Endothelial adapter proteins in leukocyte transmigration

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Summary
Leukocyte transendothelial migration (TEM) requires endothelial signalling. This signalling is initiated by clustering of cell-surface adhesion molecules and transmitted into the endothelium by a group of associated or co-clustered adapter proteins. These adapter proteins, such as cortactin and filamin, connect the adhesion molecules to the actin cytoskeleton as well as to signalling enzymes and downstream pathways. This short review aims to define common themes in adapter protein binding in endothelial cells and to propose critical functions that are exerted by these adapters in leukocyte transendothelial migration.

Keywords
Adhesion receptors, endothelial cells, signal transduction, inflammation, leukocyte trafficking / recruitment

Introduction
The multi-step process of leukocyte transendothelial migration (TEM) is orchestrated by an ever increasing number of molecules, including selectins, integrins and their ligands on the endothelium, that mediate rolling, firm adhesion and diapedesis (1). One would predict that these adhesive interactions with different affinities and spatiotemporal characteristics would suffice to control both heterotypic cell-cell contact and leukocyte chemotaxis, the two key processes that drive TEM. However, numerous studies, published over the past 10–15 years, have uncovered an additional requirement for endothelial intracellular signalling to allow efficient TEM (2). This signalling is initiated through endothelial selectins and integrin-ligands, which, apart from the fact that these are transmembrane proteins, do not resemble signalling receptors at all. Yet, their clustering activates various critical intracellular signalling pathways. This brief review focuses on the adapter proteins that interact with endothelial adhesion molecules and on their contribution to adhesion molecule clustering, cytoskeletal dynamics and recruitment of regulatory enzymes, events that have all been implicated in TEM. Detailed discussion on the relevance of various leukocyte and adhesion molecules, as well as on the various downstream signalling pathways induced in endothelial cells can be found elsewhere (2–5).

Endothelial adapter proteins associated with leukocyte adhesion receptors
Studies on endothelial signalling by adhesion molecules and the concomitant analysis of associated adapter proteins have been most successful for E-selectin and ICAM-1. This is not to say that other adhesion molecules, such as VCAM-1, do not bind to endothelial adapter proteins. In fact, most, if not all, transmembrane adhesion molecules, irrespective of the length of their intracellular domain, are likely to interact with one or several intracellular adapters, to allow linkage to the sub-cortical actin cytoskeleton and to specific signalling cascades (Fig. 1).

E-selectin
Cytokine-activated endothelial cells express primarily E- and P-selectin, as well as selectin-ligands, such as CD34 and PSGL-1. Whereas data on endothelial P-selectin-mediated signalling are minimal, a series of studies have focused on E-selectin signalling in endothelial cells, establishing its link to the actin cytoskeleton in response to antibody-induced clustering.

Based on the finding that clustered E-selectin associates to the endothelial actin cytoskeleton, Yoshida et al. (6) tested if actin-binding proteins also bound to E-selectin. This analysis re-
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Figure 1: Schematic summary of the consequences of endothelial adhesion molecule clustering. VCAM1, ICAM1, ICAM2 or E-selectin clustering leads to association with one or more endothelial adapter proteins, of which ERM proteins, filamin (FLN), α-actinin and cortactin are shown as examples. Recruitment of these adapter proteins leads to various proximal events, such as src activation (1) which can induce cortactin phosphorylation, activation of RhoGTPases through recruitment of specific GEFs (2), and actin polymerization and crosslinking (3). These events, or a subset thereof, further promote clustering, thus representing a positive feedback loop (4). At the same time, additional downstream signalling pathways are activated (5) that have been implicated in the regulation of endothelial cell-cell contact as well as of transcription.

ICAM-1
Recent work from the Sanchez-Madrid lab has identified tetraspanin-enriched microdomains, coined endothelial adhesive platforms, in the apical endothelial membrane (17). Upon leukocyte binding, these domains coalesce and recruit ICAM-1 as well as VCAM-1, increasing the avidity of these integrin ligands and promoting firm leukocyte adhesion. For this strong adhesion, linkage of ICAM-1 to the actin cytoskeleton is important. The original work from the Couraud lab (18) showed that ICAM-1 crosslinking in brain endothelial cells induces association of ICAM-1 to cortactin and the induction of its tyrosine phosphorylation through activation of Src. Later studies showed that cytoskeletal dynamics are important for cortactin phosphorylation, whereas at the same time, activation of RhoA was downstream from this event. The biological relevance of the Src-cortactin pathway relates to the clustering of ICAM-1 (similar as for E-selectin) and transendothelial migration of PMN (9, 19, 20). Cortactin is known to bind to both the Arp2/3 complex as well as to actin and as such represents an important linker regulating actin dynamics (21). Cortactin has also been implicated in assembly of paxillin-containing adhesion structures (22) which may be very analogous to its role in ICAM-1 clustering. In fact, Etienne et al. showed that crosslinking of ICAM-1 results in the phosphorylation of paxillin, FAK and p130Cas (23). These findings indicate that the signalling proteins that are assembled and activated}

PSGL-1
As for PSGL-1 signalling in endothelial cells, no direct information is available, presumably because its expression levels are not very high and its expression in endothelial cells has only recently been recognized (11, 12). In leukocytes, however, PSGL-1 has been shown to associate to moesin through a juxtamembrane region that comprises various Ser-residues as well as a stretch of basic residues. This same region is also critical for PSGL-1 targeting to the uropod in polarized, migrating T-cells (13, 14). Subsequent studies from the Sanchez-Madrid lab showed that moesin and ezrin mediate recruitment of Syk to PSGL-1 (15). It is well conceivable that also in endothelial cells ERM (Ezrin-Radixin-Moesin) proteins bind PSGL-1, but the consequences and relevance for, e.g. monocyte adhesion (12, 16) and TEM remain to be established.

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downstream from ICAM-1 clustering are, like for E-selectin, similar to those found in focal adhesions. In addition to cortactin, ICAM-1 is known to be in close proximity to and even associate with members of the ERM-protein family (24). These authors used transfected Cos7 cells and peptides, encoding the ICAM-1-2 intracellular regions to establish these interactions. ERM binding may be cell-type specific, as Greenwood et al. did not find a direct association of ICAM-1 with ERM proteins in brain endothelial cells (25), whereas others did in HUVEC (26). Alternatively, the (biochemical) approach as used in these studies may also affect the efficiency by which these interactions are detected in whole cells. Functionally, the role for ERM proteins is primarily to target adhesion molecules to relevant membrane domains or structures (27). In endothelial cells, ERM proteins target ICAM-1 to microvilli, and in fact, the formation of these microvilli is mediated by ERM proteins. The localization of ICAM-1 to microvilli is mediated by the basic stretch in the ICAM-1 intracellular region, and expression of ICAM-1 mutants in Cos7 cells suggests that this region mediates adhesion and migration of human peripheral blood leukocytes (28). These findings are in line with FRAP analysis showing that ICAM-1-GFP lacking its intracellular domain, in contrast to full-length ICAM-1, is not reduced in its lateral mobility upon crosslinking. This further indicates that this region mediates connection of ICAM-1 to the cortical actin cytoskeleton (19).

Next to the ERM proteins, ICAM-1 (as well as ICAM-2 [29, 30] and integrins [31]) binds to α-actinin through basic residues in the ICAM-1 juxtamembrane region (32, 33). The association of α-actinin -1 and -4 was shown to be functionally relevant, since reducing α-actinin expression in HUVEC partially blocked neutrophil transendothelial migration (33). α-actinin is a homodimeric actin crosslinking molecule that allows myosin motor activity to mediate contractility. Moreover, α-actinin likely regulates cytoskeletal stiffness and as a result may be critical in the anchoring of ICAM-1-bound leukocytes to the endothelial cell surface. Subsequent force generation to allow locomotion on the endothelial membrane is also a feature of the ICAM-1-α-actinin connection. In good agreement with this, α-actinin has been observed to localize in endothelial docking structures (26).

Using a peptide-based approach to search for ICAM-1 binding proteins, we recently found that the 280 kDa actin-binding protein filamin B directly associates to the intracellular domain of ICAM-1 (34). This association is promoted by ICAM-1 clustering and, conversely, ICAM-1 clustering induced by antibody-coated beads required expression of filamin B. Moreover, FRAP studies in transfected Hela cells showed that filamin B controls the lateral mobility of ICAM-1 in the plane of the membrane and reducing filamin B expression in activated HUVEC impaired leukocyte TEM. Surprisingly, these results were obtained while filamin A was still expressed, suggesting that these homologous filamin proteins serve specific functions in human endothelium. Thus, the function of filamin B appears to be to link ICAM-1 to the cortical actin cytoskeleton, allowing ICAM-1 clustering, thereby providing a strong ‘anchor’ for adherent and migrating leukocytes.

Most interesting is the connection between filamin and caveolin-1 (Cav1). We observed that, when the ICAM-1 intracellular domain was used to capture filamin B and filamin A, also Cav1 was isolated (34). Earlier, filamin A was found to bind to Cav1 (35) and in fact, our own studies indicated that Cav1 associates preferentially to filamin A, rather than to filamin B. Cav1 has been implicated selectively in transcellular rather than paracellular TEM of lymphocytes across activated HUVEC (36). In these studies, reduced expression of Cav1 resulted in a shift towards the use of the transcellular route of TEM, but not in an overall reduction in transmigration. Although an attractive idea, it remains to be determined whether a filamin A-Cav1 complex selectively mediates transcellular leukocyte TEM.

A so far unique interaction concerns the SH3-domain containing Rho-GEF (SGEF). Biochemical studies showed that SGEF directly associates, through its SH3 domain, to the most C-terminal part of ICAM-1, which contains class I and class III SH3 recognition sites (KxxPxxP and RxxK, respectively) (37, 38). Upon clustering of ICAM-1, the small GTPase RhoG is activated, presumably by SGEF (39), and dorsal ruffles are formed that assemble a docking structure around adherent leukocytes (26, 37). Studies to further establish the relevance for the direct ICAM-1-SGEF interaction and for RhoG in leukocyte TEM is currently ongoing. In addition to SGEF, the Rho/Rac GEF Trio may also be activated downstream from ICAM-1 clustering, since it associates to filamin (40). Whether Trio plays a role in ICAM-1-mediated leukocyte adhesion is not known. Recently, it became clear that activation of ICAM-1, or lymphocyte adhesion, studied in HUVEC as well as in brain microvascular endothelial cells, results in increased phosphorylation of VE-cadherin, mediated by Src kinase and Pyk2. This phosphorylation results in reduced VE-cadherin function and promotes lymphocyte and neutrophil TEM (41, 42). Alcaide et al. confirmed and extended these findings by proposing a regulatory role for p120catenin, which downregulates or prevents VE-cadherin phosphorylation on Tyr658, resulting in reduced TEM of PMN (38). These data indicate that the signalling that is induced by ICAM-1 clustering directly affects junctional integrity through VE-cadherin tyrosine phosphorylation, facilitating the paracellular passage of leukocytes.

**VCAM-I**

It is well established that VCAM-1 can induce signalling in endothelial cells upon clustering (2, 43). Unfortunately, there is very little information on specific adapter proteins binding to its intracellular domain. Barreiro et al. (26) identified the interaction of VCAM-1 with ezrin and moesin using immunoprecipitation of full-length VCAM-1 from activated HUVEC. We have not been successful so far in identifying VCAM-1-binding proteins using a peptide encoding the intracellular region or using the full length VCAM-1 protein. The VCAM-1 intracellular domain is only 19 amino acids short and harbours a Type I PDZ domain binding motif (S-x-V-COOH). It may well be that VCAM-1-binding proteins are limited in number or bind with low affinity, precluding clear-cut identification in biochemical assays. Perhaps a directed search for proteins that carry a PDZ domain might represent a successful strategy.
Other IgCAMs
Like VCAM-1, the JAM family members also carry a PDZ-domain binding motif in their C-terminus. However, whereas the JAMs are known to associate to polarity proteins such as ZO1, AF-6/afadin (44, 45) and PAR-3 (46), such interactions are as yet unknown for VCAM-1. Whether this relates to sequence specificity or to the localization of the junctional JAMs versus the apical VCAM-1 or to both remains to be identified. Intriguingly, loss of JAM-C reduced acto-myosin-driven contractility, basal permeability and resulted in a Rap1-dependent stabilization of VE-cadherin-mediated cell-cell adhesion. Moreover, angiogenesis in vivo was also impaired by soluble JAM-C protein. Thus, JAM-C promotes endothelial permeability (47).

The tight junctional IgCAM ESAM (48, 49) has a type I PDZ-domain binding motif and associates to another tight junctional adapter protein MAGI-1. Reduction of ESAM expression by siRNA decreased the basal activity of the small GTPase RhoA, whereas other GTPases such as Rac1, Cdc42 and Rap1 were unaffected. Moreover, loss of ESAM blocked neutrophil, but not lymphocyte, diapedesis. Together, this indicates that ESAM regulates leukocyte transendothelial migration by the induction of RhoA-mediated endothelial signalling towards cell-cell junctions (46). To what extent MAGI-1 is involved in this pathway is not known, although its interaction with α-actinin (50) indicates also here a potentially relevant link to the actin cytoskeleton.

Another junctional IgCAM which is required for efficient neutrophil TEM is PECAM-1/CD31 (51). In contrast to other IgCAMs, PECAM-1 has a large intracellular domain and has extensive signalling capacities, mediated primarily by its two ITIM domains. These associate to SHP1 and SHP2 tyrosine phosphatases, which regulate a large number of downstream events following PECAM-1 clustering, although the relevance of this for neutrophil TEM has been questioned (52). Information on adapter protein binding to PECAM-1 is scarce. There are no data on ERM protein binding to PECAM-1 in endothelial cells, although in platelets, moesin binding to PECAM-1 has been observed (53). Intriguingly, binding of beta- and gamma-catenin to PECAM-1 has been reported (54, 55), which may regulate the connection of PECAM-1 to the cortical actin cytoskeleton as well as to VE-cadherin. As yet, however, the direct relevance for leukocyte TEM is unknown. PECAM-1 has been shown to localize to a ‘lateral border recycling compartment’ (LBRC) which was found to recycle continuously, directing endothelial membrane towards site of monocyte diapedesis (56). This membrane traffic is driven by microtubules, requires the activity of kinesin motor proteins (57) and is regulated by src kinase (58). The membrane traffic from the LBRC to the junctional membrane is required for PECAM-dependent as well as PECAM-independent TEM and thus represents a novel endothelial function that regulates leukocyte diapedesis in general.

Finally, signalling by the junctional transmembrane phosphatase VE-PTP is also regulated during diapedesis (59). VE-PTP is constitutively associated to VE-cadherin, but the complex dissociates upon neutrophil or lymphocyte binding to activated endothelium, albeit that the critical endothelial adhesion molecule involved is presently unidentified. The consequence of this dissociation is increased tyrosine phosphorylation of junctional proteins such as VE-cadherin and in particular plakoglobin. These events reduce VE-cadherin function and lead to increased permeability and enhanced leukocyte diapedesis (59).

Role for the endothelial adapters in leukocyte TEM
The clustering of endothelial adhesion molecules such as ICAM-1 and VCAM-1, following the binding to leukocyte integrins, triggers actin rearrangements. Wojciak-Stothard et al. showed that F-actin is critical for this clustering and for proper functioning of adhesion molecules such as ICAM-1 (60). ERM-proteins link transmembrane proteins to the actin cytoskeleton and localize to microvilli on the apical membrane. Ezrin was originally identified as a substrate of protein kinases and is positively regulated, ‘unfolded’, by PIP2 binding (27). Similar to these events, also ERM translocation to the plasma membrane and consequent formation of microvilli is dependent on RhoA signalling (61). Conversely, ERM proteins can also activate Rho signalling by binding to RhoGDI and inducing release of inactive RhoA which is subsequently available for GEF-mediated activation (62, 63). Since RhoGDI is known to bind to PIP-5-Kinase which can associate to Rac1 (65, 66), all these regulatory proteins may in fact be in close proximity at the plasma membrane and participate in a positive feedback loop. In lymphocytes, ICAM-3 binds to moesin at the uropod (67). Detailed biochemical analysis revealed that ERM-proteins bind to a positively charged amino acid cluster in the juxta-membrane cytoplasmic domain of transmembrane proteins such as CD43 and CD44 (68, 69). The recent study by Oh et al. (28) confirmed that the positively charged amino acid stretch RRKIKK in the intracellular tail of ICAM-1 is critical for the binding of ezrin to ICAM-1, when expressed in Cos7 cells. In contrast, Romero et al. did not find a direct interaction between ICAM-1 and ezrin or moesin in brain endothelial cells, although these proteins did co-localize (23). In line with this, we found no detectable interaction between ezrin and ICAM-1 in HUVEC (JDvB and PHL, unpublished data). Thus, although ERM proteins are likely important for ICAM-1 clustering and/or function, whether these proteins directly interact in endothelial cells remains to be established.

The 280 kDa ICAM-1-binding protein filamin binds actin as a dimer (70). This results in formation of a loose three-dimensional meshwork (Fig. 2), which underlies the plasma membrane and supports the surface of the cell. This “gel-like” actin-rich structure that is induced by filamin is important for the formation of membrane lamella at the cellular periphery (71). Filamin is also present at focal adhesions, regulating the interaction between integrins to the actin cytoskeleton. Feng et al. reported the importance for filamin in endothelial cells by showing that mutations in the human filamin A gene cause cardiovascular defects (72). Filamin not only serves as an actin-binding protein but also binds to Rac1, RhoA, Cdc42 and RalA (73). Moreover, filamin A links the guanine-nucleotide exchange factor (GEF) Trio to the actin cytoskeleton (40) and thus may act as a scaffold for the spatial organization of Rho-GTPase-mediated signalling pathways. This is in good agreement with data from our group.
showing that the formation of endothelial docking structures depends on the presence of filamin (26, 34). Mamamoto et al. showed that, in microvascular endothelial cells, filamin may promote the accumulation of p190RhoGAP in lipid rafts, keeping RhoA inactive and allowing the cell to spread (74). Since clustering of ICAM-1 induces the formation of lipid rafts concomitant with tyrosine phosphorylation of cortactin (9, 75), these findings suggest an important regulatory role for filamin in the formation of ICAM-1-positive lipid rafts and subsequent ICAM-1 clustering and signalling.

$\alpha$-actinin (102 kD), like filamin, binds to actin as a dimer and is also found in focal adhesions (76). However, actin filaments that are crosslinked by $\alpha$-actinin are separated by greater distances than those crosslinked by filamin (Fig. 2). This increased spacing allows motor proteins such as myosin to interact with the actin filaments. Interestingly, in contrast to ezrin, PIP2 reduces $\alpha$-actinin activation in human non-muscle cells (77). Moreover, Celli et al. found that $\alpha$-actinin binds to the basic and hydrophobic amino acid stretch of the intracellular domain of ICAM-1, including the RKIKK motif that may mediate the binding to ezrin (28, 33). However, ezrin and $\alpha$-actinin cannot bind simultaneously to the same ICAM-1 molecule. At low calcium concentrations, the binding of $\alpha$-actinin to actin is optimal (78), whereas ICAM-1-mediated adhesion of lymphocytes to the endothelium raises intracellular calcium concentrations (79). Thus, increased levels of PIP2 together with high intracellular calcium levels, induced upon ICAM-1 clustering, may replace $\alpha$-actinin for ezrin at the tail of ICAM-1, resulting in ICAM-1 reorganization.

Cortactin is an 60–65kD actin-binding protein that binds a large number of signalling proteins, including the actin-nucleation complex Arp2/3 (80). Cortactin is a well-established substrate for tyrosine kinases, including Src, but can also be phosphorylated by Ser/Thr kinases such as PAK. Cortactin phosphorylation creates binding sites for specific signalling proteins, such as dynamin2, affects its cleavage by calpain and potentially its actin-crosslinking activity and has now also been implicated in Arp2/3-mediated actin polymerization (81). Rac1 activity induces cortactin recruitment to cortical actin, where it promotes further actin polymerisation. Cortactin promotes the formation of a branched actin network that is required for protrusive activity (Fig. 2). Thus, adherent leukocytes may, through Rac1 and Src activation, regulate the ICAM-1-associated cortactin such that local actin polymerisation and membrane protrusion is induced. Cortactin therefore may, together with filamin and ERM proteins, promote the formation of endothelial docking structures which explains its requirement for TEM (19, 20, 82).

**Concluding remarks**

From the above overview, some common themes and ideas emerge. First of all, it is most likely that all endothelial adhesion molecules, engaged upon leukocyte binding, are indirectly connected to the actin cytoskeleton via one or more adapter proteins. These adapter proteins appear to serve at least three roles (Fig. 1):

- To mediate the ‘anchoring’ of transmembrane adhesion molecules to the endothelial cortical actin cytoskeleton. This is important for force transduction to the adherent leukocyte which conversely is required for proper adhesion to the endothelial cell surface. Without such adhesion, spreading and migration of adherent leukocytes would be very inefficient.
Now, why is there not simply one specific adapter for each transmembrane adhesion molecule? Although this question remains unanswered, it is relevant to realize that: (1) different adapters in the sequential differences in adapter protein binding. Clustered molecules (60). This clearly represents an important positive feedback loop. As detailed above, there are several studies that have shown that adapter proteins such as cortactin or filamin are required for efficient clustering of, e.g., ICAM-1 around adherent cells (19, 34). Interfering with this feed-back loop inhibits TEM.

To transduce signals into the endothelial cells. This can be by linking IgCAMs or selectins to specific tyrosine or lipid kinases, phosphatases, to RhoGEFs or through actin dynamics. Localized activation of small GTPases will constitute a positive feedback loop by promoting membrane protrusion, recruitment of additional endothelial adhesion molecules and adapter proteins and actin, which results into further clustering of adhesion molecules (60).

References


