF involves its interaction with the heterotrimeric G protein Gα13. p115RhoGEF stimulates the GTPase activity of Gα12 and Gα13, whilst in return, Gα13 stimulates p115RhoGEF to catalyse nucleotide exchange on Rho (Hart et al., 1998; Schmidt and Hall, 2002). This mechanism of activation of p115RhoGEF has been described for thrombin-induced Rho activation, as well as Rho-dependent stress fibre formation in response to LPA, thrombin and thromboxane A2 (Kaibuchi, 1999).

The PH domain can also regulate GEF activity through localisation of the protein to membranes or the cytoskeletal structures. Indeed, some GEF PH domain-deletion mutants are inactive, but can be rescued by addition of a CAAX motif, which targets the protein to the plasma membrane (reviewed in Schmidt and Hall, 2002). The PH domains of Dbl and Lbc are required for their localisation to stress fibres (Zheng et al., 1996; Olson et al., 1997; Schmidt and Hall, 2002). GEFs can also be recruited to the correct localisation independent of the PH domain, for example, the recruitment of Sos to the plasma membrane is mediated by adaptor proteins Grb2 and Shc, but not its PH domain, although the PH domain of Sos1 does associate with the plasma membrane (Schmidt and Hall, 2002). Other proteins are also correctly localised through adaptor proteins. For example, Vav is recruited to receptor complexes by SH2/SH3-dependent interactions with adaptor proteins (Bustelo, 2000).
There are several potential mechanisms for GEF activation by TNF-α. As the TNFR1 and 2 are not G protein-coupled receptors, it is unlikely that a Rho-GEF would be activated by the α or β subunits of a heterotrimeric G protein. It is therefore likely that the mechanism for activation of a GEF downstream of TNF-α receptors is via protein-protein interaction, GEF phosphorylation, and/or re-localisation of the GEF to the receptor complex via some other mechanism or recruitment to the site of stress fibre formation. The PI 3-kinase target PKB/Akt is activated in response to TNF-α (MacEwan, 2001), although the mechanism of PI 3-kinase activation has not been shown.

The adaptor protein Grb2 is known to associate with TNFR1 through its SH3 domain and a proline-rich region within the receptor (Hildt et al., 1999). PI 3-kinase might also associate with the receptor via its SH3 domain or alternatively could bind to Grb2 for its recruitment. Several GEFs are known to be activated by binding to the PI 3-kinase product, PIP₃. These include Vav, Dbl and Sos (Das et al., 2000; Russo et al., 2001; Schmidt and Hall, 2002). PI 3-kinase can act upstream of Rac (Ridley, 2001). The recruitment and activation of PI 3-kinase, and subsequent PIP₃ formation could result in GEF activation, followed by activation of Rac and cytoskeletal reorganisation. Indeed, PI 3-kinase activation of the Rac GEFs, Tiam1, Sos and Vav, has been shown to induce Rac-mediated membrane ruffling (Flemming et al., 2000; Das et al., 2000).

No specific Rho-GEF has yet been cited as a potential component in TNF-α-induced Rho signalling or stress fibre formation. Nevertheless, one Rho-GEF, GEF-H1, has recently been described, which not only activates Rho, but also localises to the tight junction (Benais-Pont et al., 2003). Two isoforms of this protein have been identified and are due to alternative splicing. GEF-H1 was originally identified as a microtubule-associated protein in unpolarized cells (Ren et al., 1998) and has subsequently been shown to localise to cell-cell contacts in MDCK cells in interphase and also to spindle microtubules during mitosis (Benais-Pont et al., 2003). Furthermore, GEF-H1 localises to different actin structures such as stress fibres (fibroblasts) and cortical actin (MDCK cells). Overexpression of GEF-H1 activates RhoA and increases paracellular permeability, without altering tight junction intramembrane strands or TER. Conversely, a reduction in protein levels of GEF-H1 using RNA interference reduces paracellular
Chapter 1

Introduction

permeability (Benais-Pont et al., 2003). This indicates that GEF-H1 could be an important regulator of permeability. It remains to be seen whether GEF-H1 regulates Rho in response to TNF-α in endothelial cells, and if this contributes to TNF-α-induced permeability changes.

1.11 Aims of Project

The objectives of this project were to characterise the effects of TNF-α on endothelial cell morphology, F-actin and paracellular permeability. The mechanisms through which TNF-α might alter endothelial permeability were to be investigated, specifically the role of Rho GTPases, cytoskeletal rearrangement and signalling to the tight junction proteins.
Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Materials

2.1.1 Reagents and Kits

- ABI PRISM d Rhodamine Terminator
  - PE Applied Biosystems
  - www.appliedbiosystems.com

- Cycle Sequencing Ready Reaction Kit
  - Bio-Rad Laboratories Ltd
  - www.biorad.com

- Bio-Rad protein Assay
  - Bio-Rad Laboratories Ltd
  - www.biorad.com

- DNA Markers II and IV
  - Roche
  - www.roche.com

- ECL Reagent
  - Amersham Pharmacia
  - www.amershambiosciences.com

- Electroporation cuvettes
  - Bio-Rad Laboratories Ltd
  - www.biorad.com

- Oligonucleotides
  - Genosys, Sigma Aldrich Company, Ltd
  - www.sigmaaldrich.com

- QIAprep Miniprep and Maxiprep Kits
  - Qiagen Inc
  - www.qiagen.com

- RPN 756 rainbow molecular weight marker
  - Amersham Pharmacia
  - www.amershambiosciences.com

- Super RX medical X-ray film
  - Fuji
  - www.fujimed.com

- Trypsin/EDTA
  - Calbiochem
  - www.emdbiosciences.com

- Transwell filters, 0.4 μm pore size
  - Costar (3460)
  - www.fishersci.com

- FITC-dextran, Mr 42,000
  - Sigma Aldrich Company, Ltd
  - www.sigmaaldrich.com

- Dako Mounting Medium
  - Dako Corporation
  - www.ump.com/dako.html
### Materials and Methods

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
<th>Website</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qiaex II gel extraction kit (150)</td>
<td>Qiagen Inc</td>
<td><a href="http://www.qiagen.com">www.qiagen.com</a></td>
</tr>
<tr>
<td>Dyex 2.0 spin kit (250)</td>
<td>Qiagen Inc</td>
<td><a href="http://www.qiagen.com">www.qiagen.com</a></td>
</tr>
<tr>
<td>EBM-2 endothelial cells medium and bullet-kits</td>
<td>Calbiochem</td>
<td><a href="http://www.emdbiosciences.com">www.emdbiosciences.com</a></td>
</tr>
<tr>
<td>HF7c and SFY526 Saccharomyces cerevisiae yeast strains</td>
<td>Clontech</td>
<td><a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a></td>
</tr>
<tr>
<td>Bacto peptone</td>
<td>BD Bioscience (211677)</td>
<td><a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a></td>
</tr>
<tr>
<td>Yeast extract</td>
<td>Difco (212750)</td>
<td><a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a></td>
</tr>
<tr>
<td>Bacto agar</td>
<td>BD Bioscience (214010)</td>
<td><a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a></td>
</tr>
<tr>
<td>Dextrose</td>
<td>Difco (215510)</td>
<td><a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a></td>
</tr>
<tr>
<td>Yeast nitrogen base without amino acids</td>
<td>Difco (0919-07)</td>
<td><a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a></td>
</tr>
<tr>
<td>Amino acid bases</td>
<td>Clontech:</td>
<td></td>
</tr>
<tr>
<td>0.74 g/L -Trp (63413)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.69 g/L -Leu (8605-1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.62 g/L -Trp/-Leu (8610-1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium dodecyl sulphate (SDS)</td>
<td>Invitrogen (15525-017)</td>
<td><a href="http://www.invitrogen.com">www.invitrogen.com</a></td>
</tr>
<tr>
<td>Salmon sperm carrier DNA</td>
<td>Ambion (9680)</td>
<td><a href="http://www.ambion.com">www.ambion.com</a></td>
</tr>
<tr>
<td>Circular filter papers, 70 mm</td>
<td>Whatman (1001070)</td>
<td><a href="http://www.fishersci.com">www.fishersci.com</a></td>
</tr>
</tbody>
</table>
3-aminotriazole (3AT) | Sigma Aldrich Company, Ltd (A-8056)  
www.sigmaaldrich.com
Lithium acetate (LiAc) | Sigma Aldrich Company, Ltd (L-6883)  
www.sigmaaldrich.com
PEG-4000 (polyethylene glycol) | Sigma Aldrich Company, Ltd (P-6840)  
www.sigmaaldrich.com
Dimethylsulphate (DMSO) | Sigma Aldrich Company, Ltd (D-8779)  
www.sigmaaldrich.com
X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) | Sigma Aldrich Company, Ltd (B-9146)  
www.sigmaaldrich.com
Chloroform:isoamylalcohol (24:1) | Sigma Aldrich Company, Ltd (C-0549)  
www.sigmaaldrich.com
β-mercaptoethanol | Sigma Aldrich Company, Ltd (M-6250)  
www.sigmaaldrich.com
Glass beads 425-600 μm | Sigma Aldrich Company, Ltd (6-8772)  
www.sigmaaldrich.com
Phenol | Sigma Aldrich Company, Ltd (A-9506)  
www.sigmaaldrich.com
CPRG (chlorophenol red-β-D-galactopyranoside) | Sigma Aldrich Company, Ltd (P-4557)  
www.sigmaaldrich.com
L-Aspartate (hemi-Mg salt) | Roche (884 308)  
www.roche.com
Glutathione sepharose, protein A-sepharose | Amersham Bioscience  
www.amershambiosciences.com
and protein G-sepharose |
### Materials and Methods

#### 2.1.2 Buffers and solutions

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP40 Lysis buffer</td>
<td>1 % NP40, 150 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 1 mM Na₃VO₄, 25 mM NaF, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotonin</td>
</tr>
<tr>
<td>SDS-buffer (for insoluble fraction)</td>
<td>0.025 M Hepes, 4 mM EDTA pH 8.0, 0.025 M NaF, 1 % SDS, 1 mM Na₃VO₄</td>
</tr>
<tr>
<td>50 X TAE</td>
<td>2 M Tris-HCL, pH 8.0, 50 mM EDTA, pH 8.0, 5.7 % acetic acid</td>
</tr>
<tr>
<td>6 X Lamelli Sample buffer</td>
<td>10 % SDS, 30 % Glycerol, 2 % β-mercaptoethanol, 100 nM Tris pH 6.8, 0.25 % bromophenol blue</td>
</tr>
<tr>
<td>10 X DNA loading buffer</td>
<td>80 % glycerol, 0.25 % bromophenol blue, 0.25 % Xylene cyanol FF</td>
</tr>
<tr>
<td>Coomasie Stainer</td>
<td>0.1 % coomasie brilliant blue, 20 % ethanol, 0.5 % acetic acid</td>
</tr>
<tr>
<td>Coomasie Destainer</td>
<td>20 % methanol, 0.5 % acetic acid</td>
</tr>
<tr>
<td>10 X SDS-PAGE running buffer</td>
<td>20 mM glycine, 25 mM Tris-HCl, 0.1 % (w/v) SDS</td>
</tr>
<tr>
<td>SDS-separating gel (lower)</td>
<td>0.38 M Tris HCl pH 8.8, 0.1 % (w/v) SDS, 0.05 % (v/v) APS, 0.05 % (v/v) temed, appropriate amount of acrylamide: 6 %, 7.5 %, 9 %, 10 % or 12 %</td>
</tr>
</tbody>
</table>
### Chapter 2

**Materials and Methods**

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stacking gel (upper)</td>
<td>0.1% Tris-HCl (pH 6.8), 4% acrylamide, 1% SDS, 0.5% APS (v/v), 0.1% (v/v) temed.</td>
</tr>
<tr>
<td>10 X Transfer buffer</td>
<td>20% methanol, 1.44% (w/v) glycine, 0.3% Tris-HCl</td>
</tr>
<tr>
<td>TBS/Tween washing buffer</td>
<td>20 mM Tris-HCl pH 7.6, 130 mM NaCl, 0.05% Tween-20</td>
</tr>
<tr>
<td>Silver stain developer</td>
<td>2% sodium carbonate (Na₂CO₃), 0.04% formaldehyde</td>
</tr>
<tr>
<td>Ampicillin 100 X stock</td>
<td>100 mg/ml in dd H₂O</td>
</tr>
<tr>
<td>L-broth</td>
<td>25 g/L Millers LB broth base (Invitrogen)</td>
</tr>
<tr>
<td>LB-Agar</td>
<td>2% bacterial agar in L-broth (20 g/L)</td>
</tr>
<tr>
<td>Stripping Buffer</td>
<td>2% (w/v) SDS, 77 mM Tris pH 6.8, 0.68% β-mercaptoethanol (v/v).</td>
</tr>
<tr>
<td>PBS</td>
<td>137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 2 mM K₂HPO₄</td>
</tr>
<tr>
<td>Sodium thiosulphate sensitization buffer</td>
<td>0.02% (w/v) Na₂S₂O₃ in ddH₂O</td>
</tr>
<tr>
<td>Silver nitrate solution</td>
<td>0.15% (w/v) AgNO₃ in ddH₂O</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>3.7% formaldehyde in PBS</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate (Sigma), 10% stock made up in ddH₂O and stored at -20 °C</td>
</tr>
</tbody>
</table>
Chapter 2

**Materials and Methods**

- **PMSF**: Phenyl methyl sulfonyl fluoride (Sigma), 1 M stock prepared in isopropanol and stored at -20 °C
- **NaF**: Sodium fluoride (Sigma), 1 M stock made up in ddH₂O and stored at -20 °C
- **Na₃VO₄**: Sodium orthovalidate (Sigma), 100 mM stock prepared in ddH₂O and stored at -20 °C
- **DTT**: Dithiothreitol, 1 M stock made up in ddH₂O and stored at -20 °C
- **IPTG**: Isoprpryl β-D-thiogalactopyranoside, 0.5 M stock made up in ddH₂O and stored at -20 °C
- **YPD media and plates**: A total of 950 ml was made from 20 g/L Bacto peptone, 10 g/L Yeast extract only or with 20 g/L bacto agar in H₂O. The pH was adjusted to 5.8 and autoclaved at 121 °C for 15 mins. 2 % sterile dextrose was added to the cooled YPD media from a dextrose stock of 40 %.
- **SD synthetic media and plates**: A total of 850 ml was made from 6.7 g/L Yeast nitrogen base without amino acids, with or without 20 g/L agar made up in ddH₂O. A single NaOH pellet was also
Chapter 2

Materials and Methods

Z-buffer

16.1 g/L Na$_2$HPO$_4$.7H$_2$O, 5.50 g/L Na$_2$HPO$_4$. H$_2$O, 0.75 g/L KCl, 0.246 g/L MgSO$_4$.7H$_2$O and the pH was adjusted to 7.0

10 X TE buffer

0.1 M Tris-HCl, 10 mM EDTA, pH 7.5 and autoclaved.

10 X LiAc

1 M lithium acetate, pH 7.5 (with diluted acetic acid), autoclaved

1 X TE/LiAc

1 X TE and 1 X LiAC (ie. 1 ml of each in 10 mls)

X-gal stock

20 mg/ml X-gal in DMF (N, N dimethylformamide), stored in the dark at -20°C

Z buffer / X-gal solution

100 ml Z buffer, 0.27 ml β-mercaptoethanol, 1.67 ml X-gal stock

10 X Amino acid base stocks

10 X stocks made up from the amino acid bases were filter sterilised at stored at 4 °C
Chapter 2

Materials and Methods

Buffer 1 for CRPG screen
For 100 ml, 2.38 g Hepes, 0.9 g NaCl, 0.065 g L-Aspartate (hemi-Mg salt), 1.0 g BSA, 50.0 µl Tween-20 in ddH₂O, adjusted to pH 7.25-7.30, filter sterilised and stored at 4 °C for up to 3 months.

Buffer 2 for CPRG screen
27.1 mg of CPRG in 20 ml of buffer 1 (final CRPG concentration of 2.23 mM). Filter sterilised and stored in the dark for up to 3 months.

MP buffer
100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1% SDS

PEG-400 stock
50 % PEG-400 made up in ddH₂O, autoclaved and stored at room temperature

Dextrose stock
40 % dextrose made up in dd H₂O was autoclaved and stored at 4°C

Rho pull-down lysis buffer
50 mM Tris pH 7.5, 1% Triton-X-100, 0.5 % sodium decholate, 0.1% SDS, 500 mM NaCl, 100 mM MgCl₂, 10 µg/ml leupeptin, 10 µg/ml aprotonin, 1 mM PMSF, 10% glycerol, 1 mM DTT

5 X Rac and Cdc42 pull down buffer
125 mM Hepes pH 7.5, 750 mM NaCl, 5% NP40, 50 mM MgCl₂, 5 mM EDTA, 10 % glycerol. To make 1 X buffer, this is diluted into 10 % glycerol, plus 25 mM NaF, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotonin
Chapter 2

Materials and Methods

0.2% Triton-X-100 pre-extraction buffer

0.2% Triton-X-100, 100 mM KCl, 3 mM MgCl₂, 1 mM CaCl₂, 300 mM sucrose, 10 mM Hepes
## 2.1.3 Primary Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Reactivity</th>
<th>Western dilution</th>
<th>Approx. size (kDA)</th>
<th>IF dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>VE-cadherin</td>
<td>Mouse monoclonal</td>
<td>1/500</td>
<td>100</td>
<td>-</td>
<td>R and D systems (<a href="http://www.rndsystms.com">www.rndsystms.com</a>)</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Rabbit polyclonal</td>
<td>1/500</td>
<td>225</td>
<td>1/250</td>
<td>Zymed (<a href="http://www.zymed.com">www.zymed.com</a>)</td>
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<tr>
<td>Myosin II</td>
<td>Rabbit polyclonal</td>
<td>1/4000</td>
<td>200</td>
<td>1/200</td>
<td>Biogenesis (<a href="http://www.biogenesis.co.uk">www.biogenesis.co.uk</a>)</td>
</tr>
<tr>
<td>GEF-H1/JuGEF</td>
<td>Mouse monoclonal</td>
<td>1/500</td>
<td>100</td>
<td>1/50</td>
<td>Gift - Maria Balda</td>
</tr>
<tr>
<td>Claudin-5</td>
<td>Mouse monoclonal</td>
<td>1/200</td>
<td>22</td>
<td>1/50</td>
<td>Zymed (<a href="http://www.zymed.com">www.zymed.com</a>)</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Rabbit polyclonal</td>
<td>1/4000</td>
<td>92</td>
<td>1/200</td>
<td>BD Transduction laboratories (<a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a>)</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Rabbit polyclonal</td>
<td>1/1000</td>
<td>92</td>
<td>-</td>
<td>BD Transduction laboratories (<a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a>)</td>
</tr>
<tr>
<td>p120</td>
<td>Mouse monoclonal</td>
<td>1/1000</td>
<td>92</td>
<td>-</td>
<td>BD Transduction laboratories (<a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a>)</td>
</tr>
<tr>
<td>RockI</td>
<td>Mouse monoclonal</td>
<td>1/500</td>
<td>200</td>
<td>-</td>
<td>BD Transduction laboratories (<a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a>)</td>
</tr>
<tr>
<td>RockII</td>
<td>Mouse monoclonal</td>
<td>1/2000</td>
<td>200</td>
<td>-</td>
<td>BD Transduction laboratories (<a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a>)</td>
</tr>
<tr>
<td>Rac1</td>
<td>Mouse monoclonal</td>
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<td>-20</td>
<td>-</td>
<td>Upstate biotechnology (<a href="http://www.upstate.com">www.upstate.com</a>)</td>
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<tr>
<td>RhoA</td>
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<td>21</td>
<td>-</td>
<td>Transduction laboratories</td>
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<tr>
<td>RhoA</td>
<td>Rabbit polyclonal</td>
<td>1/250</td>
<td>21</td>
<td>-</td>
<td>BD Transduction laboratories (<a href="http://www.bdbiosciences.com">www.bdbiosciences.com</a>)</td>
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<tr>
<td>RhoE</td>
<td>Mouse monoclonal - shift seen with phosphorylation (activation)</td>
<td>1/1000</td>
<td>30 (just below)</td>
<td>-</td>
<td>Gift - Kirsi Riento</td>
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</table>
### Materials and Methods

#### Antibody Reactivity

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Reactivity</th>
<th>Western dilution</th>
<th>Approx. size (kDa)</th>
<th>IF dilution</th>
<th>Source</th>
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<tbody>
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<td>30-45</td>
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<tr>
<td>pMLC (T/S)</td>
<td>Rabbit polyclonal</td>
<td>1/200</td>
<td>20 doublet</td>
<td>-</td>
<td>Upstate biotechnology (<a href="http://www.upstate.com">www.upstate.com</a>)</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>Rabbit polyclonal</td>
<td>1/500</td>
<td>Tetramer 464 kDa under non-reducing conditions (non SDS-PAGE), 115 kDa under SDS-PAGE</td>
<td>1/250</td>
<td>MP biomedicals (formally ICN/Cappel) (<a href="http://www.mpbio.com">www.mpbio.com</a>)</td>
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<tr>
<td>β-galactosidase</td>
<td>Mouse monoclonal</td>
<td>-</td>
<td>115</td>
<td>1/100</td>
<td>Gift (Kelly Nikolaidou)</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>Mouse monoclonal</td>
<td>1/2000</td>
<td>55</td>
<td>-</td>
<td>Sigma (<a href="http://www.sigmaaldrich.com">www.sigmaaldrich.com</a>)</td>
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<td>moesin</td>
<td>Goat polyclonal</td>
<td>1/1000</td>
<td>~70</td>
<td>-</td>
<td>Santa Cruz (<a href="http://www.scbt.com">www.scbt.com</a>)</td>
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<td>c-myc</td>
<td>Mouse monoclonal</td>
<td>The tag is 11kDa</td>
<td>The tag is 11kDa</td>
<td>1/250</td>
<td>Santa Cruz (<a href="http://www.scbt.com">www.scbt.com</a>)</td>
</tr>
<tr>
<td>Occludin, c term</td>
<td>Rabbit polyclonal</td>
<td>1/250</td>
<td>65</td>
<td>1/100</td>
<td>Zymed (<a href="http://www.zymed.com">www.zymed.com</a>)</td>
</tr>
<tr>
<td>Occludin, c term</td>
<td>Mouse monoclonal</td>
<td>1/251</td>
<td>66</td>
<td>1/101</td>
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<td>1/200</td>
<td>18-20</td>
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<tr>
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<td>Mouse monoclonal - shift seen with phosphorylation (activation)</td>
<td>-</td>
<td>-</td>
<td>1/200</td>
<td>Sigma (<a href="http://www.sigmaaldrich.com">www.sigmaaldrich.com</a>)</td>
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### Antibody Reactivity

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<td>Sigma (<a href="http://www.sigmaaldrich.com">www.sigmaaldrich.com</a>)</td>
</tr>
</tbody>
</table>

### 2.1.4 Secondary Antibodies

- Horse radish peroxidase conjugated (HRP) -anti-mouse, rabbit, rat: Amersham Pharmacia, www.amershambiosciences.com
- HRP-anti-goat: Santa Cruz, www.scbt.com
- FITC or TRITC conjugated anti-rabbit and anti-mouse antibodies: Southern Biotechnology Associates, www.southernbiotech.com
- Cy5-conjugated goat anti-rabbit and anti-mouse antibodies: Jackson immuno research, www.jacksonimmuno.com
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2.1.5 Plasmids

pGEX-2T Glutathione S-transferase fusion vector used to create GST-fusion proteins (Amersham Pharmacia).

pGBT9 GAL4 fusion vector used to create GAL4 fusion proteins for the yeast two-hybrid experiments (Clontech, www.bdbiosciences.com).
2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Growing, passaging and stimulating cells

Pooled human umbilical vein endothelial cells (HUVECs) were cultured in EBM-2 culture medium with growth factors (EBM-2 Bulletkit), 3% fetal calf serum (FCS), penicillin (100 IU/ml) and streptomycin (100 µg/ml) (full medium). Cells were cultured on plastic flasks (25 cm$^2$ or 75 cm$^2$), glass coverslips or filters that had previously been coated with 10 µg/ml fibronectin for 20 min (flasks) or overnight (coverslips and filters). It was found that HUVECs grew far better on flasks than on dishes, so at no time were dishes used for culture or experiments. For passaging, cells were washed once in PBS and incubated with 5 ml trypsin/EDTA at 37 °C until the cells detached. Trypsin/EDTA was inactivated by dilution with 5 ml of full EBM-2 medium and the cells pelleted by centrifugation at 200 g. Cells were usually split 1:3 or 1:4 depending on their growth rate. For coverslips and filters, HUVECs were seeded at high density (1/4 of a confluent 75 cm$^2$ flask onto 12 coverslips or 12 filters). Cells were used up to a maximum of four passages. For experiments, cells were starved in EBM-2 culture medium supplemented with 1% FCS and penicillin/streptomycin, but lacking growth factors (‘starvation medium’) for 2-4 h prior to stimulation.

For HEK293 cells, these were cultured on 75 cm$^2$ or 162 cm$^2$ flasks in DMEM medium (Gibco) containing 10% FCS, penicillin (100 IU/ml) and streptomycin (100 µg/ml). Fresh medium was applied to these cells every two days and they were usually split 1:10.

2.2.1.2 Thawing of cells

HUVECs were removed from liquid nitrogen and defrosted at 37 °C. Cells were diluted into 10 ml of EBM-2 full medium and plated onto a 75 cm$^2$ tissue culture flask that had been coated with 10 µg/ml fibronectin for 20 min. Fresh medium was applied to the cells every 2 days.
2.2.1.3 TNF-α stimulation of HUVECs

Confluent HUVECs were starved for 2 h and usually stimulated with 10 ng/ml TNF-α for 10 min or 24 h. Alternative TNF-α concentrations and incubation times were also used.

2.2.1.4 Transfection of HUVECs

HUVECs were transfected using recombinant adenoviruses. Purified adenoviruses were applied to the cells at a multiplicity of infection (MOI) of 500. The MOI is defined as the number of virions per cell and the number of virions is equivalent to the number of plaque forming units (found from the plaque forming assay). This calculation is shown below. Usually 3 μl was added per coverslip, and 30 μl was added per 25 cm² flask.

\[
\text{MOI} = \frac{\text{number of virions (or PFU)}}{\text{number of cells}}
\]

Number of virions = \(10^{10}\) per ml = \(3 \times 10^7\) per 3 μl

Number of cells / coverslip \(\sim 60,000\)

So MOI for 3 μl = \(3 \times 10^7 / 60,000 = 500\)

Cells were usually infected with the viruses in full medium for 90 min, washed with PBS and placed into full medium or starvation medium overnight to allow protein expression.

2.2.2. Adenovirus preparation

2.2.2.1 Background to obtaining the adenoviruses

Recombinant adenoviruses carrying vectors encoding N19RhoA, N17Rac1 and N17Cdc42 were generated by Sandra Potempa (Wojciak-Stothard et al., 2001). Ad-β-gal was a kind gift from Glaxowellcome and Ad-GFP was a kinds gift from Brian Foxwell (Kennedy institute of Rheumatology).

Recombinant adenoviruses were generated by transfection of an admid vector containing the required gene into E.coli K12 strain, which was already transformed with a virus homing plasmid and a vector encoding a transposase. The virus homing vector contains 'gutless' Ad5 adenovirus genome that has E1 and E3 viral gene cassettes deleted, rendering the virus replication-deficient. The \(E.coli lacZ\) gene
encoding β-galactosidase, was substituted into the E1 position. Transposition of the required gene occurs from the admid vector into the Ad homing vector, which results in disruption of the β-galactosidase gene. Successfully transposed clones were selected for, the adenoviral DNA purified, and linearized before transfection into HEK293 cells (embryonic kidney). This helper cell line contains sheared Ad5 DNA and constitutively expresses the Ad-virus E1 gene allowing for DNA replication and virus production to occur. Virions were amplified (as described below) in HEK293 cells and purified to produce adenovirus stocks used to infect HUVECs.

2.2.2.2 Adenovirus propagation and purification
The following procedure was used to propagate Ad-N17cdc42 and Ad-N19RhoA. Other viruses used were propagated by Carole Fages (Ad-β-gal), Beata Wojciak-Stothard (Ad-GFP), Varuni Kanagasundaram (Ad-GFP) and Lynn Williams (Ad-N17Rac1). One well of a six-well plate containing 80-100% confluent HEK293 cells in 600 μl of serum-free DMEM was infected with 200 μl of archive adenovirus (cell lysate from previous amplifications). Cells were incubated for 1-2 h prior to addition of 200 μl of DMEM containing 10% FCS. Cells were incubated for 1-2 days. Cells were collected from the plate by gentle pipetting (P1000gilson pipette), placed into a 50 ml tube and freeze-thawed three times (37°C and liquid nitrogen or place at –20°C). The lysate was added to 5 ml of serum-free medium and applied to 80-100% confluent HEK293 cells grown in a 75 cm² flask. Cells were incubated for 1 h at 37°C and further diluted with 5 ml of DMEM containing 4% FCS. Cells were incubated for 1-2 days at 37°C. The flask was tapped to detach the cells, and the cells were transferred into a 50-ml tube. Cells were freeze-thawed as before, and diluted to 100 ml with serum-free DMEM. The lysate was used to infect 10 x 162 cm² flasks of HEK293 cells (10 ml of lysate per flask). The cells were infected for 2 h at 37°C, and a further 10 ml of DMEM containing 4% FCS was added to each flask. The cells were incubated at 37°C until the cells became non-adherent (but not starting to lyse). The cells were harvested into 50-ml flasks and collected by centrifugation at 13000 g for 5 min. Cells were resuspended in 10 ml of serum-free DMEM and pooled into one 50 ml tube. Cells were re-pelleted and resuspended in 10 ml of 0.1 M Tris pH 8.0. The cells were lysed by freeze-thawing three times, and the chromatin was sheared by passing through a 19 G needle 4-5 times. The cell debris was pelleted by
centrifugation at 2000 rpm in a Sorvall RT 6000D centrifuge and a Sorvall H1000B rotor. The supernatant was placed into a new 50 ml tube, the volume recorded and increased to 11.4 ml with 0.1 M Tris, pH 8.0 and transferred to Sorvall ultracentrifuge tubes, using a needle and syringe. All air bubbles were removed, and the tube collar was screwed into place. Tubes were balanced and centrifuged at 180 000 g overnight at 4 °C. The collar was removed and the tube was clamped to a clamp stand to enable removal of the virus band using a syringe. Sometimes two viral bands were observed, and both of these were removed. The virus material was then transferred into a fresh Sorvall ultracentrifuge tube and filled with 1.34 g/ml of CsCl. The tube collar was screwed into place and the overnight centrifugation was repeated. A PD10 column was equilibrated with 25 ml PBS. The tube was recovered from the ultracentrifuge and the viral band obtained as before. The viral material was applied to the PD10 column followed by PBS if required, to make a total volume of 2.5 ml. The PBS flow-through was collected and discarded. To elute the purified virus, 3.5 ml of PBS was applied to the column and collected in a bijou tube. The solution was then passed through a 0.22 μ filter using a needle and syringe, collected in a fresh bijou tube and diluted with 10 drops of sterile 100% glycerol were added (final concentration 10%). Purified virus was aliquoted and stored at -80 °C.

### 2.2.2.3 Adenovirus plaque-forming assay

In a 48-well plate, 990 μl of serum-free medium was applied to the first well, followed by 1.1 ml in the second well and 1 ml in the following 4 wells. A serial dilution of the virus was performed by addition of 10 μl of virus stock to the first well, transfer of 10 μl from the first to the second well, and transfer of 11 μl from the second well to the third well. 111 μl was transferred sequentially to the remaining four wells. This resulted in dilutions of $10^{-6}$, $10^{-7}$, $10^{-8}$, $10^{-9}$, $10^{-10}$. The $10^{-2}$ and $10^{-4}$ dilutions were retained for the replication competence test (see below). HEK293 cells were grown in a 6 well-plate and each well was infected with one of the dilutions. One well was left uninfected and diluted with serum-free DMEM as a negative control. Cells were incubated for 2 h at 37 °C. 1.5% seaplaque agarose was dissolved ddH$_2$O and maintained at 37 °C. This was diluted into MEM (1:1). The medium was removed from the cells and 3 ml of the agarose/MEM was applied to each well. The cells were incubated for 7-8 days to allow plaques to develop. One
plaque (plaque forming unit or PFU) in the $10^{10}$ diluted well gives a concentration of $10 \times 10^{10}$ pfu/ml, which is a relatively high concentration and was usually observed.

### 2.2.2.3 Adenovirus replication competent test

Theoretically, the recombinant adenoviruses should be replication-deficient as they lack the E1 and E3 viral genes. However, amplification of the viruses in E1-containing-HEK293 cells introduces the potential for homologous recombination between the E1 gene and the recombinant Ad DNA so that replication competent adenovirus (RCA) can be produced. This was tested each time Ad DNA was amplified by using a plaque-forming assay on A549 cells (human lung cancer) that have no E1 gene, so only infection by RCA could produce plaques. To do this, one 75 cm$^2$ flask of A549 cells were infected with both the $10^{-2}$ and $10^{-4}$ dilutions of adenovirus left from the adenovirus plaque forming assay. Cells were incubated for 1-2 h at 37 °C and the virus removed and replaced with DMEM containing 10% FCS. The cells were observed over the following 7-10 days for a cytopathic effect upon the cells.

### 2.2.3 Biochemical techniques

#### 2.2.3.1 Cell lysis and NP40-soluble/insoluble fractionation

One 25 cm$^2$ tissue culture flask of confluent HUVECs (1.5-2 million cells) was lysed in 500 μl NP40-lysis buffer, and the lysate transferred into an eppendorf tube. The lysate was rotated at 4 °C for 30 min prior to centrifugation at 13000 g for 30 min at 4 °C in a micro-centrifuge. The supernatant (NP40-soluble fraction) was obtained and transferred into a fresh tube. The insoluble pellet was either discarded or transferred in 100 μl of SDS-lysis buffer into a homogeniser and grounded 5 times with minimal foaming. The plunger was rinsed into the homogenate with 900 μl NP40-lysis buffer and the homogenate was transferred into a fresh eppendorf tube. The homogenate was passed through a 26-gauge needle 10 times with minimal foaming before incubation at 4 °C with rotation for 30 min. The solution was centrifuged at 13000 g for 30 min at 4 °C and the supernatant (NP40-insoluble fraction) retained.
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2.2.3.2 Biorad protein assay

A 5 μl sample of the cell lysate was diluted into 800 μl of ddH₂O. 200 μl of Biorad protein assay dye was added and the solution mixed and incubated for 10 min at room temperature. The blank consisted of 800 μl ddH₂O and 200 μl Biorad protein dye only. The absorbance of each sample was determined at 595 nm (Bradford assay) and the protein concentration (μg/ml) found using a calibration curve made from known concentrations of BSA.

2.2.3.3 SDS-PAGE

Proteins were separated by SDS-PAGE using minigel apparatus (Mini-Protean II Cell, Biorad). A basal separating gel of 1.5 mm or 0.75 mm thickness was cast, which contained 6%, 7.5%, 9%, 10% or 12% acrylamide according to the size of the proteins of interest. The gel was overlayed with ddH₂O and set at room temperature. After the separating gel had set, a second stacking gel was poured over it and a comb was inserted around which the stacking gel set, creating lanes for protein loading. The combs were removed and the gel was placed into an electrophoresis tank containing 1X running buffer.

Prior to the samples being loaded, the amount of total protein for each sample was determined using a Biorad protein assay, and if necessary, the samples were diluted in ddH₂O or lysis buffer to equal protein concentrations. Samples were boiled (100°C) for 5 min in the presence of 6X Laemmli sample buffer (1:6 dilution), briefly pulsed in a micro-centrifuge and loaded on to the gel. 5 μl of rainbow marker (RPN 756, Amersham) in the presence of 6X sample buffer was also loaded into one of the lanes. Current was applied to the gel (100 volts) until the marker appeared past the stack, and then the voltage was increased to 150 volts until the dye front reached the bottom of the gel.

2.2.3.4 Western blotting

Proteins separated by SDS-PAGE were transferred from the gel onto a PVDF membrane (Immobilon-P) by wet transfer. To do this, the membrane was briefly immersed in methanol and equilibrated in 1X transfer buffer. The membrane was placed over the gel and both were sandwiched between the two sheets of Whatman 3MM paper and two sponges that had also been soaked in 1X transfer buffer and
placed in a Mini-Protean II case. Air bubbles were squeezed out and the case was
inserted into a Mini-Protean II Cell, filled with 1 X transfer buffer. Proteins were
transferred at 100 V for 1 h. The membrane was blocked in 10 ml of 5% non-fat
dried milk or 5% BSA in TBS/Tween-20 for at least 30 min. The blot was
subsequently incubated with 0.1 – 0.5 µg/ml primary antibody in 1% BSA in
TBS/Tween-20 for 1 h at room temperature or overnight with rocking. The blot was
washed for 30 min in TBS/Tween-20 with two changes. It was then incubated with
HRP-conjugated secondary antibodies (1/2000) for 1 h at room temperature with
rocking. The blot was washed for 30 min in TBS/Tween-20 with two changes and
developed with ECL for 1 min and exposed to X-ray film.

2.2.3.5 Stripping western blots
Blots were placed in 10 ml of stripping buffer and placed securely into a water bath
set at 65 °C for 20 min with gentle rotation. Blots were washed in TBS/Tween-20 and
placed back into the water bath in TBS/Tween-20 for a further 20 min. Blots were
removed from the water bath and washed in fresh TBS/Tween with shaking at room
temperature for 20 min, before re-blocking in 5% BSA in TBS/Tween-20 for 30 min
at room temperature or overnight at 4 °C. Blots were then re-probed with the
antibodies of interest. Fresh blots could be stripped and re-probed up to 4 or 5 times,
but stripping and re-probing of older blots was found to be less successful.

2.2.3.6 Coomassie Blue and silver staining
For Coomassie blue staining, gels were stained in 0.025% Coomassie Brilliant Blue
for a minimum of 2 h and destained using coomassie destainer for 2 h or until the
bands became visible. For silver staining, gels were fixed in fixing buffer for 20 min
at room temperature, or overnight at 4 °C with shaking. The gel was washed twice in
ddH₂O for 20 min and sensitised in 0.02% sodium thiosulphate sensitization buffer. It
was then washed twice in ddH₂O for 1 min and incubated with cold 0.15% silver
nitrate solution in the dark at 4 °C for 20 min. Residual silver nitrate was removed by
washing twice with ddH₂O for 1 min and the bands were developed using developing
buffer. The reaction was stopped with 5% acetic acid. Gels were then scanned
directly or dried overnight at room temperature between two sheets of gel drying film
(5 x 28 cm, Promega Corporation) that had previously been soaked in tap water.
2.2.3.7 GST-cOcc and GST-nOcc protein purification

BL 21 *E. coli* (Stratagene) containing plasmids coding for the GST-fusion proteins (nOcc or cOcc) were inoculated into 100 ml L-broth containing 100 µg/ml ampicillin and the culture was incubated at 37°C overnight with shaking. The culture was diluted 1:10 into fresh L-broth containing 50 µg/ml ampicillin and grown for 1 h. Protein expression was induced with 0.2 mM IPTG for 3 h at 37°C with shaking. Cells were pelleted by centrifugation at 4000 g for 10 min at 4°C and the cells were resuspended in 6 ml of cold bacterial lysis buffer (50 mM Tris pH 7.5; 50 mM NaCl; 5 mM MgCl₂; 1 mM DTT; 1 mM PMSF). The suspension was sonicated five times for 10 seconds with a 50-second break between each burst, then centrifuged for 10 min at 10,000 rpm in a Sorvall SS-34 rotor at 4°C. 500 µl of glutathione sepharose beads were washed three times in 1 ml of the bacterial lysis buffer prior to being incubated with the bacterial supernatant for 1 h at 4°C. The beads were centrifuged, washed three times with 5 ml of cold bacterial lysis buffer that lacked PMSF, resuspended in storage-buffer (1:1) and stored at -80°C. In order to calculate the amount of purified protein, a 5 µl sample of the beads was boiled in 6 X Laemllii sample buffer for 5 min and loaded onto a 12% gel for SDS-PAGE along side known amounts of BSA. The gel was Coomassie blue-stained and the amount of protein was estimated by comparison to the known standards.

2.2.3.8 GST-WASP-PBD and GST-Rhotekin-RBD purification

A scraping of a bacterial glycerol stock containing the plasmid (pGEX-2T) coding for the GST-WASP-PBD or GST-Rhotekin-RBD (kindly provided by Beata Wojciak-Stothard) was inoculated, amplified and protein expression induced and purified as described for GST-cOcc and -nOcc, except that a different bacterial lysis buffers and storage buffers were used. For GST-WASP-PBD the lysis buffer contained 50 mM Tris pH 8; 2 mM MgCl₂; 10% glycerol; 20% sucrose; 2 mM DTT; 1 mM PMSF; 10 µg/ml aprotonin; 10 µg/ml leupeptin and 10 µg/ml pepstatin A. The storage buffer contained 10 mM Tris pH 8; 1 mM DTT; 100 mM NaCl; 50% glycerol. For GST-Rhotekin-RBD, the lysis buffer contained 50 mM Tris pH 7.5, 1% Triton-X-100, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 10 mg/ml aprotonin, 10 mg/ml leupeptin, 1 mM PMSF. The wash buffer contained 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM
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MgCl₂ and 1 mM DTT. The storage buffer for GST-Rhotekin-RBD was the same as the washing buffer with an additional 10% glycerol.

2.2.3.9 GTPase activation assays (pull-downs)

The method originally described by Ren and Schwartz (2000) was followed for these experiments. Glutathione sepharose-coupled GST-fusion proteins of rhotekin, the p21-binding domain (PBD) of PAK (Upstate Biotechnology) or the CRIB domain of WASp were used for Rho, Rac and Cdc42 pull-downs respectively. 20 µg of the fusion proteins were used per pull-down. One 25 cm² tissue culture flask of confluent HUVECs was used per pull-down (1.5-2 million cells). Cells were starved for 2 h and stimulated prior to rapid lysis in the pull-down lysis buffer. Cells were scraped immediately, transferred into an eppendorf tube and centrifuged at 13000 g for 3 min at 4°C. A 50 µl sample of the whole cell lysate was obtained from the supernatant and the remaining supernatant was incubated with the bead-sludge at 4°C with rotation for 1 h. It is imperative that between cell lysis and incubation with the beads that the cell lysates are kept on ice and placed on to the beads as rapidly as possible, to minimise GTP hydrolysis to GDP (GTPase inactivation). For the positive and negative controls, prior to incubation with the beads, the supernatant was incubated for 15 min at 37 °C with either GTPγS or GDPβS respectively, in the presence of 200 mM EDTA. The reaction was stopped in 60 mM MgCl₂ and the lysate placed on the beads and incubated as described above. The beads were pulsed briefly in a cooled centrifuge and the unbound supernatant discarded. The beads were washed three times in pull-down lysis buffer and placed in 30 µl 6X sample buffer. Beads were boiled for 5 min and pulsed in a centrifuge prior to the 30 µl sample separated by SDS-PAGE (12% gel) and western blotting. To ensure equal protein loading in SDS-PAGE, a biorad protein assay was conducted using a sample from the whole cell lysate, and the same amount of protein for the pull-downs and for the whole cell lysates were used in each lane of the polyacrylamide gel. This was subsequently confirmed by stripping the blot and reprobing the whole cell lysate lanes with either an anti-α-tubulin or an anti-β-actin antibody.
2.2.3.10 GST-cOcc and nOcc pull down experiments

Gst-fusion proteins were purified as described above. A maximum of 20 µg of the fusion protein was used per pull down (usually approximately 10-20 µl of the bead sludge) and the beads were washed 3 times in Triton-X-100 lysis buffer before being used in the pull down. One 25 cm³ flask of confluent HUVECs (1.5-2 million cells) was used per pull down, except in large-scale pull downs where 4x T75 flasks (~15 million cells) per pull down were used. Cells were lysed in Triton-X-100 lysis buffer, scraped and incubated for 30 min at 4°C for 30 min. Lysates were centrifuged at 13000 g for 30 min at 4°C and the soluble fraction retained for the pull down. 50 µl of the soluble lysate was kept as whole cell lysate to use in SDS-PAGE. The total protein content for each sample was determined using a Biorad protein assay, and an equal amount of protein from each sample was incubated with the bead-sludge for 1 h with rotation at 4 °C. The beads were briefly pulsed in a centrifuge and the unbound lysate discarded. The beads were washed 3 times in Triton-X-100 lysis buffer, and resuspended in 30 µl of 6X sample buffer. The beads were then boiled for 5 min before loading onto SDS-PAGE. The entire 30 µl of the sample was loaded per lane.

2.2.3.11 Immunoprecipitation (IP)

5 µg of antibody was used per IP. Mouse monoclonal (IgG₁) and rabbit polyclonal antibodies were coupled to a 20 µl sludge of protein A or protein G sepharose respectively. One 25 cm³ tissue culture flask of confluent HUVECs was used per IP (1.5-2 million cells). Cells were lysed in 500 µl of NP40-lysis buffer, scraped into an eppendorf tube and rotated at 4 °C for 30 min. Lysates were centrifuged at 13000 g for 30 min at 4 °C and the supernatants retained. The insoluble pellet was either discarded or solubilised according to the sol/insol fractionation method (2.2.3.1). A 50 µl whole cell lysate sample of each of the soluble and insoluble fractions was retained for SDS-PAGE. The fractions were subject to a Biorad protein assay and an equal amount of protein for each sample was incubated with the sepharose/antibody sludge for 1 h at 4 °C with rotation. The samples were briefly pulsed in a micro-centrifuge and the unbound supernatant was either discarded or a 50 µl sample retained for SDS-PAGE. The bead-sludge was washed five times with NP40-lysis buffer, and then placed into 30 µl of 6X sample buffer. Beads were boiled for 5 min
and pulsed in a micro-centrifuge prior to the entire 30 µl sample being used in SDS-PAGE and western blotting. Equal protein amounts of each of the whole cell lysates and unbound sample were used in SDS-PAGE.

2.2.3.12 Permeability assays
HUVECs were cultured to confluency on Transwell filters (Costar). Monolayers that failed to form completely or began to overlap were disregarded. Growth medium (0.5 ml) was applied to the apical chamber and 1.5 ml to the lower chamber. Cells were starved for 2 h in the presence of 1% BSA in starvation medium. In each experiment, control monolayers were left unstimulated, and the permeability of the stimulated monolayers were compared to the permeability of the control monolayers. TNF-α or thrombin were applied apically to the cells and permeability monitored by FITC-dextran that was applied to the apical cell surface at a concentration of 0.1 mg/ml. FITC dextran was applied either 30 min before the stimulant, to allow the monolayers to equilibrate, or was applied at a set time during the TNF-α incubation (eg. the last h of a 24 h incubation). Samples of the basal medium (250 µl) were removed at time points from both the stimulated cells (eg. TNF-α) and the unstimulated cells (controls) and transferred into a black 96-well plate. To maintain a 1.5 ml volume in the lower chamber (to immerse the cells), the removed volume was replaced with 250 µl of starvation medium. Although this dilutes the subsequent readings taken from the same well, the readings from the control monolayers are also being diluted, so the ratio of control to stimulated is not altered. Fluorescence of the samples was determined using a Perkin Elmer Fluorimeter at an excitation wavelength of 485 nm and a detection wavelength of 535 nm. Each condition was done in triplicate per experiment (including the control) and the mean fluorescence was determined from these. Increased permeability was then plotted as a percentage of the control monolayers ± standard deviation.

2.2.4. Cell staining

2.2.4.1 Immunofluorescent staining
For ZO-1, VE-cadherin, β-catenin, myosin II, c-myc, GFP, β-galactosidase, ICAM-1 and F-actin staining, cells were fixed in 3.7% formaldehyde for 10 min at room temperature. Cells were washed for a minimum of 15 min in PBS with 2 changes and
permeabilised in 0.2% Triton-X-100 for 5 min at room temperature. For claudin-5, GEF-H1 and JAM-A, cells were fixed and permeabilised in cold 100% methanol for 30 min on ice. Cells were then placed in cold 100% acetone for 5 min. After permeabilisation, all cells were washed with PBS for a minimum of 15 min and blocked in 1% BSA in PBS for 30 min. Primary antibody was applied to the cells at between 4 an 20 µg/ml in 1% BSA/PBS for 1 h at room temperature. Cells were washed for a minimum of 15 min with PBS and incubated simultaneously with the secondary antibody (1/200 dilution) and TRITC-phalloidin (1/400 dilution) where applicable, for 1 h at room temperature. Cells were washed for a minimum of 15 min with PBS and mounted onto glass slides using Dako fluorescent mounting medium.

For occludin and β-actin staining, cells were pre-extracted in chilled 0.2% Triton-X-100 pre-extraction buffer for 2 min on ice. Cells were fixed and permeabilised in 95% ethanol for 30 min on ice. Cells were then placed in 100% acetone for 1 min on ice, the acetone removed and the cells air-dried at room temperature for a maximum of 2 min. Cells were washed for 15 min with PBS and blocked in 3% BSA/PBS for at least 2 h. Mouse or rabbit anti-occludin antibodies (1/50 dilution) was applied to the cells in the presence of 3% BSA/PBS overnight at 4°C. Cells were washed in PBS for at least 15 min, and incubated with mouse anti-β-actin antibody (cy3-conjugated) in 3% BSA/PBS for 1 h at room temperature. Cells were washed, incubated with a secondary anti-mouse IgG antibody and mounted as described above.

2.2.4.2 Confocal microscopy

A Zeiss Confocal laser scanning microscope was used with LSM 510 software and an LSM 510 (Zeiss) mounted over an affinity-corrected Axioplan microscope (Zeiss) fitted with a x10 eyepiece, using a x 40 1.3 NA oil immersion objective. Image files were collected as a matrix of 1024 x1024 pixels describing the average of 8 frames scanned at 0.062 Hz where FITC, TRITC and Cy5 were excited at 488 nm, 543 nm and 633 nm and visualised with a 540 ± 25, 608 ± 32, and 690 ± 30 nm band pass filter, respectively. The channels of interchannel crosstalk were insignificant, as previously determined by Dr Alan Entwistle (Wojciak-Stothard et al., 1998).

2.2.4.3 Time lapse microscopy
HUVECs were seaded on to fibronectin-coated 35 mm dishes and grown to confluency. A heated stage was used to maintain the temperature between 30 and 37 °C. Cell images were collected with a KPM1E/K-S10 CCD camera (Hitachi Denshi, Japan), a Zeiss Axiovert 135 microscope and a 10 X water immersion objective every 10 min for 24 hours using Tempus software (Kinetic Imaging Ltd, Liverpool, UK). The movie was created using ImageJ software (http://rsb.info.nih.gov/ij/).

2.2.5 DNA methods

2.2.5.1 Designing cDNA primers
Primers were designed for use in PCR to obtain the N-terminus (nOcc) and C-terminus (cOcc) of occludin. Primers were designed according to the rules that 1) the 5' primer is written the same as the mRNA; 2) The 3' primer is written in the reverse and complementary to the mRNA; 3) The restriction enzyme sites are written as they are (Biolaboratories book); 4) The translation start codon (ATG) is removed (as there is one in the pGEX vector); 5) Addition of a stop codon into the 3' primer is optional, and is inserted between the restriction enzyme recognition site and the insert primer sequence in the reverse and complementary to the mRNA (i.e. CTA); 6) The primer length should give an annealing temperature of 50-60 °C (C = 4 °C, G = 4 °C, T = 2 °C, A = 2 °C); 7) The 5' primers must be in frame; 8) The 5' and 3' restriction enzyme sites of the primers must be compatible with the chosen vector; 9) The insert must not contain internal restriction enzyme sites the same as those used for the primers.

nOcc Primers for GST-cOcc

5' Primer and BamHI cleavage site:
CGG//GATCCCGTCATCCAGGCCTCTTGAAAG

3' Primer and BamHI cleavage site:
CGG//GATCCCTAAGGAGAGGTCCATTTGTAG

cOcc Primers for GST-cOcc

5' Primer and BamHI cleavage site:
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CGG//GATCCTTGGAAAAACTCGAAGAAAGATG

3’ Primer and EcoRI cleavage site:

CGG//AATTCCTATGTTTTCTGTCTATCATAG

nOcc Primers for pGBT9-nOcc

5’ Primer and BamHI cleavage site:

CGG//GATCCGTCATCCAGGCCTCTTGAAAG

3’ Primer and PstI cleavage site

CGG//GATCCCTATGTTTTCTGTCTATCATAG

cOcc Primers for pGBT9-cOcc

5’ Primer and EcoRI cleavage site:

5’ CGG // AATTCGTGAAAAACTCGAAGAAAGATG

3’ Primer and BamHI cleavage site

3’ CGG//GATCCCTATGTTTTCTGTCTATCATAG

// indicates the restriction enzyme site; bold font indicates the stop codons.

2.2.5.2 Generation of GST-cOcc and GST-nOcc

For the GST-fusion protein constructs, a BamHI recognition site was engineered into the 5’ and 3’ primers for nOcc and a 5’ BamHI and 3’ EcoRI restriction site was engineered into the 5’ and 3’ primers for cOcc respectively. These primers were ordered from Genosys and used for PCR to obtain the cOcc and nOcc PCR products. Full length occludin cDNA in a Bluescript vector kindly provided by Tina Van Itallie was used as the template DNA. These PCR products were purified, digested and ligated into a pGEX-2T vector, which encodes GST and produces a GST-fusion protein when expressed in E. coli.

2.2.5.3 Generation of pGBT9-cOcc and pGBT9-nOcc
For the yeast-two-hybrid experiment, nOcc and cOcc were ligated into the pGBT9 vector. To do this, nOcc was obtained through PCR using a 5’ primer that contained a BamHI restriction enzyme site and a 3’ primer containing a PstI site. Additionally, cOcc was obtained using a 5’ primer that contained an EcoRI restriction enzyme site and a 3’ primer containing a BamHI site. These PCR products were purified and then individually digested and ligated into pGBT9.

2.2.5.4 Polymerase Chain Reaction (PCR)

Four PCR reaction mixtures were made each time PCR was performed. These include a control lacking template DNA; a control lacking primer 1; a control lacking primer 2 and a complete reaction mixture. The complete reaction mixture of a total volume of 95 μl consisted of 0.1 nmol of each primer (2 μl of 50 nmol/ml), 10 μl of 10 X Vent buffer (Thermopol), 10 ng of template DNA (usually 1 μl of a 1:10 dilution from a mini-prep) and 200 μM of dNTPs was prepared and overlayed with mineral oil. This was subjected to a hot start of 94 °C for 1 min and then 80 °C for 30 min to ensure complete denaturation before Vent polymerase was added. This was added as 5 μl of a 1:10 dilution.

2.2.5.5 Ethanol precipitation of DNA

To ethanol precipitate DNA, 3 M Sodium acetate (pH 5.1) (1/10th of the volume of the DNA mixture) was added, followed by 100% cold ethanol to 2.5 times the volume. This was placed at −70°C for 20 min or at −20°C overnight, followed by centrifugation at 13000 g for 15 min at 4°C. The supernatant was decanted off and the pellet washed in 200 μl of 70% ice-cold ethanol. This was centrifuged for 5 min, at 4°C and 13000 g. Again, the supernatant was decanted off and the pellet was dried in a speed-vac for 2 min or until the pellet became white. This was redissolved in ddH2O or TE.

2.2.5.6 Transforming DNA into E. coli

Between 10 and 50 ng of plasmid DNA or 5 μl of the ligation mixture was used per transformation. Competent bacterial cells (BL-21 or XL-1 blue) were defrosted on ice, gently mixed and 100 μl were aliquoted into 15 ml polypropylene falcon tubes. β-mercaptoethanol (1.7 μl) was added to each tube, the tubes were swirled and
incubated on ice for 10 min. The DNA was added to each aliquot and incubated on ice for a further 30 min. The tubes were heat-pulsed at 42 °C for 45 seconds and then incubated on ice for 2 min, before the addition of 0.9 ml L-broth (no antibiotics). The tubes were incubated for 1 h at 37 °C with shaking, the cells pelleted and resuspended in 100 μl of L-broth and spread onto antibiotic plates (usually containing ampicillin, but this depended upon the vector). The plates were incubated overnight at 37 °C.

2.2.5.7 Restriction digestion and Cip treatment
For DNA digestion, usually 5 μg of DNA (ethanol precipitated if the product of PCR) was used per reaction. Between 3 and 5 units of the restriction enzyme was used per μg of DNA and the enzymes were used at a maximum of 10% of the total reaction volume. BamHI, EcoRI, XhoI, Pst-I were obtained from New England Biolabs (NEB) and used with the appropriate buffer recommended by NEB. The restriction digest was performed at 37 °C for 2 h. In order to prevent vector re-ligation, 1 μl of calf-intestinal phosphatase (Cip) was added to the digestion mixture for the final 15 - 30 min of the digestion. A sample of the digestion reaction (or all of it if a ligation was to be done) was electrophoresed on a 1% agarose gel.

2.2.5.8 Agarose gel electrophoresis
Agarose gels (1%) were prepared by dilution of 0.5 g of Ultrapure agarose into 50 ml of 1X TAE buffer. This was dissolved in a microwave oven and allowed to cool to 50 °C prior to the addition of 0.5 μg/ml ethidium bromide. Ethidium bromide intercalates between the DNA and fluoresces under U.V. light. The gel was poured into a gel tank, a comb inserted to create wells for loading the DNA and allowed to set. The gel was immersed in 1X TAE buffer and samples loaded in the presence of 6 X DNA loading buffer. Two sets of DNA markers (Roche) were loaded into two of the wells (10 μl of each). The DNA was separated by 8-10 V/cm for between 30 min and 1 h and the bands were visualised using 254 nm short wave U.V. light.

2.2.5.9 DNA purification from agarose gels
The DNA bands were visualised over a U.V. box (254 nm) and excised from the gel with a sterile surgical blade. The DNA was purified using a Qiaex II gel extraction kit (Qiagen). The excised gel was incubated with three times its volume in sodium
iodide and 10 μl of glass milk per 1 μl of DNA (usually 10 μl) at 50 °C for 10 min or until the gel had dissolved. The mixture was vortexed every 2 min during this incubation to keep the glass beads in suspension. The sample was pelleted and the supernatant removed and discarded. The pellet was washed twice with buffer QX1 and twice with buffer PE, air-dried (speed-vac), but not over-dried, and resuspended in 20 μl dd H₂O. Usually, to check the purification, 3 μl of the DNA was run on a 1% agarose gel, to determine the amount of DNA present and how much to use for a DNA ligation.

2.2.5.10 DNA ligation
Vector-insert DNA ligations were performed overnight at 16°C. For sticky-end ligations, a 5-fold excess of insert to vector was used, whereas for blunt-ended ligations, a 10-20 fold excess of insert to vector was used. T4 DNA ligase was used at 200 units per ligation reaction in the recommended NEB ligation buffer. Usually, 5 μl of this ligation was used for a bacterial transformation, followed by plasmid purification (mini-prep) and sequencing and/or restriction digestion to ensure the ligation has been successful.

2.2.5.11 DNA sequencing
The ABI Rhodamine DyeTerminator kit was used to prepare samples for sequencing. Usually, 200-500 ng of double stranded DNA (11 μl from a mini-prep) or 30-90 ng of DNA as a PCR product was used per reaction. A 20 μl reaction mixture containing the DNA, 8.0 μl of terminator ready reaction mix and 3.2 pmol of primer was prepared and overlayed with mineral oil. The mixture was exposed to a rapid thermal ramp to 96 °C for 30 sec (denaturing); a rapid thermal ramp to 50 °C for 15 sec (annealing) and a further thermal ramp to 60 °C for 4 min (extension). This cycle was repeated a total of 25 times. The reactions were then purified using a DyeEx 2.0 Spin Kit (Qiagen) and dried using a speed vac for 20-30 min. Samples were then analysed in-house on an ABI sequencer (run by Athena Nikitopoulou). Alternatively, purified DNA (10 pM) was dried in a speed-vac and sent to MWG-Biotechnology for sequencing.

2.2.5.12 DNA purification from E. coli (mini/maxi preps)
For smaller-scale DNA purifications, Qiagen QIAprep mini-prep kits were used. These yield up to 10 μg of plasmid DNA. Single bacterial colonies were picked from the selection plates and inoculated into 5 ml of LB containing ampicillin (or other antibiotics depending on the plasmid). These were incubated overnight at 37 °C with shaking. Of these, 2 ml of each overnight culture was centrifuged at 10000 g for 30 sec and the supernatant was removed by aspiration. Cell resuspension solution (200 μl) was added and the pellet resuspended prior to the addition of 250 μl of cell lysis solution. The tubes were gently inverted 10 times and 250 μl of neutralisation solution was added to stop the reaction. The cell debris was pelleted for 5 min at 10000 g and the supernatant was applied to a spin filter. Quantum prep matrix was added (200 μl) and mixed and the filters were placed into an eppendorf tube and centrifuged for 30 sec at 10000 g. The filtrate was removed and 500 μl of wash buffer was added to the filter. This was centrifuged for a further 30 sec at 10000 g. The filtrate was discarded and the washing step repeated, followed by a full 2 min centrifugation at 10000 g. The filtrate was discarded and the DNA was eluted into a fresh eppendorf tube by the addition of 50 μl of ddH2O to the filter and a 1 min centrifugation at 10000 g.

For larger DNA purifications, Qiagen maxi-preps were used. Maxi-preps yield up to 700 μg of DNA. For these, a single colony was picked from a selection plate and inoculated into 100 ml of L-broth containing ampicillin (or an alternative antibiotic depending on the plasmid) and was incubated at 37 °C overnight with shaking. The bacteria were transferred into 50-ml falcon tubes and harvested by centrifugation at 3000 rpm in a Beckman centrifuge (J series 4.2 rotor) for 20 min. The bacteria were resuspended in 10 ml of buffer P1, followed by the addition of 10 ml of buffer P2. The solution was gently mixed by inversion 4-6 times and incubated at room temperature for 5 min. Chilled buffer P3 was added (10 ml) and the solution was mixed by gentle inversion 4-6 times before incubation on ice for 20 min. The cell debris was pelleted by centrifugation at 10000 rpm in a Sorvall RC5C centrifuge (rotor SS-34) for 30 min. The supernatant containing the plasmid DNA was promptly removed and applied to a Qiagen-tip 500 column that had been pre-equilibrated with 10 ml of buffer QBT. The column was washed by addition of 2 x 30 ml of buffer QC and the DNA was eluted using 15 ml of buffer QF. DNA was precipitated by addition
of 10.5 ml of room-temperature iopropanol. The solution was mixed and centrifuged in 50-ml falcon tubes at 3000 rpm in a Beckman centrifuge (J series 4.2 rotor) for 30 min. The supernatant was decanted off and the DNA pellet was washed with 5 ml of room-temperature 70% ethanol, prior to centrifugation at 3000 rpm in a Beckman centrifuge (J series 4.2 rotor) for 20 min. The supernatant was gently decanted off and the pellet transferred into an eppendorf tube using 1 ml of 70% ethanol. This was pelleted by a brief centrifugation and the ethanol was removed. The pellet was dried in a speed-vac until it became white (but not overdried). The pellet was redissolved in 250 µl of TE.

2.2.5.13 Obtaining DNA concentrations

To obtain the concentration of DNA from the Maxi-preps, the absorbance was read in a Biorad SmartSpec spectrophotometer at 260 nm using a quartz cuvette. The DNA was usually diluted 1/200 into 1 ml of ddH₂O. An A₂₆₀ of 1 is equivalent to 50 µg of DNA. Therefore;

\[
[\text{DNA}] (\mu\text{g/ml}) = A_{260} \times \text{dilution factor (usually 200)} \times 50 \mu\text{g/ml}. 
\]

2.2.6 Yeast procedures

2.2.6.1 Phenol/chloroform plasmid DNA extraction and ethanol precipitation

One large yeast colony was picked into 200 µl of yeast MP buffer (100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 0.1% SDS). Glass beads were added until just below the meniscus and vortexed vigorously for 1 min. Phenol (400 µl) was added mixed, and centrifuged for 2 min at maximum speed. The top layer was transferred into a new tube. MP buffer (160 µl) was added again to the beads, mixed, centrifuged and the top layer combined with the first one. Chloroform:isoamylalcohol (24:1) (400 µl) was added to this, centrifuged and the top layer transferred into a fresh tube. Sodium azide (40 µl of 3 M) was added and then 1 ml of cold 99% ethanol. This was pelleted in a centrifuge for 5 min at 4°C. The pellet should be red in appearance. The pellet was washed in 200 µl 70% cold ethanol for 15 min at -20°C, centrifuged for 5 min at maximum speed and the pellet resuspended in 50 µl 1XTE.
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2.2.6.2 Yeast glycerol stocks
Several well-growing pink yeast colonies were scraped into a 1 ml solution of 50 μl of 40% dextrose, 100 μl of 10X Trp amino acid base, 330 μl of 75% sterile glycerol (to a final 25%), and 520 μl of SD media (without dextrose). The suspension was vortexed and stored at -80 °C.

2.2.6.3 Storing yeast plates
Yeast grown on selection plates that needed to be kept were sealed with parafilm and kept at 4 °C for up to 2 months. After this time they were re-streaked or transferred by blotting with filter paper onto fresh plates.

2.2.6.4 Yeast transformation #1
HF7c yeast was streaked from glycerol stocks onto a YPD media plate and incubated for 2 - 3 days at 30°C. Lots of colonies were scraped into a microcentrifuge tube containing ddH₂O until it became cloudy and red. The OD₆₀₀ was measured (A₆₀₀ = 2x10⁷ cells). The microcentrifuge tube was centrifuged at 1000 x g for 5 min, supernatant removed and cells resuspended in 1x TE/LiAc (made from a sterile 10X stock of TE and 10X stock of LiAc adjusted to pH 7.5 with dilute acetic acid). Cells were centrifuged at 1000 x g for 5 min and resuspended to 1-2x10⁹/ml TE/LiAc. Carrier DNA was boiled for 10-15 min and cooled on ice to ensure it became single stranded. One transformation contained 50 μl of the yeast suspension, 1 μg bait DNA, 5 μl of 10 mg/ml carrier DNA and 300 μl of 40% sterile PEG in TE/LiAc. The suspension was incubated for 30 min at 30°C with shaking. 40 μl 100% DMSO was added and the tube inverted. Cells were heat shocked at 42°C for 15 min, centrifuged at 2000 g for 3 min, the supernatant removed and cells washed in 1X TE. Cells were centrifuged again at 1000 g for 5 min, resuspended in 100 μl 1x TE and plated onto a -Trp/SD media selection plate. The plate was placed at 30°C for approximately 3 days.

2.2.6.5 Yeast transformation #2
Yeast cells transformed with bait vector and growing on a -Trp/SD selection plate were inoculated into liquid SD medium plus -Trp amino acid base and grown overnight to 1-2x10⁷ cells/ml (A₆₀₀ 1 = ~ 2x10⁷ cells). Cells were diluted into fresh
YPD medium and regrown to 1-2 x10⁷ cells per ml. Cells were then centrifuged at 1,000 x g for 5 min, washed in sterile H₂O, transferred to 1.5 ml microcentrifuge tubes and re-pelleted. The yeast was then washed with 1X TE/LiAc and resuspended to 2x10⁹ cells/ml. Carrier DNA was boiled for 10-15 min and immediately placed on ice. 50 μl of the yeast suspension was mixed with 1 μg of transforming DNA, and 50 μg (5 μl of 10 mg/ml) carrier DNA in a microcentrifuge tube. 300 μl of sterile 40% PEG-4000 in 1X TE/LiAc was added and the suspension incubated at 30°C for 30 min. 40 μl DMSO was added and cells heat-shocked for 15 min at 42°C. The yeast was then centrifuged for 5 sec at 1000 x g, resuspended in 1 ml 1xTE and a 1/200 dilution plated onto a -Trp/-Leu/SD selection plate before the remaining suspension was plated to -Trp/-Leu/-His/SD selection plate. Plates were placed at 30°C for 3-5 days.

NB. This protocol is for one transformation and was scaled up to 10 or 100 transformations per day as required for screening the library.

2.2.6.6 X-gal screen

Fresh yeast colonies were grown at 30 °C for 2-4 days. A single circular filter paper was placed into a clean 150-mm plate and soaked in 2.5 ml Z-buffer/X-gal solution containing 100 ml Z-buffer, 0.27 ml β-mercaptoethanol and 1.67 ml X-gal (from a stock of 20 mg/ml X-gal in N, N-dimethylformamide). Using forceps, a clean dry filter paper was placed over the surface of the fresh yeast colonies and rubbed gently to ensure the colonies clung to the filter. Holes were created in the filter paper using a needle so that the orientation of the ‘lift’ could be recalled later. The filter paper was gently peeled back and placed into a pool of liquid nitrogen for 10 seconds with the colonies facing upwards. The filter was then allowed to thaw completely before being placed, colonies facing upwards, onto the pre-soaked filter. The filter was incubated overnight at 30 °C. Colonies that had turned blue on the filter paper were identified on the original plate using the orientating holes.

2.2.6.7 Rejection of bait vector

One yeast colony was inoculated into 5 ml liquid SD medium containing -Leu amino acid base. As Trp is present in this medium, the yeasts no longer require the selective
advantage of the trp3-containing plasmid and are inclined to expel it. This suspension was incubated at 30 °C for 3 days, diluted 1/1000 into dddH₂O and 10 μl plated onto –Leu/SD selection plates. Plates were placed at 30 °C for approximately 3 days. One colony was picked from each plate and re-streaked both to a –Leu/SD selection plate and to a –Leu/-Trp plate. Plates were placed at 30 °C for approximately 3 days and then compared to identify yeasts that have thrown out the bait (yeasts without the bait vector can only grow in the presence of Trp). If unsuccessful, this procedure was repeated and the yeasts incubated for a longer time in the presence of Trp to encourage the bait plasmid to be expelled.

2.2.6.8 Isolation of plasmid DNA from yeast and phenol/chloroform purification

The pACT2 vector in HF7c yeast containing the library clones of interest were isolated as follows: One large yeast colony was picked and placed into 200 μl of yeast MP buffer. Glass beads were added up to just below the level of the liquid and vortexed vigorously for 1 min. Phenol was added (400 μl), mixed and the solution was centrifuged for 2 min at 10000 g. The top layer was transferred to a fresh tube. MP buffer (160 μl) was added to the glass beads, mixed and centrifuged for 2 min at 10000 g. The top layer was removed and combined with the first layer. The removed layers were mixed with 400 μl of chloroform:isoamylalcohol (24:1), centrifuged 2 min at 10000 g and the top layer transferred into a fresh tube. Sodium azide (40 μl of 3 M) was added, followed by 1 ml of cold 100% ethanol. The DNA was pelleted in a centrifuge at 4°C for 2 min at 10000 g. The pellet was washed with 200 ul of 70% ethanol for 15 min at –20 °C, pelleted for 5 min and the supernatant removed. The DNA pellet was resuspended in 50 μl of TE.

2.2.6.9 Plasmid DNA transformation into E. coli by electroporation

Plasmid DNA (pACT2) purified from the HF7c yeast was transformed into E. coli for amplification to enable DNA sequencing. To do this, sterile electroporation cuvettes (0.1 cm gap) were cooled on ice. Electroporation competent cells (XL1-blue MRF’ Electroporation-Competent Cells; Stratagene) were thawed on ice, mixed and aliquoted (40 μl) to pre-chilled eppendorf tubes. 2-4 μl of plasmid DNA (10 pg) that had been purified from the yeast was added to the cells and the cell-DNA mix was
transferred into the chilled electroporation cuvettes. The cuvette was tapped to settle the fluid to the bottom, and the cuvette was dried and placed into the electroporation chamber. The samples were pulsed once using a Biorad electroporator set at 1700 V (17 kV.cm), a resistance of 200 Ω and a capacitance of 25 μF. The samples were quickly resuspended in 960 μl of L-broth that was warmed to 37 °C and transferred into 14 ml BD falcon polypropylene round-bottom tubes (BD Biosciences). The tubes were incubated at 37 °C for 1 h with shaking at 225-250 rpm. The transformation was plated on to L-Broth agar plates containing ampicillin and incubated overnight at 37 °C.

2.2.6.10 CPRG colour change assay

One well-growing SFY526 yeast colony was picked and grown in 1 ml SD/-Trp/-Leu medium overnight at 30 °C with shaking. 4 ml of YPD medium was added to the cells and they were grown for an additional 3 h or until the OD₆₀₀ reached 0.5-0.8. 1.5 ml of this culture was centrifuged at 10000 g for 30 sec, the supernatant removed and the cells re suspended in 1 ml of buffer 1. Cells were vortexed, pulsed and resuspended in 300 μl of buffer 1. Of this, 100 μl was used for the CPRG assay. The cells were lysed by freeze-thawing (37°C water bath and liquid nitrogen) three times. 0.7 ml of CRPG in buffer 1 (i.e. Buffer 2) was added and the suspension vortexed. This was the starting point. As soon as a colour change occurred (yellow to red), the reaction was stopped using 0.5 ml of 3 mM ZnCl₂ and the A₅₇₈ recorded.
3 TNF-α-Induced Changes in the Morphology and Permeability of HUVECs

3.1 Introduction

TNF-α is a pro-inflammatory cytokine that is released upon pathogenic infection and among many other functions, activates endothelial cells to express adhesion molecules that are required for recruitment of immune cells to the site of infection. It is known to activate several signalling proteins within endothelial cells such as kinases, lipases, phosphatases and reactive oxygen species (MacEwan, 2002) (Chapter 1). Some effects of TNF-α in endothelial cell tight junctions and permeability have been described, although most of these data come from bovine endothelial cells. TNF-α has been reported to increase endothelial cell permeability. Increased macromolecular permeability has been reported by 1 h of TNF-α exposure to bovine aortic endothelial cells (Brett et al., 1989), by 2 h in bovine pulmonary arterial endothelial cells (BPAECs) (Goldblum et al., 1990 and 1993) and by 4 h in bovine pulmonary microvessel endothelial cells (BPMVEC) (Ferro et al., 1997 and 2000), although in another report, no increase in albumin permeability was observed at 6 hours in these cells (Ishii et al., 1992). In BPAECs, TNF-α also induces a time and dose dependent decrease in TER, which begins after 4-5 h of exposure, is maximal at 10 h, but persists for 24-48 h (Petrache et al., 2001). There is one report of a rapid permeability increase in HUVECs in response to TNF-α, which occurred at 15 min and was measured using 125I-albumin (Ferrero et al., 2001). There is also another report of TNF-α-induced permeability to FITC-dextran at 1 h in HUVECs (Nwariaku et al., 2002). Cytoskeletal reorganisation is known to be involved in increased endothelial permeability. The formation of actin stress fibres, actomyosin interaction and cellular contractility have been shown to result in intercellular gap formation and loss of barrier function in response to vasoactive agents such as histamine and thrombin (Ehringer et al., 1996; van Hinsbergh, 1997; van Nieuw Amerongen et al., 1998).

There are several reports of cytoskeletal rearrangements that correlate with the formation of intercellular gaps and increased permeability induced by TNF-α in endothelial cells (Brett et al., 1989; Camussi et al., 1991; Goldblum et al., 1993). Goldblum et al. described a thinning of cytoplasmic F-actin filaments, an increase in peripheral F-actin and intercellular gap formation.
of cytoplasmic F-actin filaments, an increase in peripheral F-actin and intercellular gap formation in BPAEC (Goldblum et al., 1993). Others have reported a loss of peripheral F-actin, an increase in actin stress fibres, cell retraction and intercellular gap formation in BAECs (Brett et al., 1989). In HUVECs, TNF-α at 100 ng/ml induces an early response of membrane ruffling, filopodia and actin stress fibre formation (Wojciak-Stothard et al., 1998). These events in HUVECs have not yet been shown to be correlated with, or be responsible for, increased permeability. In addition, the prolonged effects of TNF-α on the actin cytoskeleton and permeability have not been fully characterised.

The inter-endothelial barrier to macromolecules, solutes and blood cells is formed and regulated by tight junctions. Changes in paracellular permeability have been attributed to signalling to tight junction proteins, which, for example, results in junctional protein relocalization (Nwariaku et al., 2002) or degradation (Wachtel et al., 1999). The effects of TNF-α on junctional proteins in endothelial cells has not been extensively studied. TNF-α has been reported to induce tyrosine phosphorylation of the adherens junction protein VE-cadherin, as well as a reduction in the association of this protein with the plasma membrane in endothelial cells (Petrache et al., 2003; Nwariaku et al., 2002; Wojciak-stothard et al., 1998). Platelet endothelial cell adhesion molecule-1 (PECAM) phosphorylation and redistribution has also been observed in response to TNF-α in HUVECs (Ferrero et al., 1996), which accompanied an increase in macromolecular permeability. In addition, a combination of TNF-α and IFN-γ partially reduces PECAM protein levels on the cell surface and lowers PECAM mRNA levels (Rival et al., 1996) as well as inducing JAM-A redistribution away from the tight junction without changing its overall cell-surface expression (Ozaki et al., 1999). These events are associated with a reduction in leukocyte transmigration, but macromolecular permeability was not assessed. Occludin protein levels have been reported to be down-regulated in response to TNF-α in astrocytes, although the functional relevance of these is not clear as no functional permeability barrier is formed in these cells (Wachtel et al., 2001). TNF-α has also been reported to reduce expression from the occludin promoter in HT-29/B6 intestinal cells (Mankertz et al., 2000, 2002). There is little evidence for effects of TNF-α on other endothelial tight junction proteins.
The aim of this chapter is to characterise the effects of TNF-α on HUVEC morphology and tight junction protein expression levels and localization. In addition, the effects of TNF-α on HUVEC F-actin organization and barrier dysfunction have been examined.
3.2 Results

3.2.1 TNF-α changes the morphology of confluent HUVECs

The effects of thrombin and histamine on HUVEC morphology has been well-characterised (Ehringer et al., 1996), whereas less is known about the morphological responses endothelial cells to TNF-α. To examine this, changes in HUVEC morphology during TNF-α stimulation were examined by time-lapse microscopy (supplementary CD). Phase-contrast stills from a time-lapse video are shown in Fig. 3.1. Individual control cells were rounded and formed a tight monolayer that had a 'honeycomb' or 'cobblestone'-like appearance. There were few gaps in the monolayer. After TNF-α stimulation (10 ng/ml) for 10 min, no detectable changes were observed compared to the control. In contrast, after 6 h of TNF-α stimulation, the cells had begun to elongate. By 24 h of TNF-α stimulation the monolayer had substantially altered. Individual cells were elongated, with an accompanying formation of gaps between some cells.
TNF-α changes the morphology of HUVEC monolayers

Confluent HUVECs were examined for changes in cell morphology in response to TNF-α. The phase contrast micrographs taken from a time-lapse video are shown for unstimulated cells and cells treated with 10 ng/ml TNF-α for 10 min, 6 h and 24 h. Cells were cultured on fibronectin-coated tissue culture plastic and starved for 2 h prior to stimulation. Arrow indicates elongated cells, arrowhead shows intercellular gaps. Bar = 40 μm.
Chapter 3

TNF-α-Induced Changes in the Morphology and Permeability of HUVECs

3.2.2 TNF-α induces stress fibre formation and changes ZO-1 localisation

Thrombin is known to induce rapid cytoskeletal changes and increased permeability in endothelial cells (Bogatcheva et al., 2002) and so the response of HUVECs to TNF-α was compared to the response of HUVECs to thrombin. Confluent HUVECs were stimulated with TNF-α (10 ng/ml) for 10 min or 24 h or with thrombin (2 units/ml) for 10 min.

Confocal Z-stacks were merged to show the overall F-actin distribution within the cells. These showed a strong induction of cortical F-actin in thrombin treated cells, which was accompanied by the formation of many intercellular gaps (Fig 3.2). In contrast, TNF-α induced a very subtle increases in cortical F-actin at 10 min and the formation of tiny gaps between some adjacent cells (Fig. 3.2). Prolonged TNF-α exposure induced the formation of thick actin stress fibres that traversed the cells in the direction of the cell elongation.

Single confocal sections of cells co-stained for F-actin and ZO-1 were taken at the level of the tight junction to examine changes to tight junctions and the junctional associated F-actin (Fig. 3.3). In control cells the majority of F-actin was aligned in bundles parallel with cell-cell borders and thin lines of F-actin co-localised with ZO-1. Some fine F-actin fibres also traversed the cells. At some cell corners between three or more cells bright ‘knots’ or ‘stars’ of F-actin were present between the cells and in these regions ZO-1 staining was fragmented or absent. Elsewhere, ZO-1 formed a continuous line at the cell-cell junction, with some faint staining within the cell-body. TNF-α (100 ng/ml) caused thickening of the F-actin at the cell periphery by 1 h and increased the number of bright F-actin ‘knots’. Sometimes small gaps were observed between cells. ZO-1 was retained at tight junctions, but was distinctly more disjointed, particularly at F-actin ‘knots’. After 4 h of stimulation, the cells had fewer F-actin knots, but still had thickened cortical F-actin and discontinuous ZO-1. By 6 h, a stronger and more obvious response was observed compared to earlier times. The cells had begun to elongate and some cells overlapped each other. There was less peripheral F-actin and F-actin stress fibres traversed the cells. ZO-1 was retained at junctions, but was fragmented in places. By 10 h, F-actin organisation was similar to 6 h. ZO-1 was disjointed and lost where areas of cell-cell contact had been abolished. There was a slight increase in cytoplasmic ZO-1 staining compared to control cells. Exposure to TNF-α for 24 and 30 h caused a further increase in the number of actin stress fibres that traversed the cells and the cells alignment. Small gaps were still present between cells and some cells had F-actin rich
processes linking them to neighbouring cells. ZO-1 remaining at the cell periphery localized in these processes, but was absent where there was no cell-cell contact. There was also some increase in perinuclear ZO-1 compared to control cells (Fig. 3.3 inserts).

Overall these observations indicate that the morphology of TNF-α–stimulated cells changes over 24 h. The initial response occurs up to one hour and involves thickening of cortical F-actin and alterations to tight junctions as indicated. From 6 and 30 h cells progressively elongate and re-align, stress fibres form and ZO-1 becomes disjointed.
Chapter 3  

**TNF-α-Induced Changes in the Morphology and Permeability of HUVECs**

**TNF-α (10 ng/ml)**
**Thrombin 2 units/ml**

---

**Figure 3.2**

**TNF-α alters cell morphology and F-actin in HUVECs**

Confocal micrographs are shown of confluent HUVECs. Cells were starved for 2 h and either left unstimulated or treated with 10 ng/ml TNF-α for 10 min or 24 h or with 2 units/ml thrombin for 10 min. Cells were fixed and stained for F-actin and 11 confocal Z-stacks were taken over a depth of 4.0 μm and with an interval of 0.4 μm between each stack. The stacks were projected into one image. White arrow, stress fibres; white arrowhead, intercellular gaps; red arrow, an area of thickened cortical F-actin; bar = 20 μm.
Figure 3.3
TNF-α (100 ng/ml)

F-actin | ZO-1 | Merged

Control

1 h

4 h

6 h
TNF-α induces stress fibre formation and changes the organization of ZO-1

Confocal micrographs are shown of confluent HUVECs. Cells were starved for 2 h and either left unstimulated or treated with 100 ng/ml TNF-α for 1 h, 4 h, 6 h, 10 h, 24 h or 30 h. Cells were fixed and stained for F-actin (red) and ZO-1 (green). Insets (merged images) show lower magnification images. Con, no TNF-α; white arrow, stress fibres; white arrowhead, cortical F-actin thickening; yellow arrow, F-actin ‘knots’; yellow arrowheads, disjointed ZO-1; red arrow, actin processes; red arrowhead, an area of overlapping cells; bar = 20 μm.
3.2.3 The response of HUVECs to TNF-α depends on the concentration applied
To determine whether TNF-α-induced changes to the actin cytoskeleton and ZO-1 were affected by dose, different concentrations of TNF-α were applied to the cells for 10 min and for 24 h (Fig. 3.4). At 10 min, all concentrations of TNF-α tested induced slight disruption of ZO-1, but the strongest response was observed with 10 and 100 ng/ml TNF-α (Fig. 3.4A). At these higher doses, the cells were rounder than the control cells and more knots of F-actin were seen than in the control cells. Overall, there was a slight increase in cortical actin and disjointed ZO-1 with increasing doses of TNF-α, but these effects were very subtle. At 24 h, even the lower concentrations of 0.1 and 1.0 ng/ml caused HUVECs to elongate and form actin stress fibres across the cells (Fig. 3.4B). These doses, however, did not induce the formation of gaps within the monolayer, nor did they cause ZO-1 to become disjointed. Higher doses of 10 and 100 ng/ml also induced actin stress fibre formation, as well as some gaps between the cells. ZO-1 localization at cell-cell junctions was increasingly disrupted at higher concentrations of TNF-α, and there was a slight increase in cytoplasmic ZO-1. It also decreased at tri-cellular corners and areas where elongated cells met at poles. ICAM-1 protein levels were also examined as a measure of endothelial cell activation by TNF-α (Fig. 3.5). ICAM-1 protein was induced even with the lowest dose tested. More ICAM-1 protein was induced with increasing concentrations of TNF-α and a downward shift of the bands also accompanied the increasing amounts of TNF-α. This shift might be due to different post-translational modification of the protein.
Figure 3.4 Actin

A) Control

TNF-α-Induced Changes in the Morphology and Permeability of HUVECs

0.1 ng/ml

1 ng/ml

10 ng/ml

100 ng/ml
Figure 3.4

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<th>Merge</th>
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Figure 3.4

Dose response of early and Late TNF-α- induced changes in ZO-1 and F-actin localization

Confluent HUVECs were starved for 2 h and either left unstimulated or exposed to 0.1, 1, 10 or 100 ng/ml TNF-α for 10 min (A) or 24 h (B). Cells were fixed and stained for F-actin (red) and ZO-1 (green). White arrow, F-actin ‘knots’; white arrowhead, disjointed ZO-1; red arrow, gaps; Bar = 20 μm.
Figure 3.5  
**ICAM-1 protein is induced in response to TNF-α**

Confluent HUVECs were starved for 2 h and either left untreated or were stimulated with 0.1, 1, 10, or 100 ng/ml of TNF-α for 6 h. Cells were lysed and the NP40-soluble fraction obtained. Proteins were separated by SDS-PAGE and ICAM-1 was detected using western blotting (Upper panel). Equal total protein loading in each lane was demonstrated by re-probing the blot with an anti-ERK1 antibody (lower panel). Control: no TNF-α stimulation.
3.2.4 TNF-α increases HUVEC permeability to FITC-dextran

The formation of intercellular gaps and alterations in ZO-1 localisation implied that the barrier function of the endothelial monolayer might be compromised by TNF-α. To test this, a paracellular permeability assay was employed using a fluorescent marker of 40 kDa. This was present throughout the TNF-α incubation to give a measure of the continuous changes induced by TNF-α. No significant increase in permeability was detected up to 1 h after TNF-α stimulation (Fig. 3.6A). 8 to 24 h exposure to TNF-α, however, did result in a highly significant increase in permeability. This did not change significantly past 24 h of TNF-α exposure. The maximal increase observed over the control was ~2.20-fold. Thrombin is known to induce a prolonged increase in endothelial monolayer permeability compared with histamine (van Nieuw Ameringen et al., 1998; van Hinsburgh, 2002), and so was used as a positive control experiment. Thrombin-induced permeability was measured under the same conditions as the TNF-α permeability test (Fig. 3.6B). Thrombin induced an increase in HUVEC permeability to FITC-dextran beginning at 10 min, which peaked at 30 min. By 4 h the permeability had returned to basal levels (Fig. 3.6B). This time course is consistent with reports in the literature (Lum et al., 1992; Drenckhahn and Ness, 1997). Interestingly, the maximum permeability observed at 30 min was ~4.3-fold more than the control monolayers. Thrombin therefore induces a much more rapid and more acute increase in permeability than TNF-α.

As the morphological response of the HUVECs to TNF-α depended upon the concentration applied, it was also likely that the paracellular permeability increase was dependent on the concentration of TNF-α used. Consistent with the previous observations, no significant increase in HUVEC permeability was observed up to 6 h at any of the doses tested (Fig. 3.7). However, at 20, 24, 28 and 30 h, both 10 ng/ml and 100 ng/ml TNF-α induced increased HUVEC permeability, whereas, in contrast to morphology, 0.1 ng/ml had no effect. The largest increase was with 100 ng/ml TNF-α, which gave a ~2.5-fold increase in permeability compared to control, was reached at 24 h and did not significantly changed up to 30 h.
Figure 3.6

A) TNF-α-Induced Changes in the Morphology and Permeability of HUVECs

For both control and TNF-α monolayers

Sample removed (250 μl) from lower chamber at time points

250 μl medium reapplied to retain 1.5 ml volume

B) Data used

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C) TNF-α

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<td>30</td>
<td>500</td>
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Figure 3.6
TNF-α-induced permeability is less rapid and more sustained than thrombin-induced permeability

(A) shows the method used for measuring paracellular permeability. HUVECs were seeded onto fibronectin-coated filters and cultured until confluent. Control cells were left unstimulated. The permeability of TNF-α treated monolayers was compared to the permeability of the control cells at each time point. FITC-dextran was added at a concentration of 0.1 mg/ml to the apical cell surface 30 min prior to addition of the stimulant. (B) shows the data collected for the graph in (C) and is shown as an example of how the graphs were created. The data shown is the mean fluorescence of three monolayers each for the control and TNF-α-treated cells. Cells were starved for 2 h prior to the addition of 100 ng/ml TNF-α for up to 30 h (C) or with 2 units/ml thrombin for up to 4 h (D). TNF-α-induced permeability is representative of 3 separate experiments. Permeability is shown as a percentage of the control monolayers ± SD. *P ≤ 0.05 compared with control, **P ≤ 0.01 compared with control.
Figure 3.7

Dose response of TNF-α-induced permeability

Confluent HUVECs were starved for 2 h and stimulated with 0.1, 10 or 100 ng/ml TNF-α for up to 30 h and permeability was measured using 40-kDa FITC-dextran. FITC-dextran was added at a concentration of 0.1 mg/ml to the apical cell surface 30 min prior to addition of the stimulant. Each point is the mean permeability of three monolayers. Permeability is shown as a percentage of the control ± SD. *P = ≤ 0.05 compared with control, **P = ≤ 0.001 compared with control.
3.2.5 TNF-α transiently increases HUVEC permeability at 10 min

Although TNF-α-induced changes to the actin cytoskeleton and to the adherens junction protein, VE-cadherin, have been previously reported to occur before 1 h (Wojciak-Stothard et al., 1998) and changes in endothelial morphology where observed here at 10 min (Fig. 3.2), macromolecular permeability has not been determined at these early times. The permeability of HUVECs to FITC-dextran was therefore measured after stimulation with 10 ng/ml TNF-α up to 60 min (Fig. 3.8A). A small increase in permeability was observed at 10 min. A second method was used to assess permeability at this time, whereby the FITC-dextran was added after precisely 10 min of TNF-α exposure, and the permeability was measured at time-points subsequent to this (Fig. 3.8B). This method was used in case an accumulation of FITC-dextran during the equilibrium period was masking an increase in permeability. No increase in permeability was detected. It is possible that HUVEC monolayers demonstrate size selectability towards macromolecules allowed to transmigrate between the cells. Indeed, there are reports that monolayer macromolecular permeability and transendothelial resistance are differentially regulated (McCarthy et al., 2000; Van Itallie et al., 2001). In order to test whether there is an increase in HUVEC permeability, undetectable using the 40-kDa FITC-dextran marker, a smaller, 4-kDa FITC-dextran marker was used to test permeability of HUVECs up to 30 h after the addition of TNF-α (10 ng/ml) (Fig. 3.9). No significant increase in permeability was detected up to 6 h, but between 8 and 30 h permeability increased significantly. This increase was smaller than that detected using 40-kDa FITC-dextran, which may reflect a greater ability of the 4-kDa FITC-dextran to permeate the control monolayers (i.e. higher background). As a small increase in permeability at 10 min was detected in some experiments, but not in the others, permeability was tested again at 10 min and 24 h in multiple separate experiments (six) and the data was pooled to make the graph in Fig. 3.10. In this experiment, a small, but significant increase in permeability was detected after 10 min of TNF-α stimulation, followed by a larger increase after 24 h. This indicates that there is a small increase in permeability at 10 min in response to TNF-α, but it is only just within the means of detection using FITC-dextran.
Figure 3.8

TNF-α transiently increases HUVEC permeability at 10 min

Permeability of confluent HUVEC monolayers cultured on Transwell filters was determined using 40-kDa FITC-dextran. Cells were starved for 2 h prior to the addition of 10 ng/ml TNF-α. FITC-dextran was added at a concentration of 0.1 mg/ml to the apical cell surface either 30 min prior to the addition of TNF-α (A) or after 10 min of TNF-α stimulation (B). Samples were removed from the basal chamber at the times indicated. Each time point is the mean of three monolayers. Permeability is shown as a percentage of the control monolayers ± SD. *$P=\leq0.05$ compared with control, **$P=\leq0.01$ compared with control.
Figure 3.9

**TNF-α increases HUVEC permeability to 4-kDa-FITC dextran**

Permeability was measured in HUVECs using 4-kDa FITC-dextran. Cells were cultured on Transwell filters and starved for 2 h prior to stimulation with 10 ng/ml TNF-α. FITC-dextran applied to the apical cell surface at the same time as the stimulant. Each time point is the mean of three monolayers. Permeability is shown as a percentage of the control monolayers ± SD. $^*P = \leq 0.05$, $^{**}P = \leq 0.01$ compared with control (no TNF-α).
TNF-α-Induced Changes in the Morphology and Permeability of HUVECs

Fig. 3.10

TNF-α increases permeability in HUVECs

Graph represents pooled data from 6 different experiments, showing the mean of six independent monolayers per time point. The permeability of confluent HUVEC monolayers cultured on Transwell filters was determined using 40-kDa FITC-dextran. Cells were starved for 2 h prior to the addition of 10 ng/ml TNF-α for up to 24 h. Control cells were left unstimulated. FITC-dextran was applied to the apical chamber 30 min prior to the addition of TNF-α and samples of the basal medium were taken for fluorescent measurements at the time points indicated. Permeability is shown as a percentage of control ± SD. * = p ≤ 0.05 compared to control; ** = p ≤ 0.01 compared to control.
3.2.6 TNF-α-induces some cell death

TNF-α has been shown to induce apoptosis in HUVECs, but only in the presence of cyclohexamide, an inhibitor of protein synthesis (Zen and Karson et al., 1999; Pohlman et al., 1989; Polunovsky et al., 1994). To ensure that the increases in permeability were not due to an increase in cell death, the number of dead, detached cells were counted as a measure of cell death using cells treated with TNF-α for 24 h or left in starvation medium only for 24 h (Fig. 3.11A). Both cells at passage 2 and passage 3 were examined. The number of dead, detached cells varied from culture to culture, but overall, there was a slight increase in the number of detached cells where TNF-α had been applied. This was not statistically significant. In addition, the total amount of protein of unstimulated cells, and those that were exposed to TNF-α for 24 h was determined using a Biorad-protein assay (Fig. 3.11B). There was a slight decrease in the amount of protein in those monolayers that had been treated with TNF-α and this was found to be statistically significant. This decrease could be caused by loss of cells or suppression of protein synthesis by TNF-α.
Figure 3.11
TNF-α induces some cell death

Cells were starved for 2 h and either left untreated, or were stimulated with 10 ng/ml TNF-α for 24 h. To determine the number of non-viable detached cells (A), a sample of medium was removed from the cells and incubated with Trypan blue for 2 min. Cells that had absorbed the dye were counted using a hemacytometer. The mean of nine monolayers is shown for each condition. To determine the total protein content (B), cells were lysed and the insoluble fraction pelleted by centrifugation. A sample of the soluble fraction was used in a Bio-rad protein assay. The mean of eight HUVEC monolayers is shown for each condition ± SD. **P = ≤ 0.01 compared with control.
3.2.7 TNF-α-induced changes to HUVEC morphology, F-actin and ZO-1 are not reversible

Thrombin is able to induce a rapid and acute increase in HUVEC permeability (Fig. 3.6), but the cells are also able to fully recover and re-establish their inter-endothelial barrier (Ehringer et al., 1996; Kouklis et al., 2004). To examine whether HUVECs can recover from TNF-α-induced changes, cells were incubated with TNF-α for 24 h in low serum, washed and allowed to recover for 24 h in starvation medium (Fig. 3.12A). There was no observable difference between TNF-α-treated cells that were washed or unwashed, indicating that TNF-α induces changes to the HUVEC monolayer that are not reversible in 24 h. To ensure that the lack of recovery was not due to the length of time in low serum starvation medium (1% FCS for a total of 50 h), this experiment was repeated in full growth medium (2% serum plus growth factors and supplements) (Fig. 3.12B). Under these conditions, the morphological response to TNF-α was weaker. The cells were not so elongated and had fewer stress fibres. Even so, the cells did not regained their ‘cobblestone morphology’ after washing.
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TNF-α-Induced Changes in the Morphology and Permeability of HUVECs

A) Starvation Medium

Control

TNF-α 24 h

Recovery

B) Full Serum

Control

TNF-α 24 h

Recovery
Figure 3.12

TNF-α-induced changes to HUVEC morphology, F-actin organization and ZO-1 localization are not reversible

Confluent HUVECs were incubated in 1% FCS starvation medium (A) or full 2% FCS growth medium (B) for 2 h and either left untreated (control), treated with 10 ng/ml TNF-α alone for 24 h, or stimulated for 24 h, washed twice in PBS, and placed back in starvation medium (A) or growth medium (B) for a further 24 h (A) (recovery). Cells were then fixed and stained for F-actin (red) and ZO-1 (green). Arrow indicates elongated cells; arrowhead shows stress fibres. Bar = 20 μm.
Chapter 3

**TNF-α-Induced Changes in the Morphology and Permeability of HUVECs**

### 3.2.8 TNF-α induces changes to the tight junction proteins occludin, claudin-5, GEF-H1 and JAM-A

To identify potential changes that might be responsible for the increase in permeability, other junctional proteins were examined after TNF-α exposure. Protein localization and protein levels of the tight junction components occludin, claudin-5, GEF-H1 and JAM-A were examined by confocal microscopy and western blotting.

#### 3.2.8.1 Occludin

Two antibodies were used to examine occludin localization. Both these antibodies were raised against the occludin C-terminal-150 amino acids (see Chapter 5 for picture of occludin) and both the anti-rabbit and the anti-mouse antibodies showed that occludin localization changed very little with 10 min of TNF-α exposure, although in some cases it appeared more disjointed than the controls, similar to ZO-1 (Fig. 3.13A and B). After 24 h, the anti-rabbit antibody-stained occludin was lost from the junctions of all cells and was presumably redistributed either into the cytoplasm or on the cell membrane (Fig 3.13 A). The anti-mouse antibody-stained occludin was also lost from cell-cell junctions, but formed parallel lines that traversed the cells, strikingly similar to TNF-α-induced actin stress fibres (Fig. 3.13B). Co-staining with β-actin revealed that occludin did indeed co-localize with actin along these fibres (Fig. 13C and D). In western blots of occludin, three distinct bands were detected of approximately 65 kDa, 48 kDa and 35 kDa (Fig. 3.14). Both 10 ng/ml (Fig. 3.14A, C) and 100 ng/ml TNF-α increased the level of the lower bands after 4 and 6 h (Fig. 3.14B and C), and all three bands were reduced to levels below that of the control by 24 h (Fig. 3.14A and B). These may be degradation products that are increased after exposure to TNF-α. Furthermore, the decrease in all three bands by 24 h indicates that occludin protein is downregulated in response to long-term TNF-α exposure.

155
Figure 3.13

TNF-α-Induced Changes in the Morphology and Permeability of HUVECs
Figure 3.13

TNF-α induces changes to the localisation of occludin

Confluent HUVECs were starved for 2 h and either left untreated or exposed to 10 ng/ml TNF-α for 10 min or 24 h. Cells were fixed and stained for occludin using an anti-rabbit occludin antibody (A), or an anti-mouse occludin antibody (B). TNF-α-induced co-localisation between occludin and actin stress fibres is shown in (C) and a part of the figure is shown at higher magnification (D). For (C) and (D), cells were starved for 2 h and exposed to 10 ng/ml TNF-α for 24 h. HUVECs were pre-extracted in 0.2% pre-extraction buffer (Chapter 2), fixed and permeabilised in 95% ethanol and stained for total actin and for occludin using a mouse monoclonal antibody. N.B. Phalloidin staining with this fixation procedure was not possible. Arrow in (B) indicates occludin parallel lines; arrow in (D) indicates occludin and F-actin co-localization; Bar = 20 μm.
Figure 3.14

**A)**

10 ng/ml

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Occludin

ERK 1

**B)**

100 ng/ml

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Occludin

ERK 1

**C)**

6 h

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Occludin

ERK-1
Figure 3.14

TNF-α induces apparent occludin degradation and a decrease in occludin protein levels

Confluent HUVECs were starved for 2 h and either left untreated or exposed to 10 ng/ml TNF-α for 10 min or 24 h (A) or 100 ng/ml TNF-α for 4 h, 10 h or 24 h (B) or 0.1, 1, 10 and 100 ng/ml TNF-α for 6 h. Cells were lysed and the proteins were separated and identified by SDS-PAGE and western blotting using a rabbit polyclonal anti-occludin antibody. Three independent experiments are shown in (A). Blots were stripped and re-probed with an anti-ERK-1 antibody to show equal loading between lanes.
3.2.8.2 Claudin-5

Claudin-5 is an endothelial-specific claudin isoform, which has been implicated in regulation of barrier function (Morita et al., 1999; Inai et al., 1999). In unstimulated cells, claudin-5 expression varied from cell to cell, with some cells expressing little or undetectable levels, whilst others show high expression levels at the tight junction and/or in the cytoplasm (Fig. 3.15A and 3.21). Frequently, punctate staining in the cytoplasm was observed, suggestive of vesicular localization. After 10 min of TNF-α, claudin-5 localisation was similar to the control, although there may have been slightly more cytoplasmic staining. At 24 h, more cells appeared to lack detectable claudin-5 staining, although this was not quantified and no change in protein levels compared to control or 10 min stimulation was detected by western blotting at this time (Fig. 3.13B). Claudin-5 was still observed at some cell-cell junctions at 24 h.

3.2.8.3 JAM-A

JAMs are tight junction-associated integral membrane proteins that have been implicated in the regulation of diapedesis and barrier function (Muller et al., 2003; Martin-Padura et al., 1998). In control cells, JAM-A was present at cell-cell junctions, with some punctate cytoplasmic staining (Fig. 3.16A). There was no change in this JAM-A localisation after 10 min TNF-α exposure. However, by 24 h, JAM-A was not detectable at cell-cell junctions, but was present across the cell body. JAM-A may have been redistributed over the cell membrane or internalised. In some cells, JAM-A was clustered along one cell edge. No change in JAM-A protein levels was detected by western blotting (Fig. 3.16B).
Figure 3.15

TNF-α-induced changes to claudin-5

Confluent HUVECs were starved for 2 h and either left untreated or exposed to 10 ng/ml TNF-α for 10 min or 24 h before fixation and staining for claudin-5 (A). An accompanying western blot of three independent experiments is shown in (B). For these, parallel cultures were stimulated with TNF-α, lysed and the proteins were separated and identified by SDS-PAGE and western blotting using an anti-claudin-5 antibody. The blot was stripped and reprobed with an anti-ERK 1 antibody to check total protein levels in each lane. Arrow in (A) indicates punctate claudin-5. Insets in (A) show a lower magnification. Bar = 20 μm.
Figure 3.16

TNF-α-induced changes to JAM-A

Confluent HUVECs were starved for 2 h and either left untreated or exposed to 10 ng/ml TNF-α for 10 min or 24 h before fixation and staining for claudin-5 (A). An accompanying western blot of three independent experiments is shown in (B). For these, parallel cultures were stimulated with TNF-α, lysed and the proteins were separated and identified by SDS-PAGE and western blotting using an anti-JAM-A antibody. The blot was stripped and reprobed with an anti-ERK 1 antibody to check total protein levels in each lane. Insets in (A) show a lower magnification. Bar = 20 μm.
3.2.4 GEF-H1 and ZO-1

GEF-H1 (also known as Ju-GEF) is a Rho-GEF protein that has been shown to localize to epithelial cell-cell junctions, and can activate Rho (Benais-Pont et al., 2003). This protein is therefore potentially important for localized Rho activation and cortical F-actin contraction, and may mediate increased permeability. Although GEF-H1 mostly co-localized with ZO-1 in control cells (Fig. 3.17A), it was not always present in the strands of ZO-1 (arrow). There appeared to be little change in the GEF-H1 junctional localization after 10 min of TNF-α stimulation, whereas by 24 h a proportion had been lost from the cell periphery and GEF-H1 appeared as a finer line than in control cells and exhibited similar fragmentation to ZO-1 (Fig. 3.17A). No change in GEF-H1 or ZO-1 protein levels were detected in western blotting (Fig. 3.17B). The doublet observed in ZO-1 western blots is likely to be the α⁺ and α⁻ isoforms (Chapter 1) because the antibody is raised to a sequence that is N-terminal to the α⁺/α⁻ splice site of ZO-1 (Zymed data sheet 61-7300) (Fig. 3.17B).

3.2.8.5 Adherens junctions

The function of the tight junction depends upon the integrity of the adherens junctions. To determine whether TNF-α affects the adheren junctions, β-catenin and VE-cadherin localizations were examined (Fig. 3.18A). In both cases, stimulation with TNF-α for 10 min induced no discernable changes to the junctional localization, but after 24 h, TNF-α caused the staining to become disjointed and sometimes punctate at the junctions. Neither β-catenin nor VE-cadherin were lost from cell junctions. However, VE-cadherin and β-catenin exhibited thick areas of reticular junctional staining in both the control cells and cells treated with TNF-α for 10 min. This is similar to the localisation of PECAM (Mamdouh et al. 2003) (may arise in areas of cell-cell overlap). Reticular staining was lost at 24 h. No change in total protein level of either β-catenin or VE-cadherin were detected (Fig. 3.18B).
Chapter 3

TNF-α-Induced Changes in the Morphology and Permeability of HUVECs

Figure 3.17

A) GEF-H1   ZO-1   Merged

B) 

<table>
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<tr>
<th></th>
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<th>10 m</th>
<th>24 h</th>
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GEF-H1
ZO-1
ERM
Figure 3.17

TNF-α induced changes to GEF-H1 and ZO-1

Confluent HUVECs were starved for 2 h and either left untreated or exposed to 10 ng/ml TNF-α for 10 min or 24 h before fixation and staining for GEF-H1 and ZO-1.

Accompanying western blots of three independent experiments for each protein are shown in (B). For these, parallel cultures were stimulated with TNF-α, lysed and the proteins were separated and identified by SDS-PAGE and western blotting using anti-GEF-H1 and ZO-1 antibodies. The blot was stripped and reprobed with an anti-ERM antibody to check protein levels in each lane. The doublet seen with the anti-ERM antibody is ezrin (upper band) and moesin (lower band) (Santa Cruz Biotechnology data sheet, SC-6410). Arrow in (A) indicates an area lacking GEF-H1 and ZO-1 co-localization. Insets in (A) show a lower magnification. Bar = 20 μm.
Chapter 3

**TNF-α-Induced Changes in the Morphology and Permeability of HUVECs**

**Figure 3.18**

A) Control / V E -C a d h e r in / L p-cadherin.

B) TNF-a-Induced Changes in the Morphology and Permeability of HUVECs

10 min

24 h

- VE-Cadherin

- β-catenin

- ERK

- ERM
Chapter 3  

TNF-α-Induced Changes in the Morphology and Permeability of HUVECs

Figure 3.18

TNF–α induced changes to β-catenin and VE-cadherin

Confluent HUVECs were starved for 2 h and either left untreated or exposed to 10 ng/ml TNF-α for 10 min or 24 h before fixation and staining for β-catenin or VE-cadherin (A). Accompanying western blots of three independent experiments for each protein are shown in (B). For these, parallel cultures were stimulated with TNF-α, lysed and the proteins were separated and identified by SDS-PAGE and western blotting using anti-β-catenin and anti-VE-cadherin antibodies. Arrow in (A) indicates reticular β-catenin staining. Insets in (A) show a lower magnification. Bar = 20 μm.
3.2.9 TNF-α alters tight junction protein association with the NP40-insoluble pool

There is some evidence in the literature that changes in the actin-cytoskeletal pool to which tight junction proteins associate may affect permeability. The detergent-insoluble fraction contains the majority of F-actin and its associated proteins (Tsukamoto et al., 1997). To test whether TNF-α can alter which fraction the tight junction proteins associate with, cell lysates were separated into NP40-soluble and -insoluble pools and western blotting was used to detect the functional proteins of interest (Fig. 3.19). There was a substantial amount of ZO-1, claudin-5, occludin and GEF-H1 in the control insoluble fractions, whereas very little JAM-A was present in the insoluble fraction. Association of ZO-1 and occludin with the insoluble fraction decreased slightly by 24 h of TNF-α stimulation, whereas this association was not altered for GEF-H1, JAM-A or claudin-5.
TNF-α-Induced Changes in the Morphology and Permeability of HUVECs

Figure 3.19

<table>
<thead>
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<tr>
<td>ERM</td>
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| 45   |         |     |      |      |
| JAM-A (Short exposure) |
| 45   |         |     |      |      |
| JAM-A (Long exposure) |
| 66   |         |     |      |      |
| Occludin |
| 45   |         |     |      |      |
| β-actin |

| 20   |         |     |      |      |
| Claudin-5 (Short exposure) |
| 20   |         |     |      |      |
| Claudin-5 (Long exposure) |
| 45   |         |     |      |      |
| β-actin |
Figure 3.19

**TNF-α alters tight junction protein association with the NP40-insoluble pool**

Confluent HUVECs were starved for 2 h and either left untreated or stimulated with TNF-α for 4 h, 10 h or 24 h. Cells were lysed in 1% NP40-containing buffer and the insoluble fraction obtained by centrifugation and further solubilisation in 0.1% SDS-containing buffer. Proteins were detected using SDS-PAGE and western blotting for claudin-5, GEF-H1, occludin and JAM-A. The blots were stripped and re-probed with anti-ERM and anti-β-actin antibodies to show the total protein levels in each lane. Blots are representative of two independent experiments for each protein. ‘S’ indicates NP40-soluble fraction; ‘I’ indicates NP40-insoluble fraction.
3.2.10 Claudin-5 and β-catenin co-immunoprecipitate, but β-catenin binds to multiple antibodies

The cell-cell junctions in endothelial cells are thought to be linked, rather than forming discrete junctional compartments (Section 1.2.1). To test this in HUVECs, the association between the tight junction protein, claudin-5 and the adherens junction protein, β-catenin, was examined using immunoprecipitation experiments (Fig. 3.20). This was done both by immunoprecipitation of claudin-5 and immunoblotting for β-catenin (Fig. 3.20A-D) and vice versa (Fig. 3.20G-J). Cell lysates were precleared prior to immunoprecipitation to reduce non-specific binding to the antibody during the experiment. Although β-catenin did co-immunoprecipitate with claudin-5 in the NP40-soluble fractions, it also co-immunoprecipitated with the mouse IgG and the anti-Flag antibodies used in the preclearing steps and with the anti-c-myc and anti-ZO-1 antibodies, which were used as negative controls (Fig. 3.20B and D). Increasing doses of TNF-α slightly increased the amount of co-precipitated protein (Fig. 3.20D). Furthermore, reprobing of the blots showed that the related p120-catenin also co-immunoprecipitated with ZO-1, as well as with claudin-5. No co-immunoprecipitation was observed using the insoluble fractions, nor was claudin-5 detected in blots of β-catenin immunoprecipitates. These data indicate that although β-catenin and p120-catenin bind to claudin-5, the catenins are sticky proteins, and this interaction may not necessarily occur in vivo.

3.2.11 β-catenin and claudin-5 co-localize at cell junctions

To further evaluate the potential interaction between β-catenin and claudin-5, the localisation of both proteins was studied in unstimulated HUVECs (Fig. 3.21). There was good co-localization between β-catenin and claudin-5 in some areas of cell-cell contact, but not in all of the junctions. β-catenin was not observed to localize to areas containing the punctate cytoplasmic claudin-5. HUVECs are relatively flat cells and there was no discernable segregation of the co-localized proteins along the lateral membrane (yellow staining in the Z-stacks). These results show that β-catenin and ZO-1 co-localize at some cell-cell junctions and that in HUVECs, it is not possible to distinguish between adherens
and tight junctions on confocal Z-stacks, unlike in epithelial cells, where the tight junctions are clearly separate from the adherens junctions.
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TNF-α-Induced Changes in the Morphology and Permeability of HUVECs

Figure 3.20

A) LYSE

PRECLEAR

IMMUNOPRECIPITATE (IP)

IMMUNO-BLOT (IB)

B) NP40-soluble

C) NP40-insoluble

Claudin-5 IP and β-catenin western blot

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<td>α-p120</td>
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TNF-α-α

IB: β-catenin (rabbit)

IB: β-catenin (rabbit)
Chapter 3  

TNF-α-Induced Changes in the Morphology and Permeability of HUVECs

Figure 3.20

Claudin-5 IP and β-catenin and p120 western blots

D)  

- **kDa** 
  - 97-
  - 100 µg/ml
  - 10 µg/ml
  - 1 µg/ml
  - Control

IB: β-catenin (rabbit)

IB: p120

E)  

- **kDa** 
  - 97-

IB: β-catenin

IB: p120
Chapter 3

TNF-α-Induced Changes in the Morphology and Permeability of HUVECs

Figure 3.20

β-catenin IP

--- NP40-soluble --- NP40-insoluble ---

F) [Images of Western blots showing protein bands for TNF-α-induced changes in HUVECs]

IB: Claudin-5 (mouse)

Heavy chain

Light chain

H) Stripped and reprobed

IB: β-catenin (rabbit)

30 min 10 min

I) Stripped and reprobed

IB: β-catenin (rabbit)

30 min 10 min

TNF-α
Figure 3.20

α-catenin co-immunoprecipitates with claudin-5, but β-catenin bind to multiple antibodies

Three immunoprecipitation experiments were conducted as outlined in (A). Confluent HUVECs were starved for 2 h and either left unstimulated or were stimulated with 10 ng/ml TNF-β for 10 min or 24 h (B and C) or for 10 min and 30 min (F, G, H, I) or for 6 h with increasing doses of TNF-β (D and E). Cells were lysed and the NP40-soluble and insoluble fractions were separated. In (B) and (C) lysates were pre-cleared using an anti-FLAG antibody. The pre-cleared lysate was then either incubated with an anti-myc antibody (negative control), or with an anti-claudin-5 antibody. Co-immunoprecipitated protein was detected using SDS-PAGE and western blotting with an anti-rabbit-β-catenin antibody. In (D), lysates were incubated directly (no pre-clearing) with either an anti-ZO-1 antibody (negative control) or an anti-claudin-5 antibody. Co-immunoprecipitated protein was detected using SDS-PAGE and western blotting with an anti-rabbit-β-catenin antibody. The blot was then stripped and re-probed for p120 (D). Total protein levels for β-catenin and p120 are shown in (E). In (F) and (G), lysates were pre-cleared using a mouse anti-IgG antibody and then were either incubated with an anti-CD59 antibody (kind gift from Dr Jaime Millan, negative control) or with an anti-β-catenin antibody. Co-immunoprecipitated protein was detected using SDS-PAGE and western blotting using an anti-claudin-5 antibody. The blots were then stripped and reprobed for β-catenin (H, I). ‘WCL’ indicates whole-cell lysate; ‘IP’ indicates immunoprecipitation; ‘IB’ indicates immuno-blot, control indicates = no TNF-α stimulation; red arrow, correct size of claudin-5; black arrow, correct size of ZO-1; dash, no TNF-α stimulation.
Figure 3.21
Claudin-5 and β-catenin co-localize at cell junctions

Confluent HUVECs were starved for 2 h and fixed and stained for β-catenin (green) and claudin-5 (red). 11 confocal Z-stacks were taken, spanning of 4.0 μm, with a 0.4 μm interval between each image. The Z-stack images were projected into one image. The planes of the Z-section are shown by the red, green and blue lines. Yellow/orange colour indicates areas of co-localization between the two proteins. Arrows indicate some areas of co-localization. Bars = 20 μm, except in the merged image where bar = 10 μm.
3.3 Discussion

In this chapter, the effects of TNF-α on HUVEC tight junction proteins, permeability, F-actin and cell morphology have been characterised. TNF-α induced progressive changes to HUVECs over 24 h. At early time-points it induced relatively subtle responses, including a small and transient increase in permeability and at later time points (4-24 h), it induced a stronger response that was accompanied by a greater increase in permeability.

The increase in permeability that was detected by 10-30 min, was very small and was only just detectable using FITC-dextran after multiple experiments. Other groups have also reported early increases in HUVEC permeability in response to TNF-α. For example, an increase was detected using 125I-albumin in HUVECs after 15 min of TNF-α stimulation (Ferrero et al., 2001). A radioactively labelled tracer is probably more sensitive than FITC-dextran. Indeed FITC-dextran must reach a threshold of accumulation before it can be detected. Changes in TER can be more rapidly detected than changes in macromolecular permeability (compare van Nieuw Amerongen, et al; 1998; Ehringer et al., 1996), but unfortunately, HUVECs in culture do not have high electrical resistance, which makes it difficult to assess changes in TER in response to stimuli. TNF-α treatment for 10 min also induced thickening of the cortical F-actin and the formation of tiny gaps, which have previously been attributed to the activation of Rho, Rac and Cdc42 (Wojciak-Stothard et al., 1998). This effect was stronger where higher doses of TNF-α were applied.

The long-term response to TNF-α was much stronger that the early response and involved the formation of thick actin stress fibres, cell elongation, intercellular gap formation and permeability increases. There was detectably less total protein in lysates treated with TNF-α for 24 h, which may indicate a global down-regulation of protein expression after TNF-α stimulation. This is probably not due to apoptosis as others have reported a lack of apoptosis in response to TNF-α in HUVECs up to 24 h and 100 ng/ml TNF-α (Zen and Karsan, 1999). However, apoptosis could have been further examined in several ways. Translocation of phosphatidylserine from the inner to the outer membrane occurs in cells undergoing apoptosis. This can be recognised by annexin V conjugated to a fluorescent label and allows quantification of apoptosis. Nuclear breakdown and changes to cell genome, such as condensation of
TNF-α-Induced Changes in the Morphology and Permeability of HUVECs

chromatin and DNA degradation, also occurs during apoptosis and these can be measured using flow cytometry. DNA fragmentation can also be observed using gel electrophoresis. In addition, caspase cleavage indicates apoptosis and cleavage of a fluorogenic substrate can be used to determine caspase activity. Furthermore, caspases are synthesized as zymogens and these undergo cleavage during activation, which can be monitored by immunoblotting (Bonifacino et al., 2003). Mitochondrial changes during apoptosis can also be measured by using, for example, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide). Mitochondrial dehydrogenase from viable cells is able to cleave MTT resulting in a colour change that can be spectrophotometrically measured (Zen and Karson et al., 1999). Many other methods for identifying cell apoptosis are described elsewhere (Bonifacino et al., 2003).

Stress fibre formation is characteristic of activation of Rho (Ridley and Hall, 1992) and Rho may therefore be activated during long-term TNF-α exposure (Chapter 4). The actin cytoskeleton is important for the maintenance of endothelial barrier function, as disruption of actin using cytochalasin B or D increases endothelial permeability (Shasby et al., 1982). Alterations in the junction-associated actin could account for the increase in permeability. Likewise, cytoskeletal remodelling is linked to changes in cell shape and could be responsible for the cell elongation and intercellular gap formation within the monolayer. This in turn could result in increased permeability. Due to the time it takes for these changes to occur, it is likely that transcriptional changes are involved in this response. Microarrays have identified some potential candidates for transcriptional modulation by TNF-α that might affect the cytoskeleton and/or permeability. For example, the actin-binding proteins, filamin B, moesin and connexin-43 are transcriptionally upregulated by TNF-α stimulation for 16 h. Conversely, thymosin-β10, tropomyosin and the smooth muscle isoform of myosin regulatory light chain 2 are all downregulated approximately 2-fold after TNF-α stimulation for 2 h (Zhou et al., 2002).

Only the higher doses of TNF-α of 10 and 100 ng/ml were sufficient to increase permeability. A dose of 0.1 ng/ml caused cell elongation, but no intercellular gap formation or tight junction disruption, which is consistent with a lack of increased permeability with this dose. A dose of 0.1 ng/ml, however, was sufficient to induce ICAM-1 expression (Fig.3.5). Doses of TNF-α up to 20 ng/ml are physiologically relevant. This is based upon the observation that non-lethal
injection of endotoxin into human volunteers produces a serum TNF-α concentration of 0.358 ng/ml (± 0.166 ng/ml), whereas lethal doses of endotoxin injected into baboons produce a plasma TNF-α concentration of 20.5 ng/ml (± 9.89 ng/ml) (Hesse et al., 1988). A localised region of inflammation with high TNF-α expression, which may be the case in atherosclerosis or septic shock, may induce barrier dysfunction as described here. Indeed, it has been shown that the overall biological effect of TNF-α is primarily determined by the body compartment in which it is produced, and not the serum level (Tracey and Cerami, 1993). Otherwise, low doses of TNF-α that are sufficient to induce the expression of adhesion molecules (ICAM-1), might not be enough to cause barrier breakdown and increased permeability in vivo. Since cell elongation induced by 0.1 ng/ml TNF-α was not accompanied by an increase in permeability, this morphological change cannot be responsible for TNF-α-induce permeability. Only where ZO-1 localization was altered and gaps were present was an increase in permeability observed. This indicates that the morphological changes are less important for the breakdown of barrier function than the loss of junctional integrity and cell-cell adhesion.

It is possible that the permeability increase at 24 h results from signalling directly to the tight junction proteins, independently of, or simultaneously with, the cytoskeletal changes. Increased permeability is often associated with loss or redistribution of the tight junction proteins. Occludin was completely removed from the tight junction after long-term TNF-α stimulation and this was also largely the case for JAM-A, which is consistent with another report; in this case, it was redistributed on the cell surface after TNF-α exposure (Ozaki et al., 1999). Both these proteins have been implicated in maintaining barrier function (McCarthy et al., 1996; Martin-Padura et al., 1998) and occludin forms part of the tight junction fibril itself, therefore the removal of these proteins from the junction could perturb barrier function. Western blotting of occludin revealed the appearance of faster migrating forms in cells treated with TNF-α. It is likely that these forms are degradation products of occludin. Similar-sized bands have previously been reported to appear in response to tyrosine phosphatase inhibition, and have been attributed to the action of a metalloproteinase. Interestingly, in this report the appearance of these bands coincided with increased HUVEC permeability and occludin redistribution from the junctions (Wachtel et al., 1999). Taken together, it could be that TNF-α activates a protein
Chapter 3

TNF-α-Induced Changes in the Morphology and Permeability of HUVECs

kinase, or inhibits a protein tyrosine phosphatase, which results in activation of a metalloproteinase and cleavage of occludin. Cleavage events have been described for other membrane proteins. The γ-secretase complex is a multiprotein complex containing a multipass transmembrane protein, presinilin, which contains an aspartyl protease catalytic core. The γ-secretase complex cleaves several integral membrane proteins, including Notch and the junctional protein, E-cadherin (Steiner and Haass 2000; Fortini, 2002). Pre-cleavage of Notch by ADAM/TACE (a disintegrin and metalloprotease/TNF-α-converting enzyme) is required for γ-secretase activity. This event leaves a short extracellular stalk, which may dictate the cleavage site of presinilin (Fortini, 2002). Pre-cleavage of occludin by a metalloproteinase could therefore also regulate further cleavage by the γ-secretase complex.

The C-terminus of occludin has been shown to associate with several signalling and structural proteins at the tight junction (Chapter 1), and cleavage of this region could have implications for the structural integrity of the junctional complex and barrier function. However, co-localization of occludin with the newly formed actin stress fibres implies that a portion of occludin retains its F-actin binding region, and is perhaps redistributed across the cell membrane. This is supported by the observation that there was still an association of occludin with the cytoskeletal fraction after 24 h TNF-α, although it is reduced. Since it was discovered that tight junction fibrils can form in the absence of occludin (Saitou et al., 1998) and that some cell types do not form tight junctions but nevertheless express occludin (Wachtel et al., 2001), the function of occludin has been called into question. Given that T-cells also express occludin (Alexander et al., 1998), it is conceivable that activated HUVECs redistribute occludin across the cell surface so that it can form homotypic interactions with occludin expressed on T-cells, and may have a role in cell-cell adhesion, and/or cell guidance in transendothelial migration. A similar scenario has been described for JAM-A. JAM-A that has been redistributed over the endothelial cell surface by combined treatment of TNF-α and IFN-γ (Rival et al., 1996) co-operates with ICAM-1 in arresting memory T-cells on the cell surface. This is achieved by the lymphocyte LFA-1 interaction with both JAM-A and ICAM-1 on the endothelium.

Control cells expressed claudin-5 to variable extents across the cell monolayer, both at the cell-cell junctions and within the cell-body. This has not been previously reported and could explain
why HUVECs form looser junctions than other endothelial cell types, for example endothelial cells from the blood-brain barrier. Long-term TNF-α treatment caused an increase in this variability and a greater number of cells appeared to lack claudin-5 expression at the tight junction, although there was no change in overall protein levels. It is possible that claudin-5 was redistributed away from the tight junctions of some cells, but is retained within the plasma membrane. Claudin-1 mRNA has been detected in HUVECs (Martin et al., 2002), but there are no reports of its protein expression in HUVECs to date. Claudin-1 protein expression was examined here, but was not detected. Given that occludin was completely relocalized, and that claudin-5 was retained at the cell-cell junctions of neighbouring cells that expressed it, it may be that occludin is more important for barrier regulation in HUVECs than claudin-5.

This is the first report of GEF-H1 expression in endothelial cells. In MDCK cells it has been observed at the cell-cell junctions, and has been shown to activate RhoA (Benais-Pont et al., 2003). A similar localization was observed in HUVECs and it was retained at the cell junctions during a 10 min TNF-α stimulation and the majority was retained at the junctions during a 24 h stimulation. This is a potentially important signalling molecule that might locally activate RhoA to induce cortical actin contraction and gap formation. It would be interesting to determine whether GEF-H1 responds to stimuli such as TNF-α.

Increased permeability can also be a result of increased transcellular transport (transcytosis) through the cell monolayer (Simionescu, 2002). There is not a lot of evidence for TNF-α effects on transcellular transport in endothelial cells, however, TNF-α has been shown to increase lactoferrin (an iron binding protein involved in host defence against infection) transcytosis in bovine brain capillary endothelial cells (BBCECs) (Fillebeen et al., 1999). In addition, TNF-α induces increased low density lipoprotein uptake and a decrease in transferrin-receptor mediated endocytosis in BBCECs (Descamps et al., 1997). Transcellular transport has been proposed to involve caveolae. Caveolae are membrane invaginations, that have a high cholesterol content and contain caveolin expression, and are thought to organise the plasma membrane and associated proteins into specific regions which are important for signalling complexes, endocytosis and vesicle transport (Simionescu, 2002). TNF-α has been shown to decrease caveolin-1 levels in HUVECs under shear stress, although levels return to normal after


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20 h (Sun et al., 2002b). These results indicate that TNF-α is able to modulate transcellular routes, as well as paracellular pathways.

HUVECs are relatively flat cells compared to epithelial cells and the interendothelial junctions are thought to be linked. Claudin-5 co-precipitated with the adherens junction protein β-catenin and p120. However, β-catenin also co-precipitated with the negative control and preclearing antibodies. A GST-pull down experiment might clarify whether or not this interaction is real. Claudin-5 and β-catenin also co-localized at some cell-cell junctions, but not in all junctional areas. The lack of a distinction between claudin-5 and β-catenin along the lateral membrane in the Z-stacks indicates that discrete junctions may not form in these cells and it is likely, therefore, that some cross-talk does occur between the cell junctions. It is noteworthy, however that claudin-5 did not show areas of reticular staining, like that observed with β-catenin and VE-cadherin (Fig. 3.18), which may be one way to distinguish between the junctions.

It would be interesting to pursue the signalling mechanisms that result in the formation of actin stress fibres and the changes to the junctional proteins, and to establish whether either or both of these events contribute to TNF-α-induced permeability.
Chapter 4

Cytoskeletal Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological Changes

4  Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological changes

4.1 Introduction

The previous chapter characterised the effects of TNF-α on the actin cytoskeleton and permeability in HUVECs. The early changes are consistent with previous reports both in HUVECs and in other endothelial cells (Petrache et al., 2001; Wojciak-Stothard et al., 1998; Goldblum et al., 1990 and 1993; Brett et al., 1989). In many experiments actin rearrangement and altered permeability coincide. For example, actin stress fibre formation, contraction and gap formation induced by TNF-α were reported to correlate with decreased barrier function in bovine aortic endothelial cells (Brett et al., 1989).

Furthermore, F-actin-disrupting agents have been shown to increase endothelial permeability (Shasby, et al., 1982) and F-actin-stabilizing agents protect against increased permeability (Goldblum et al., 1993; Phillips et al., 1989; Alexander et al., 1988; Hippenstiel et al., 1995). There is, therefore, a role for the actin cytoskeleton in the regulation of barrier function.

TNF-α has been reported to induce rapid formation of lamellipodia, filopodia and subsequently actin stress fibres, intercellular gaps and VE-cadherin dispersion and perinuclear accumulation in HUVECs (Wojciak-Stothard et al., 1998). In bovine pulmonary endothelial cells, TNF-α also induces myosin light chain phosphorylation, which can be reduced both with the MLCK inhibitor, ML-7 and with the Rho kinase inhibitor, Y-27632 (Petrache et al., 2001). ML-7 is a relatively specific MLCK inhibitor, although at higher concentrations it can also inhibit PKA and PKC (Calbiochem). TNF-α-induced stress fibre formation and contractility in HUVECs can also be inhibited using ML-7 or butanedione-2-monoxime (BDM), which inhibits muscle myosin ATPase activity (Wojciak-Stothard et al., 1998), also indicating a role for MLCK and contractility in TNF-α-induced cytoskeletal changes. However, neither ML-7 nor Y-27632 are able to inhibit TNF-α-induced barrier disruption in bovine pulmonary endothelial cells (Petrache et al., 2001). Recent evidence from the same group and others has demonstrated a role for the microtubule network in TNF-α-induced permeability changes (Petrache et al.,
2003; Molony et al., 1991). Microtubule disruption has previously been shown to act via Rho and MLC to induce endothelial barrier disruption (Verin et al., 2001); inhibition of microtubule polymerisation using nocodazole or vinblastine results in decreased TER, increased stress fibre formation and Rho-dependent MLC phosphorylation. Conversely, both decreased TER and MLC phosphorylation are attenuated by the microtubule stabilizer, paclitaxel. Paclitaxel also attenuates TNF-α-induced permeability, stress fibres and adherens junction disruption (Petrache et al., 2003).

Constitutively active Rho induces the formation of actin stress fibres and intercellular gaps, which are similar to reported early TNF-α-induced changes (Wojciak-Stothard et al., 1998). Constitutively active Cdc42 and Rac induced similar responses although to a lesser extent. Furthermore, dominant negative Rho, Rac or Cdc42 inhibit TNF-α-induced stress fibres and contraction and partially prevent VE-cadherin dispersion. It is therefore possible that all three GTPases contribute to TNF-α-induced permeability both through cytoskeletal changes and signalling to adherens junctions, possibly by their sequential activation (Nobes et al., 1995). TNF-α activation of Cdc42 has been suggested to be upstream of Rac1, which in turn activates RhoA (Wojciak-Stothard et al., 1998; van Wetering et al., 2002), although experiments to demonstrate this conclusively in endothelial cells are lacking.

Although the effects of Rho, Rac and Cdc42 on the endothelial cytoskeleton and morphology have been extensively characterised, the role of these proteins in TNF-α-induced permeability has not been examined. This chapter investigates whether signaling pathways known to regulate actomyosin contraction and cytoskeletal reorganisation in response to thrombin are activated in response to TNF-α and whether such pathways also regulate TNF-α-induced barrier dysfunction and tight junction protein redistribution in HUVECs.
Chapter 4

Cytoskeletal Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological Changes

4.2 Results

4.2.1 RhoA but not Rac1 or Cdc42 is transiently activated by TNF-α

Rho regulates the formation of actin stress fibres in many cell types (Ridley & Hall, 1992). As TNF-α caused early subtle changes to F-actin, and caused the formation of thick actin stress fibres at 24 h (Chapter 3), it seemed likely that Rho is involved in these responses. To test this, Rho pull-down experiments using the Rho-binding domain (RBD) of the Rho-effector protein, Rhotekin, was used to extract GTP-loaded Rho from cells that had been treated with or without TNF-α (Fig. 4.1) (Ren and Schwartz, 2000). RhoA was activated by TNF-α at 1 min and 10 min, but this activation was reduced by 24 h, despite the presence of stress fibres at this time (Fig. 4.2). The extent of this activation varied from experiment to experiment, but the overall trend was clear (Fig. 4.2B). In some cases, total protein levels of RhoA were reduced, but so too were the total protein levels of the loading control, α-tubulin, indicating a global reduction in protein levels (see also section 3.2.6). Rac and Cdc42 have been implicated in early TNF-α responses (Wojciak-Stothard et al., 1998). To determine whether either GTPase is activated by TNF-α exposure, pull-down experiments were used (Fig. 4.3 & 4.4). Rac1 pull-down experiments used the p21-binding domain (PBD) of the Rac effector protein, PAK (Fig. 4.3) and Cdc42 pull-down experiments used the Cdc42/Rac interactive binding (CRIB) domain of the Cdc42 effector protein, WASP (Fig. 4.4). There was some variability from experiment to experiment, but overall there was no consistent Rac1 or Cdc42 activation by TNF-α at 10 min or 24 h, nor did total protein levels of Rac or Cdc42 change. These results indicate that activation of RhoA, Rac or Cdc42 does not account for the increase in stress fibres at 24 h, but that RhoA activation at early times may account for increased cortical stress fibres observed at 10 min of TNF-α stimulation (Fig. 3.2).
Figure 4.1

Rho pull-down experiments

An overview of the pull-down experiments is shown above. Only activated, GTP-bound RhoA will associate with its effector, Rhotekin and so this protein is used to purify GTP-bound RhoA as a measure of RhoA activity.
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Cytoskeletal Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological Changes

Figure 4.2

RhoA pull-downs

A)  

B)  

C)  

188
Chapter 4  Cytoskeletal Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological Changes

D)  

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<td>WCL 45</td>
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E)  

Graph showing RhoA Activity (% of control) over time:
- 10 min
- 24 h
Figure 4.2
The Effects of TNF-α on RhoA activity

Cells were starved for 2 h and either left untreated or stimulated with 10 ng/ml TNF-α for the times indicated. Cells were rapidly lysed and GTP-bound-RhoA was purified from cell lysates by incubation with the Rho binding domain of Rhotekin. Incubation of the cell lysate with GTPγS or GDPβS was used as positive and negative controls respectively. GTPγS binds to small GTPases and maintains them in their active conformations because it cannot be hydrolysed, whereas GDPβS binds to and maintains small GTPases in their inactive conformation. Protein was detected by SDS-PAGE, western blotting and immuno-detection using an anti-RhoA antibody. Four independent experiments are shown (A-D). Whole cell lysate (WCL) samples show the total RhoA protein levels in each sample and protein loading in each lane of the gel is shown by α-tubulin levels. Pull down (PD) indicates GTP-bound RhoA. The graphs depicting the activity of RhoA was created by normalisation of the active bands of RhoA to the RhoA WCL controls using densitometry. The mean activity of three separate experiments was used to create the graph in (E). A student t-test was used to test the statistical relevance of the activity at 10 min and 24 h for the graph in (E), but no statistical significance was observed. For 10 min, this is due the variation of the extent of activation from experiment to experiment (i.e. large standard deviations), but the overall trend was one of RhoA activation at 10 min. Dashes indicate lanes that are irrelevant to this experiment, and are shown in Chapter 5.
Chapter 4

Cytoskeletal Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological Changes

Figure 4.3

Rac1 pull-downs

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β-actin
**Figure 4.3**

The Effects of TNF-α on Rac1 activity

Cells were starved for 2 h and either left untreated or stimulated with 10 ng/ml TNF-α for the times indicated. Cells were rapidly lysed and GTP-bound-Rac was obtained from cell lysates using the PBD domain of PAK-1. GTPγS or GDPβS were used as positive and negative controls respectively (see Fig. 4.2). Protein was detected by SDS-PAGE, western blotting and immuno-detection using an anti-Rac1 antibody. Three independent experiments are shown (A-C). Whole cell lysate (WCL) samples are shown to demonstrate the total Rac1 protein levels in each sample and in (C), protein loading is indicated by western blotting using an anti-β-actin antibody. Graphs depicting the activity of Rac1 were created by normalisation of the active bands of Rac1 to the WCL controls using densitometry. Frequently, two bands were detected in the WCL of Rac1. The upper band is of the correct size and was taken to be Rac1 (and was used for the normalisation). The lower band may be a degradation product or Rac1 splice variant. Pull down (PD) indicates GTP-bound Rac1. Dashes indicate lanes that are irrelevant to this experiment and are shown in Chapter 5.
Cytoskeletal Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological Changes

Figure 4.4
Cdc42 pull-downs

A) GTPγS

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B) GTPγS

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C) GTPγS

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α-tubulin
Chapter 4  
Cytoskeletal Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological Changes

D)

![Western Blot Analysis](image)

E)

![Western Blot Analysis](image)

F)

![Graph](image)
Figure 4.4

The Effects of TNF-α on Cdc42 activity

Cells were starved for 2 h and either left untreated or stimulated with 10 ng/ml TNF-α for the times indicated. Cells were rapidly lysed and GTP-bound-Cdc42 was purified from cell lysates using the CRIB domain of WASP. GTPγS or GDPβS were used as positive and negative controls respectively (see Fig 4.2). Protein was detected by SDS-PAGE, western blotting and immuno-detection using an anti-Cdc42 antibody. Five independent experiments are shown (A-E). Whole cell lysate (WCL) samples are shown to indicate the total Cdc42 protein levels in each sample and overall protein loading in each lane of the gel is shown by α-tubulin levels. Pull down (PD) indicates GTP bound Cdc42.

Graphs depicting the activity of Cdc42 were created by normalisation of the active bands of Cdc42 to the WCL controls using densitometry. The mean activity of three separate experiments (A, C and D) was used to create the graph in (F). A student t-test was used to test the statistical relevance of the activity at 10 min and 24 h for the graph in (E), but no statistical significance was observed. Dashes indicate lanes that are irrelevant to this experiment, and are shown in Chapter 5.
4.2.2 Effects of Rho, ROCK and MLCK inhibition on resting HUVECs

Rho, ROCK and MLCK have each been shown to modulate the actin cytoskeleton and regulate endothelial permeability (Wojciak-Stothard et al., 2003). To see whether they are involved in maintaining cortical actin and the cell-cell junctions, the effects of Rho, ROCK and MLCK inhibition on F-actin and ZO-1 distribution in resting HUVECs were examined. To inhibit Rho, cells were treated with C3 transferase (C. botulinum C3 exoenzyme), which is an ADP-ribosyltransferase that specifically ADP-ribosylates and inhibits Rho (Aktories and Hall, 1989; Balsh, Channing and Hall, 1995). C3 transferase targets the Asp 41 of RhoA, B and C, but not other Rho family members, and this interferes with downstream signalling of Rho. Where C3 transferase was added to the cell culture medium, it appeared to be taken up by clusters of cells (non-specific endocytosis, Channing and Hall, 1995) and not universally across the monolayer (Arrow in Fig. 4.5). These cells were easily detected due to their complete lack of actin stress fibres. In resting cells, those treated with C3 transferase for 4 h or 24 h contained a single line of F-actin at the cell periphery and lacked any basal stress fibres across the cell body or at the cell-cell junctions. ZO-1 tightly co-localised with this single F-actin band and was continuous at the cell-cell junctions (Fig. 4.5).

The Rho effector molecule, ROCK, stimulates actomyosin interaction and contraction (Kureishi et al., 1997; Amano et al., 1996; Riento and Ridley, 2003) and is involved in thrombin-induced actin cytoskeletal reorganisation and permeability (Carbajal et al., 2000). To investigate whether ROCK is also involved in maintaining cortical F-actin and ZO-1 distribution at the cell-cell junctions, the ROCK inhibitor, Y-27632 (Uehata et al., 1997) was used. Y-27632 is a relatively specific inhibitor of ROCK I and II, although it has also been shown to inhibit PRK2 at similar concentrations to those used for ROCK II inhibition (Uehata et al., 1997; Davies et al., 2000). Y-27632 (5 μM) alone induced ‘mesh-like’ F-actin and disrupted ZO-1 staining at early time points (Fig. 4.5). After 24 h it abolished actin cables within the cell body, but a thin line of F-actin was maintained at the cell periphery of some cells and some cells still contained fine F-actin fibres (Fig. 4.5). ZO-1 co-localised with the thin line of F-actin at cell-cell junctions.
The MLCK inhibitor compound, ML-7 (Saitoh et al., 1987) was used to inhibit MLCK in resting HUVECs. At higher concentrations, ML-7 can also inhibit PKA (21 μM) and PKC (42 μM) (Calbiochem). To avoid this, a maximum concentration of 10 μM was used. ML-7 at a concentration of 5 μM had no detectable effect on unstimulated HUVECs after 40 min (data not shown), but did remove basal stress fibres from the cell body of some cells after 24 h (Fig. 4.5). A higher concentration of 10 μM induced some changes after 40 min, although on some occasions this concentration induced cell-blebbing and some cell detachment. Cells treated with 10 μM ML-7 alone usually had a single strong line of F-actin at the cell periphery, and lacked basal stress fibres within the cell body or at the cell-cell junctions (Fig. 4.5). ZO-1 co-localised with this line of F-actin (Fig. 4.5).
Figure 4.5

Cytoskeletal Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological Changes

Control

Y-27632 40 min

C3 4 h

ML-7 10 μM 40 min
Figure 4.5

Rho, ROCK and MLCK inhibition affect the actin cytoskeleton and ZO-1 in unstimulated HUVECs

Confluent HUVECs were starved for 2 h and either exposed to Y-27632 (5 μM) for 40 min or 24 h. For those treated with ML-7, the inhibitor was added either for 40 min (10 μM) or was added for 24 h (5 μM). For cells treated with C3 transferase, this inhibitor was applied at a concentration of 15 μg/ml in 0.1% glycerol for 4 h. Glycerol was found to increase cell uptake of C3 transferase (unpublished observations, Dr Beata Wojciak-Stothard) and was added for this reason. Inhibitor concentrations were chosen based on previous publications using HUVECs (Wojciak-Stothard et al., 1998, 2001). Cells were fixed and stained for F-actin (red) and ZO-1 (green). C3 indicates C3 transferase; white
arrows, fine actin cables ('mesh-like'); white arrowheads, a single line of F-actin; red arrows, continuous junctional ZO-1; red arrowheads, disjointed ZO-1. Bar = 20 μm.
4.2.3 The effects of Rho, ROCK and MLCK inhibition on early TNF-α-induced changes to the actin cytoskeleton and to ZO-1 localisation

As each of the inhibitors (ML-7, ROCK and C3 transferase) had affected cortical F-actin and ZO-1 distribution in resting HUVECs, it was possible that MLCK, ROCK and Rho were also involved in TNF-α-induced cytoskeletal changes and changes to the localisation of ZO-1. To test this, each of the inhibitors were applied to the cells prior to a 10 min incubation with TNF-α. C3 transferase inhibited cortical F-actin thickening induced by 10 min of TNF-α stimulation. Similar results were observed whether C3 transferase was applied apically to the cells (Fig. 4.6), or when it was microinjected (Fig. 4.8). These results suggest that Rho is required for early TNF-α-induced cytoskeletal reorganisation.

Similar to unstimulated HUVECs, ROCK inhibition prior to TNF-α stimulation for 10 min caused punctate and ‘mesh-like’ F-actin to form. Furthermore, ZO-1 staining at the cell periphery was discontinuous. Similar results were seen where a higher dose of TNF-α was used (Fig. 4.9). Moreover, Y-27632-treated cells do not look the same as C3 transferase-treated cells, implying that other proteins downstream of Rho may also be involved. This indicates that ROCK may contribute to cortical stress fibre formation, but is probably not crucial for the disruption of ZO-1.

ML-7 (10 μM) inhibited gap formation, ZO-1 redistribution and cortical F-actin thickening induced by TNF-α at 10 min (Fig. 4.6), indicating a role for MLCK in early TNF-α-induced changes to F-actin and ZO-1.
Cytoskeletal Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological Changes

Figure 4.6

Control

TNF-α 10 min

TNF-α 10 min + Y-27632 40 min

TNF-α 10 min C3 4 h

TNF-α 10 min + ML-7 10 μM 40 min
Chapter 4

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Figure 4.6

Inhibition of Rho and ML-7 prevents early TNF-α-induced changes to F-actin and ZO-1

HUVEC were grown to confluency and starved for 2 h and either left untreated or were incubated with 10 ng/ml TNF-α for 10 min. Where Y-27632 was used, it was applied at a concentration of 5 μM for 30 min prior to the addition of 10 ng/ml TNF-α for 10 min. For those treated with ML-7 (10 μM), the inhibitor was added for 30 min prior to the addition of TNF-α for 10 min. For cells treated with C3 transferase, this inhibitor was applied at a concentration of 15 μg/ml in 0.1% glycerol 4 h prior to the addition of 10 ng/ml TNF-α for 10 min. Cells were fixed and stained for F-actin (red) and ZO-1 (green). C3 indicates C3 transferase; white arrows, fine actin cables (‘mesh-like’); white arrowheads, single line of F-actin; red arrows, continuous junctional ZO-1; Bar = 20 μm.
4.2.4 Effects of Rho, ROCK and MLCK inhibition on late TNF-α-induced responses

The effects of Rho, ROCK and MLCK inhibition on late TNF-α-induced cytoskeletal and ZO-1 responses were also examined. C3 transferase abolished TNF-α-induced actin stress fibres and loss of ZO-1 from the cell-cell junctions at 24 h (Fig. 4.7). The lack of stress fibres across the cell body was clearly observed in confocal Z-stacks (Fig. 4.10). This indicates a role for Rho in late TNF-α cytoskeletal reorganisation and disruption of continuous junctional ZO-1 localisation.

ROCK inhibition using Y-27632 prevented TNF-α-induced stress fibre formation at 24 h and punctate and mesh-like F-actin were present instead (Fig. 4.7). The loss of TNF-α-induced actin stress fibres was also clearly visible in confocal Z-stacks (Fig. 4.10). Cell elongation induced by TNF-α was also completely blocked by ROCK inhibition. ZO-1 localisation to cell-cell junctions was not continuous, but instead formed punctate patches at the cell periphery in the presence of Y-27632, which localised with the mesh-like F-actin staining (Fig 4.7). ROCK is therefore involved in stress fibre formation and cell elongation, but may not be critical in dismantelling tight junctions. Similar results were seen where a higher dose of TNF-α was used (Fig. 4.9) and also if Y-27632 was applied to cells for the last hour of the 24 h TNF-α incubation (Fig 4.7). Overall, ROCK inhibition did not restore cells to control-like F-actin and ZO-1 localisation.

Y-27632 only partially blocked the TNF-α-responses, indicating that ROCK is involved in the TNF-α-induced changes, but is not solely responsible for all of them. Furthermore, cells treated with Y-27632 did not inhibit TNF-α-induced responses to the same extent as C3 transferase (compare in Fig. 4.7). This implies that other proteins downstream of RhoA might be involved in the TNF-α-induced changes, such as formins (Chapter 1).

ML-7 did not prevent stress fibre formation when applied to cells at a concentration of 5 µM for the entire 24-h TNF-α stimulation. Here, cells were elongated and contained strong actin stress fibres and disjointed ZO-1 as observed with TNF-α alone. To control for degradation of ML-7 that may have occurred over the 24 h period, ML-7 was applied...
at 10 μM for the final hour of a 24 h incubation. ML-7 was not able to inhibit the TNF-α-induced stress fibres (Fig. 4.7) and the cells contained thick actin stress fibres across the cell body and at the cell border. These results indicate that MLCK is important for early cellular responses to TNF-α, but is not important for maintaining long-term TNF-α-induced changes.
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Cytoskeletal Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological Changes

Figure 4.7

F-actin | ZO-1 | Merge
---|---|---
Control

TNF-α 24 h

TNF-α 24 h + Y-27632 24 h

TNF-α 24 h + Y-27632 last 1 h

TNF-α 24 h + C3 last 4 h
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Figure 4.7
Rho and ROCK, but not MLCK are important for late TNF-α-induced changes to HUVEC morphology, F-actin and ZO-1 distribution

HUVECs were grown to confluency and starved for 2 h and either left untreated or were incubated with 10 ng/ml TNF-α for 24 h. Where Y-27632 was used, it was applied at a concentration of 5 μM either for 30 min prior to the addition of 10 ng/ml TNF-α for 24 h, or was added to the cells for the final hour of the 24 h TNF-α incubation. For those treated with ML-7, the inhibitor (5 μM) was added 30 min prior to the addition of TNF-α for 24 h, or for the last hour of the 24 h TNF-α incubation (10 μM ML-7). For cells treated with C3 transferase, this was applied at a concentration of 15 μg/ml in 0.1% glycerol for the final 4 h of the 24 h TNF-α incubation. Cells were fixed and stained for F-actin (red) and ZO-1 (green). C3 indicates C3 transferase; white arrows, fine actin cables (‘mesh-like’); white arrowheads, single line of F-actin; red arrows, continuous junctional ZO-1, red arrowheads, disjointed ZO-1. Bar = 20 μm.
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Figure 4.8

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Figure 4.8

**Microinjected C3 transferase inhibits TNF-α-induced stress fibres and ZO-1 disruption**

Confluent HUVECs in full medium were microinjected (Ritu Garg) with 5 μg/ml C3 transferase in the presence of mouse-IgG in order to visualize the successfully injected cells. Where cells were stimulated with TNF-α, cells were either stimulated with TNF-α for 23.5 h prior to the microinjection, or they were injected with C3 transferase 30 min prior to the 10 min TNF-α incubation. Cells were fixed and stained for F-actin (red), ZO-1 (green) and IgG (blue). White arrows indicate fine actin cables; white arrowheads, single line of F-actin; red arrows, continuous junctional ZO-1. Bar = 20 μm
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Figure 4.9

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Figure 4.9

**Y-27632 inhibits higher doses of TNF-α**

Confluent HUVECs were starved for 2 h and either left untreated, or were stimulated with 100 ng/ml TNF-α for 10 min or 24 h. Where Y-27632 was used, this was applied to the cells 30 min prior to the addition of TNF-α. Cells were fixed and stained for F-actin (red) and ZO-1 (green) and examined under a confocal microscope. White arrows indicate fine actin cables; white arrowheads, single line of F-actin; red arrows, continuous junctional ZO-1, red arrowheads, disjointed ZO-1. Bar = 20 μm.
Figure 4.10

Y-27632 and C3 transferase inhibit TNF-α induced stress fibres

Confluent HUVECs were starved for 2 h and either left unstimulated (Con) or were stimulated with 10 ng/ml TNF-α for 24 hr. Where Y-27632 (5 μM) was used, this was applied for the final hour of the 24 h TNF-α incubation. Where C3 transferase (15 μg/ml) was used, this was applied apically for the final 4 hour of the 24 h TNF-α incubation in the presence of 0.1 % glycerol. Where ML-7 was used, it was applied at a concentration of 10 μM for the final h of the 24 h incubation. Cells were fixed and stained for F-actin and 11 confocal Z-stacks were taken of 4.0 μm and with an interval of 0.4 μm between each stack. These were merged to show the F-actin throughout the cell body. Arrow indicates a cell that has taken up C3 transferase. Bar = 20 μm.
4.2.5 Myosin II co-localises with TNF-α-induced actin stress fibres

Thrombin-induced permeability has largely been attributed to actomyosin interaction and contraction. It was therefore of interest to see whether the TNF-α-induced early cortical F-actin and late F-actin cables contained myosin II. To determine this, HUVECs were immunofluorescently stained for F-actin and myosin II. In resting HUVECs, myosin II was largely diffuse across the cell, but was excluded from the nucleus and localised with basal F-actin fibres (Fig. 4.11). TNF-α stimulation for 10 min induced thickening of the cortical F-actin, and this co-localised with myosin II at the cell periphery, although some myosin II was still present in the cytoplasm. In cells stimulated for 24 h with TNF-α, myosin II localised to F-actin stress fibres. The presence of both actin and myosin II within these cables confirms that they are stress fibres.
Figure 4.11

Myosin II co-localises with TNF-α-induced actin stress fibres

Cells were cultured on fibronectin-coated coverslips until confluent and starved for 2 h. Cells were either left untreated, or treated with 10 ng/ml TNF-α for 10 min or 24 h. Cells were fixed and stained for F-actin (red) and myosin II (green). White arrows indicate some areas of co-localisation between F-actin and myosin II. Bar = 20 μm.
4.2.6 TNF-α transiently increases phosphorylation of MLC on Ser^{19}

MLC phosphorylation is associated with increased endothelial permeability (Garcia et al., 1995, van Nieuw Amerongen et al., 1998). MLCK has been shown to phosphorylate MLC on Thr^{18} and Ser^{19}, whilst ROCK has been shown to phosphorylate Ser^{19} (Seto et al., 1996, Amano et al., 1996). Phosphorylation of MLC on Ser^{19} was examined by western blotting with a phospho-specific antibody. Phosphorylation was increased after TNF-α stimulation for 10 and 30 min and was lost by 4-24 h (Fig. 4.12). Total MLC protein levels were also downregulated by long-term TNF-α exposure and decreased below control levels. Thrombin is known to induce MLC Ser^{19} phosphorylation and is shown as a positive control (Fig. 4.12 C).

4.2.7 MLC Ser^{19} phosphorylation is dependent upon ROCK

ROCK is known to phosphorylate MLC on Ser^{19} (Amano et al., 1996). Y-27632 was used to inhibit ROCK to determine whether it is also responsible for MLC phosphorylation downstream of TNF-α. ROCK is required for MLC Ser^{19} phosphorylation by TNF-α as Y-27632 abolished the phosphorylation observed with TNF-α at 10 min (Fig. 4.13A and B). However, Y-27632 inhibition did not affect long-term loss of Ser^{19} phosphorylation (24 h) or MLC downregulation. Neither C3 transferase nor ML-7 were capable of preventing MLC phosphorylation (Fig. 4.13 B). This implies that ROCK is involved in this phosphorylation event, whereas RhoA and MLCK are not so important.

4.2.8 ROCK and RhoE protein levels do not change in response to TNF-α

As RhoA activity was not altered at 24 h, it is possible that other proteins that regulate the actin cytoskeleton are altered by TNF-α, resulting in the formation of stress fibres. This response is likely to involve transcriptional changes due to the length of time it takes for the stress fibres to appear (Chapter 3). A Possible candidate is ROCK because ROCK is known to induce stress fibres downstream of Rho (Riento & Ridley, 2003) and ROCK protein expression therefore, might be induced. To test whether TNF-α induces an increase in the protein levels of ROCK I or II, a western blot was performed. ROCK I and II protein levels remained unchanged after 24 h of TNF-α stimulation (Fig. 4.14).
Another protein that might be regulated by TNF-α is RhoE. RhoE negatively regulates actin stress fibre formation by binding to the N-terminal region of ROCK and inhibiting ROCK activity (Riento et al., 2003; Riento & Ridley, 2003). It is possible, therefore, that TNF-α might down regulate this protein, which in turn could cause an increase in stress fibres. Western blotting, however, revealed that total RhoE protein levels were not altered by TNF-α (Fig. 4.14).
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C)

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Figure 4.12

Myosin light chain (MLC) is Ser^{19} phosphorylated by TNF-α and thrombin and is down regulated by TNF-α

Confluent HUVECs were starved for 2 h and either left unstimulated or were treated with either 10 ng/ml TNF-α (A) or with 2 U/ml thrombin (B) for the times indicated. Cells were lysed and proteins separated by SDS-PAGE and detected by western blotting with a Ser^{19} MLC phospho-specific antibody. Total protein levels of MLC were also examined and Rac1 levels (A) are shown to demonstrate equal protein loading in each lane.
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Figure 4.13

MLC Ser\(^{19}\) phosphorylation is dependent upon ROCK and Rho, but not MLCK

Confluent HUVECs were starved for 2 h (A) or 4 h (B) and either left untreated or stimulated with 10 ng/ml TNF-α for the times indicated. For cells treated with Y-27632 (Y) only, 5 μM of Y-27632 was applied for 24 h. For stimulation with TNF-α as well as Y-27632, cells were incubated with Y-27632 for 30 min prior to the addition of TNF-α for 10 min, or 24 h (A). Alternatively, Y-27632 was added to the cells for the final hour of the 24-h TNF-α incubation (A and B). For cells treated with ML-7, this inhibitor was added at a concentration of 10 μM, 30 min prior to the addition of TNF-α (B). For cells treated with 15 μM C3 transferase (C3), this inhibitor was present during the 4 h starvation time and 10 min TNF-α stimulation (B). Cells were lysed and proteins separated and identified by SDS-PAGE and western blotting. MLC phosphorylation was detected using a Ser\(^{19}\) phospho-specific antibody. Total MLC levels are shown (MLC), and Rac1 levels are shown to demonstrate equal protein loading in each lane. M = minutes, h = hours.
Cytoskeletal Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological Changes

Figure 4.14
ROCK and RhoE protein levels do not change in response to TNF-α

Cells were starved for 2 h and either left untreated, or stimulated with 10 ng/ml TNF-α for 4 h, 10 h or 24 h (ROCK I and II). Proteins were separated and analysed using SDS-PAGE and western blotting. Alternatively, cells were starved for 2 h and stimulated with TNF-α for 10 min or 24 h (A). Myosin II and ERK 1 were used to show protein loading in each lane. RhoE was detected using either a mouse or rabbit primary antibody, both of which are shown.
4.2.9 ROCK, RhoA, stress fibres and MLCK are not required for TNF-α-induced increased permeability

As ROCK inhibition significantly inhibited the late TNF-α-induced effects on cell morphology and F-actin reorganisation and early MLC phosphorylation, it was possible that Y-27632 also inhibited TNF-α-induced paracellular permeability. To test this, HUVEC monolayers were pre-incubated with Y-27632 before and during the addition of TNF-α for 24 h and their permeability to FITC-dextran was assessed. In this experiment, FITC-dextran was present throughout the TNF-α incubation to give a measure of the continuous permeability changes to the monolayer. Although Y-27632 eliminated the actin stress fibres and cell elongation induced by TNF-α, inhibition of ROCK had no significant inhibitory effect on TNF-α-induced permeability from 8 to 30 h (Fig 4.15). Y-27632 alone slightly lowered the basal permeability of HUVECs by 30 min, but the permeability of Y-27632-treated cells was equal to control cell permeability at 24 h (Fig. 4.15). The effect of Y-27632 on TNF-α-induced HUVEC permeability was further studied in two different ways. In both instances, Y-27632 was applied to the cells for the final hour of the 24 h TNF-α incubation. In the first experiment, FITC-dextran was applied 30 min prior to the addition of TNF-α and permeability was measured before the addition of Y-27632 (23 h) and at the end of the 24 h TNF-α stimulation (24 h) (Fig. 4.16B). This gives a measure of the continuous changes to the permeability over the 24 h period. However, due to the large accumulation of FITC-dextran during the 24 h period, it was possible that any change in permeability was being masked by the high background levels. To overcome this, in the second type of experiment, FITC-dextran was applied after 24 h of TNF-α stimulation and readings were taken 1 hour sequentially to this (Fig.4.16C). This gives a measure of the more acute changes taking place over a 1 h period. In both experiments, the addition of Y-27632 for the final hour of a 24-h TNF-α incubation had no significant inhibitory effect on TNF-α-induced permeability, in fact permeability was slightly augmented (Fig. 4.16C).

Both methods for examining permeability were also used to test the effects of ML-7 on TNF-α-induced permeability. MLCK has been implicated in thrombin-induced increased
permeability due to its phosphorylation of MLC (Garcia et al., 1995). This could also be true for TNF-α. However, consistent with the lack of effect on cell morphology (Fig. 4.7), MLCK inhibition did not prevent TNF-α-induced permeability in either experiment (Fig. 4.16 B & C). ML-7 alone also did not significantly alter the permeability of HUVECs up to 24 h. To test whether Rho is also involved in the permeability increases caused by TNF-α, C3 transferase was used (Fig. 4.16 C). For this inhibitor, FITC-dextran was applied after 24 h of TNF-α stimulation and a reading was taken 1 h subsequently to this. Where C3 transferase was present for the final 4 h of a 24 h TNF-α stimulation it did not inhibit TNF-α-induced permeability.
Figure 4.15

ROCK inhibition does not affect TNF-α-induced permeability

The permeability of confluent HUVEC monolayers cultured on Transwell filters was determined using 40-kDa FITC-dextran. Cells were starved for 2 h prior to the addition of 100 ng/ml TNF-α for 24 h. Cells treated with Y-27632 were pre-incubated with 5 μM Y-27632 for 30 min before TNF-α was applied. Control cells were left unstimulated. FITC-dextran was applied to the apical chamber at the same time as TNF-α and samples of the basal medium were taken for fluorescence measurements at the time points indicated. Each condition is the mean of three monolayers and is shown as a percentage of control ± SD. * = p ≤ 0.05 compared to control; ** = p ≤ 0.01 compared to control; * = p ≤ 0.05 compared to TNF-α; ** = p ≤ 0.01 compared to TNF-α.
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Cytoskeletal Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological Changes

A)

![Graph A]

B)

![Graph B]
Cytoskeletal Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological Changes

Figure 4.16

Rho, ROCK, MLCK and actin stress fibres are not required for TNF-α induced increased permeability

The permeability of confluent HUVEC monolayers cultured on Transwell filters were determined using 40-kDa FITC-dextran. Cells were starved for 2 h prior to the addition of 10 ng/ml TNF-α for 24 h. For cells treated with C3 transferase, this was added at a concentration of 15 μg/ml for the final 4 h of the 24 h TNF-α incubation. For those treated with 10 μM ML-7 or 5 μM Y-27632, the inhibitors were added for the last hour of the 24 h TNF-α incubation. Control monolayers were placed into starvation media only. FITC-dextran was applied either 30 min prior the addition of the TNF-α incubation and samples taken at the times indicated (A & B), or was applied after the 24 h TNF-α incubation and samples subsequently taken from the lower chamber after 1 h (C). Each condition is the mean of a minimum of three monolayers and is shown as a percentage of control ± SD. * = p ≤ 0.05 compared to control; ** = p ≤ 0.01 compared to control; * = p ≤ 0.05 compared to TNF-α; ** = p ≤ 0.01 compared to TNF-α.
4.2.10 Dominant negative Rac1, but not dominant negative RhoA or Cdc42 significantly inhibit TNF-α-induced stress fibres and morphological changes

To clarify the involvement of Rho, Rac and Cdc42 in TNF-α-induced stress fibre assembly and morphological changes, adenoviruses carrying the cDNA for dominant negative RhoA (Ad-N19RhoA), Rac1 (Ad-N17Rac1) or Cdc42 (Ad-N17Cdc42) (Wojciak-Stothard et al., 2001) were applied to cells prior to a 24-h TNF-α incubation. In contrast to C3 transferase, dominant negative RhoA did not significantly inhibit TNF-α-induced stress fibre assembly and cell elongation at 24-h (Fig. 4.17), although some restoration of ZO-1 to a smooth junctional localisation was observed. Similarly, dominant negative Cdc42 did not inhibit TNF-α-induced changes (Fig. 4.17). Only dominant negative Rac1 profoundly altered the cellular response to TNF-α (Fig. 4.17). In these cells, actin stress fibre formation was completely abolished, and only a single line of peripheral F-actin remained at cell-cell junctions. ZO-1 was largely lost from cell-cell junctions and in some cells clustered in the cytoplasm (Fig. 4.17). The cells were also less elongated than control TNF-α-treated cells. Interestingly, β-galactosidase-expressing viruses (Ad-β-gal), which were used as a control in this experiment, inhibited the TNF-α responses. This may be due to signalling by the viruses themselves (Chapter 6). As inhibition of RhoA with C3 transferase had abolished TNF-α-induced stress fibres (Fig. 4.4), it seemed inconsistent that the dominant negative RhoA did not also inhibit this response. It is possible that the dominant negative RhoA was not sufficiently expressed to inhibit TNF-α-induced changes. Indeed, Ad-β-gal itself is sufficient to activate RhoA (Chapter 6), indicating that Ad-N19RhoA might be activating RhoA upon infection, and also expressing dominant negative RhoA cDNA.
Cytoskeletal Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological Changes

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<td><strong>TNF-α 24 h &amp; N17Cdc42</strong></td>
<td><img src="image" alt="TNF-α 24 h &amp; N17Cdc42 F-actin" /></td>
<td><img src="image" alt="TNF-α 24 h &amp; N17Cdc42 ZO-1" /></td>
<td><img src="image" alt="TNF-α 24 h &amp; N17Cdc42 Merge" /></td>
</tr>
<tr>
<td><strong>TNF-α 24 h &amp; N17Rac1</strong></td>
<td><img src="image" alt="TNF-α 24 h &amp; N17Rac1 F-actin" /></td>
<td><img src="image" alt="TNF-α 24 h &amp; N17Rac1 ZO-1" /></td>
<td><img src="image" alt="TNF-α 24 h &amp; N17Rac1 Merge" /></td>
</tr>
</tbody>
</table>
Dominant negative Rac1, but not dominant negative RhoA or Cdc42, significantly inhibits TNF-α-induced stress fibres and morphological changes

HUVEC monolayers were cultured on fibronectin-coated coverslips until confluent and were exposed to Ad-N17Rac1, Ad-N19RhoA, Ad-N17Cdc42 at a MOI of 500 for 90 min before being washed in PBS and placed in full medium for 18 h. Cells were then starved for 2 h and treated with 10 ng/ml TNF-α for a further 24 h. Cells were fixed and stained for expression of the GTPases using 9E10 anti-myc epitope antibody (blue). This was possible because the expressed proteins contain a myc epitope at their N-terminus. ZO-1 (green) and F-actin (red). Bar = 20 μm.
4.2.11 N17Cdc42 and N19RhoA, but not N17Rac1 reduce TNF-α-induced permeability

Dominant negative RhoA, Rac1 or Cdc42 were used to test the involvement of Rho GTPases in TNF-α-induced permeability. Despite a lack of inhibition of stress fibre formation, N17Cdc42 and N19RhoA partially inhibited the increase in permeability, but not fully. However, Ad-β-gal also reduced the permeability increase. N17Rac1 had no effect on TNF-α-induced permeability (Fig. 4.18).
Ad-β-gal, N17Cdc42 and N19RhoA but not N17Rac1 reduce TNF-α-induced permeability

The permeability of confluent HUVEC monolayers were determined using 40-kDa FITC-dextran. HUVECs were cultured to confluency on Transwell filters and exposed to either Ad-N17Rac1, Ad-N19RhoA, Ad-N17Cdc42 or Ad-β-gal (b-gal) at a MOI of 500 for 90 min. Cells were washed in PBS and placed in full medium for 18 h. Cells were then starved for 2 h and treated with 10 ng/ml TNF-α for a further 24 h. FITC-dextran was applied to the apical surface of the monolayer simultaneously with the TNF-α and samples of the basal media were taken at 24 h for fluorescence measurements. Each condition is the mean of six independent monolayers from two separate experiments. Permeability is shown as a percentage of control ± SD. * = p ≤ 0.05 compared to control, ** = p ≤ 0.01 compared to control, * = p ≤ 0.05 compared to TNF-α, ** = p ≤ 0.01 compared to TNF-α.
4.3 Discussion

Using the specific inhibitors C3 transferase, Y-27632 and ML-7 the roles of Rho, ROCK and MLCK were investigated in TNF-α-induced actin cytoskeletal, junctional and permeability changes in HUVECs, both at early and late time points.

The early TNF-α-induced thickening of the cortical F-actin is accompanied by phosphorylation of MLC and redistribution of myosin-II to the cell periphery where it co-localises with F-actin. This suggests that intercellular gap formation and increased permeability may be linked with cytoskeletal contractility, similarly to the mechanism for thrombin (Garcia et al., 1995; Bogatcheva et al., 2002). Consistent with this hypothesis are the observations that Rho, ROCK and MLCK inhibition had some inhibitory effects upon early TNF-α-induced cytoskeletal reorganization and C3 transferase and ML-7 restored ZO-1 to continuous junctional staining. Furthermore, RhoA is activated by TNF-α at early time-points and ROCK inhibition prevents MLC phosphorylation. However, the response of HUVECs to TNF-α is not as strong as their response to thrombin (Fig. 3.2), which at a concentration of 1 unit/ml induces actin stress fibres, gap formation and a 10-fold increase in permeability to horseradish peroxidase by 15 min (Essler et al., 1998). This indicates that different signalling mechanisms may be involved. Suprisingly, neither C3 transferase nor ML-7 inhibited MLC Ser^{19} phosphorylation. There are several possible explanations for this. TNF-α might also signal to ROCK in a Rho-independent manner, for example by relieving ROCK inhibition, which may sufficiently activate ROCK to phosphorylate MLC in the presence of C3 transferase. Alternatively, as C3 transferase was absorbed by clusters of cells and not by the monolayer as a whole, it may not have been present in enough cells to induce a detectable inhibitory effect on MLC phosphorylation.

MLCK has been shown to phosphorylate both Thr^{18} and Ser^{19} of MLC (Ikebe et al., 1985, 1986, 1988; Hixenbaugh et al., 1997) and so it could be that its primary target in HUVECs is Thr^{18}, whereas ROCK is more important for MLC Ser^{19} phosphorylation. Phosphorylation of both sites may be required for stress fibre formation and bundling at the cell periphery, which could be why an inhibition of either protein inhibits cortical F-
Cytoskeletal Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological Changes

actin thickening. In support of this, in smooth muscle cells, Ser^{19} phosphorylation alone may not be sufficient to induce contractility, as without calcium to activate MLCK, there is minimal MLC phosphorylation and contraction (reviewed in Riento and Ridley, 2003). It would be interesting to investigate the mechanism for MLCK activation in response to TNF-α, as no increase in cytosolic calcium is detected with this cytokine (Tiruppathi et al., 2001; Schutze et al., 1991).

It is interesting to note the importance for MLCK in early TNF-α-induced actin rearrangements, but the lack of its involvement in late stress fibre formation, morphological changes or permeability. Furthermore, contractility may play a lesser role in the late TNF-α-induced permeability compared to the earlier responses, as MLC is not phosphorylated at this time and its protein levels are down-regulated. This is consistent with observations from microarrays that report the downregulation of the smooth muscle isoform of myosin regulatory light chain 2 mRNA by approximately 2-fold after TNF-α stimulation for 2 h in HUVECs (Zhou et al., 2002). It is possible that MLC is targeted for degradation. Interestingly, total myosin II protein levels were unchanged (Fig. 4.14) and myosin II co-localised with the F-actin cables, which confirms that they are stress fibres. These results imply that myosin II may be important for stress fibre stabilisation, but contractility of the fibres is not required after 24 h of TNF-α treatment.

No increase in RhoA activity is observed to correlate temporally with actin stress fibre formation at 24 h. Basal Rho activity is required for the maintenance these stress fibres, as C3 transferase abolishes them. As cells treated with Y-27632 alone or simultaneously with TNF-α do not resemble cells treated with C3 transferase, it is likely that another Rho effector is involved in the formation of TNF-α-induced stress fibres. Such an effector might be upregulated by TNF-α and low RhoA activity might be sufficient for its activation. In this regard, ROCK and RhoE protein levels did not change, demonstrating a lack of transcriptional or translational regulation of these proteins by TNF-α. Another good candidate is the formin family (Chapter 1). Formins are Rho GTPase effector proteins that can induce the formation of F-actin cables, even if overexpressed in the presence of C3 transferase (Watanabe et al., 1997) (Chapter 1). Simultaneous activation
of both ROCK and mDia1 are required to induce stress fibre organisation that is indistinguishable from those induced by active Rho in HeLa cells (Watanabe et al., 1999). It is, therefore possible that a formin may contribute to stress fibre formation downstream of TNF-α in HUVECs.

Rac has been implicated in maintaining barrier function. Furthermore, precise Rac activity is required for proper barrier maintenance, as either Rac activation or inhibition alters permeability (Wojciak-Stothard et al., 2001, van Wetering et al., 2002). Recently, Rac1 activation has been shown to contribute to VEGF-induced permeability in vivo (Eriksson et al., 2003). Although no change in Rac1 activity was detected here, dominant negative Rac1 dramatically abolished stress fibres and cell elongation, and reduced cortical F-actin to a single peripheral band. However, it did not restore ZO-1 to the cell-cell junctions; in fact it induced removal of ZO-1 from the cell periphery and in some cases ZO-1 was observed on vesicular structures. Consistent with this apparent loss of tight junction integrity, dominant negative Rac1 did not inhibit TNF-α–induced permeability and in one experiment N17Rac1 itself increased basal permeability (data not shown), which is consistent with other reports (Wojciak-Stothard et al., 2001; van Wetering et al., 2002). These results imply that Rac1 may contribute to TNF-α-induced cytoskeletal reorganisation, but is also required for tight junction barrier maintenance, and may fine-tune the extent of barrier breakdown.

Dominant negative RhoA, like dominant negative Cdc42 had a negligible effect on TNF-α–induced stress fibres. This is inconsistent with the strong effect of C3 transferase. It is possible that N19RhoA was not sufficiently expressed in these cells to induce an inhibitory effect on the stress fibres. Another possibility is an effect by the viruses that were used for the transfection. Ad-β-gal itself activates RhoA (Chapter 6) and it is possible that with Ad-N19RhoA, the viruses activate endogenous RhoA, whereas the construct that they are carrying expresses dominant negative RhoA. This might account for a lesser effect by the dominant negative construct, as the endogenous RhoA might simultaneously be activated.
None of the inhibitors used were sufficient to inhibit long-term TNF-α-induced permeability. The lack of inhibition by ML-7 is consistent with the observations that ML-7 did not prevent cell-elongation or gap formation and indicates that MLCK may not be important for long-term TNF-α effects. Y-27632 only partially prevented ZO-1 redistribution from the cell-cell junctions after 24 h of TNF-α exposure, which is consistent with a lack of its effects on TNF-α-induced permeability. Rho inhibition with C3 transferase also did not inhibit TNF-α-permeability, despite its inhibitory effects on stress fibres and ZO-1 relocalisation, although Ad-N19RhoA did slightly reduce TNF-α-induced permeability. This indicates that signalling pathways independent of Rho are also important for TNF-α-induced permeability.

Two models for the early and late responses of HUVECs to TNF-α, based on these results are shown in Figure 4.19.
Chapter 4

Cytoskeletal Mechanisms Involved in TNF-α-induced Permeability Increases and Morphological Changes

A) Early TNF-α-induced Permeability

- Actin polymerisation
- RhoA
- ROCK
- mDia
- MLC
- Stress Fibre formation
- Contractility
- Tight Junction Proteins
- Permeability

B) Late TNF-α-induced Permeability

- Actin polymerisation
- RhoA
- ROCK
- MLC
- Stress Fibre formation
- Tight Junction Proteins
- Permeability

N.B. Crosses indicate a lack of activation of that pathway
Figure 4.19

Model for early TNF-α-induced permeability (A)

TNF-α-induces a small and transient early increase in permeability, which returns to basal levels by 1 h (Fig. 3.8). MLC phosphorylation, cortical stress fibre formation and contractility are all important for the early TNF-α-induced responses. TNF-α signals to, and activates RhoA within 1 min (pull-down assays). RhoA activates ROCK, and probably other regulatory proteins such as Dia1/2, which regulate the cytoskeleton in order to co-ordinate actin polymerisation, and the thickening of cortical F-actin. Rho may cause the tight junction proteins to become disjointed either by cytoskeletal re-arrangements or signalling independently of the actin cytoskeleton (Rho inhibition prevents stress fibre formation and ZO-1 redistribution at the cell junctions). ROCK phosphorylates MLC on Ser\(^19\) (ROCK inhibition prevents MLC Ser\(^19\) phosphorylation in western blots). This contributes to stress fibre formation and/or contractility (ROCK inhibition partially prevents stress fibre formation). TNF-α also activates MLCK by an unknown mechanism. MLCK is important for stress fibre formation and ZO-1 redistribution at cell-cell junctions (MLCK inhibition prevents stress fibre formation and ZO-1 redistribution). It may do this through phosphorylation of MLC, but not on MLC Ser\(^19\) (MLCK inhibition does not prevent MLC Ser\(^19\) phosphorylation in western blots). It may phosphorylate MLC on Thr\(^18\). The result of these signals is intercellular gap formation and a small increase in permeability (which is difficult to detect using FITC-dextran - Chapter 3).

Model for late TNF-α-induced permeability (B)

Late TNF-α-induced permeability is delayed and sustained. Actin stress fibres form that traverse the cells and some tight junction proteins are redistributed away from the cell junctions (Chapter 3). Stress fibre formation and contractility are less important for increased permeability than at earlier times (Removal of stress fibres with C3 transferase or Y-27632 does not restore basal permeability; MLC is not phosphorylated; MLC is down-regulated). Although RhoA is not activated by TNF-α at later times, it is required for stress fibre formation and ZO-1 re-localisation way from the tight junctions.
(inhibition of Rho removes stress fibres and restores ZO-1 to the tight junctions), but this is only partially dependent upon ROCK. ROCK inhibition removes stress fibres, but leaves a fine F-actin mesh-work; ROCK inhibition does not completely restore ZO-1 to tight junctions). However, Rho alone is not sufficient to increase permeability (C3 transferase does not inhibit increased permeability, N19RhoA slightly reduced it), indicating that signalling independently of Rho is occurring. This is not via MLCK (MLCK inhibition does not prevent stress fibre formation, cell elongation, ZO-1 redistribution or increased permeability).
5 Identification of Novel Interacting partners of Occludin

5.1 Introduction

Occludin is a ~65 kDa integral membrane protein present at the tight junction (Furuse et al., 1993). Occludin exhibits adhesive, barrier and fence function roles and has been shown to bind to several neighbouring tight junction proteins, as well as some signalling molecules (Chapter 1). Occludin contains a coiled-coiled domain within its C-terminal region to which many of its associating proteins bind (Fig 5.1), including the MAGUK proteins ZO-1, -2 and -3 (Furuse et al., 1994), F-actin (Wittchen et al; 1999; Nusrat et al., 2000), PKC-ζ, c-Yes, p85 regulatory subunit of PI 3-kinase, connexin-26 and other occludin molecules (Nusrat et al., 2000). Casein kinase 2 (CK2) and p34cdc2/cyclinB phosphorylate the occludin E-domain in vitro (C-terminal cytoplasmic domain, Furuse et al., 1994, Cordenonsi et al., 1999), which encompasses the coiled-coiled region (amino acids 396-428 of \(X. laevis\) occludin, Cordenonsi et al., 1999 and Leu\(^{440}\) – Glu\(^{469}\) in human occludin, Ando-Akatsuka et al., 1996; Nusrat et al., 2000) and cingulin and has been shown to bind to the C-terminal region of occludin, although the specific regions are not known (Cordenonsi et al., 1999, 1997). The intracellular N-terminus of occludin also regulates permeability, as truncation of this domain results in increased paracellular permeability and decreased TER (Bamforth et al., 1999). Finally, the ubiquitin protein ligase, Itch, associates with the occludin N-terminus (Traweger et al., 2002). This is so far the only protein known to bind to this region of occludin.

This chapter describes a yeast two-hybrid screen (5.2) and GST-pull down experiments (5.3) employed to identify novel binding partners for occludin. The initial aim was to identify further proteins that interact with the N-terminal cytoplasmic domain, and also with any region within the C-terminal cytoplasmic domain, not just the short coiled-coiled domain. In this respect it is noteworthy that the coiled-coiled region is approximately 29 amino acids in length (Ando-Akatsuka et al., 1996; Nusrat et al., 2000), whereas the entire C-terminus is a total of 258 amino acids, providing ample scope for association with other proteins outside of this \(\alpha\)-helical region. Occludin-interacting partners are of interest.
Figure 5.1

Structure of human occludin

Occludin is an integral membrane protein with cytoplasmic N- and C-terminal regions, both of which were used for a yeast two-hybrid screen for interacting protein partners. Acidic (solid circle), basic (cross-hatch) and uncharged (open circle) residues at neutral pH are indicated. Glycines are represented as (triangle) and tyrosines as (inverted triangle); red numbering (1-4) indicates the transmembrane regions; red letters (A-E) represents the occludin domains designated by Furuse et al., 1994; area between the black lines is the coiled-coil region. Modified from Mitic and Anderson, 1998.
because of their potential roles in regulating occludin function at the tight junction, and also to further our understanding of the composition of, and signalling to, the tight junction itself.

5.1.1 Principles of a yeast two-hybrid screen

A yeast two-hybrid screen is a transcriptional assay for detecting protein interactions \textit{in vivo} in \textit{Saccharomyces cerevisiae}. It can therefore be used to detect novel protein-protein interactions. The DNA binding domain (DNA-BD) of the transcription factor, GAL4 is fused to the bait protein and used to screen a library containing potential interaction proteins that are fused to the activation domain (AD) of GAL4. If the bait and library proteins interact, then the DNA-BD and AD are brought into close proximity, which activates transcription of a reporter gene (Fields et al., 1989, Fig. 5.2.A).

Yeast promoters consist of a minimal promoter region called the TATA box, which designates the transcription start site and determines the basal levels of transcription. Also present are cis-acting elements (operators) to which regulatory proteins can bind and influence transcriptional levels. In yeast, the TATA box is usually located 25 base pairs upstream of the start of transcription, and is usually in relatively close proximity to the cis-acting element. There may also be more than one TATA box present upstream of the start site, such as the \textit{S. cerevisiae his3} gene, which contains two TATA boxes, one of which is regulated and the other of which is constitutive (Chen et al., 1988; Mahadevan et al., 1990).

One type of cis-acting element is the upstream activating sequence (UAS). This sequence can be in several orientations, but in order to regulate its target gene it must not be more than a few hundred base pairs from the TATA box. Specific transcription factors can recognise the UAS and enhance transcription from the downstream TATA box. It is also possible for more than one UAS to be present upstream of the yeast coding region.

The HF7c strain of \textit{S. cerevisiae} used in this screen carries two reporter genes, the \textit{his3} and \textit{lacZ} genes, both of which contain a UAS that is regulated by GAL4 (Fig 5.2) (Feilotter et al., 1994). In the case of \textit{his3}, the UAS and the minimal promoter originate from the native
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Identification of novel interacting partners of occludin

Figure 5.2

A schematic diagram showing bait and library gene products interacting in HF7c S. cerevisiae, which brings the GAL4 DNA-BD and AD into close proximity and activates transcription of the reporter genes, his3 and lacZ (A). The selection advantages conferred to the yeast when both vectors are present and an interaction between bait and library gene products occurs are shown in (B). Only yeast expressing HIS3, TRP1 and LEU2 can survive on plates lacking histidine, tryptophan and leucine.
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\textit{GAL1} promoter region. For \textit{lacZ}, the UAS is a synthetic 17'\textsuperscript{mer} and its minimal promoter is from the yeast cytochrome C1 (\textit{cycl}) gene, which is a weak promoter and so weak protein interactions may not be observed unless a highly sensitive \(\beta\)-galactosidase assay is employed (Feilotter et al., 1994; Yeast protocols handbook, Clontech).

The native yeast \textit{his3} promoter region contains a UAS site recognised by the transcriptional activator GCN4 and two TATA boxes. In HF7c, this entire region has been replaced as described above, resulting in tight regulation of the \textit{his3} reporter in this strain. Furthermore, HF7c yeast lacks \textit{leu2} and \textit{trp1} genes, providing auxotrophic markers for selection of cells containing the DNA binding and activation vectors (Feilotter et al., 1994).

Finally, HF7c also contains deletions of endogenous \textit{gal4} and \textit{gal80} genes. This is important as in the presence of galactose, endogenous GAL4 will bind to its UAS in order to activate transcription of galactose metabolism genes. Also, in the absence of galactose, GAL80 will bind to GAL4 and suppress gene expression (Johnston et al., 1994; Yeast protocols handbook, Clontech). To prevent endogenous GAL80 and GAL4 from interfering with the reporter gene transcription, their genes have been removed (Feilotter et al., 1994).
5.2 Results

5.2.1 Cloning of the Bait Vector

The first step in a yeast two-hybrid screen is the cloning of the desired bait sequence into the bait vector, in this case pGBT9 (Fig. 5.3). This vector contains a multiple cloning site into which the bait sequence is cloned to produce the GAL4-DNA-BD-bait fusion protein. This protein expression is driven by the constitutive \textit{adh1} promoter, which leads to expression of sequences under its control during logarithmic growth of host \textit{S. cerevisiae}. pGBT9 also contains a \textit{trp1} gene, which enables yeast carrying this plasmid to grow on selection plates that lack tryptophan amino acid (Fig. 5.2B). DNA encoding the N-terminal 60 amino acids of occludin (Fig. 5.1 and 5.4) was cloned into the pGBT9 vector, in frame, at the BamHI/PstI restriction enzyme site. This vector was named pGBT9-Nocc. Separately, DNA encoding the C-terminal 258 amino acids of occludin (Fig. 5.4) was cloned into this vector, also in frame, at the EcoRI/BamHI restriction enzyme sites and was called pGBT9-Cocc. The vectors were then sequenced to ensure that the inserts had ligated successfully and were indeed in the correct frame and orientation.
Figure 5.3

The pGBT9 vector and multiple cloning site.

This vector produces GAL4BD-bait fusion protein. Yeast containing this vector can express TRP1 and survive on –Trp selection plates. From www.bdbiosciences.com
Figure 5.4

A)
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901  gtg tct gca gcc aca cag gag gtg cct tca ccc cca tct gac tat
301  V S A G T Q D V P S P S D Y
945

946  gtg gaa aga gtt gag act ccc atg gcc tca tac tct tcc aat ggc aaa
316  V E R V D S P M A Y S S S N G K
990

991  gtg aat gag aag cgg ttt tat cca gag tct tcc tat aaa tcc agc
331  V N D K R F Y P E S S Y K S T
1035

1036  ccc gtt cct gaa gtg gtt cag gag ctt cca tta act tcp cct gtg
346  P V P E V V E B L P L T S P V
1080

1081  gat gac ttc agg cag cct cgt tac acg agc ggt ggt aac ttt gag
361  D D F R Q P Y S S G N P E
1125

1126  aca cct tca aca aga gcc cct gcc aag gga aca gca gga agg tca
376  T P S K R A P A K G R A G R S
1170

1171  aag aga aca gag cca aat cac tat gag aca gac tac aca act ggc
391  K R T E Q D H Y T E D Y T T G
1215

1216  ggc gag tcc tgt gat gag ctt gag gag gag tgg atc aag gaa tta
406  G E S C D E L E E D W I R E Y
1260

1261  cca cct act act tca gat cca cca aca cag cta tac aag aag aat
421  P P I T S D Q Q R Q L Y K R N
1305

1306  ttt gag act ggc cta cag gag cca aac tta cca tca gaa cct
436  F D T G L Q E Y K S L Q S E L
1350

1351  gat gag atc aat aaa gaa ctc tcc cgt tgg gat aag gaa tgg gat
451  D B I N K L S R L D K E L D
1395

1396  gac tat aga gaa gaa agt gaa gag tac atg gct gct gat gaa
466  D Y R E E S E E Y M A A A D E
1440

1441  tac aat aga ctg aag cca gtg aag gga tct gca gat tat aca agt
481  Y N R L K Q V K G S A D Y K S
1485

1486  aag aag aat cat tgc aag cag tta aag acg aca aaa tgg tca cag atc
496  X X H N C K Q L K S K L S H I
510

1531  aag aag atg gtt gga gat tat gat aga cag aca aca tag aag gct
511  K K M V G D Y D R Q K T * K A
555

1576  gat gcc aag tgt ttt gag aaa tta agt atc tga cat ctc tgc aat
526  D A K L P E K L S I * H L C N
580

1621  ctt ctc aga aga cag aag act atg gac cat aac ccc gga agc cca
541  L L R R Q M T L D H N P G S Q
625

1666  acc tct gtg agc atc aca aag ttt tgg gtt gct tta aca tca tca
556  T S V S I T K F W V A L T S S
670

1711  gta tgt aag cat att ata aat cgc ttt tga taa tca act ggg ctt
571  V L K H F I N R P * S T G L
725

1756  aac aac ttc aat taa gga ttt tat gct tta aac att ggt tct tgt
586  N N S N * G F Y A L N I G S C
780

1801  att aag aat gaa ata ctg ttt gag gtt ttt aag cct taa agg aag
601  I K N E I L F E V P K P * R K
835

245
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B)

\textit{N terminus}

\textbf{5' Primer and BamHI cleavage site:}

\begin{align*}
5' & \\
\text{CGG} & \text{//GATCCGTCATCCAGGCCTCTTGAAAG}
\end{align*}

\textbf{3' Primer and PstI cleavage site}

\begin{align*}
3' & \\
\text{CGG} & \text{//GATCCCTATGTTTTCTGTCTATCATAG}
\end{align*}

60 AA
180 nucleotides

C)

\textit{C terminus}

\textbf{5' Primer and EcoRI cleavage site:}

\begin{align*}
5' & \\
\text{CGG} & \text{//AATTCGTGAAAAACTCGAAGAAAGATG}
\end{align*}

\textbf{3' Primer and BamHI cleavage site}

\begin{align*}
3' & \\
\text{CGG} & \text{//GATCCCTATGTTTTCTGTCTATCATAG}
\end{align*}

259 AA
777 Nucleotides

\textbf{Figure 5.4}

The full length cDNA sequence for occludin is shown in (A). Grey highlight denotes the 5' and 3' primer sequences used for N-terminus cloning; red highlight denotes the 5' and 3' primer sequences used for C-terminus cloning; bold indicates the occludin coiled-coiled region. The primer sequences and their cleavage sites (//) for both N and C-terminus occludin are shown in (B) and (C) respectively.
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5.2.2 Library vector
The cDNA library used in this screen is created from human lung mRNA pooled from two caucasian females. This library was selected from a choice of lung, leukemia, lymphocyte or prostate libraries. The lung library was chosen as it is the richest of these in endothelial cells. cDNAs were cloned by Clontech into a pACT2 vector (Fig. 5.5) at the XhoI/EcoRI multiple cloning site (MCS) using a 5’ adaptor sequence containing an EcoRI restriction enzyme site and a XhoI-(dT)15 primer (Fig. 5.6). This results in partial cDNAs being cloned into the vector. Also, pACT2 contains a leu2 gene, which enables yeast carrying this plasmid to grow on selection plates that lack leucine (Fig. 5.2B).
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Figure 5.5
The library vector and multiple cloning site.
This produces GAL4AD-library fusion protein. Yeast with this vector can express LEU2 and survive on -Leu selection plates. From www.bdbiosciences.com
Figure 5.6
Making the cDNA library
This procedure was done by Clontech. Lung mRNAs are primed at the 3' end with an Oligo T primer containing an XhoI restriction enzyme site. Reverse transcriptase generates DNA complementary to the mRNA, resulting in an mRNA/cDNA hybrid. RNase H I nicks the mRNA and DNA polymerase repairs gaps in the cDNA. T4 DNA polymerase uses the series of primers left by RNase H for synthesis of a second DNA strand. A large molar excess of linker molecule containing an EcoRI restriction enzyme site is added and T4 DNA ligase joins the blunt end of the linker molecule with the 5' and 3' ends. Restriction digestion of the cDNA removes the unwanted EcoRI site at the 3' end and results in a double stranded cDNA molecule with sticky EcoRI 5' end and XhoI 3' ends. This cDNA is now ligated into the multiple cloning site (MCS) of pACT2.
5.2.2 Yeast transformation #1 – bait vector
In order to screen for interacting partners, the bait vector must first be transfected into the *S. cerevisiae* yeast strain, HF7c. Briefly, HF7c yeast cells were transformed with either 0.1 μg pGBT9-Nocc or pGBT9-Cocc using TE/LiAc transformation protocol #1 as described in Chapter 2 (Fig. 5.7A). The yeast were then plated onto -Trp/SD agar selection plates and incubated at 30 °C for 2-3 days. Only successful transformants containing the pGBT9 vector are capable of synthesizing TRP1 and growing on the limiting synthetic medium lacking Trp. One colony was picked from the selection plate and streaked onto a -Trp/-Leu/SD plate as a control measure. Transformed HF7c should be unable to grow on this double selection plate as they lack the ability to grow on medium lacking Leu. This was the case for yeast colonies containing both pGBT9-Nocc and pGBT9-Cocc. Glycerol stocks of pGBT9-transformed HF7c were prepared.

5.2.3 Yeast transformation #2 – library vector transformation efficiency
In order to test the library clones for interaction partners, the cDNA library should ideally be completely screened three times. This is calculated using the transformation efficiency of each transformation (Fig. 5.7B). Assuming that one library clone is taken up per yeast cell, the transformation efficiency describes the number of library clones that have been taken up by the yeast cells (and therefore forms a yeast colony) per μg of library DNA per transformation. Hence it is a measure of the number of library clones that have been tested for a bait interaction. As the number of independent cDNA clones within the library are known, it is therefore possible to calculate how many clones have been tested and how many further transformations need to be done to screen the whole library. The calculation for the initial transformation efficiency is shown in Table 5.1.

It is important that the transformation efficiency of pGBT9-containing yeast is optimised for the uptake of the library vector, so that the screen can be completed as quickly as possible. This is done by TE/LiAc yeast transformation using different amounts of library-plasmid DNA and determining the smallest amount of DNA that produces the most yeast colonies on -Trp/-Leu/SD selection plates. Only successful transformants containing the pGBT9 vector and the ACT2 vector are capable of synthesizing TRP1 and LEU2 and growth on the double
selection plates. In the case of the pGBT9-Cocc-yeast, 1 µg of library DNA was the smallest amount of DNA to produce the maximum amount of yeast clones, whereas 2 µg of library DNA was the smallest amount of DNA to produce the maximum amount of yeast clones when transformed into pGBT9-Nocc (Table 5.1A and B).
Figure 5.7

A) YPD media – all amino acids

Wash, make competent with TE/LiAC + bait plasmid
Incubate 30 °C

42 °C

Heat shock

Plate to -Trp media. Only transformed yeast containing bait plasmid can grow

Make glycerol stock

Control test:
Restreak one colony to -Trp/-Leu. Should be unable to grow as they cannot make LEU2
**Figure 5.7**  

**B)**

- Incubate overnight 100 ml
- Dilute to 2 x 500 ml and incubate ~ 5 h to correct growth phase and density
- X ~ 20 50 ml tubes
- Plate to -Trp/-Leu media. Only transformed yeast containing bait plasmid and library plasmid can grow. Use to work out transformation efficiency.
- Restreak healthy colonies (pink, well growing) to -Trp/-Leu media. Store these yeasts at 4 °C until entire library is screened.

- Infuse 2050 ml tubes
- Pellet cells
- 10 transformations per 50 ml tube
- Wash, make competent with TE/LiAC + library plasmid
- Incubate 30 °C
- Heat shock
- Plate to -Trp/-Leu media. Only transformed yeast containing bait plasmid and library plasmid can grow. Use to work out transformation efficiency.
- Restreak healthy colonies (pink, well growing) to -Trp/-Leu media. Store these yeasts at 4 °C until entire library is screened.

Repeat procedure until entire library is screened 3 times.
Figure 5.7

Schematics of the yeast transformation.

Schematic diagram displaying the steps involved in large-scale screening for interacting proteins in a yeast two-hybrid. (A) shows transformation protocol #1. (B) shows transformation protocol #2. See text for details.
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A)  
C terminus (pGBT9cOcc)  
<table>
<thead>
<tr>
<th>Amount of library DNA (µg)</th>
<th>Number of colonies on - Trp/-Leu plate</th>
<th>Colonies/µg</th>
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<tr>
<td>0.5</td>
<td>14</td>
<td>28</td>
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<td>30</td>
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<td>5</td>
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</table>

B)  
N terminus (pGBT9-nOcc)  
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<th>Amount of library DNA (µg)</th>
<th>Number of colonies on - Trp/-Leu plate</th>
<th>Colonies/µg</th>
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</thead>
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<td>5</td>
<td>44</td>
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C)  
<table>
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<tr>
<th>Library DNA (µg)</th>
<th>Number of colonies on double selection plates*</th>
<th>Number of Transformations</th>
<th>Number of triple selection plates (2 transformations/plate)</th>
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<td>Initial transformation efficiency</td>
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<td>1</td>
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<tr>
<td>To screen library once</td>
<td>550</td>
<td>3.3x10^8</td>
<td>550</td>
</tr>
<tr>
<td>To screen library three times</td>
<td>1560</td>
<td>9.9x10^8</td>
<td>1560</td>
</tr>
</tbody>
</table>
Table 5.1

Transformation efficiency tables

1 μg was the smallest amount of library DNA that produced the maximum amount of yeast clones when transformed into the pGBT9-Cocc-yeast (A), whereas 2 μg of the library DNA produced the maximum amount of yeast clones when transformed into pGBT9-Nocc-yeast (B). The calculations to determine the how many transformations were required to screen the library once and three times are shown in (C). This is an initial approximation based on the first transformation efficiency calculated. The transformation efficiency varies from day to day (see Discussion) and as a control, the efficiency was calculated from a -Leu/-Trp/SD selection plate for each transformation done. The calculation in (C) is based on the assumption that there are 3.3x10^6 independent clones present in the cDNA library (Clontech library data sheet).

* Calculated from a 1/200 dilution plated on to double selection plates: 1 μg of library DNA gave 30 yeast colonies on a -Trp/-Leu/SD selection plate after a 1/200 dilution.
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5.2.4 Screening for Interactions #1: Histidine production

The TE/LiAc transformation protocol was followed for the screen for interactions (Chapter 2 and Fig. 5.7B). Initially, 10 transformations, each of 50 ml yeast suspension in YPD media, were done to ensure the transformations were working before scaling up to 1 L YPD, resulting in approximately 100 transformations on to 50 -His/-Leu/-Trp/SD selection plates (2 transformations per plate). Each day yeast transformations were done, 1% of one transformation was plated onto a -Trp/-Leu/SD selection plate to check the transformation efficiency. Only yeast cells containing both the library plasmid and the bait plasmid are able to produce TRP1 and LEU2 and can grow on the double selection plates, which usually became visible after 2-3 days. Only yeast colonies where bait and library proteins are interacting can induce transcription of \textit{his3} and grow on the triple selection plates, which usually took between 3 and 5 days. After this time, healthy colonies (pink and well-growing) were picked from the triple plates, restreaked onto double plates and incubated for 3 days. These plates were then stored at 4 °C until the library screen was complete (Fig. 5.7B).

Unfortunately, pGBT9-Nocc plus the library vector resulted in too many colonies to count on the triple selection plates. This indicates that N-terminal occludin may itself induce transcription of \textit{his3} without binding to any other protein. This screen was terminated and only the pGBT9-Cocc screen was continued.

5.2.4.1 Leaky \textit{his3} expression and the use of 3-amino-triazole (3AT)

\textit{his3} has some leaky expression in many yeast strains (basal expression), which allows yeast that do not contain an interacting partner to grow on triple selection plates. This can be inhibited by using 3-amino-triazole (3AT), a competitive inhibitor of the \textit{his3} gene product. 3AT increases the stringency compared with that of medium simply lacking histidine and thus requires the yeast to produce more histidine in order to survive (Odom et al., 2002). However, this needs to be titrated to ensure that the correct concentration is used, as too much would eliminate detection of weak binding partners, whereas too little allows larger numbers of false positives.
Due to variable, and sometimes low, transformation efficiencies, the amount of yeast cells per transformation was doubled. This improved the transformation efficiency, but also increased the background lawn of small transparent/white yeast colonies on the triple plates. To overcome this problem, 3AT was used in the selection plates. Triple selection plates containing 0.25 mM, 0.5 mM, 1 mM, 1.5 mM and 2.5 mM and 5 mM 3AT were used to test the appropriate amount to use. 0.25 and 0.5 mM 3AT plates still had background similar to plates without 3AT. 1, 1.5 and 2.5 mM 3AT eliminated the background, but only white colonies were produced, indicating that the yeast containing putative interaction partners were struggling to grow (as the colonies should be pink/red) and that weaker partners may be unable to grow altogether. 5 mM 3AT plates contained no background and no colonies. 1 mM 3AT was therefore chosen for subsequent transformations, as it was the lowest dose to reduce background, but still allowed possible interaction partners to grow, although the colonies were usually white.

Colonies that were able to grow on 1 mM 3AT triple selection plates were used in the X-gal screen for lacZ gene expression.
5.2.5 Screening for Interactions #2: X-gal screen

Interacting bait and library proteins are also able to induce transcription of the lacZ reporter gene present in the HF7c yeast strain. The protein product of lacZ is β-galactosidase, a protein able to cleave X-gal, which results in a visible blue colour. Yeast colonies that had been selected and stored from the screen above were used in this second screen for β-galactosidase activity (Fig. 5.8), as described in Chapter 2.

Of the thirty-two colonies able to survive on triple plates in the first screen, eight were able to produce a blue colour when exposed to X-gal (Fig. 5.9A). These were designated A-H.

5.2.6 Rejection of bait vector

In order to identify the library cDNA clones that are successful in both the interaction screens, the pACT2 vector containing the library clone must be purified from the yeast and sequenced. To prevent interference in this procedure by the bait-containing plasmid, the yeast is grown under conditions favouring expulsion of this pGBT9 vector from the yeast (Chapter 2). All eight yeast colonies successfully threw out the bait vector. This was determined by their ability to grow on plates lacking Leu (as the yeast still contains leu2-containing pACT2), but their inability to grow on plates lacking Leu and Trp (as they lack the trp1-containing pGBT9).

5.2.7 Isolation of library cDNA from pACT2

The pACT2 vector was purified from the HF7c yeast strain using phenol/chloroform extraction and ethanol precipitation (Chapter 2). After purification of the eight library vectors from the HF7c yeast, the plasmids were successfully transformed into E. coli and two bacterial colonies were picked from each bacterial plate. These picked clones were designated A1, A2, B1, B2 and so forth. DNA purified from each colony was digested with BamHI and XhoI to excise the library cDNA insert from the vector (Fig. 5.9B). The size of the inserts varied from plate to plate, but in each case, the two clones picked from the same plate contained inserts of the same size.
Yeast that survived -Trp/-His/-Leu in previous screen and have been stored

Filter paper
Overlay yeast

Mark the orientation with needle, so that colonies can be identified later

Peel off filter paper
Plunge into liquid N₂ 10 sec
This lyses the yeast cells

Place filter paper into a fresh petri dish containing X-gal
Incubate overnight

Few hours / next morning: If there are interacting proteins and β-galactosidase has been produced, X-gal is cleaved and the yeast turns blue

Return to original plate to identify and restreak the yeast containing interacting proteins

Figure 5.8
X-gal screen
Schematic diagram displaying the steps involved in X-gal screen for interacting partners. See text for details.
Figure 5.9

X-gal screen and library cDNA excision from pACT2

(A) shows a representative example of some yeast clones that produced a blue colour in the X-gal screen. This screen was repeated twice. (B) shows DNA digests of the library cDNA.
from the pACT2 vector from the yeast clones that produced a blue colour in the X-gal screen. BamH1 and Xho1 restriction enzymes were used for the restriction digest. same plate (eg. A1 and A2) produced the same insert size when digested (as they contained the same insert). The vector is 8.1 kb, and a band of this size was observed in each digest.
5.2.8 Sequencing, BLAST database search and binding partner identification

All the clones contained approximately the correct size for the vector and an insert after restriction enzyme digestion and were sequenced using a forward pACT2 plasmid primer from 5' of the insert (Fig. 5.10) (Chapter 2). cDNA sequences A2, B1, B2, C1, D1, E1, F1, G2 and H2 were then applied to the BLAST database (NCBI) which should identify known cDNAs containing these sequences and hence the bait-interacting proteins (Table 5.2).

Three sequences were identified as Casein kinase I ε (δ isoforms is very similar to CKIε and may also be a match), Unc-51 like kinase (ULK-1) and Ski-interacting protein (Skip). These are therefore potential occludin-interacting proteins. The cDNA and amino acid similarity between each of the clones and the identified proteins is shown in Fig. 5.11 (CKIs), Fig. 5.12 (CKIδ), Fig. 5.13 (ULK-1) and Fig. 5.14 (Skip).
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Figure 5.10

A) 5' CACTACAATGGATGATG

B)  
**Clone A1 - Homo sapiens PAC clone RP5-837C9**

**Clone B1 - Library clone has inversely ligated into the vector**

**Clone B2 - Library clone has inversely ligated into the vector**
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**Clone C1 – Ski-interacting Protein**
AACCCAAAAAAGAGATCTCTATGGCTTACCCATACGATGTCCAGATTACGCTA
GCTTGGGTGTCATATGGACATTGAG GCAGCCCGGAGATCCGAAATTTGCTTTTTTTG
CTGACTGCAACTGCTAATCTCAAGGACCAGCTGGAAGGAAATAGGCTAAGCTTAGATTG
AACCCAAAAAAGAGATCTCTATGGCTTACCCATACGATGTTCCAGATTACGCTA
GCTTGGGTGTCATATGGACATTGAG GCAGCCCGGAGATCCGAAATTTGCTTTTTTTG
CTGACTGCAACTGCTAATCTCAAGGACCAGCTGGAAGGAAATAGGCTAAGCTTAGATTG
AACCCAAAAAAGAGATCTCTATGGCTTACCCATACGATGTTCCAGATTACGCTA
GCTTGGGTGTCATATGGACATTGAG GCAGCCCGGAGATCCGAAATTTGCTTTTTTTG
CTGACTGCAACTGCTAATCTCAAGGACCAGCTGGAAGGAAATAGGCTAAGCTTAGATTG

**Clone D1 – Human DNA sequence from clone RP11-10C13 on chromosome 10**
AACCCAAAAAAGAGATCTCTATGGCTTACCCATACGATGTTCCAGATTACGCTAGCTTGGG
TGGTCATATGGACATTGAG GCAGCCCGGAGATCCGAAATTTGCTTTTTTTTGTAAATTTGTTTAAGTTCTTTATAGTTTCT
GGATATCAGCATATGAAAAATGTTACTTAGAAGCATAGCCTCTCTCTCCTGGAACCCCTACTGTTTTAC
TAAAGGAGGTTACGTCAGCTGGAAAATTTAGTTCTGACATTTTCAGTACACGACAT
AAGAGTGGTTCATCTCATGTTAATAGAATGGTTAGATGAAATAAATAGAAGGAGACCCTCGC
TGCATTATTAGTCAATTACAGTACTAGCTTTAATGTCTTCTTTTAC
TAAAGGAGGTTACGTCAGCTGGAAAATTTAGTTCTGACATTTTCAGTACACGACAT
AAGAGTGGTTCATCTCATGTTAATAGAATGGTTAGATGAAATAAATAGAAGGAGACCCTCGC
TGCATTATTAGTCAATTACAGTACTAGCTTTAATGTCTTCTTTTAC

**Clone E1 –Library clone has inversely ligated into the vector**
AAGAGGATCTCTCTAGGCTTACCCATACGATGTCCAGATTACGCTAAGCTTTGGG
TGTTCATATGGCAGAGGCAGCGGACGGATCCGAAATTTGCTTTTTTTTTTGAAATTTGTTTATAGTTTCT
GCATTACTGAGTTTTTATTTTTTTTTTTTTTTTTTTGAAATTTGTTTATAGTTTCT
GCATTACTGAGTTTTTATTTTTTTTTTTTTTTTTTTGAAATTTGTTTATAGTTTCT
GCATTACTGAGTTTTTATTTTTTTTTTTTTTTTTTTGAAATTTGTTTATAGTTTCT
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Clone F1 – Casein kinase 1ε
ACCCACACAAAAAGAGATCTCTATGGCTTACCACATACGATGGTTCCAGATT
TCGCTAGCTGCTTGGTGCATATGGCCATGGAGGCCCCGGGGATCCGAATTCGCGAT
GCCGCGTCGACCTGGGTGGTCATATGGCCATGGAGGCCCCGGGGATCCGAATTCGCG

Clone G2 – Vector sequence only
AAAAGAGATCTCTATGGCTTACCACATACGATGGTTCCAGAATACGCTAGCTTGGG
TGTTCA

Clone H2 – ULK-1, double adaptor sequence
ACCCACACAAAAAGAGATCTCTATGGCTTACCACATACGATGGTTCCAGATT
GCCGCGTCGACCTGGGTGGTCATATGGCCATGGAGGCCCCGGGGATCCGAATTCGCG
ATCCGCTAGCTTGGGTGGTCATATGGCCATGGAGGCCCCGGGGATCCGAATTCGCG

Figure 5.10
The pACT2 forward primer (A) was used to sequence clones A1, B1, B2, C1, D1, E1, F1, G2 and H2 (B). Grey is the 5’ adaptor sequence immediately adjacent to the library cDNA.
Where appropriate, underlining indicates stop codons within frame 1. Note that some clones have a double adaptor sequence.
### Table 5.2

**BLAST search and protein identification**

Summary table of the sequencing and BLAST search.

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<th>cDNA</th>
<th>Sequencing Result</th>
<th>BLAST Result</th>
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<td>Homo-sapiens PAC clone RPS-837C9 (probably untranslated RNA)</td>
</tr>
<tr>
<td>B1</td>
<td>Library clone has inversely ligated into the vector</td>
<td>-</td>
</tr>
<tr>
<td>B2</td>
<td>Vector sequence only</td>
<td>-</td>
</tr>
<tr>
<td>C1</td>
<td>Library clone correctly ligated</td>
<td>SKI-interacting protein</td>
</tr>
<tr>
<td>D1</td>
<td>Library clone correctly ligated</td>
<td>Human DNA sequence from clone RP11-10C13 on chromosome 10 (Possibly an unknown gene)</td>
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<td>Library clone has inversely ligated into the vector</td>
<td>-</td>
</tr>
<tr>
<td>F1</td>
<td>Library clone correctly ligated</td>
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<tr>
<td>G2</td>
<td>Vector sequence only</td>
<td>-</td>
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<tr>
<td>H2</td>
<td>Library clone correctly ligated</td>
<td>ULK-1 up to first stop codon</td>
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Figure 5.11 Sequence comparison between Clone F1 and Casein Kinase Iε (CKIε)

A)

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B)

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Figure 5.11

Clone F1 is identical to Casein Kinase Iε (CKIε)

(A) shows the cDNA sequence similarity between clone F1 and CKIε. Blue is the human CKIε coding sequence (accession number BC006490). Red is the sequence of clone F1. Identical sequence is shown by parallel bar. The purple box is an internal EcoRI site and one internal XhoI site is present (boxed). The translation start codons (atg) and stop codons (tga) are underlined. The amino acid similarity between clone F1 (in frame 1) and CKI is shown in (B). Green is human CKIδ amino acid sequence; Blue is human CKIε amino acid sequence; Red is clone F1 amino acid sequence; Grey box is the catalytic domain.
Figure 5.12
Sequence comparison between Clone F1 and Casein Kinase Iδ (CKIδ)

Clone F1 is similar to Casein Kinase Iδ
Blue is the human casein kinase Iδ coding sequence (accession number NM_001893);
Red is the sequence of clone F1; identical sequence is shown by parallel bar; the translation
start codons (atg) and stop codons (tga) are underlined. Note there is greater cDNA
sequence homology between clone F1 and CKIδ (Fig. 5.11) than with CKIδ.
Figure 5.13  Sequence comparison between Clone H2 and ULK-1

A)
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Figure 5.13

Clone H2 is identical to ULK-1

The cDNA sequence identity between clone H2 and ULK-1 is shown in (A). Start codons (ATG) and stop codons (TGA) are marked. Blue is the ULK-1 coding sequence; Red is clone H2 sequence: identical sequence is shown by parallel bars; the translation start codon (atg) and stop codon (tga) are underlined. The amino acid similarity between clone H2 and ULK-1 is shown in (B). Grey box is the catalytic domain; Red is clone H2 amino acid sequence. Note that this is the translation of clone H2 in frame 2; black is the amino acid sequence of human ULK-1 (accession number NM_003565). NB. No internal EcoRI or XhoI sites are present.
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Figure 5.14 Sequence comparison between Clone C1 and Skip

A)

```
1  cgctcgcgct  ggaagaagcg  gagaagatg  gcgcctacca  gctttttacc  tgacacctct
   CGCTCGCGCT  GGAAGAAGCG  GAAAGAATGT  GCCTACCA  GCTTTTACC  TGAACCTACT

61  cagctatctc  aggaccagct  tgaggctgaa  gaaaggccaa  gatcccaagag  atccgccag
   CAGCTATCTC  AGGACCAGCT  TGGAGCTGAA  GAAAGGCCAA  GATCCCAAGAG  ATCCGCCAG

121  acctcactgg  ttcctctcccg  aagagaacct  cccccgtacg  gataccggaa  aggctgggata
   ACCTCACTGG  TTCCTCTCCCG  AAGAGAACCCT  CCCCCGTACG  GATACCGGAA  AGGCTGGAATA

181  cctcgtttat  tagaggattt  tggagatgga  ggtgcttttc  cagagatcca  tgtggccag
   CCTCGTTTAT  TAGAGGATT  TGGAGATGGA  GGTGCTTTTC  CAGAGATCCA  TGGGCCCAAG

241  ttgaagggaa  aaattaaata  tgatgcaatt  gctcgacaag  gacagtcaaa  agacaaggtc
   TCTGAAGGAA  AAATTAAATA  TGATGCAATT  GCTCGACAAG  GACAGTCAAA  AGACAAGGTC

301  atttatatca  aatcacctga  cctggcttcca  aagggggtta  tgaatgcaga  tgcctggtat
   ATTTATATCA  AATCACCTGA  CCTGGCTTCCA  AAGGGGGTTA  TGAATGCAGA  TGCCTGGGAT

361  gcaatgaggta  aatcactgga  aatacactga  ccttgcttcca  aagggaggtta  tgaatgcaga
   GCATAAGGTA  AAATACTGG  AATACTGGA  CCTGGCTTCCA  AAGGGAGGTTA  TGAATGCAGA

421  ctcgaaagcc  ccgtgcaagaa  agtcatataa  gagatacagag  aagacatcaaa  agagccagttc
   TCTGAAGAGCC  CCTGCAAGAA  AGTATATAAA  GAGATACAGAG  AAGACATCAAA  AAGACCATCTC

481  ggaaaaatctg  tatcacagaa  cctgctgctc  agtatatccg  atacacacca  tgcctggtat
   GAAAAATCTG  TATCACAGAA  CCTGCTGCCTC  AGTATATCCG  ATACACCACCA  TGCCTGGGAT

541  gcgctgctct  agtatattc  atacacacca  ttcctgcaag  gagctggcatt  caaactctgga
   GCCTGGCTCT  AGTATATTCC  ATACACCACCA  TTCCTGCAAG  GAGCTGGCATT  CAACCTCGGA

601  gcctccagag  ggttctttagc  gatgctagaa  attcgacgaa  atccccagag  gcctccagag
   GCCTCCAGAG  GGTTCCTTAGC  GATGCCTAGAA  ATTCGACGAA  ATCCCGAGAG  GCCTCCAGAG
```

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1321 gtttatgtc aagcctggag aggtgttaaa gatagatatta taggcccgtt
1381 aaaaatcgg acaagcctggag acttagagtac aagacaggg ccagaggaac
1441 tttgttcctc aacagctggag cagaggttaga cagagacccccc acaagagcag
1501 agtacctgttt gctgttttt gaccaaaaag cacacagtcg
1561 gtttcgctcct aacaggtggtt ggtgagcagc cagacagctgg acaagagcag
1621 aagcagagc aacagtttttt gttatgctttt caaatagttc ggtgagcagc cagacagctgg acaagagcag
1681 gttcagtttc ttatatcagc agtactgtgc cccatacata aacgctggtc acaagagcag
1741 gttgcagttgc ccaaaaagtg atagttaattt ttatgctttt caaatagttc ggtgagcagc cagacagctgg acaagagcag
1801 gtatcagtttt ttatatcagc agtactgtgc cccatacata aacgctggtc acaagagcag
1861 gcattttttttt ttatatcagc agtactgtgc cccatacata aacgctggtc acaagagcag
1921 tagttttttttt ttatatcagc agtactgtgc cccatacata aacgctggtc acaagagcag
1981 ccacagagc aagcagagc aacagctggag cacagctggag cagaggttaga cagagacccccc acaagagcag
2041 gttcagttttttt ttatatcagc agtactgtgc cccatacata aacgctggtc acaagagcag
2101 gtttaaattt tttatatcagc agtactgtgc cccatacata aacgctggtc acaagagcag

**Figure 5.14**

Clone C1 is identical to Skip

The cDNA sequence identity between clone C1 and Skip is shown in (A). The amino acid sequence identity between clone C1 (frame 1) and Skip is shown in (B). Red is clone C1 sequence; black is Skip sequence (accession number NM_012245); identical sequence is shown by parallel bars; the translation start codon (atg) and stop codon (tag) are underlined.

N.B. no internal EcoRI or XhoI sites are present.
5.2.9 Binding partner verification

5.2.9.1 CPRG liquid colour change assay

In order to verify that the interactions between cOcc and Skip, CKIε and ULK-1 were real, a second in vivo reporter assay was used. pACT2-library clones C1 (Skip), F1 (CKIε) and H2 (ULK-1) were individually transformed into a different yeast strain, SFY526 ('single transfectants'). Separately, each of the pACT2-library clones were also co-transformed with pGBT9-cOcc in to SFY526 cells ('double transfectants'). This yeast strain contains a lacZ reporter gene, which is regulated by GAL4. Single library transfectants should be unable to induce β-galactosidase (β-gal) expression in these yeast, whereas in the co-transfected yeast, if an interaction with cOcc occurs, then β-gal should be expressed. Chlorophenol red-β-d-galactopyranoside (CPRG) is a chromogenic chemical that is modified by β-gal resulting in a colour change from yellow to red, which is measured by the absorbance at OD$_{578}$. This was used to detect β-gal expression. The single yeast transfectants were grown on -Leu/SD selection plates and the double transfectants were grown -Leu/-Trp/SD selection plates and healthy pink colonies were scraped and used for overnight liquid cultures. The cells were broken open according to the Clontech instructions (Chapter 2) and the samples were incubated with CPRG until a colour change occurred, at which point the reactions were stopped and the OD$_{578}$ taken. For each of the single transfectants, no colour change was observed after 48 h. This indicates that none of pACT2-Skip, -CKIε or -ULK-1 are able to induce gene transcription in the absence of pGBT9-cOcc. SFY526 yeast carrying both pGBT9-cOcc and pACT2-Skip produced a colour change after 136 min (2 h 16 min). pGBT9-cOcc and pACT2-CKIε produced a colour change after 1050 min (17 h 30 min). pGBT9-cOcc and pACT2-ULK-1 did not produce a visible colour change and the reaction was stopped after 48 h. The strength of the β-gal induction can be quantitated from the time it took for the colour change to occur and the extent of the colour change (found from OD$_{578}$ when the reactions were stopped), and is expressed as β-gal units. 1 unit of β-gal is defined as the amount which hydrolyses 1 μM of CPRG to chlorophenol red and D-galactose per min per cell (Clontech instructions). An OD$_{578}$ of between 0.25 and 1.8 is within the linear
range of the assay (Clontech). The results of this calculation are shown in Table 5.3B and Fig. 5.15.

Although ULK-1 failed to induce a colour change in the CPRG liquid assay, it is noteworthy that the β-gal filter assay, which first indicated an interaction between occludin and ULK-1 is more sensitive than the CPRG liquid assay. Also, when compared to the single transfectants, there is a statistically significant increase in β-gal units in the double transfectants (Fig. 5.15).

5.2.9.2 GST-cOcc pull down assay

A yeast two-hybrid screen offers just one method of identifying potentially interacting proteins in vivo. This screen must be complemented with other biochemical techniques to verify that the interaction is real. To test whether CKIε can interact with cOcc in vitro, a GST-pull down assay was performed. Unfortunately, due to a lack of commercial antibodies at this time, it was not possible to test Skip or ULK-1 in this way. A GST-cOcc fusion protein was incubated with HUVEC cell lysates and tested for an association with CKIε using SDS-PAGE and western blotting with an anti-CKIε antibody. CKIε was not detected where lysates had been incubated with GST alone, or in samples consisting of GST-cOcc and buffer only (no cell lysate). However, CKIε was detected where HUVEC cell lysates had been incubated with GST-cOcc (Fig. 5.16). This indicates that CKIε not only associates with cOcc in two different yeast strains, but that endogenous CKIε in endothelial cells is also capable of binding to cOcc in vitro. Furthermore, the CKIε antibody specifically detected two bands in the whole cell lysates. These might represent two different CKI isoforms. Interestingly, GST-cOcc specifically bound to the upper of these two bands, indicating that an association with occludin may be specific to one CKI isoform (probably CKIε as found in the yeast-two-hybrid screen).
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Table 5.3

A)

\[ \beta \text{gal units} = 1000 \times \text{OD}_{578} \cdot (T \cdot V \cdot \text{OD}_{600}) \]

\( T = \) time elapsed (min) of incubation
\( V = 0.1 \times \) concentration factor*
\( \text{OD}_{600} = A_{600} \) of 1 ml culture

*The concentration factor is determined at the start of the experiment as follows (see Chapter 2 for details):
1. Grow liquid cultures to mid-log phase \( \text{OD}_{600} = 0.8 \)
2. Centrifuge 1.5 ml of the cell culture and resuspend pellet in 300 \( \mu l \)
3. This gives a concentration factor of 5

<table>
<thead>
<tr>
<th></th>
<th>( A_{600} ) of 1ml</th>
<th>Conc factor</th>
<th>Time elapsed (min)</th>
<th>( \text{OD}_{578} )</th>
<th>( \beta \text{gal units} )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Single transfecants</strong></td>
<td>Skip</td>
<td>0.6</td>
<td>5</td>
<td>2880</td>
<td>0.037, 0.032, 0.036</td>
</tr>
<tr>
<td>CKIe</td>
<td></td>
<td>0.9</td>
<td>5</td>
<td>2880</td>
<td>0.035, 0.028, 0.043</td>
</tr>
<tr>
<td>ULK-1</td>
<td></td>
<td>0.88</td>
<td>5</td>
<td>2880</td>
<td>0.346, 0.318, 0.284</td>
</tr>
<tr>
<td><strong>Double transfecants</strong></td>
<td>Skip</td>
<td>1.18</td>
<td>5</td>
<td>136</td>
<td>0.204, 0.200, 0.197</td>
</tr>
<tr>
<td>CKIe</td>
<td></td>
<td>0.64</td>
<td>5</td>
<td>1050</td>
<td>0.096, 0.104, 0.081</td>
</tr>
<tr>
<td>ULK-1</td>
<td></td>
<td>0.78</td>
<td>5</td>
<td>2880</td>
<td></td>
</tr>
</tbody>
</table>

B)
Table 5.3

CRPG Colour Change Assay

Each of the library clones (pACT2-Skip, -CKIε and -ULK-1) were tested in triplicate for an interaction with cOcc in SFY526 yeast using a CPRG colour change assay according to the Clontech protocol (Chapter 2). SFY526 yeast were either transformed with the library plasmid only (single transfectants) or with both the library plasmid and bait plasmid (pGBT9-cOcc) (double transfectants). The strength of the induction of β-gal was determined by calculating the number of β-gal units. The formula for this is shown in (A) and the calculation for each transfectant is shown in (B). The experiment was repeated twice. Conc = concentration
**Figure 5.15**

Graph depicting the induction of β-gal by the interaction between cOcc and Skip, CKIε and ULK-1.

Each of the library clones (pACT2-Skip, -CKIε and -ULK-1) were tested in triplicate for an interaction with cOcc in SFY526 yeast using a CPRG colour change assay according to the Clontech protocol (Chapter 2). SFY526 yeast were either transformed with the library plasmid only (single) or with both the library plasmid and pGBT9-cOcc (double). The strength of the induction of β-gal was quantitated by calculating the number of β-gal units (Table 5.3) and were plotted in the above graph. Mean values are shown ± SD.

** = p ≤ 0.01 compared to the single transfectant in a student t-test.
Figure 5.16

A) Short exposure

B) Long exposure

C) Coomassie blue-stained gel
Figure 5.16

CKIε binds to cOcc in vitro

Confluent HUVECs were starved for 2 h, lysed and incubated with either GST only or with a GST-cOcc fusion protein for 1 h. GST-cOcc was also incubated with lysis buffer only (no cell lysate) as a control for associating *E. coli* proteins resulting from the bacterial GST-fusion protein preparation. CKIε association to occludin was detected using SDS-PAGE and western blotting with an anti-CKIε antibody. The gel was subsequently stained using Coomassie blue to show the presence of the GST or the GST-fusion protein in each lane, and to demonstrate equal amounts of GST or GST-cOcc were used for each pull down. N.B. A non-specific protein band was recognised by the anti-CKIε antibody in the GST-cOcc − buffer lane. This protein migrates more slowly than either of the proteins recognised by the anti-CKIε antibody in the whole cell lysates, and was therefore not thought to be CKIε.
5.3 GST pull-down experiments

An alternative strategy for identifying novel occludin-interacting proteins was employed. For this, GST fusion proteins of the N-terminal cytoplasmic region of occludin (nOcc) and the C-terminal cytosplasmic domain (cOcc) of occludin were constructed. These were used in a GST pull-down experiments to extract from HUVEC cell lysates, proteins that associate with the nOcc or cOcc. cDNAs encoding nOcc or cOcc were obtained by PCR, cloned into the GST-expression vector, pGEX-2T, and the GST-fusion proteins of nOcc and cOcc were expressed and purified as described in Chapter 2. 20 μg of fusion protein and approximately 5 million HUVECs were used for each pull down. Lysates were prepared from cells that were either left unstimulated or were stimulated with TNF-α, to detect any differences in the binding partners that might be induced with this cytokine. As negative controls, GST only was also incubated with HUVEC cell lysates, and GST-nOcc and GST-cOcc were incubated with buffer only (no lysate) to identify non-specific bands from the bacteria used to grow up the fusion constructs. Associating proteins were identified by SDS-PAGE and silver staining of the gels. For cOcc, four proteins of interest were observed at ~200, 70, 50 and 35 kDa respectively (Fig. 5.17A). All of these were present in both control lysates and lysates from TNF-α-stimulated cells, and a 30-kDa band was observed only in the control lysates, and not in those from cells that had been stimulated with TNF-α. None of these bands were present in the negative control lanes. For nOcc, three very faint bands were observed in lysates from control and TNF-α-stimulated cells (of ~65, 70 and 80 kDa respectively) (Fig. 5.17B). These were also not seen in the negative control lanes. Each band of interest was excised from the gel and submitted for mass spectrometry analysis and protein identification. Unfortunately, mass spectrometry failed to identify any of the bands. The experiment was scaled up to increase the amount of protein in the lanes by using a greater number of cells (~15 million) and the ~80 kDa band that was found in the GST-nOcc pull-down was re-submitted for identification. This band was successfully identified. In fact two proteins were found in this band: Phosphofructokinase, platelet type (NCBI acc. # NP_002618) and ATP-dependent DNA helicase II (NCBI acc. # NP_066964), which has some homology to Ku heterodimer. DNA helicases are responsible for unwinding DNA to enable replication and transcription (von Hippel and Delagoutte, 2003) and the Ku heterodimer protein has a role in the repair of DNA double-stranded breaks (Doherty and
Jackson, 2001). These interactions were not deemed relevant enough to be pursued at this time and the experiment was discontinued.
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A)

![Image of protein gel analysis](image)

B)

![Image of protein gel analysis](image)
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Figure 5.17

Confluent HUVECs were starved for 2 h and either left unstimulated or were stimulated with TNF-α for 24 h. Cells were lysed and incubated with 20 μg of GST only, or GST-cOcc (A) or GST-nOcc (B). As negative controls GST-cOcc and GST-nOcc were also incubated with buffer only (no lysate), and lysates were incubated with GST only. Bands of interest are indicated by the arrows.
5.4 Discussion

Three novel occludin binding partners have been identified using a yeast two-hybrid screen. Casein kinase 1ε, ULK1 and SKI-interacting protein were each selected from the cDNA lung library based upon their ability to bind to the C-terminal cytoplasmic domain of occludin and induce transcription of two reporter gene in yeast. In the case of CK1ε, the in vivo interaction has also been verified using biochemical techniques.

5.4.1 Troubleshooting

Yeast two-hybrid experiments are by no means to be considered a quick and singular route to identifying protein-protein interactions. The stages involved can be time-consuming, particularly screening for HIS3 production, whereby good yeast transformation efficiencies can be difficult to maintain: the lower the transformation efficiency, the more times the experiment needs to be repeated to ensure the entire library is screened. In the screen described here, the transformation efficiencies varied greatly – the best being 944,000 independent colonies screened in one day, and the worst resulting in just 5000 being screened. Some yeast cells are simply more difficult to make competent than others, and so variability can be introduced merely by picking a particular colony from a master plate. It is therefore advisable to try to obtain a stock of yeast cells known to be good for transformation (Criekinge and Beyaert, 1999). Although each library screen used bait-transformed yeast from a single glycerol stock, this glycerol stock contained several colonies from the original bait transformation, and so was a possible source of variability in the competent yeast.

Other important factors for good transformations are the yeast cell density and total volume. The HF7c strain needs to be in the logarithmic growing phase, which can be monitored by reading the cell density at A600. Too many cells in the transformation will also drop efficiency and be difficult to spread evenly onto plates. Too many cells per plate will result in high background, as yeast can grow on the corpses of other dead yeast cells, without expressing selectable markers. At times, where transformation efficiencies were low, the volume of cells used per transformation was increased and this did result in better
efficiencies, but also higher background. This problem was limited to a workable level by using 3AT in the selection plates.

Finally, the amount of library DNA used is important, as too much DNA will saturate the yeast, and can lead to more than one library plasmid being taken up by a single yeast cell. This causes problems with the subsequent analysis. This did not seem to present itself as a problem in this screen, as 1 \( \mu \)g DNA was used per transformation as initially established as the least amount of DNA required for a good transformation efficiency.

Another obstacle that was encountered relatively early on in the screen was autoactivation of \textit{his3} expression by the N-terminal domain of occludin. This was indicated by the sheer number of cells able to grow on the triple plates. The occludin N-terminal screen was not continued because of this reason and because the C-terminal screen was looking more promising. Had time of permitted it, 3AT in the selection plates could have been used to limit this expression, or a smaller region of the N-terminal domain could have been cloned in the hope of eliminating the autoactivating region of the clone.

\subsection*{5.4.2 The interacting partners}

\textbf{5.4.2.1 Casein kinase I \( \varepsilon \) and \( \delta \) (CKI \( \varepsilon \) and \( \delta \))}

Casein kinase I (CKI) is a Ser/Thr protein kinase of which there are seven mammalian isoforms: \( \alpha, \beta, \gamma_1, \gamma_2, \gamma_3, \delta \) and \( \varepsilon \) (Fig. 5.18). There are four splice variants of CKI\( \alpha \), designated CKI\( \alpha L \), \( \alpha_2 \), \( \alpha_2L \) and \( \alpha_3 \). Splice variants have also been shown for CKI\( \gamma_3 \). The isoforms share a conserved kinase domain, but have variability in their amino and carboxy terminal regions, which could be important for substrate recognition, localisation and/or regulation. CKI has been detected in the nucleus, cytosol, plasma membrane and microsomes (Gross & Anderson, 1998). Some features of the isoforms are shown in Table 5.4.

The casein kinase I family are a group of of phosphate-directed kinases that act upon proteins that have been previously phosphorylated by other kinases (Gross & Anderson,
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1998). This negative charge requirement is provided best by the sequence S/T/Y(P)X 1-2 S/T/Y, where S/T/Y(P) is any phosphorylated serine, threonine or tyrosine residue and X is any amino acid. This sequence is quite common. CKI has thus been shown to phosphorylate many of proteins in vitro, for example, insulin growth factor, TNFR2 and the β-adrenergic receptor (Gross & Anderson, 1998). Some isoforms phosphorylate tyrosines as well, such as the erythrocyte CKI, yeast CKI isoforms HRR25p, Hhp1 and Hhp2, and *Xenopus* CKIα (Gross & Anderson, 1998). Whether the remaining mammalian isoforms are also dual-specificity kinases remains to be seen.

CKI is mostly constitutively active but can be regulated by compartmentalisation of specific isoforms. For example, phosphatidylinositol 4,5-bisphosphate (PIP₂) binding retains CKIα at the membrane by binding to its phosphate-binding domain (Xu et al., 1995, Brockman et al., 1991). Interestingly, autophosphorylation of CKI isoforms ε and δ creates pseudo-substrate sites that inhibits CKI activity (Gross and Anderson, 1998; Cegielska et al., 1998; Graves et al., 1995; Gietzen et al., 1999, Rivers et al., 1998). This regulation is specific to the extended C-terminal region of these isoforms, which is not present in other isoforms such as CKIα. However, *Drosophila* CKIα is also negatively regulated by phosphorylation (Santos et al., 1996). So far six auto-phosphorylated residues have been found between His^{317} and Pro^{342} of CKIδ, all of which have an inhibitory effect on CKIδ activity (Graves et al., 1995). Dephosphorylation of CKIε has been shown to involve calcium-dependent activation of calcineurin downstream of the group I metabotropic glutamate receptors (mGluRs) (Liu et al., 2002). The αL insert of CKIα splice variants regulates substrate binding and turnover, possibly by altering the conformation of the substrate binding pocket and catalytic site (Gross & Anderson, 1998).

At the cell surface CKI may be involved in signalling from, and desensitisation of, activated receptors. CKI inhibition blocks TNF-α-mediated apoptosis (Beyaert et al., 1995). Furthermore, CKI phosphorylates the 3-muscarinic receptor and rhodopsin in a stimulus dependent manner (Tobin et al., 1997) and CKI phosphorylates the activated insulin receptor, which attenuates the receptor activity (Rapuano et al., 1991). The most
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calculated functional effects in vivo of CKI phosphorylations are DNA repair (Santos et al., 1996, Ho et al., 1997), growth and cell cycle progression (Knippschild et al., 1997; Gross et al., 1997; Wang et al., 1996; Hoekstra et al., 1991; Ho et al., 1997) and vesicle trafficking (Panek et al., 1997; Wang et al., 1996).

Both CKI ε and δ are potential candidates for occludin-binding as shown in this yeast two-hybrid screen, although clone F1 shows most cDNA homology to CKIε. CKIε shows most homology to CKIδ, particularly in the catalytic domain (98% homology) and they both contain an extended C-terminal region which has 40% identity. CKIε has been implicated in positively regulating the Wnt signalling pathway (Sakanaka, 2000; Polakis, 2002). In the absence of Wnt (a secreted ligand), β-catenin is found in a complex containing glycogen synthase kinase 3β (GSK3β), adenomatous polyposis coli (APC) and axin. GSK3β phosphorylates β-catenin, which targets it for ubiquitination and degradation. When Wnt binds to the frizzled receptor, dishevelled (Dvl/Dsh) is hyperphosphorylated and activated. Dvl activation leads to inhibition of GSK3β, decreased phosphorylation of axin, APC, and β-catenin, and stabilization of β-catenin. β-catenin is then translocated to the nucleus where it binds to LEF/TCF proteins and stimulates expression of Wnt-responsive genes. CKIε is a positive regulator of the Wnt-β-catenin pathway as its overexpression has been shown to promote expression of Wnt-responsive genes and inhibitors, or RNA-interference of CKIε inhibits the accumulation of β-catenin in response to Wnt signalling (Sakanaka et al., 2000; Cong et al., 2000). Furthermore, CKIε phosphorylates APC and axin in vitro, which may destabilise the degradation complex in vivo (Gao et al., 2002), and CKIε can phosphorylate LEF/TCF, which stimulates the association between LEF/TCF and β-catenin (Lee et al., 2001). CKIε has also recently been shown to be dephosphorylated and activated by Wnt (Swiatek et al., 2004).

CKIε and δ are implicated in DNA repair and cell cycle progression. An inhibitor of these casein kinases inhibits cytokinesis and causes mitotic arrest (Behrend et al., 2000) and both CKIε and δ phosphorylate p53 in vivo and in vitro (Knippschild et al., 1997, Dumaz et al., 1999). p53 is a tumour supressor protein that becomes activated by a variety of cellular
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Stresses such as DNA damage or heat shock can lead to cell cycle arrest, which prevents proliferation of genetically damaged cells (Knippschild et al., 1997). Furthermore, CKIδ and ε, which are most homologous to the yeast HRR25, are able to rescue a growth defect of HRR25-lacking yeast (Fish et al., 1995, Gross review, 1998). CKIε also targets the SV40 large T antigen, IκBα, and Ets-1 (a transcription factor) (Cegielska et al., 1998). These phosphorylation events depend upon relief of CKIε autophosphorylation of the C-terminal extension by phosphatases. CKIδ has also been shown to be involved in vesicular trafficking and spindle formation (Milne et al., 2001, Behrend et al., 2000). CKIε so far has been identified in colon carcinoma, cervical carcinoma, embryonic kidney (293) cell lines, and neuroblastoma cell lines (Fish et al., 1995). CKIδ was originally identified in rat testis (Graves et al., 1993). There are no reports so far for endothelial expression of CKIδ, but a commercially available CKIε antibody has detected CKIε in endothelial cells (BD Transduction Laboratories). This is the first report of CKIε expression in HUVECs.

The most relevant reference in the literature to the finding of CKIε or δ in this yeast two-hybrid screen is the targeting of the gap junction protein, connexin-49 and connexin-43 by CKI (Cheng et al., 1999, Cooper et al., 2002). In fact connexin-43 co-immunoprecipitates with, and is directly phosphorylated in vitro by CKIδ on 5 potential serine residues. Inhibition of CKIε or δ isoforms using the specific inhibitor, IC261, results in decreased connexin-43 phosphorylation. The pan-CKI inhibitor, CKI-7, results in accumulation of connexin-43 at the plasma membrane rather than gap junctions in rat kidney cells. This inhibitor also reduced the ability of the cells to transfer the dye, lucifer yellow, which is a test of gap junction function (Cooper et al., 2002). Endothelial cells also express connexin-43, and other connexins, such as connexin-32 and connexin-26 have been shown to associate directly with occludin (Kojima et al., 1999; Nusrat et al., 2000). It is therefore tempting to speculate that a complex could form between connexin-43, occludin and CKI, which results in CKI phosphorylation of both proteins.

There are in fact 8 potential CKI phosphorylation sites within the C-terminal cytoplasmic domain of human occludin used in this yeast two-hybrid screen, one of which resides within
the coiled-coil region (Fig. 5.19). Human occludin shares 48% identity in the C-terminal tail with *Xenopus* occludin, for which another group did not detect phosphorylation by CKI in vitro (Cordenonsi et al., 1999). This may be due to a lack of previously phosphorylated residues within the 5 *Xenopus* consensus sites, which as described above, are required for CKI phosphorylation (Fig. 5.19). This group also did not specify the isoform of CKI used. This is important for target specificity of the CKI isoforms. Occludin is a phosphoprotein and occludin localisation to the tight junction is modulated by its phosphorylation (Chapter 1). Thus occludin is a good candidate target for CKI and phosphorylation of specific occludin residues by CKI might regulate barrier function.
Figure 5.18

Schematic diagram of the different CKI isoforms

CKIα and δ share an extended C-terminal region (40% homology between them). The anti-CKIε antibody used for the GST pull-downs (Fig 5.16) was raised to the C-terminal region (The exact region is unspecified, BD Transduction laboratories)
### Table 5.4

**Comparison of the different CKI isoforms**

The size, structural features and known tissue distributions and splice variants for each CKI isoform are summarised. AA = amino acid
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A)
Human Occludin

265 VKTRKMDRDYSNWLSDKHEIYDEQPPNVVEEWVKNVSA
AGTQDVSPPSDYVERVSPMAYSSNGKVKNDKRFPESYSK
STPVPEVQELPLTSPVDFRQPARYSSGGNFETPSKRAPAK
GRAGRSKRTEQDHYETDYTTGECDELEDWIREYPPITS
DOQRQLKRNFDTGQLYEKSLSQELDEINKLSRDLKELD
DYRESEEYMAAAYENRLKQVKGSAKSKKNHCQLKL
SKLSHIKKMVGDYDRQKSTop 523

- T-329 SAGTQDV
- S-337 SPPSDYV
- T-349 SPMETYAS
- S-370 SSYKSTPV
- T-419 SKRTEQD
- T-430 TDYTTGGE
- S-474 SLSQELD
- S-536 SKLSHIK

B)
Xenopus Occludin

247KTRKINQYGKTNLWKKNHYEDGDPQVEQWVKNVAESAPALSDYNEKVD
GSVADYRSANGVQAYPSQNNISHPIAEEELPLKEDYGMSPRHYSSSSDATTKKAPPK
KRPGKPRRLDTRNEmGYNTGEGSADELEDSDWSEYPITQTKORQYEYKQEFAS
DLHEYKRLOELDELSKIPVPSLNRELGQSRRKDEEYRTVADKYNRLKEIKSSAD
YRNKKKRCRKLTKLNHIKQMVSNYDKSTOP 493

- S-365 SSSSDAT
- T-388 SDLDTNEG
- S-399 TGGESADE
- S-410 SWDSEYP
- S-465 SRKDEEY

Figure 5.19
Potential phosphorylation sites for protein kinase CKI within the C-terminal region of Occludin
The eight potential phosphorylation sites within the C-terminal region of human occludin (A). This is compared with the five potential sites within Xenopus occludin (B). CKI consensus sequence is S(p)/T(p)-X_2-3-S/T-X. Note the N-terminal S/T must be pre-
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phosphorylated. Underlining indicates potential CKI phosphorylation sites. Bold indicates coiled-coil domain.

(Pinna and Ruzzene (1996), from:
http://www.cbs.dtu.dk/databases/PhosphoBase/predict/consensus_sequences.php)
5.4.2.2 UNC-51 like kinase-1 (ULK-1)

*unc-51* was first described as an *unc* (uncoordinated) gene, which affects the movement of *Caenorhabditis elegans* (Brenner et al., 1974). Mutations in this gene result in abnormal axonal elongation and axonal structures. They also have defects in membranous structures, including enlarged axon diameter and abnormal vesicles and cisterna-like structures within axons (Brenner et al., 1974; Hedgecock et al., 1985). This gene was later shown to encode a Ser/Thr protein kinase (Ogura et al., 1994). Subsequently the *Saccharomyces cerevisiae* protein kinase homologue, Apg1p/Aut3p, was found (Matsuura et al., 1997), as well as mouse homologue, UNC-51-like kinase-1 (ULK-1) and a second member, mouse ULK-2 (Yan et al., 1998, 1999) and finally the human ULK-1 (Kuroyanagi et al., 1998). This protein family is characterised by an N-terminal kinase region, an intervening proline-serine rich region and a conserved C-terminal domain (Yan et al., 1998, Kuroyanagi et al., 1998). Mouse ULK-1 has been shown to be autophosphorylated within its proline-serine-rich domain (Yan et al., 1998). The human ULK-1 mRNA was detected mostly in heart and skeletal muscle, and to a lesser extent in brain, placenta and pancreas, and less still in lung, liver and kidney (Kuroyanagi et al., 1998), whereas UNC-51 is specifically detected in the nervous system of *C. elegans*.

Okazaki et al. (2000) showed that ULK-1 localises to the Golgi, rough endoplasmic reticulum (ER) and smooth ER in rat liver fractions, but that had a punctate localisation distinct from the ER or Golgi as observed by immunofluorescent staining of HeLa cells. This group also obtained two ULK-1-binding proteins using a yeast two-hybrid screen. These were the GABA-A receptor-associated protein (GABARAP) and Golgi-associated ATPase Enhancer of 16 kDa (GATE-16). These have roles in receptor and Golgi transport respectively. It is noteworthy that ULK-1 contains an ER membrane retention signal at its N-terminus (Okazaki et al., 2000). Most recently, mouse ULK-1 has been shown to be essential for neurite extension of cerebellar granule neurons by use of a dominant negative ULK-1 construct (Tomoda et al., 1999).

There have been no studies to date to show whether ULK-1 is expressed in endothelial cells, nor on ULK-1 function, regulation or protein targets, thus it is difficult to suggest what role

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It may have in associating with occludin. Even so, occludin is known to be phosphorylated, and this event is thought to be associated with occludin localisation to the tight junction (Chapter 1) and occludin could therefore potentially be a target for phosphorylation of ULK-1, perhaps as it passes through the Golgi en route to the tight junction.

**5.4.2.3 SKI-interacting protein (Skip)**

SKI-interacting protein/nuclear coactivator-62 kDa (Skip/NcoA62) was first identified as binding partner of the retroviral and cellular transcription factor, Ski. Skip localises to the nucleus of multiple tissues and contains a nuclear localisation sequence near its C-terminus (Zhang et al., 2003). It has been shown to be required for *C. elegans* viability and development (Kostrouchova et al., 2002). It is homologous to the *Drosophila melanogaster* protein Bx42, which is associated with chromatin in salivary glands (Dahl et al., 1998). Skip is a coactivator protein for nuclear hormone receptors, including the vitamin D receptor (VDR), and it enhances VDR transcriptional activity (Barry et al., 2003). Skip also acts as a transcriptional co-activator on some cellular and viral promoters (Prathapam et al., 2001) and associates with E7, the major transforming protein of human papillomavirus (Prathapam et al., 2001) and the tumour suppressor Rb, the product of the retinoblastoma gene, and Skip abrogates Rb’s ability to repress gene expression (Prathapam et al., 2002).

Skip has also been shown to associate with the DNA-binding proteins Smad 2 and 3 (Leong et al., 2001), the DNA-binding protein CBF1 (Zhou et al., 2000) and poly (A)-binding protein 2 (PABP2) (Kim et al., 2001). Finally, Skip has been reported to associate with components of the spliceosome (Zhang et al., 2003).

As Skip has a role in gene transcription, it is possible that in the yeast two-hybrid screen, it has fulfilled its role as a co-activator to induce *his3* and *lacZ* transcription without associating with occludin. This is unlikely to be the case, however, as expression of Skip alone in SFY526 yeast was not sufficient to induce a colour change in the CPRG liquid assay, yet co-expression of C-terminal occludin and Skip did induce a colour change in this assay and therefore confirms their interaction (Fig. 5.15).
Some receptor proteins such as Notch have been shown upon activation to be cleaved, and for that cleavage product to migrate to the nucleus and modulate gene transcription (Struhl et al., 1998; Kramer et al., 2000 review; Iso et al., 2003 review). It is possible that this is also true of a portion of the C-terminus of occludin. Indeed, in a recent publication, occludin appeared to partially localise to the nucleus, although no reference to this observation was made in the publication text (Stamatovic et al., 2003). Although the C-terminal region of occludin does not contain an obvious classical basic-type nuclear localisation sequence (NLS), (monopartite basic NLS = (K/R)4-6, from Large T antigen; bipartite basic NLS = (K/R)2 X10-12 (K/R)3, from nucleoplasmin), it does associate with ZO proteins, which do contain an NLS (Chapter 1) and could function to shuttle a portion of occludin to the nucleus. Moreover, possible cleavage products of occludin can be seen in western blots upon HUVEC stimulation with TNF-α (Chapter 3). Furthermore, cell-adhesion molecules, including E-cadherin, which also localises to intercellular junctions, are also known to be cleaved by the γ-secretase complex (Fortini et al., 2002; Steiner and Haass, 2000 review). Taken together, these observations make a plausible case for occludin C-terminal cleavage and fragment re-localisation to the nucleus, where an interaction with Skip might occur.
6 Adenoviruses Inhibit TNF-α Responses in HUVECs

6.1.1 Introduction
Adenoviruses (Ad) are known to cause acute upper respiratory tract infections, as well as ocular and gastrointestinal diseases and they infect a wide range of cell types as well as dividing and non-dividing cells. Adenoviruses are a family of non-enveloped viruses that carry a double-stranded DNA genome of 36 kb. There are 51 different human adenovirus serotypes, each classified into six subgroups A to F depending on their biological and genetic make-up and their ability to induce tumours in mice. The well-studied Ad2 and Ad5 are within subgroup C, which along with D, E and F are non-oncogenic (www.micro.msb.le.ac.uk/335/Adenoviruses.html).

6.1.2 The adenovirus structure
The Ad-DNA and central core is surrounded by a capsid of icosahedral symmetry, about 90 Å in diameter (Fig. 6.1). This shell is comprised of 240 subunits of the major coat protein, hexon and they form the faces of the icosahedron. There are also 12 copies of a penton base protein, which itself is made out of five identical polypeptide subunits and from this penton base projects a trimeric fibre protein, the ends of which form a fibre knob domain (Durmort et al., 2001; Nemerow et al., 1999).

6.1.3 The adenovirus genome and central core
The virus genome consists of linear, double-stranded DNA that contains terminal repeat sequences at the end of each strand. This genome can potentially encode 30-40 genes. The core of the particle also contains proteins that associate with the DNA: Terminal protein (TP) is covalently attached to the 5’ ends of the DNA strands and the very basic protein VII and Mu protein associate non-covalently with the DNA. Protein V links the DNA to the capsid via protein VI. A virus-encoded protease is also present, and is required for the processing of structural proteins that make the virus infectious (Russell et al., 2000).
Adenoviruses are non-enveloped icosahedral particles.

The capsid consists of a total of 252 capsomers. 240 of these are hexavalent and 12 (situated at the apices) are pentavalent (penton base). A penton fibre projects from each penton base (A) and they are involved in viral attachment to the host cell. Fibres easily become detached during preparation for electron microscopy (B) (http://web.uct.ac.za/depts/mmi/stannard/adeno.html; Linda Stannard, of the Department of Medical Microbiology, University of Cape Town). (C) shows the structure of adenoviruses and associated proteins (Russell, 2000).
6.1.4 Adenovirus attachment and infection

The first steps for Ad infection are viral attachment and penetration into the host cell. Initial absorption is mediated by the fibre knob domain and its interaction with the coxsackie and adenovirus receptor (CAR) (Chapter 1; Bergelson et al., 1997; Roelvink et al., 1998 and 1999; Nemerow et al., 2000). This receptor has been identified as the receptor for all Ad viruses excluding members of the subgroup B (Roelvink et al., 1999). The cytoplasmic domain of CAR is not required for viral entry, suggesting that signalling from this receptor is not necessary for virus infection (Wang and Bergelson, 1999). Subgroup C adenoviruses are also able to utilise the major histocompatibility complex class I (MHC I) as a receptor for viral attachment (Hong et al., 1997) and Ad37 is capable of binding to the sialoglycoprotein receptor (Russell, 2000, Armbser et al., 2000). Integrins \( \alpha_5\beta_1 \) and \( \alpha_5\beta_3 \) act as co-receptors with CAR as they recognise the RGD sequence present in the penton base protein and are required for particle internalisation (Wickham et al., 1993; Chiu et al., 1999). The virus enters the host cell via clathrin-mediated endocytosis (Wang et al., 1998), which involves activation of PI 3-kinase (Li et al., 1998a) and the Rho family GTPases (Li et al., 1998b; Rauma et al., 1999). Once the virus particle has been endocytosed and is present within endosomes, the virus protease destabilises the capsid by cleaving protein VI (Greber et al., 1996). This disrupted virus particle is then transported to the nuclear membrane where the virus genome (DNA and associated proteins) enters the nucleus through nuclear pores in a process involving host cell dynein and microtubules (Russell, 2000). Viral proteins can be detected in the nucleus within 1-2 h of virus exposure (Dales and Chardonnet, 1973; Greber et al., 1997; Matthews and Russell, 1998; Russell, 2000).

6.1.5 Viral gene transcription, DNA replication and escape from the host cell

Viral gene transcription involves two main phases: early and late, with DNA replication occurring between the two phases (Fig. 6.2; Russell, 1999). The earliest transcripts produced are E1A, E1B, E2A, E2B, E3 and E4, which undergo complex splicing events to generate viral proteins. These early genes mediate viral gene expression, drive quiescent cells into cell cycle progression (to achieve S phase for DNA synthesis) and counteract host anti-viral responses (McNees et al., 2002). Adenoviruses also transcribe a set of RNAs called viral-
associated RNAs (VA RNAs), which are not transcribed and are thought to have roles in viral growth, protein synthesis of the host cell, and combating host defences (Russel 2000; Ma et al., 1996; Sharp et al., 1993). Late proteins are more concerned with the assembly of virus progeny and release from the host cell: After DNA replication has occurred from the virus DNA origins of replication (the ITRs), the 5 late gene transcripts (L1 to L5) are activated and are involved in producing structural components and packaging of virus progeny in the nucleus. This depends on the major late promoter (MLP), which is utilised by the binding of virus gene products of Iva2 and IX genes. Packaging of the virus depends on the AT-rich region called a packaging signal present in the virus DNA. Finally, the nuclear membrane is destabilised and permeabilised, releasing the virions into the cytoplasm (Rao et al., 1996; Tollefson et al., 1996a and b). The virus then escapes from the cell by lysis of the plasma membrane (Russell, 2000).
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Figure 6.2

Transcription of the adenovirus genome.
The early transcripts are in green, the late in blue. Arrows indicate the direction of transcription. The gene locations of the VA RNAs are shown in brown. MLP; major late promoter (Russell, 2000).
6.1.6 Laboratory uses of adenoviruses
A good method of transfecting HUVECs and other cell types is by using recombinant adenoviruses. These viruses are replication defective because their E1 and E3 gene cassettes have been removed and replaced with the gene of interest. Such recombinant adenoviruses require a helper cell line for their propagation (e.g., HEK293 cells) (Chapter 2). Transfection efficiencies of 80% and greater can be achieved using adenoviruses (Wojciak-Stothard et al., 2001; Russell, 2000). Chapter 3 describes the effects of TNF-α on HUVEC morphology, permeability and tight junction proteins. In order to examine the involvement of the Rho GTPase family of proteins in these effects, adenoviruses that carry dominant negative constructs of RhoA, Rac1 or Cdc42 were used to investigate TNF-α–induced permeability and signalling in HUVECs. As controls, viruses carrying β-galactosidase (Ad-β-gal) or GFP (Ad-GFP) were also employed. However, when Ad-β-gal or Ad-GFP recombinant viruses were applied to HUVECs in the initial experiments, a striking effect on HUVEC morphology was observed and these results are described in this chapter.
6.2 Results

6.2.1 β–galactosidase-carrying-adenoviruses (Ad-β–gal) block TNF-α–induced morphological changes

Chapter 3 described the typical response of HUVECs to TNF-α after 24 h. This response comprises cell elongation, gap formation, stress fibre formation and ZO-1 redistribution from the tight junction. In order to test the involvement of the Rho family of small GTPases in this response, dominant negative constructs of Rho, Rac and Cdc42 were to be transfected into HUVECs using adenoviruses prior to the addition of TNF-α. Ad-β–gal or Ad-GFP were to be used as controls to ensure that the viruses themselves do not affect the cells. Interestingly, exposure of HUVECs to Ad-β–gal at a multiplicity of infection (MOI) of 500 (this is the ratio of virions to cells; Chapter 2) prior to addition of TNF-α completely abolished the TNF-α–induced morphological changes (Fig.6.3). Cells lacked the thick, TNF-α–induced actin stress fibres, and instead punctate F-actin was frequently observed in the cell body (Fig.6.4) and a single line of F-actin was present at the cell periphery. The cells appeared rounder, with more continuous ZO-1 at the tight junctions compared to TNF-α–treated cells. In fact this inhibition was quite similar to the effect that ML-7 had on HUVECs (Fig. 4.5). Similar observations were made where Ad-β–gal was applied to the cells in the absence of TNF-α.
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Figure 6.3

F-Actin | ZO-1 | Merge & β-gal

Control

TNFα 18 h

Ad-β-gal

TNFα 18 h
Ad-β-gal

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Figure 6.3

Ad-β-gal inhibits TNF-α-induced morphological changes and actin stress fibres

Confluent HUVECs were either left uninfected ('control' and 'TNF-α') or were infected with Ad-β-gal (Ad-β-gal and Ad-β-gal / TNF-α). For cells infected with Ad-β-gal without TNF-α stimulation ('Ad-β-gal'), the virus was applied for 90 min and the cells were washed in PBS and transferred into starvation medium for a total of 22 h. For cells that were infected with Ad-β-gal and also stimulated with TNF-α (Ad-β-gal / TNF-α), the virus was applied for 90 min and the cells were washed in PBS and transferred into starvation medium for 4 h prior to the addition of 10 ng/ml TNF-α for 18 h. Control cells were starved for 2 h prior to fixation and uninfected cells treated with TNF-α were starved for 4 h and exposed to TNF-α for 18 h ('TNF-α'). Cells were stained for ZO-1 (green), F-actin (red), and β-gal (blue) (detected using an anti-β-galactosidase antibody). Bar = 20 μm. White arrowhead indicates punctate F-actin; white arrows show a single line of cortical F-actin; red arrow, continuous junctional ZO-1. Inset on merged image shows a lower magnification of that region.
Figure 6.4

F-Actin

Control

TNN-α 18 h

Ad-β-gal

TNN-α 18 h
Ad-β-gal

β-gal expression
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**Figure 6.4**

**Analysis of the actin cytoskeleton in HUVECs infected with Ad-β-gal**

Confluent HUVECs were either left uninfected (‘control’ and ‘TNF-α’) or were infected with Ad-β-gal (‘Ad-β-gal’ and ‘Ad-β-gal / TNF-α’). For cells infected with Ad-β-gal without TNF-α stimulation (‘Ad-β-gal’), the virus was applied for 90 min and the cells were washed in PBS and transferred into starvation medium for a total of 22 h. For cells that were infected with Ad-β-gal and also stimulated with TNF-α (‘Ad-β-gal / TNF-α’), the virus was applied for 90 min and the cells were washed in PBS and transferred into starvation medium for 4 h prior to the addition of 10 ng/ml TNF-α for 18 h. Control cells were starved for 2 h prior to fixation and uninfected cells treated with TNF-α were starved for 4 h and exposed to TNF-α for 18 h (‘TNF-α’). F-actin (red), and β-gal (blue) and a series of and 11 confocal Z-stacks were taken over a depth of 4.0 μm and with an interval of 0.4 μm between each stack. The Z-stacks were merged to show the F-actin throughout the cell body. White arrows = single line of cortical F-actin; white arrowheads = punctate F-actin. Bar = 20 μm.
6.2.2 Ad-β–gal partially blocks the TNF–α morphological response if cells are stimulated by TNF-α before the viruses infect

In order to test whether the viruses are able to prevent TNF-α from inducing morphological changes in HUVECs even if they are added after TNF-α has been applied, TNF-α was added to the cells 30 min prior to the addition of Ad-β-gal. Ad-β–gal was able to partially inhibit TNF-α-induced stress fibres under these circumstances (Fig. 6.5). Overall, HUVECs reacted more weakly when cells were infected with virus compared to TNF-α only. Some cell elongation had occurred in infected cells, gaps were present, but most cells lacked actin stress fibres and instead exhibited punctate actin staining. This indicates that the viruses may interfere with early TNF-α-signalling or that the viruses need time to infect or for protein synthesis to occur before they are inhibitory to TNF-α responses.

6.2.3 Ad-β-gal does not inhibit TNF-α-induced ICAM-1 expression

To determine whether Ad-β-gal prevents all signalling from the TNF-α receptors, cells were infected with Ad-β-gal, stimulated with TNF-α for 24 h and stained for ICAM-1 (Fig. 6.6). ICAM-1 is well known to be upregulated in response to TNF-α (Chapter 3; Pober, 1987). TNF-α induced an increase in ICAM-1 expression in HUVECs, despite their lack of stress fibres. This indicates that the Ad-β-gal viruses are blocking a specific pathway downstream of TNF–α– receptor engagement, whilst other pathways leading to ICAM-1 expression are unaffected.
Figure 6.5  

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Adenoviruses Inhibit TNF-α responses in HUVECs

Figure 6.5

Ad-β-gal only partially blocks the TNF-α morphological response if cells are stimulated by TNF-α before the viruses infect

HUVECs were cultured to confluency and starved for 2 h. Cells infected with Ad-β-gal only were exposed to the virus for 90 min, the cells were washed in PBS, and placed back into starvation medium. Alternatively, cells were starved for 2 h and pre-stimulated with TNF-α for 30 min before Ad-β-gal was applied to the cells for 90 min in the continued presence of TNF-α. The cells were washed in PBS and placed back into TNF-α for a further 16 h. Control cells were starved for 2 h before fixation and cells treated with TNF-α in the absence of virus were starved for 2 h and stimulated with 10 ng/ml TNF-α for 18 h. Cells were stained for β-gal (blue) and F-actin (red). Arrow shows a cell lacking stress fibres, and exhibiting punctate F-actin. Bar = 20 μm.
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Fig. 6.6

- F-Actin
- ICAM-1
- Merge & β-gal expression
Fig. 6.6
Ad-β-gal does not inhibit TNF-α-induced ICAM-1 expression
Confluent HUVECs were infected with Ad-β-gal for 90 min, washed in PBS and transferred into starvation medium for 4 h. Cells were then either left unstimulated or exposed to 10 ng/ml TNF-α for 18 h. Alternatively, non-infected cells were starved for 4 h and then fixed or stimulated with TNF-α for 18 h. Cells were stained for ICAM-1 (green), β-gal (blue) and F-actin (red).
6.2.4 GFP-carrying-adenovirus (Ad-GFP) also inhibits TNF-α-induced responses

To eliminate the possibility that the effects of Ad-β-gal are due to the β-galactosidase expression itself, Ad-GFP was applied to cells either before or after TNF-α stimulation (Fig. 6.7). In both cases, the cortical F-actin appeared as a single thick line at the cell periphery, with some punctate actin staining in the cytoplasm. TNF-α-induced actin stress fibres and cell elongation were inhibited by the Ad-GFP (Fig. 6.7). As previously observed with Ad-β-gal, basal stress fibres were removed where virus alone was applied to the cells. The higher the dose of TNF-α applied to HUVECs, the stronger the response of the cells, particularly in the induction of actin stress fibres (Chapter 3). To determine whether a higher dose of TNF-α could inhibit any effect of Ad-GFP, and also to check that TNF-α is inducing the expected response at all doses, different doses of TNF-α were tested (Fig. 6.8). Ad-GFP strongly inhibited the TNF-α–induced responses at all doses tested, even at 100 ng/ml. To see whether the inhibitory effect of the adenoviruses depends upon the number of virions applied, 3 different MOIs were applied to cells prior to addition of TNF-α for 24 h. A MOI of 200 of Ad-GFP weakly inhibited TNF-α–induced stress fibres and elongation (Fig. 6.9). In these cells approximately 95% of cells were expressing GFP. This inhibitory effect was lost where an MOI of 20, 2 or 0.2 was used. An MOI of 20 produced weak GFP expression in approximately 50% of the cells and an MOI of 2 produced very low expression in about 5% of the cells (this could only be seen when the detector gain of the microscope was increased). The lower MOI of 0.2 produced no visible GFP expression. Cells exposed to this level of viruses exhibited a more typical TNF-α response of cell elongation, gaps and stress fibre formation.
Figure 6.7: F-actin and GFP expression

- **Control**
- **Ad-GFP**
- **TNF-α 18 h**
- **Ad-GFP 1st TNF-α 18 h**
- **TNF-α 1st Ad-GFP**
Figure 6.7
Ad-GFP inhibits TNF-α-induced morphological changes
HUVECs were starved for 2 h and either fixed or stimulated with TNF-α for 18 h. Where virus was applied to the cells before or without stimulation, cells were infected with Ad-GFP for 90 min, washed with PBS and allowed to express protein for 4 h in starvation medium. Cells were then either placed back in to starvation medium or were stimulated with TNF-α for 18 h. Where cells were stimulated prior to the addition of virus, HUVECs were starved for 2 h and then stimulated with TNF-α for 30 min. Ad-GFP was added in the continued presence of TNF-α for 90 min before cells were washed in PBS, and transferred back into starvation media containing 10 ng/ml TNF-α for a further 18 h. Cells were fixed and stained for F-actin (red). GFP is shown in green. Arrows = single line of cortical F-actin; arrowhead = puncate F-actin.
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Figure 6.8

<table>
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<th>F-actin</th>
<th>ZO-1</th>
<th>Merge &amp; GFP expression</th>
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</thead>
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<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>TNF-α 1 ng/ml</td>
<td>TNF-α 1 ng/ml</td>
<td>TNF-α 1 ng/ml</td>
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<td>TNF-α 1 ng/ml</td>
</tr>
<tr>
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<td>Ad-GFP</td>
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</table>
Chapter 6

Adenoviruses Inhibit TNF-\(\alpha\) responses in HUVECs

Figure 6.8

<table>
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<th>F-actin</th>
<th>ZO-1</th>
<th>Merge &amp; GFP expression</th>
</tr>
</thead>
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<td><img src="image2.png" alt="Image" /></td>
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<tr>
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</tr>
<tr>
<td><strong>TNF-(\alpha) 100 ng/ml</strong></td>
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<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td><strong>TNF-(\alpha) 100 ng/ml</strong> Ad-GFP</td>
<td><img src="image7.png" alt="Image" /></td>
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</table>
Higher doses of TNF-α do not inhibit Ad-GFP effects

Confluent HUVECs were either uninfected or infected with Ad-GFP for 90 min and then were placed in starvation media for 4 h. Cells were then left unstimulated or were stimulated with 1 ng/ml, 10 ng/ml, or 100 ng/ml TNF-α for 18 h. Control cells were starved for 2 h before fixation. Cells were fixed and stained for ZO-1 (blue) and F-actin (red). Bar = 20 μm; insets (merged image) show a lower magnification of the same area.
Adenoviruses Inhibit TNF-α responses in HUVECs

Figure 6.9

Control

F-actin

ZO-1

Merge &
GFP expression

TNF-α 18 h

Ad-GFP
MOI 200

TNF-α 18 h

Ad-GFP
MOI 20

TNF-α 18 h

Ad-GFP
MOI 2

TNF-α 18 h

Ad-GFP
MOI 0.2
Chapter 6

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Figure 6.9

The inhibitory effect of Ad-GFP is lost at low MOIs

Confluent HUVECs were either left untreated or exposed to Ad-GFP at a MOI of 200, 20, 2 or 0.2 and the virus was allowed to infect for 90 min. Cells were then washed in PBS and transfered into starvation medium for 4 h, and then stimulated with 10 ng/ml TNF-α for 18 h. Cells treated with TNF-α alone were starved for 4 h and stimulated for 18 h. Control cells were starved for 2 h prior to fixation. Cells were fixed and stained for ZO-1 (blue) and F-actin (red). Images were collected using the same microscope detector gain in each case. GFP expressing cells are in green. White arrows indicate single line of cortical F-actin; white arrowhead, punctate F-actin; red arrows, stress fibres; orange arrows, disjointed or absent ZO-1; orange arrowheads, continuous junctional ZO-1; bar = 20 μm.
6.2.5 Ad-β-gal and Ad-GFP affects the tight junctions

In order to determine whether Ad-β-gal or Ad-GFP can alter the tight junction proteins, cells were treated with Ad-β-gal or Ad-GFP and immunofluorescently stained for GEF-H1, claudin-5 or ZO-1. Ad-GFP alone resulted in greater and homogenous junctional staining of all three junctional proteins (Fig. 6.10A). Similar results were observed for ZO-1 where Ad–GFP was used (Fig. 6.10B). To determine whether Ad-β-gal can increase the protein levels of GEF-H1 or claudin-5, western blotting was used to assess levels of these proteins in cells infected with Ad-β-gal only or in conjunction with TNF-α. Ad-β-gal alone or with TNF-α did not induce detectable increases in claudin-5 or GEF-H1 (Fig. 6.10A).
Adenoviruses Inhibit TNF-α responses in HUVECs

Figure 6.10

A) Ad-β-gal

Claudin-5

GEF-H1

ZO-1

B) Ad-GFP

ZO-1
Figure 6.10

Ad-β-gal and Ad-GFP assemble the tight junction proteins to the junction

Cells were either left uninfected or infected with Ad-β-gal (A) or Ad-GFP (B) for 90 min, washed with PBS and placed in to starvation medium for 18 h. Uninfected cells were starved for 4 h before fixation. Cells were methanol-fixed (GEF-H1 and claudin-5) or fixed in 3.7% formaldehyde (ZO-1) and stained for GEF-H1, claudin-5, or ZO-1 and β-gal (blue) (inset). GFP is shown in green. Methanol-fixed cells were not able to be stained for β-gal, due to the fixation procedure. Arrows = disjointed tight junction protein staining or thin line of GEF-H1; arrowheads = continuous tight junction staining. Bar = 20 μm. For the western blots, confluent HUVECs were infected with Ad-β-gal for 90 min, washed with PBS and either placed in starvation medium in the absence of TNF-α, or were starved for 4 h prior to the addition of TNF-α for 18 h (Ad-β-gal / TNF-α). Alternatively, control cells were starved for 2 h before cell lysis, or for 4 h before the addition of TNF-α for 18 h. Protein was separated by SDS-PAGE and claudin-5 or GEF-H1 were detected by western blotting. The claudin-5 blot was stripped and reprobed for ERK-1 and the GEF-H1 blot was stripped and reprobed with an anti-ERM antibody to show equal protein loading across the lanes. In the western blots C = control; β = Ad-β-gal; T = TNF-α; β/T = Ad-β-gal and TNF-α.
6.2.6 RhoA and Rac1 are activated by Ad-β–gal alone and in conjunction with TNF-α

As the viruses blocked the formation of actin stress fibres, which are known to be induced by active Rho (Ridley & Hall, 1992), a Rho pull-down assay was used to detect changes in activation of this GTPase. Surprisingly, instead of reducing basal Rho activity, Ad-β–gal reproducibly activated RhoA after 18 h (Fig. 6.11). This was also observed when cells were infected with Ad-β–gal and subsequently activated by TNF-α for 18 h. Some cells infected with Ad-β–gal and stimulated with TNF-α appeared to ruffle (Fig. 6.12). This has previously been observed in non-confluent epithelial cells infected with Ad-β–gal (Li et al., 1998). This indicated that Rac1 might be activated by the viruses, as Rac1 is known to induce lamellipodium formation and membrane ruffling (Ridley et al., 1992) and Rac is known to be involved in adenovirus entry into cells. This has been shown using *C. difficile* toxin B, which selectively inhibits Rho GTPases by glycosylation of Threonine 37 in Rho, or the corresponding position in Rac or Cdc42 (Just et al., 1995). Both *C. difficile* toxin B and dominant negative Rac inhibit Ad2 internalisation in epithelial cells (Li et al., 1998). To test whether Rac1 is activated by Ad-β–gal, Rac pull-down assays were used to test Rac1 activity after Ad-β–gal infection. In two out of three experiments, a small increase in Rac1 activity was detected where cells had been infected with Ad-β–gal (Fig. 6.13). Rac1 activity was also observed in all experiments where cells had been infected with Ad-β–gal and stimulated with TNF-α. In contrast, Ad-β–gal with or without TNF-α did not activate the small GTPase Cdc42 (one experiment only) (Fig 6.14).
Adenoviruses Inhibit TNF-α responses in HUVECs

Figure 6.11

A) kDa GTPs Control Ad/β-gal TNF-α Ad/β-gal RhoA
PD 20 -
WCL 20 -
WCL 45 -

B) kDa GTPs Control Ad/β-gal TNF-α Ad/β-gal RhoA
PD 20 -
WCL 20 -
WCL 45 -

C) kDa GTPs Control Ad/β-gal TNF-α Ad/β-gal RhoA
PD 20 -
WCL 20 -
WCL 45 -

α-tubulin
Figure 6.11

Ad-β-gal activates RhoA

Confluent HUVECs were either left uninfected (control and GTPγS), or were infected with Ad-β-gal for 90 min, washed with PBS and placed into starvation medium for 18 h (Ad-β-gal) or for 4 h prior to the addition of TNF-α for 10 min or 18 h (TNF/Ad-β-gal).

Uninfected cells were starved for 2 h prior to cell lysis. GTP-bound-RhoA was purified from cell lysates by incubation with the Rho-binding domain of Rhotekin. Incubation of the cell lysate with GTPγS or GDPβS was used as positive and negative controls respectively. GTPγS irreversibly binds to and activates small GTPases, whereas GDPβS binds to and inactivates small GTPases. Protein was detected by SDS-PAGE, western blotting and immuno-detection using an anti-RhoA-antibody. Whole cell lysate samples are also shown to demonstrate total RhoA protein levels in each sample and overall protein loading in each lane of the gel is shown by α-tubulin levels. Three independent experiments are shown.

Graphs depicting the activity of RhoA were created by normalisation of the bands of active RhoA to the WCL controls using densitometry and the graph in (D) shows the mean of three experiments (A-C) ± SD. Pull down (PD) indicates GTP-bound RhoA. N.B. Although Ad-β-gal consistently increased RhoA activity, the extent of this activation varied from experiment to experiment. This results in the large error bars in (D) and means that the differences are not statistically significant in a student t-test.
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**Figure 6.12**

**Ad-β-gal induces some membrane ruffling**

Representative micrograph showing F-actin-containing ruffles some times observed in cells infected with Ad-β-gal and stimulated with TNF-α. Confluent HUVECs were infected with AD-β-gal for 90 min, washed in PBS and allowed to express for 4 h in starvation medium. TNF-α (10 ng/ml) was then applied to the cells for a further 18 h. Cells were fixed and stained for F-actin and a series of confocal Z-stack images taken and merged to show the F-actin throughout the cell body. Bar = 20 μm.
Chapter 6

Adenoviruses Inhibit TNF-α responses in HUVECs

Fig. 6.13

A) 

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- Rac1
- α-tubulin

PD ——— WCL

B) 

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- Rac1
- β-actin

PD ——— WCL

C) 

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- Rac1
- β-actin

PD ——— WCL
Adenoviruses Inhibit TNF-α responses in HUVECs

D)

Fig. 6.13
Ad-β-gal activates Rac1

Confluent HUVECs were either left uninfected (control and GTPγS), or were infected with Ad-β-gal for 90 min, washed with PBS and allowed to express for 18 h (Ad-β-gal) or allowed to express for 4 h prior to the addition of TNF-α for 18 h (TNF/Ad-β-gal). Control cells were starved for 2 h prior to cell lysis. Cells were rapidly lysed and GTP-bound-Rac was obtained from cell lysates using the PBD domain of PAK-1. Incubation of the cell lysate with GTPγS was used as positive controls. GTPγS irreversibly binds to and activates small GTPases. Protein was detected by SDS-PAGE, western blotting and immunodetection using an anti-Rac1 antibody. Three independent experiments are shown (A-C). Whole cell lysate (WCL) samples are shown to demonstrate the total Rac1 protein levels in each sample and overall protein loading in each lane of the gel is shown by α-tubulin or β-actin levels. Graphs depicting the activity of Rac1 were created by normalisation of the bands of active Rac1 to the WCL controls using densitometry and the graph in (D) shows the mean of three experiments (A-C) ± SD. N.B. Although Ad-β-gal consistently increased Rac1 activity in the presence of TNF-α, the extent of this activation varied from experiment to experiment. This results in the large error bars in (D), and makes it difficult to obtain statistical significance in a T-test. PD indicates Pull down; WCL, whole cell lysate.
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Fig. 6.14
Ad-β-gal does not activate Cdc42

Confluent HUVECs were either left uninfected (control), or were infected with Ad-β-gal for 90 min, washed with PBS and allowed to express for 18 h (Ad-β-gal) or allowed to express for 4 h prior to the addition of TNF-α for 18 h (TNF-α / Ad-β-gal). Control cells were starved for 2 h prior to lysis. Cells were rapidly lysed and GTP-bound-Cdc42 was purified from cell lysates using the CRIB domain of WASP. Protein was detected by SDS-PAGE, western blotting and immuno-detection using an anti-Cdc42 antibody. Whole cell lysate (WCL) samples are shown to demonstrate the total Cdc42 protein levels in each sample and overall protein loading in each lane of the gel is shown by α-tubulin levels. Pull down (PD) indicates GTP-bound Cdc42. The graph depicts the activity of Cdc42 and was created by normalisation of the active bands of Cdc42 to the WCL controls using densitometry. N.B. This experiment was only conducted once.
6.2.7 Ad-β-gal does not change the protein levels of ROCK I

ROCK I acts downstream of Rho in the formation of actin stress fibres (Riento and Ridley, 2003). As RhoA was activated by the viruses both in the presence and in the absence of TNF-α, yet no stress fibres were observed, it was possible that the viruses were down-regulating an effector of Rho, such as ROCK. To test this, western blots were performed on cells that had been infected with Ad-β-gal and either left unstimulated, or were stimulated with TNF-α. No change in ROCK protein levels were observed, indicating that the viruses were not reducing stress fibres through altering ROCK protein levels (Fig. 6.15).
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Fig. 6.15

Ad-β-gal does not alter protein levels of ROCK I

Confluent HUVECs were either left uninfected (control and TNF-α) or were infected with Ad-β-gal for 90 min (Ad-β-gal and Ad-β-gal / TNF-α). Infected cells were then washed with PBS and either placed in starvation medium in the absence of TNF-α (Ad-β-gal), or were starved for 4 h prior to the addition of TNF-α for 18 h (Ad-β-gal / TNF-α).

Uninfected cells were starved for 4 h before fixation, or before the addition of TNF-α. Protein was separated by SDS-PAGE and ROCK I was detected by western blotting. The blot was stripped and reprobed for ERM proteins (upper band is ezrin, lower is moesin) to show equal protein loading across the lanes.

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<th>ERM</th>
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![Western Blot Image](image_url)
6.2.8 Ad-β-gal reduces TNF-α induced permeability

As Ad-GFP and Ad-β-gal increase the localisation of ZO-1, GEF-H1 and claudin-5 to the tight junctions, and activated Rho, it was possible that they also altered the transendothelial permeability. To test this, the permeability of monolayers that had been infected with Ad-β-gal was assessed (Fig. 6.16). Ad-β-gal did not significantly alter basal permeability up to 1 h after infection. However, it did reduce basal permeability after 24 h and it also significantly lowered TNF-α-induced permeability at 24 h. This indicates that Ad-β-gal modulates endothelial permeability, possibly by retaining tight junction proteins at the cell-cell junctions, and by modulating Rho GTPases and the actin cytoskeleton.
Figure 6.16
Ad-β-gal inhibits TNF-α-induced permeability

HUVEC monolayers were grown to confluency on Transwell filters and their permeability determined by flux of FITC-dextran from the apical cell surface to the lower chamber of the filters. Cell monolayers were either left uninfected (control and TNF-α), or were infected with Ad-β-gal for 90 min, washed in PBS and either left in starvation medium or starved for 4 h prior to the addition of TNF-α for 18 h. Control cells were starved for 4 h only. FITC-dextran was applied to all the monolayers 30 min prior to the addition of TNF-α, and samples of the basal medium were removed for fluorescence measurements either just before TNF-α addition (basal), or at 10 min, 30 min, 1 h and 18 h after addition of TNF-α. The mean permeability of six monolayers from two different experiments is shown as a percentage of control ± SD. ** = p ≤ 0.01 compared to control in T-tests; ** = p ≤ 0.01 compared to TNF-α at 24 h in student t-tests.
6.2.9 Ad-β-gal does not inhibit TNF-α-induced p38 phosphorylation

TNF-α has been shown to phosphorylate the MAP kinase (MAPK), p38. Active p38 is thought to contribute to TNF-α-induced stress fibre formation by phosphorylation of HSP27, an actin-capping protein, which, when phosphorylated, looses its ability to bind to F-actin. The loss of the HSP27-F-actin association allows actin polymerisation to occur. p38 is implicated in TNF-α-induced permeability through this mechanism of cytoskeletal reorganisation (Kiemer et al., 2002; Nwariaku et al., 2002; Petache et al., 2003). Atrial natriuretic peptide (ANP) is a cardiovascular hormone that has an inhibitory effect on TNF-α-induced F-actin reorganisation and permeability. ANP upregulates the MAPK phosphatase, MKP-1, which dephosphorylates p38 and inhibits TNF-α-induced p38 activation (Kiemer et al., 2002) and downstream cytoskeletal effects. As this inhibitory action of ANP on TNF-α-induced stress fibres and permeability in HUVECs resembled the observations made with Ad-β-gal, it was of interest to determine whether Ad-β-gal could also block TNF-α-induced p38 phosphorylation. To this end, western blots were performed on cells that had been exposed to Ad-β-gal and TNF-α. No inhibition of TNF-α-induced p38 phosphorylation was observed, ruling out Ad-β-gal inhibition of this signalling molecule downstream of TNF-α (Fig. 6.17).
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Figure 6.17

Ad-β-gal does not inhibit TNF-α-induced p38 phosphorylation

Confluent HUVECs were infected with Ad-β-gal for 90 min, washed in PBS and placed in starvation medium for 18 h. Cells were then stimulated with 10 ng/ml TNF-α for 10 min. Alternatively, cells were starved for 2 h and either left unstimulated (control) or exposed to TNF-α for 10 min (TNF-α). Cells were lysed and proteins separated by SDS-PAGE. Phospho-p38 was detected using western blotting. The blot was stripped and re-probed for total p38.
6.3 Discussion

Adenoviruses have been shown to be extremely effective for transfection of endothelial cells, including HUVECs. Indeed, a transfection efficiency of 80% has been reported for myc epitope-tagged proteins (Wojciak-Stothard et al., 2001), however detection of GFP is far better than for c-myc, and in monolayers infected with Ad-GFP, it appeared that 100% of were expressing GFP to some degree. HUVECs are difficult to transfect by other methods and so viral transfection is an enormously useful tool in endothelial cell research. However, adenoviruses utilise cell surface receptors, the CAR receptor and integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ as well as taking advantage of other endogenous cellular processes such as clathrin-mediated endocytosis, to achieve their ultimate goal of cell entry, protein expression and DNA replication. In doing this, the viruses may also be manipulating some cell signalling pathways in ways that have not been fully investigated. Understanding the effect of virions on cell signalling is important not only for experimental purposes, to know if the transfection procedure itself is disrupting the pathway under investigation, but also for physiological reasons, as these viruses are now being used for gene therapy and cancer treatment (Nadeau and Kamen, 2003; St George, 2003). Furthermore, little is known about the function of the CAR receptor itself, and so the viruses could prove to be a useful tool in elucidating the physiological role of this receptor.

In using adenoviruses as a tool to study endothelial tight junctions and actin reorganisation, an interesting effect of the viruses themselves was observed, either with or without TNF-\(\alpha\) stimulation. Viruses alone reduced stress fibres, enhanced junctional localisation of GEF-H1, claudin-5 and ZO-1 and lowered basal HUVEC permeability. Ad-\(\beta\)-gal also blocked TNF-\(\alpha\)-induced stress fibres and compromised TNF-\(\alpha\)-induced increased permeability. Furthermore, both RhoA and Rac1 activation was observed after Ad-\(\beta\)-gal infection. The viruses did not act as an antagonist or as a TNFR1/2 inhibitor, as ICAM-1 expression was unaffected. This also indicates that a specific TNF-\(\alpha\) pathway is inhibited by the viruses. Furthermore, GFP-adenovirus resulted in similar effects, demonstrating that the \(\beta\)-gal expression itself was not having an adverse effect upon the cells. At times, the effects on the actin cytoskeleton and cell morphology were very striking, whilst at other times the
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Inhibitory affect was not as strong, and the cells appeared more elongated, although in these cases they usually still exhibited punctate F-actin instead of actin stress fibres. This variability may be a result of different levels of infectivity, or may be dependent upon the cell age or responsiveness to TNF-α or the viruses.

TNF-α–induced permeability was reduced by Ad-β-gal. Consistent with this, the adenoviruses increased the staining of claudin-5, GEF-H1 and ZO-1 at the tight junction, although neither claudin-5 nor GEF-H1 protein levels changed. This event could be pivotal for resisting TNF-α-induced gap formation, and increased permeability and may be as a result of CAR stimulation. CAR itself is located within the tight junction, and is probably involved in barrier function, as shown with overexpression experiments, which resulted in reduced permeability (Cohen et al., 2001; Chapter 1). Furthermore, CAR expression in CHO cells has been shown to recruit ZO-1 to the cell-cell junctions (Cohen et al., 2001).

Ad-β-gal led to an increase in activation of RhoA and Rac1 18 h after infection. This activation could be downstream of CAR, although currently there are no reports of signalling between CAR and Rho, Rac or Cdc42. Ad-β-gal might also signal though the integrin receptors. One mechanism for the activation of some Rho GTPases by adenoviruses has been described in the literature. Both ERK and PI 3-Kinase signalling pathways are activated upon Ad-penton base engagement with α, integrins, but activation of PI 3-Kinase, and not ERK, is required for virus internalisation and infection (Li et al., 1998; Nemerow et al., 1999). PI 3-Kinase can act upstream of Rac and Cdc42 and it has been shown that Rac and Cdc42 are important in Ad endocytosis. Inhibition of Rho GTPases with toxin B or dominant negative Rac or Cdc42, inhibits Ad internalisation. This is thought to be because of their role in modulating the actin cytoskeleton, a model supported by experiments using cytochalasin D to disrupt the actin cytoskeleton, which also blocks Ad internalisation (Li et al., 1998a). Li et al. reported filopodium formation and ruffling of subconfluent epithelial cells exposed to Ad for 10-25 mins. HUVECs also ruffled after adenovirus infection, although this was observed at 18 h in the presence of TNF-α.

Furthermore, HUVECs exhibited reduced actin stress fibres 18 h after infection. There is one other report of reduced levels of actin stress fibres in response to adenoviral infection,
but this occurred early after infection of rat embryo cells with wild type Ad5 infection; an effect that was attributed to the E1a gene product (Jackson et al., 1985). This gene is absent from the adenoviruses used here.

Increased Rho activation is usually associated with increased actin stress fibres (Ridley and Hall, 1992). This was not observed after Ad-β-gal infection, when RhoA activity was increased. It is possible that a RhoA effector is downregulated or inhibited, which might block Rho-induced stress fibre formation. In ras-transformed fibroblasts, elevated levels of RhoA-GTP inhibit the cell cycle inhibitor protein, p21/Waf1, but Rho no longer regulates stress fibre formation because the Rho effector protein, ROCK, is downregulated (Sahai et al., 2001). Although ROCK levels were not affected by Ad-β-gal, similar inhibition of proteins that lead to stress fibre formation downstream of Rho might be occurring in response to the adenoviruses. Alternatively RhoE, a protein that disassembles stress fibres, might be induced. Another Rho-binding protein, Rhophilin-2, has recently been reported to induce stress fibre disassembly in HeLa cells (Peck et al., 2002) and may limit Rho-induced stress fibre formation. This protein could also be involved in Ad-β-gal reduction in TNF-α-induced stress fibres.

GEF-H1, a Rho-GEF that has been shown to activate Rho and which localises to the tight junction (Benais-Pont et al., 2003), shows increased localisation to the tight junction in response to Ad-β-gal. It is possible that an increase in GEF-H1 at the cell periphery is responsible for activation of a local pool of Rho, whereas other pools, which localise within the cell body and regulate stress fibre formation and tension, perhaps leading to increased permeability, remain inactive.

Ad5 adenoviruses used in these experiments lack the E1 and E3 viral gene cassettes, which renders the virus replication-deficient. The E. coli lacZ gene or GFP gene is substituted into the E1 position. Propagation of the recombinant adenoviruses is carried out in HEK293 cells, which contain sheared Ad-5 DNA and which constitutively express Ad-virus E1 gene, which is required for DNA replication and virus production to occur. This creates the possibility of homologous recombination between the recombinant-Ad DNA and Ad-5 viral
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genes. Homologous recombination resulting in the E1 gene being re-incorporated into recombinant Ad-DNA results in replication-competent adenoviruses (RCA). RCAs were controlled for using plaque assay tests (Chapter 2) and at no stage were high levels of RCAs produced. It is possible that some viruses have picked up some of the Ad-E3 gene cassette from the HEK293 cells, which could provide a possible route for interfering with TNF-α signalling, as Ad-E3 gene has been shown to subvert host defences; some of which are triggered by TNF-α pathways. Indeed, Ad- E3 can avert TNF-α–induced apoptosis (McNees et al., 2002) and similar mechanisms could account for the results reported here. This is probably unlikely, however, because recombination in HEK293 cells is very low.

In conclusion, inhibition of host inflammatory responses, such as increased endothelial permeability, would be advantageous to an invading pathogen. Adenovirus-induced recruitment of some tight junction proteins to the cell-cell junctions and reduced permeability, as well as the inhibition of TNF-α-induced permeability, stress fibre formation and cell elongation could be as a result of signalling from CAR or the integrin receptors (Fig. 6.19). Alternatively, expression of a viral protein could result in these effects. One way to distinguish between these possibilities would be to stimulate CAR with recombinant fibre knob protein only, or to stimulate the α, integrin receptors with recombinant penton base protein, both of which have previously been used, and have been shown to specifically bind each individual receptor (Molinier-Frenkel et al., 2003). Use of these viral coat proteins would eliminate any affects that viral DNA expression might have, including expression of β-gal or GFP.
Figure 6.19

Model for Adenovirus inhibition of TNF-α signalling

The adenoviruses could be inhibiting TNF-α-induced responses through CAR or integrin mediated signalling, or through expression of viral protein. Red hexagons = adenoviruses; yellow circles = TNF-α.
Chapter 7

Conclusions

7 Conclusions and Future Perspectives

7.1 TNF-α as a mediator of increased permeability and vascular disease

Increased TNF-α production is a hallmark for inflammatory responses to pathogens, such as septic shock and ARDS, and is also associated with chronic inflammatory states including atherosclerosis and rheumatoid arthritis. The results reported here demonstrate that TNF-α has a progressive and profound effect on HUVEC morphology, actin cytoskeleton and permeability, which may contribute to these conditions.

The aim of this project was to characterise the effect of TNF-α on HUVEC permeability, morphology and actin cytoskeleton. This was largely addressed by comparing TNF-α and thrombin responses, because thrombin has been well characterised for its effects on endothelial permeability. Thrombin induces a rapid (5-10 min) increase in endothelial permeability, which returns to unstimulated levels by 90 min (Kouklis et al., 2004, van Nieuw Amerongen et al., 1998, Ehringer et al., 1996; Lum et al., 1992). This increase involves the formation of actin stress fibres that traverse the cell, intercellular gap formation, MLC phosphorylation downstream of ROCK and MLCK and contractility (reviewed in Bogatcheva et al., 2002). However, the response of endothelial cells to TNF-α was very different to that of thrombin. Firstly, the response at 10 min was weaker than observed with thrombin (Fig. 3.2): there was a subtle thickening of the cortical actin, which at times was difficult to detect and only a few small intercellular gaps were observed. Secondly, permeability was increased very slightly at 10 min and this was barely within the means of detection with FITC-dextran. TNF-α seems to invoke a progressive change to the monolayer with a gradual increase in permeability that peaked at 24 h and was retained at least until 30 h. Prolonged TNF-α stimulation induced the formation of thick actin stress fibres that traversed the cell and accompanied cell elongation. Indeed, it is possible that this morphological change occurred as a result of the cytoskeletal rearrangement. Changes in the actin cytoskeleton are indicative of Rho GTPase activation and it was surprising that neither Rho, Rac nor Cdc42 activity were increased after 24 h of TNF-α stimulation, when the stress fibres were present. However, early RhoA activation indicates that it may have an earlier role, perhaps by regulating
gene expression to induce the later stress fibres. Indeed, Rho has been shown to regulate the activity of serum response factor (SRF) (Hill et al., 1995), which regulates genes under the control of serum response factor elements (SRE). In addition, recent evidence suggests that formin family members are important for unbranched actin filament nucleation and elongation (Chapter 1) and these are likely candidates for stress fibre formation in response to TNF-α. It is possible that a formin is induced by TNF-α.

With thrombin, MLC phosphorylation and contractility are an important mechanism for increased permeability (reviewed in Bogatcheva et al., 2002). It would seem that this mechanism is not so important for TNF-α induced permeability, as after 24 h of TNF-α stimulation (where permeability is at its peak), MLC phosphorylation was unchanged compared to unstimulated cells and its protein levels were downregulated. This should not be surprising, considering that at this time point cells are elongated and are not contracted, in contrast to thrombin. Importantly, lower concentrations of TNF-α induced cell elongation and stress fibre formation without an increase in permeability after 24 h of TNF-α stimulation (Chapter 3). This indicates that a loss of cell-cell junctions and probably tight junction integrity is more crucial for permeability increases than the morphological changes. This indicates that TNF-α activates signalling pathways to the tight junction that induce their disassembly e.g. removal of occludin and JAM-A from the junction (Chapter 3), rather than a physical pulling-apart of the cells induced by cell elongation. In support of this, *in vitro*, cells under flow are elongated, and resemble the morphology of HUVECs that have been exposed to TNF-α for 24 h (Drenckhahn and Ness, 1997). Increased permeability downstream of TNF-α *in vivo* (Goldblum et al., 1989) is therefore unlikely to be as a result of TNF-α-induced elongation since the cells are already elongated, and is more likely to occur, therefore, as a result of changes to the tight junctions and to cell-cell adhesion.

How might TNF-α be signalling to the tight junctions? Initial experiments (Chapter 3) demonstrated that after 24 h of stimulation, there were no changes in the protein levels of claudin-5, JAM-A, GEF-H1, ZO-1, VE-cadherin or β-catenin. Only occludin protein
levels were down-regulated, and possibly degraded. Identification of the smaller bands detected using anti-occludin antibodies in western blotting would clarify whether these are degradation products or other occludin isoforms. No shifts in the bands on western blots was detected that might indicate post-translational modifications such as phosphorylation for any of the junctional proteins screened. However, occludin and JAM-A showed substantial changes in their localisation. Follow-up experiments might address whether occludin and JAM are internalised or redistributed over the membrane, away from the tight junctions and whether they consequently no longer bind to tight junction proteins such as ZO-1, but bind to other proteins. A change in their phosphorylation status might also contribute to their redistributions and could be analysed by mass spectrometry. In the case of occludin, this may be difficult, as it is a heavily phosphorylated protein, and changes in the phosphorylation of single sites may not be easy to detect.

7.2 Tight junctions as multi-functional signalling complexes

In the early sixties, tight junctions were visualised using electron microscopy and described as intercellular membrane ‘kisses’ (Farquhar and Palade, 1963). Since this observation was made, tight junctions have been shown to be a regulatable barrier that possess gate and fence function (Gonzalez-Mariscal, 2000, van Meer et al., 1986), and more recently have been implicated in membrane trafficking (Zahraoui et al., 2000), cell-cell adhesion (Van Itallie et al., 1997), cell growth, differentiation and cell signalling (Matter and Balda, 2003). Furthermore, more than 40 proteins have been shown to localise to the junctions of epithelial and endothelial cells (Gonzalez-Mariscal, 2003). The mechanisms of barrier regulation are still unclear and are further complicated by different magnitudes of permeability changes (eg. thrombin compared to TNF-α) and solute selectivity. The identification of the claudin family has been an important step in understanding the selectivity of the tight junctions, specifically their ability to form ion-selective pores. However, the identification of specific signalling pathways from cell surface receptors to tight junction proteins that result in changes to barrier function are still lacking.
There are other possible roles for the tight junctions, which have not been extensively studied. It is tempting to speculate that the tight junctions play a role in the regulation of contact inhibition. This is defined as the stopping of cell growth when a certain density of cells has been reached. The association of ZO-1 with ZONAB has been linked with cell growth and cell density (Balda & Matter, 2000; 2003) and it is possible that the association between occludin and Skip (Chapter 5) could fulfill a similar role.

Leukocyte diapedis is the final step in leukocyte transmigration where by it squeezes between neighbouring endothelial cells to reach infected tissues. The metastasis of cancer cells is thought to occur by similar mechanisms (Voura et al., 1998). A lot of research has focused upon the roles of the adherens junctions, of PECAM and the JAMs in regulating this process. Indeed some groups have demonstrated disassembly of VE-cadherin complexes, and homotypic interactions between JAMs and PECAM on the leukocyte and the endothelium. However, the JAMs and PECAM do not constitute tight junction fibrils, and the adherens junctions are more basal that the tight junctions along the basolateral membrane. The first barrier for a leukocyte that is migrating out of the blood and through the paracellular space must, therefore, be the tight junctions. A limited number of studies (Burns et al., 2000; Kucharzik et al., 2001) have addressed the role of tight junctions in leukocyte transmigration and it is likely that future research will demonstrate a role for occludin and claudins, and maybe other tight junction proteins, in regulating diapedis and perhaps in signalling to the adherens junctions for their disassembly.

7.3 Working with HUVECs

To date there are a number of endothelial cell models that are used to investigate vascular biology. These include bovine aortic endothelial cells (BAEC), bovine pulmonary artery endothelial cells (BPEC), human coronary artery endothelial cells (HCAEC), human pulmonary microvascular endothelial cells (HPMEC), human umbilical vein endothelial cells (HUVECs), as well as a number of endothelial cell lines. Human-derived primary cells are the closest models to in vivo human vasculature and HUVECs are a well-
established laboratory cell model that are in ample supply. It was for these reasons that HUVECs were chosen for this study. However, HUVECs are not without their drawbacks, particularly as they undergo a progressive loss of cell viability and loss of marker expression with each passage (Unger et al., 2002). The presence of contaminating cells, such as fibroblasts can also pose problems, as well as the variable genetic background of the cells, which may result in a varied response of the cells from experiment to experiment. HUVECs are sensitive to the substratum on which they are cultured and were found to grow better in tissue culture flasks than in dishes or on glass or polyester filters, despite each of these being coated with fibronectin prior to cell-seeding.

HUVECs can be unpredictable in their growth and their response to starvation. At times, tight monolayers lost their junctional integrity within 1 h of being starved. This was particularly a problem for the permeability tests, where the cells were difficult to observe using a light microscope. Leaky monolayers could be detected using FITC-dextran. For this reason, where possible, a 30 min FITC-dextran equilibrium period was introduced, so that a basal permeability reading of each monolayer could be taken and leaky monolayers disregarded.

Use of some endothelial cell lines could overcome the difficulties of culturing HUVECs. The EA.hy 926 cell line is a fusion between HUVECs and the permanent epithelial human cell line, A549 (Edgell et al., 1983). These cells can be cultured in the absence of fibronectin, have tight, epithelial-like cobblestone monolayer morphology, express cell adhesion molecules, such as ICAM-1 and elongate in response to TNF-α (personal communication, Dr Jaime Millan). EA.hy 926 tight junctions, barrier function and responses to TNF-α are yet to be characterised. Another promising cell line is HPMEC-ST1.6R, which was generated by cotransfection of plasmids encoding human telomerase and the SV40 antigen into human pulmonary microvascular endothelial cells (Unger et al., 2002). These cell lines could prove to be a useful model for endothelial cell permeability research.
In vivo, endothelial cells are under shear stress from the constant flow of blood through the vasculature. This affects endothelial cell morphology and F-actin organisation (Wojciak-Stothard et al., 2003; Resnick, 2003) and could potentially alter endothelial responses to cytokines such as TNF-α. The effects of shear stress are lacking in static cell culture models and the addition of flow would be one step closer to a more physiological experimental model. Furthermore, the extracellular matrix (ECM) on which HUVECs are grown can influence the response of cells in culture. Throughout the experiments reported here, HUVECs were grown on fibronectin. Fibronectin is a known substrate for twelve specific integrins, whereas different ECM proteins, such as collagen, engage other integrins, including integrin α1β1 (Plow et al., 2000). The mechanism of cell-substratum adhesion might influence the response of HUVECs to TNF-α. Indeed, others have reported a decrease in the production of collagen and fibronectin, and expression of metalloproteinases in response to TNF-α, that might contribute to increased permeability (Partridge et al., 1992). It would be interesting to see what effect different matrices have on tight junctions and TNF-α-induced permeability.

Serum starvation (1% serum) was employed for a minimum of 2 h prior to the addition of TNF-α in all experiments reported herein. This is a standard procedure that is used to achieve basal activity of proteins that might be activated by a stimulant, allowing for the strongest change to be measured. Moreover, serum starvation removes growth factors (eg. VEGF) that might have a synergistic effect with the stimulant upon the cells. However, in vivo, it is unlikely that the production of TNF-α would occur in a serum- or growth factor-free environment, and inflammatory cytokines may act in concert to achieve the desired effect upon the endothelium. Indeed, this has been observed with a combination of TNF-α and IFN-γ in vitro (Ozaki et al., 1999). Although the model here is simplified in order to study TNF-α only, it would be interesting to determine how the effects of TNF-α on HUVEC permeability are modulated by the presence of other factors.
7.4 Future research

There are several interesting new avenues for research following on from the work reported here. In particular, the interaction between occludin and Skip would be interesting to follow up. Other junctional proteins have been linked with gene transcription through association with transcription factors (Benmerah et al., 2003). For example, ZO-1 associates with the Y-box transcription factor ZONAB and regulates cell proliferation, cell density and erbB-2 transcription (Balda & Matter, 2003) and β-catenin translocates to the nucleus in response to Wnt where it binds to LEF/TCF proteins and stimulates expression of Wnt-responsive genes. Furthermore, a cleavage product of the transmembrane protein, Notch, translocates to the nucleus and induces conversion of CSL (CBF1, Su (H), Lad-1) proteins from transcriptional repressors to activators. This upregulates expression of target genes of Notch signaling, such as HES (hairy and enhancer of split) genes (Mumm and Kopan, 2000). The DNA-binding protein CBF1 forms a complex with SKIP and the silencing mediator of retinoid and thyroid receptor (SMRT)-deacetylase complex, which results in transcriptional repression. The notch intracellular region (Notch-IC) has been reported to bind to SKIP and CBF1, which displaces the SMRT-deacetylase repressor complex, resulting in transcriptional activation (Zhou et al., 2000). A similar mechanism might occur with the occludin C-terminal region, whereby a cleavage product could migrate to the nucleus, interact with Skip, and modulate gene transcription. It will be important to verify the cOcc-Skip interaction in HUVECs using immunoprecipitation and GST-pull down assays. The specific binding region in occludin that associates with SKIP could be determined by truncation of the occludin C-terminus. These could be used in yeast two hybrid screens with SKIP in either X-gal or CPRG assays as used in Chapter 5, or in GST pull-down assays. It would also be of interest to determine the promoters and target genes downstream of cOcc-Skip. Reporter gene assays could be employed to determine whether occludin, like Notch-IC, can activate gene transcription from CBF1-responsive genes. This could be done in yeast, by expression of CBF1, a CBF1 responsive reporter gene, SKIP, SMRT and increasing amounts of cOcc, similar to that previously described (Zhou et al., 2000). Theoretically, increasing amounts of cOcc might be able to displace SMRT and induce reporter gene expression.
Chapter 7

Conclusions

It would also be interesting to follow up the interaction between CKIε and occludin. Specifically, whether CKIε can phosphorylate occludin and if so, the site of the phosphorylation and the consequences of phosphorylation could be investigated. This could be done using *in vitro* kinase assays and sites could be identified using mass spectrometry. Any sites targeted for phosphorylation by CKIε might be mutated to phospho-mimicking aspartic acid residues or alanines to mimic non-phosphorylated forms and these might be used for overexpression studies to see the effects upon occludin localisation, binding partners and permeability. Furthermore, co-localisation between occludin and CKIε could be examined and whether this changes with TNF-α exposure. Overexpression and inhibitor studies using CKIε may identify any effects this kinase has on permeability and whether this protein is activated by TNF-α. A CKIε and δ-specific inhibitor (IC261) has previously been used and might be useful in this regard (Behrend et al., 2000). CKIε may prove to be an important event in the regulation of occludin and could have implications for barrier function.

The transcriptional regulation of occludin by TNF-α might also be interesting to follow-up. At this stage it is difficult to assign the loss of occludin protein levels to degradation alone, or also to transcriptional changes. This could first be addressed by assessing the RNA levels of occludin, to see whether these are altered by TNF-α. Two promoter regions upstream of occludin have now been identified (Mankertz et al., 2002), both of which contain potential binding sites for several transcription factors (section 1.3.1.5). It would be possible to screen these known transcription factors for changes in their localisation, phosphorylation, protein levels or loss of DNA-association in response to TNF-α, which could indicate their involvement in transcriptional downregulation of occludin. Furthermore, in astrocytes, TNF-α has been shown to downregulate occludin, which is prevented by inhibition of NFκB activation (Wachtel et al., 2001). This might, therefore, also be a possible regulator of occludin in HUVECs and could be examined by inhibiting NFκB activation (Wachtel et al., 2001), or by observing NFκB re-localisation in response to TNF-α.
Another area for investigation would be the mechanism for RhoA activation. GEF-H1 would be a good potential candidate and it could be tested through overexpression of a GEF-H1 mutant that acts as a dominant negative. Such a mutant might lack the Dbl and/or pH domains that are thought to be important for GEF activity (Schmidt and Hall, 2002). If GEF-H1 is required for TNF-α-induced RhoA activation, then overexpression of a dominant negative GEF-H1 should prevent this response. Alternatively, RNA-interference could be used to inhibit GEF-H1, to see if this effects TNF-α-induced RhoA activation or increased permeability. In addition, possible effector proteins other than ROCK that are downstream of Rho in TNF-α-signalling would be important to identify. Citron kinase and Formins such as Dia1/2 are possible effectors that could contribute to stress fibre formation and contractility. The localisation of these in confluent HUVECs have not yet been reported and it is possible that they localised to tight junctions in HUVECs. They could be further studied by changes in their localisations and interactions with RhoA after TNF-α-exposure, perhaps in GST-pull down experiments (although an association is most likely to be transient). RNA interference or expression of protein mutants, such as those used to study the role of citron kinase in cytokinesis (Madule et al., 1989). Mutation of the FH2 domain of Dia1/2 formins, which is required for their actin nucleation activity, might prove to inhibit TNF-α-induced stress fibres. This would indicate a role for the formins in TNF-α-induced cytoskeletal reorganisations. Previously it has been shown that overexpression of mDia results in stress fibre formation even in the presence of C3 transferase, indicating that formins lie downstream of Rho (Wantanabe et al., 1997). In this same way, overexpression of Dia1/2 might be able to rescue HUVECs from the inhibitory effects that C3 transferase has on TNF-α-induced stress fibres and permeability, to demonstrate that they lie downstream of Rho in TNF-α responses.

The inhibitory effect observed with Ad-β-gal and Ad-GFP on TNF-α-induced responses (Chapter 6) points towards a mechanism of potential value, both therapeutically and for research purposes. TNF-α is already considered as a therapeutic target in chronic
inflammation and an anti-inflammatory agent which specifically blocks TNF-α-induced changes to endothelial cells could reduce chronic inflammation in part by reducing permeability. This warrants further investigation to understand the mechanism through which the viruses reduce basal permeability and inhibit TNF-α signalling. Of note is the striking resemblance between the appearance of the cytoskeleton where cells have been exposed to Ad-βgal or Ad-GFP and ML-7. This indicates that the viruses might inactivate MLCK. As TNF-α does not increase intracellular calcium in endothelial cells, it is unlikely that the viruses are inhibiting TNF-α responses through sequestering calcium. Alternatively, the viruses might be dephosphorylating and inactivating MLCK, as phosphorylation is a known mechanism for MLCK activation downstream of ERK1/2 (Klemke et al., 1997).

In general, other signalling pathways that are known to be activated by TNF-α might be signalling to the tight junction and have not been examined here. These include MAP kinase pathways, PI3-kinase and PKCζ. In Ras-transformed MDCK cells, occludin, claudin-1 and ZO-1 are redistributed away from the junctions, and VE-cadherin is downregulated. This can be reversed by mitogen-activated protein kinase kinase (MEK1) inhibitor PD98059 (Chen et al., 2000), indicating MAPK signalling to the tight junction proteins might induce junctional disassembly. TNF-α is known to activate MAPK signalling (section 1.10) and this is a potential mechanism for increased permeability. The PI 3-kinase effector protein, AKT/PKB, is activated in response to TNF-α, indicating activation of PI 3-kinase (MacEwan, 2001). Moreover, PI 3-kinase can act upstream of Rac (Ridley, 2001). Although no Rac activation was observed at the time points examined here in response to TNF-α, adenoviruses expressing dominant negative Rac1 did inhibit TNF-α-responses and it is possible that TNF-α modulates Rac activity through PI 3-kinase. PKC might also signal to the junctions downstream of TNF-α. PKC activation has been shown to phosphorylate occludin in MDCK cells (Andreeva et al., 2001), but leads to dephosphorylation of occludin in LLC-PK1 cells (Clarke et al., 2000). Furthermore, PKCζ and λ co-localise with ZO-1 at tight junctions (Dodane et al., 1996) and ZO-1 is phosphorylated by PKC in vitro (Stuart and Nigam, 1995). TNF-α-
induced permeability can be prevented by inhibitors and antisense oligonucleotides to PKC-α (Ferro et al., 2000). These data indicate that PKC is likely to be involved in TNF-α-signalling to tight junctions and that the outcome may be dependent upon the PKC isoform and cell type.

The adenovirus receptor is an under-studied integral tight junction protein that may be important in regulating barrier function. Furthermore, adenoviral engagement with CAR may be responsible for blocking TNF-α effects. It would be interesting to investigate the physiological function of CAR and to determine what affect CAR stimulation has on HUVEC permeability and actin cytoskeleton. This could be acheived by using the viral knob protein, which is known to mediate viral attachment by binding to CAR (Chapter 6). Use of just this domain of the virus would eliminate viral internalisation or integrin activation, and would allow the study of signalling from CAR alone. Again, overexpression studies with wild type CAR or CAR truncation mutants in endothelial cells or even a yeast two-hybrid screen with the CAR cytoplasmic region would yield information on the physiological role of this receptor, specifically its effects on permeability, other tight junction protein localisations and CAR protein-protein interactions.
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