VE-cadherin and desmoplakin are assembled into dermal microvascular endothelial intercellular junctions: a pivotal role for plakoglobin in the recruitment of desmoplakin to intercellular junctions

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SUMMARY

Vascular endothelial cells assemble adhesive intercellular junctions comprising a unique cadherin, VE-cadherin, which is coupled to the actin cytoskeleton through cytoplasmic interactions with plakoglobin, β-catenin and α-catenin. However, the potential linkage between VE-cadherin and the vimentin intermediate filament cytoskeleton is not well characterized. Recent evidence indicates that lymphatic and vascular endothelial cells express desmoplakin, a cytoplasmic desmosomal protein that attaches intermediate filaments to the plasma membrane in epithelial cells. In the present study, desmoplakin was localized to intercellular junctions in human dermal microvascular endothelial cells. To determine if VE-cadherin could associate with desmoplakin, VE-cadherin, plakoglobin, and a desmoplakin amino-terminal polypeptide (DP-NTP) were co-expressed in L-cell fibroblasts. In the presence of VE-cadherin, both plakoglobin and DP-NTP were recruited to cell-cell borders. Interestingly, β-catenin could not substitute for plakoglobin in the recruitment of DP-NTP to cell borders, and DP-NTP bound to plakoglobin but not β-catenin in the yeast two-hybrid system. In addition, DP-NTP colocalized at cell-cell borders with α-catenin in the L-cell lines, and endogenous desmoplakin and α-catenin colocalized in cultured dermal microvascular endothelial cells. This is in striking contrast to epithelial cells, where desmoplakin and α-catenin are restricted to desmosomes and adherens junctions, respectively. These results suggest that endothelial cells assemble unique junctional complexes that couple VE-cadherin to both the actin and intermediate filament cytoskeleton.

Key words: Vimentin, Cytoskeleton, Adhesion, Catenin

INTRODUCTION

Endothelial cells form a non-thrombogenic lining that functions as a semipermeable barrier between the plasma and tissue extracellular compartments (van Hinsbergh, 1997; Lum and Malik, 1996; Dejana, 1996). Individual cells that comprise the endothelial monolayer are connected to one another by adhesive contacts, termed intercellular junctions. In addition to mediating cell-cell adhesion, intercellular junctions also function as plasma membrane attachment sites for cytoskeletal networks, such as actin and intermediate filaments (Cowan and Mechanic, 1994; Cowin and Burke, 1996; Green and Jones, 1996; Garrod et al., 1996). This architectural arrangement functions to couple adhesive forces through the transmembrane glycoproteins to the cytoskeleton, thereby influencing cell shape and tissue integrity (Fuchs and Cleveland, 1998). Recent evidence indicates that in addition to mediating adhesive interactions between cells, intercellular junctions also function as macromolecular complexes that participate in signal transduction pathways that ultimately control gene expression and cell behavior (Klymkowsky and Parr, 1995; Barth et al., 1997). In this manner, adhesive intercellular junctions appear to be plasma membrane sites that integrate mechanical and chemical signaling pathways.

The mechanisms by which adhesive interactions are established between vascular endothelial cells have been studied extensively (Dejana et al., 1995; Dejana, 1996). Endothelial cells express a unique cadherin, termed VE-cadherin or cadherin-5 (Breviario et al., 1995; Ali et al., 1997). VE-cadherin mediates calcium-dependent, homophilic adhesion (Breviario et al., 1995; Ali et al., 1997). A second endothelial cadherin termed VE-cadherin-2 has recently been described (Telo et al., 1998). VE-cadherin-2 also mediates calcium-dependent adhesion, but this cadherin includes a unique cytoplasmic domain, and the role of this protein in endothelial function remains to be determined. However, the molecular components that couple the originally described VE-cadherin to the actin cytoskeleton have been studied in detail.
(Lampugnani and Dejana, 1997). Like the classical cadherins, such as E-cadherin, VE-cadherin associates with cytoplasmic proteins termed catenins. Originally identified as cadherin associated proteins and termed α, β, and γ-catenin (Ozawa et al., 1989; Ozawa and Kemler, 1992), these cytoplasmic proteins have been found to be critical in the association of cadherins with the actin cytoskeleton and the adhesive function of the cadherins. γ-Catenin is now known to be identical to plakoglobin (Cowin et al., 1986; Knudsen and Wheelock, 1992). Both β-catenin and plakoglobin bind directly to the cytoplasmic domain of the cadherins. In addition, β-catenin and plakoglobin also bind directly to α-catenin (Aberle et al., 1994; Jou et al., 1995; Nieset et al., 1997; Obama and Ozawa, 1997; Huber et al., 1997), a vinculin related protein that promotes association of the cadherin-catenin complex with the actin network (Nagafuchi et al., 1991; Herrenknecht et al., 1991). α-Catenin, in turn, binds directly to actin (Rimm et al., 1995), and to the actin binding protein α-actinin (Knudsen et al., 1995; Nieset et al., 1997). The cadherins, including VE-cadherin, are thought to be specifically attached to the actin microfilament network through these interactions.

In epithelial cells, classical cadherins assemble into actin-associated adhesive contacts termed adherens junctions. In addition to adherens junctions, epithelial cells assemble separate junctions termed desmosomes, which function as plasma membrane attachment sites for the intermediate filament network (Kowalczyk and Green, 1996; Cowin and Burke, 1996). The adhesive interface of the desmosome is thought to be formed by a distinct class of cadherins, termed desmosomal cadherins. The cytoplasmic domains of the desmosomal cadherins bind directly to plakoglobin, but not β-catenin or α-catenin (Mathur et al., 1994; Plott et al., 1994; Roh and Stanley, 1995). Instead, the desmosomal cadherins and plakoglobin associate with desmoplakin, a large cytoplasmic protein that binds directly to intermediate filament polypeptides, including keratins, vimentin, and desmin (Meng et al., 1997; Kouklis et al., 1994). Recent studies have demonstrated that the amino-terminal domain of desmoplakin binds directly to plakoglobin (Kowalczyk et al., 1997; Smith and Fuchs, 1998) and that desmoplakin is required for intermediate filament networks to associate with the cytoplasmic plaque of the desmosome (Bornslaeger et al., 1996).

Endothelial cells do not express any of the known desmosomal cadherins, and the adhesive intercellular junctions that form in endothelial cells are similar to the actin based adherens junctions that are assembled in epithelial cells. In addition to VE-cadherin, vascular endothelial cells also express N-cadherin. However, N-cadherin is localized in a diffuse pattern on the endothelial plasma membrane and VE-cadherin competitively inhibits the assembly of N-cadherin into endothelial junctions (Navarro et al., 1998). In addition, endothelial cells also express PECAM, a member of the calcium-independent immunoglobulin family of adhesion molecules. PECAM has been implicated in endothelial cell adhesion (Albelda et al., 1991; Newman et al., 1990; Newman, 1997) and has been reported to associate with β-catenin (Matsumura et al., 1997). The majority of studies focusing on endothelial intercellular junctions to date have analyzed the association of VE-cadherin with the actin cytoskeleton. However, much less is known about the potential interactions between VE-cadherin and the vimentin intermediate filament network in vascular endothelial cells. Interestingly, several recent reports have demonstrated that the intermediate filament binding protein desmoplakin is expressed in endothelial cells and colocalizes with VE-cadherin. In lymphatic endothelial cells in vivo, desmoplakin was found to be expressed and assembled into intercellular junctions termed complexus adhaerentes (Schmelz and Franke, 1993; Schmelz et al., 1994). In these junctions, desmoplakin colocalized with both plakoglobin and VE-cadherin. These investigators did not observe desmoplakin in tissue sections of blood vessels. However, analysis of human umbilical vein endothelial cells (HUVEC) demonstrated that these cells express desmoplakin mRNA and the protein was assembled into endothelial junctions and colocalized with VE-cadherin (Valiron et al., 1996). Together, these studies demonstrate that endothelial cells express desmoplakin and that desmoplakin is incorporated into intercellular junctions and colocalizes with VE-cadherin.

In the present study, desmoplakin was found to assemble into intercellular junctions of postconfluent cultures of human dermal microvascular endothelial cells. In addition, vimentin intermediate filaments were found to colocalize with desmoplakin at cell-cell borders. Using several model systems to analyze interactions between junctional proteins, VE-cadherin was found to recruit desmoplakin to cell-cell borders. Furthermore, the recruitment of desmoplakin to cell borders by VE-cadherin required plakoglobin. Interestingly, β-catenin, although closely related to plakoglobin, could not substitute for plakoglobin in the association of desmoplakin with VE-cadherin. In addition, using the yeast two-hybrid system, desmoplakin was found to bind to plakoglobin but not β-catenin. These results suggest that VE-cadherin is coupled to desmoplakin and that this complex may play a role in anchoring VE-cadherin to the vimentin intermediate filament network in endothelial cells. In addition, plakoglobin appears to play a specific and critical role in the association of VE-cadherin with desmoplakin, suggesting that one function of plakoglobin in endothelial cells may be to mediate VE-cadherin association with the vimentin cytoskeleton.

**MATERIALS AND METHODS**

**Cell culture**

Human dermal microvascular endothelial cells (HDMEC) were purchased from Clonetics (San Diego, CA) and cultured according to vendor recommendations. Mouse L-cell fibroblasts were used to establish cell lines expressing full length VE-cadherin, plakoglobin and either full length desmoplakin or an amino-terminal desmoplakin polypeptide (DP-NTP). Parental L-cells were transfected using calcium-phosphate precipitation and selected in an active concentration of 400 μg/ml G418 (Gibco BRL, Grand Island, NY). Drug resistant colonies were isolated using cloning cylinders and expression of each exogenously expressed protein was verified using both immunoblot and immunofluorescence analysis. After initial selection, cell lines were routinely passaged in 200 μg/ml G418 in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin. At least three independently derived clones were characterized for each type of cell line that was established. For transient transfection experiments, a subclone of COS 7 cells (COS 7-20) was transfected by the calcium phosphate precipitation method, rinsed after 18 hours, and processed for immunofluorescence analysis.
after 48 hours. COS cells were routinely cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin. All media and serum were obtained from Gibco BRL and tissue culture plasticware was purchased from Becton Dickinson (Lincoln Park, NJ).

cDNA constructs

Full length cDNAs for human VE-cadherin and a truncated VE-cadherin lacking the catenin binding domain were generated and subcloned into the pECE vector using the SV40 promoter as described previously (Breviario et al., 1995; Navarro et al., 1995). Human plakoglobin was expressed using the LK444 vector, a gift from Dr K. Trevor, which uses the β-actin promoter (Kowalczyk et al., 1994; Palka and Green, 1997). A cDNA encoding the first 584 amino acids of desmoplakin, producing a desmoplakin polypeptide termed DP-NTP, was generated as described previously (Bornslaeger et al., 1996; Kowalczyk et al., 1997). An epitope tagged DP-NTP with an amino-terminal FLAG epitope tag was expressed using a CMV promoter (Green et al., 1997). Full-length human β-catenin was expressed using a CMV promoter and was a generous gift from Drs P. McCrea and P. Polakis.

Barrier function assay

To monitor barrier function of various L-cell lines, cells were cultured on Transwell polycarbonate filter membranes (24 mm diameter with a 0.4 μm pore size, Corning Costar Corporation, Cambridge, MA.). In this culture system, the filter and cell layer separate an apical and basal chamber. L-cells were grown to confluence and 6 × 10^6 CPM of goat IgG (ICN, Irvine, CA) in 1.5 ml of normal growth medium was added to the apical chamber (approximately 20 ng of IgG/well). At various times, 200 μl samples were taken from the basal chamber and the media analyzed using a gamma counter. The CPM that entered the basal chamber was determined and the results expressed as a percentage of the CPM originally added to the apical chamber.

Antibodies and immunofluorescence

Cells were grown on glass coverslips to the desired stage of confluence, rinsed in phosphate buffered saline, and fixed in methanol at −20°C. VE-cadherin was monitored using mouse monoclonal antibody BV6 (Bioline, London; Breviario et al., 1995) or a commercially available cadherin-5 mouse monoclonal antibody (Transduction Laboratories, Lexington, KT). DP-NTP was detected using a rabbit polyclonal antibody NW161 directed against the desmoplakin amino-terminal domain (Bornslaeger et al., 1996) or the monoclonal antibody M2 directed against the desmoplakin carboxyl-terminal domain (Bornslaeger et al., 1996) or the monoclonal antibody M2 directed against the desmoplakin carboxyl-terminal domain (Bornslaeger et al., 1996). Full-length human β-catenin was expressed using a CMV promoter and was a generous gift from Drs P. McCrea and P. Polakis.

Immunoprecipitation and immunoblot analysis

Immunoprecipitation was carried out as described previously (Kowalczyk et al., 1994, 1997). Briefly, cells were scraped into Tris buffered saline containing 0.5% Triton X-100, vortexed and subjected to centrifugation at 14,000 g. An antibody directed against the desmoplakin amino terminus (NW161) (Bornslaeger et al., 1996) was incubated with the cell lysate for 2 hours at 4°C. Protein G-beads (Pharmacia) were then added for 2 hours at 4°C, immune complexes were captured by centrifugation, and the beads were washed five times in Tris buffered saline containing 0.5% Triton X-100 for 10 minutes with gentle rotation at 4°C. Immune complexes were released by incubation in reducing SDS-PAGE sample buffer at 95°C and analyzed by immunoblot using Enhanced Chemiluminescence (Amersham). Plakoglobin was detected using mAb 11E4, and DP-NTP was detected using monoclonal antibody M2 directed against the amino-terminal FLAG epitope tag on the DP-NTP polypeptide.

Yeast two hybrid constructs and assays

Yeast two hybrid vectors encoding the GALT4 DNA binding (pAS-CYH2) (Harper et al., 1993) or transcription activation domain (pACTII) (Bai and Elledge, 1995) were generously provided by Dr S. Elledge. Two hybrid constructs for full-length β-catenin and the carboxyl-terminal domain of α-catenin were kindly provided by Drs T-S. Jou and W. J. Nelson (Jou et al., 1995). The Dsg1 cytoplasmic domain and DP-NTP constructs were generated as described previously (Kowalczyk et al., 1997). The plakoglobin construct lacking the amino and carboxyl terminal domains was constructed as described previously (Kowalczyk et al., 1997) and subcloned into the SaFI and EcoRI sites of pACTII.

To assay for interactions between proteins, yeast (strain HF7c) were transformed with the plasmids of interest and grown on synthetic defined media lacking either tryptophan (SD−trp) for the pAS1-CYH2 vector or leucine (SD−lek) for the pACTII vector to select for transformed clones. Dual transformants were grown on medium lacking both tryptophan and leucine to select for clones that were transformed with both plasmids. Transformations were performed according to methods published in the Matchmaker™ Two Hybrid product protocol (Clontech Laboratories Inc., Palo Alto, CA) and as described previously (Kowalczyk et al., 1997). β-Galactosidase activity was monitored using 4-methylumbelliferyl β-D-galactopyranoside (4-MUG) as a substrate following previously published methods (Meng et al., 1997). Single colonies of yeast were grown in 20 ml liquid cultures at 30°C to a density of 1.0 at A600 and 100 μl of the yeast cultures were pelleted and frozen in liquid nitrogen. The yeast were then resuspended in 350 μl of Z buffer containing 50 μl of a 0.001 M MUG in 0.01 M phosphate buffer, pH 7.0. After one hour at 37°C, the reaction was terminated by adding 400 μl stop solution (0.1 M glycine, pH 10.3). β-Galactosidase activity was determined as a function of the amount of 4-MU released from 4-MUG using a fluorometric plate reader with excitation at 360 nm and emission at 450 nm. As a second reporter for interactions, colonies were also tested for growth on SD−lea−trp−his in the presence of 20 mM 3-aminotriazole (Sigma). Materials for base media and agar were obtained from Difco Laboratories, Detroit MI, and materials for synthetic defined media were purchased from Clontech Laboratories Inc.

RESULTS

Desmoplakin colocalizes at intercellular junctions with VE-cadherin in primary cultures of dermal microvascular endothelial cells

As discussed above, desmoplakin was localized in vivo to lymphatic endothelium (Schmelz and Franke, 1993; Schmelz et al., 1994) and was found to be expressed by cultured HUVEC and assembled into intercellular junctions (Valiron et al., 1996). To determine the distribution of desmoplakin in cultured microvascular endothelial cells, primary human dermal microvascular endothelial cells (HDMEC) were processed for
immunofluorescence analysis and the distribution of desmoplakin was compared to VE-cadherin, plakoglobin and α-catenin (Fig. 1). In non-confluent and newly confluent endothelial cells, desmoplakin was not present at intercellular junctions (not shown), consistent with the previous report that desmoplakin expression is upregulated in post confluent cultures of human umbilical vein endothelial cells (HUVEC) (Valiron et al., 1996). In HDMEC that were 3-5 days postconfluent, desmoplakin (B,D,F) colocalized with VE-cadherin (A), plakoglobin (C), and α-catenin (E). Although desmoplakin often colocalized with these junctional proteins, desmoplakin staining appeared less continuous. To determine the localization of desmoplakin relative to the vimentin intermediate filament network, dual label immunofluorescence analysis for desmoplakin and vimentin (Fig. 2A) and plakoglobin and vimentin (Fig. 2B) was performed. The HDMEC assembled an extensive vimentin intermediate filament network. In addition, bundles of vimentin were often found to terminate or align parallel to the plasma membrane in areas that also stained positive for desmoplakin and plakoglobin.

**VE-cadherin recruits plakoglobin and desmoplakin to cell-cell borders when co-expressed in stable L-cell fibroblast lines**

The results above suggest that desmoplakin may play a role in linking the vimentin intermediate filament network to cell-cell borders in vascular endothelial cells. In a previous study, a polypeptide comprising the first 584 amino acids of the desmoplakin amino-terminal domain (DP-NTP) was found to associate with desmosomal cadherins and plakoglobin and cluster these cadherin-plakoglobin complexes (Kowalczyk et al., 1997). To determine if the amino-terminal domain of desmoplakin also associates with VE-cadherin, we co-expressed VE-cadherin, plakoglobin and DP-NTP in stable L-cell fibroblast cell lines. Parental L-cells and neomycin resistant L-cells do not express detectable levels of plakoglobin or desmoplakin (Kowalczyk et al., 1994, 1996). Likewise, VE-cadherin was not detected by either immunofluorescence (not shown) or immunoblot (see Fig. 8A) in neomycin resistant control L-cells. In L-cell lines co-expressing VE-cadherin, plakoglobin and DP-NTP, VE-cadherin was predominantly concentrated at cell-cell borders and colocalized with both plakoglobin (Fig. 3A,B) and with DP-NTP (Fig. 3C,D). In addition, DP-NTP colocalized with the actin associated proteins α-catenin (Fig. 3E,F) and β-catenin (not shown), which are present endogenously in L-cells (Ozawa et al., 1989). No obvious difference in VE-cadherin distribution was observed in L-cells in the presence of DP-NTP compared to L-cells expressing only VE-cadherin and plakoglobin (not shown).

In addition to DP-NTP, L-cell lines co-expressing VE-cadherin, plakoglobin and full-length desmoplakin were also established (Fig. 4). Although DP-NTP expression in L-cell lines was typically homogeneous, the expression of full-length desmoplakin in the L-cell lines tended to be heterogeneous.
with only some cells within the population expressing full-length desmoplakin. In these cell lines, full-length desmoplakin (A,C) colocalized with VE-cadherin (B) and plakoglobin (D). Due to the presence of the desmoplakin carboxyl terminal domain, which aligns with and disrupts intermediate filament networks when expressed in cultured cells (Stappenbeck and Green, 1992; Stappenbeck et al., 1993), full-length desmoplakin was often detected in a filamentous staining pattern and in perinuclear aggregates. In addition, desmoplakin was also recruited to cell-cell borders in some

Fig. 2. Vimentin filaments localize along the endothelial plasma membrane at regions containing plakoglobin and desmoplakin. Human dermal microvascular endothelial cells were processed for dual label immunofluorescence using antibodies directed against vimentin (mAb V9, Sigma), plakoglobin (rabbit polyclonal antibody from J. Papkoff), or desmoplakin (rabbit polyclonal antibody NW6). The vimentin antibody was detected using a fluorescein conjugated secondary antibody and the plakoglobin and desmoplakin antibodies were detected using rhodamine conjugated secondary antibodies. Double exposures of vimentin and desmoplakin (A) or vimentin and plakoglobin (B) were taken using color slide film. Note areas of colocalization at cell-cell borders where vimentin filaments terminate at the plasma membrane. Bar, 10 µm.

Fig. 3. Immunofluorescence analysis of L-cell lines expressing VE-cadherin, plakoglobin, and DP-NTP. To determine if VE-cadherin recruits desmoplakin to cell-cell borders, L-cell lines co-expressing exogenous VE-cadherin, plakoglobin, and the amino-terminal domain of desmoplakin (DP-NTP) were established and characterized by immunofluorescence. In these cell lines, VE-cadherin (A,C) colocalized with both plakoglobin (B) and DP-NTP (D) at cell-cell borders. In addition, endogenous α-catenin (E) and β-catenin (not shown), which are up-regulated in L-cells expressing classical cadherins (Nagafuchi et al., 1991), also colocalized with DP-NTP (F) at cell-cell borders. There was no detectable VE-cadherin, plakoglobin, or desmoplakin in parental or neomycin control L-cells as determined by immunofluorescence (not shown) or immunoblot analysis (Fig. 8) (see also Kowalczyk et al., 1994, 1996, 1997). Bar, 50 µm.
Fig. 5. Analysis of barrier properties of L-cell lines expressing VE-cadherin. The ability of L-cell lines to function as a barrier to protein flux across cell monolayers was tested by growing the cells on polycarbonate filter membranes (Costar) in which the cell layer separates apical and basal chambers containing growth medium. $^{125}$I-IgG was then added to the apical chamber and the CPM that entered the basal chamber were monitored by taking samples of the basal media at various times. L-cells expressing full-length VE-cadherin and plakoglobin (L-V/Pg) or VE-cadherin, plakoglobin and DP-NTP (L-V/Pg/DP-NTP) exhibited increased barrier function compared to Neo control cells (A) or L-cells co-expressing plakoglobin and DP-NTP with a truncated VE-cadherin lacking the catenin-binding domain (L-VΔICS/Pg/DP-NTP) (B). Error bars represent s.e.m. with each point representing at least triplicate wells. Data shown are representative of at least three independently conducted experiments.

Fig. 4. Analysis of L-cell lines co-expressing VE-cadherin, plakoglobin and full-length desmoplakin. L-cell lines co-expressing VE-cadherin, plakoglobin, and full-length desmoplakin were established and analyzed by immunofluorescence microscopy. Desmoplakin (A,C,E) colocalized with VE-cadherin (B,F) and with plakoglobin (D). In untreated L-cells, desmoplakin was often observed in a filamentous staining pattern and in perinuclear aggregates (A) that colocalized with VE-cadherin (B). These aggregates are similar to those reported previously that contain both desmoplakin and intermediate filament polypeptides (Stappenbeck and Green, 1992). Desmoplakin was also observed at cell borders in some areas (see arrows in C and D). In L-cells treated with 1 $\mu$M forskolin for 24 hrs, desmoplakin staining at borders (E) was more prominent and less filamentous staining was detected, consistent with the ability of forskolin to inhibit desmoplakin interactions with intermediate filament networks (Stappenbeck et al., 1994). Bar, 50 $\mu$m.
Endothelial junction assembly

In a previous study, activation of protein kinase A by forskolin treatment resulted in the phosphorylation of a serine residue near the desmoplakin carboxyl terminal domain and the disruption of desmoplakin alignment with keratin networks (Stappenbeck et al., 1994). Therefore, L-cell lines expressing full length desmoplakin were treated with forskolin to prevent sequestration of desmoplakin along vimentin filaments (E,F). In L-cells treated with forskolin, filamentous desmoplakin staining was reduced and desmoplakin staining at borders was more pronounced (E). Again, full-length desmoplakin often colocalized with VE-cadherin (F) and plakoglobin (not shown). These data demonstrate that VE-cadherin recruits both DP-NTP and full-length desmoplakin to cell-cell borders when expressed in L-cell fibroblasts.

Endothelial intercellular junctions are thought to play a major role in regulating the flux of fluid and solutes across capillaries and into the interstitium (van Hinsbergh, 1997; Lum and Malik, 1996; Dejana, 1996). To determine if L-cells expressing VE-cadherin exhibited enhanced barrier function, the cells were cultured on polycarbonate filter membranes and the flux of radiolabelled IgG across the monolayers was measured over time (Fig. 5). L-cells expressing VE-cadherin and plakoglobin or VE-cadherin, plakoglobin and DP-NTP were compared to neomycin resistant control L-cells (Fig. 5A). The flux of IgG across monolayers of L-cells expressing cadherins (Kowalczyk et al., 1994), the levels of endogenous plakoglobin are apparently too low to support the recruitment of DP-NTP to cell-cell borders (B) or clustering of desmosomal cadherins (Kowalczyk et al., 1997). Bar, 50 μm.

**Fig. 6.** DP-NTP is not recruited to cell-cell borders in the absence of plakoglobin in stable L-cell lines. L-cell lines co-expressing VE-cadherin and DP-NTP were established and processed for immunofluorescence microscopy. VE-cadherin (A) was present at cell-cell borders, but DP-NTP (B) was diffuse in the cytoplasm and did not exhibit any colocalization with VE-cadherin. In contrast, VE-cadherin (C) colocalized with α-catenin (D) at cell-cell borders. In addition, β-catenin (E) was present at cell-cell borders and colocalized with α-catenin (F). (G) Immunoblot analysis demonstrating that these L-cell lines express VE-cadherin and DP-NTP. Neomycin control cells (lane 1), L-cells expressing VE-cadherin and plakoglobin (lane 2), and L-cells co-expressing VE-cadherin and DP-NTP (lane 3) are shown. Plakoglobin and VE-cadherin were detected by immunoblot of whole cell lysates and DP-NTP was immunoprecipitated from cell lysates with antibody NW161 against the desmoplakin amino-terminal domain and detected by immunoblot using an antibody directed against the FLAG tag (mAb M2). Plakoglobin expression was monitored using a polyclonal antibody that recognizes both mouse and human plakoglobin (Hinck et al., 1994; Kowalczyk et al., 1994). Although endogenous plakoglobin is sometimes detected in L-cells expressing cadherins (Kowalczyk et al., 1994), the levels of endogenous plakoglobin are apparently too low to support the recruitment of DP-NTP to cell-cell borders (B) or clustering of desmosomal cadherins (Kowalczyk et al., 1997). Bar, 50 μm.
truncated VE-cadherin lacking the β-catenin/plakoglobin binding domain were also tested (Fig. 5B). L-cells expressing the truncated VE-cadherin mutant did not exhibit increased barrier function compared to neo control L-cells, similar to results obtained when this mutant was expressed in CHO cells (Navarro et al., 1995). These data support the idea that VE-cadherin mediated adhesion plays an important role in the establishment of endothelial barrier function and that linkage of VE-cadherin to the cytoskeleton is critical for the establishment of this barrier.

VE-cadherin requires plakoglobin to recruit desmoplakin to cell-cell borders

In contrast to the desmosomal cadherins, which bind preferentially to plakoglobin (Plott et al., 1994), VE-cadherin has been shown to interact with both plakoglobin and β-catenin (Lampugnani et al., 1995). Therefore, we sought to determine whether DP-NTP is recruited to sites of VE-cadherin mediated adhesion by plakoglobin or β-catenin. To test this, stable L-cell lines co-expressing VE-cadherin and DP-NTP were established (Fig. 6). Although L-cells do express low levels of endogenous plakoglobin, the levels are not sufficient to support DP-NTP clustering of the desmosomal cadherins (Kowalczyk et al., 1997). In contrast, significant levels of α- and β-catenin accumulate in L-cells co-expressing classical cadherins, which dramatically upregulate catenin protein levels presumably by rescuing the catenins from rapid proteolytic degradation (Nagafuchi et al., 1991; Kowalczyk et al., 1994). In L-cells co-expressing VE-cadherin and DP-NTP, VE-cadherin accumulated at cell-cell borders (Fig. 6A). In contrast to L-cells co-expressing plakoglobin with DP-NTP and VE-cadherin (Fig. 3), in the absence of exogenous plakoglobin DP-

Fig. 7. The recruitment of DP-NTP to COS cell-cell borders by VE-cadherin requires the plakoglobin binding domain of VE-cadherin. VE-cadherin cDNA or a truncated VE-cadherin cDNA were expressed transiently in COS cells with cDNAs encoding DP-NTP and plakoglobin or β-catenin. In the absence of VE-cadherin, plakoglobin is distributed diffusely in the cytoplasm and DP-NTP is often present in aggregates in a perinuclear pattern (not shown) (Kowalczyk et al., 1997). In cells co-transfected with VE-cadherin, plakoglobin, and DP-NTP cDNAs, VE-cadherin (A) recruited DP-NTP (B) and plakoglobin (not shown) to cell-cell borders. In contrast, a mutant VE-cadherin lacking the plakoglobin binding domain, VE-CadΔICS (C), did not recruit either plakoglobin (not shown) or DP-NTP (D) to cell junctions. In addition, VE-cadherin (E) does not recruit DP-NTP (F) to cell-cell borders when co-expressed with β-catenin. Although β-catenin is recruited to cell-cell junctions (G), DP-NTP remains predominantly in cytoplasmic aggregates (H). Bar, 10 μm.
NTP remained diffusely distributed in the cytoplasm (Fig. 6B). However, VE-cadherin colocalized at intercellular borders with α-catenin (Fig. 6C and D), and extensive β-catenin staining also colocalized with α-catenin (Fig. 6E and F). Immunoblot analysis confirmed that these these cell lines co-expressed VE-cadherin and DP-NTP (G). VE-cadherin, plakoglobin, and DP-NTP were not detected in neo control L-cells (lane 1).

Fig. 8. Plakoglobin, but not β-catenin, co-immunoprecipitates with DP-NTP. Stable L-cell lines co-expressing VE-cadherin, plakoglobin, and DP-NTP were examined for expression of each protein by western blot analysis of whole cell lysates (A). VE-cadherin, plakoglobin and DP-NTP were detected only in L-cell lines transfected with the cDNAs encoding these proteins (VE-cad/Pg/DP-NTP) and were not detected in L-cells expressing only the neomycin resistance marker (Neo). In addition, immunoprecipitation of DP-NTP using NW161 from L-cell lysates co-precipitated plakoglobin, demonstrating that these proteins were in a complex in cell lines co-expressing VE-cadherin (B). Although these cells also express β-catenin, which colocalizes with VE-cadherin (see Fig. 7), no β-catenin was detected in the DP-NTP immunoprecipitations.

Fig. 9. DP-NTP binds to plakoglobin but not β-catenin in the yeast two hybrid system. To determine if the amino-terminal domain of desmoplakin binds specifically to plakoglobin, DP-NTP was tested for the ability to interact with plakoglobin and full-length β-catenin using both a β-galactosidase assay (A) and growth in the absence of histidine (B) as reporter assays for protein interactions. For the β-galactosidase assay, the galactosidase substrate MUG was used and enzyme activity was monitored as an indication of protein interactions. As reported previously, the carboxyl terminal domain of α-catenin interacts directly with β-catenin and DP-NTP binds directly to plakoglobin. As a negative control, yeast were cotransformed with DP-NTP and the desmosomal cadherin Dsg1, which do not interact directly (Kowalczyk et al., 1997). However, no interactions were detected between DP-NTP and β-catenin using either the galactosidase assay or the histidine growth assay.

Fig. 10. Model describing the possible molecular arrangements of both actin and vimentin binding proteins in endothelial intercellular junctions. VE-cadherin binds directly to both plakoglobin and β-catenin, both of which can bind directly to α-catenin, which mediates interactions with the actin filament system. Interactions between VE-cadherin and vimentin may be established by plakoglobin, which specifically associates with desmoplakin. Other proteins that are not shown may also play roles in establishing interactions between VE-cadherin and these two cytoskeletal network systems. In addition, regions of the plasma membrane may include domains in which VE-cadherin is anchored to only the actin or vimentin network.
Plakoglobin was present in L-cells co-transfected with cDNA constructs encoding plakoglobin and VE-cadherin (lane 2), but plakoglobin was not detected in L-cells co-expressing VE-cadherin and DP-NTP (lane 3).

In the L-cell lines, the junctional proteins are expressed at moderate levels. To verify that DP-NTP recruitment to cell borders could not be mediated by β-catenin when the proteins were expressed at higher levels, the recruitment of desmoplakin to intercellular junctions was monitored using transient transfection experiments in COS cells (Fig. 7). In each experiment, duplicate transfections and multiple combinations of antibodies were used for dual label immunofluorescence to verify that each protein was expressed. In the absence of VE-cadherin, plakoglobin was diffusely distributed in the cytoplasm and DP-NTP was found predominantly in punctate aggregates that were often present in a perinuclear distribution (not shown, see Kowalczyk et al., 1997). However, in the presence of VE-cadherin, both plakoglobin (not shown) and DP-NTP were redistributed to cell-cell borders and colocalized with VE-cadherin (Fig. 7A,B). To determine if the catenin-binding domain of VE-cadherin was required for the recruitment of DP-NTP to cell-cell borders, a truncated VE-cadherin lacking the plakoglobin/β-catenin binding domain was co-expressed with plakoglobin and DP-NTP (Fig. 7C,D). Although DP-NTP (Fig. 7D) and plakoglobin were expressed (not shown), DP-NTP was not recruited to cell-cell borders by the truncated VE-cadherin. In addition, DP-NTP was not recruited to cell-cell borders when VE-cadherin was co-expressed with β-catenin (Fig. 7E,F). Although exogenously expressed β-catenin (G) was recruited to cell-cell borders with VE-cadherin, DP-NTP (H) remained in cytoplasmic aggregates in the absence of exogenously expressed plakoglobin. These data indicate that plakoglobin is required for DP-NTP to be recruited to cell-cell borders when VE-cadherin is co-expressed with β-catenin and plakoglobin but not by VE-cadherin and β-catenin (G), suggesting that plakoglobin binds to VE-cadherin requires plakoglobin. Furthermore, the amino-terminal domain of desmoplakin specifically forms complexes with plakoglobin and binds directly to plakoglobin but not to β-catenin, suggesting that plakoglobin plays a specific role in the attachment of an intermediate filament binding protein to VE-cadherin. These findings raise the possibility that VE-cadherin in endothelial junctions may be coupled to both the actin microfilament system and the intermediate filament cytoskeleton.

Several studies have indicated that VE-cadherin plays an important role in adhesion, endothelial barrier function, and in angiogenesis (Ali et al., 1997; Bach et al., 1998; Matsumura et al., 1997; Vittet et al., 1997). VE-cadherin is coupled to the actin cytoskeleton by β-catenin and plakoglobin (Lampugnani et al., 1995; Navarro et al., 1995), both of which bind to the actin associated protein α-catenin (Aberle et al., 1994; Jou et al., 1995). The observations that desmoplakin is expressed in endothelial cells and that VE-cadherin recruits desmoplakin to cell-cell borders, suggest that VE-cadherin may associate with the vimentin intermediate filament network through specific cytoplasmic interactions. This is supported by the observation that VE-cadherin is assembled into intercellular junctions of lymphatic endothelium that have been termed complexus adhaerentes, the components of which appear to include VE-cadherin, plakoglobin and desmoplakin (Schmelz and Franke, 1993; Schmelz et al., 1994). Similar observations have been made in cultured HUVEC (Valiron et al., 1996), and as reported here, in cultured HDMEC. It is likely that the vimentin intermediate filament network in endothelial cells plays an important role in endothelial cell function. Endothelial cells assemble an extensive vimentin network that appears to terminate at cell-cell borders (Fig. 2), and alterations in the vimentin cytoskeleton have been associated with the degree of endothelial cell confluence (Savion et al., 1982) and barrier function (Stasek et al., 1992).

The results presented here suggest that VE-cadherin is coupled to desmoplakin by plakoglobin. This interpretation is based on the observation that DP-NTP binds directly to plakoglobin and that plakoglobin is required for the recruitment of DP-NTP to cell-cell borders by VE-cadherin in both COS cells and L-cells. When expressed in COS cells, full length VE-cadherin recruited DP-NTP to cell borders but a truncated VE-cadherin lacking the plakoglobin binding domain

**DISCUSSION**

The results of the present study indicate that the intermediate filament binding protein desmoplakin is assembled into the intercellular junctions of dermal microvascular endothelial cells. In addition, VE-cadherin recruits desmoplakin to cell-cell borders and the association of desmoplakin with VE-cadherin requires plakoglobin. Furthermore, the amino-terminal domain of desmoplakin specifically forms complexes with plakoglobin and binds directly to plakoglobin but not to β-catenin, suggesting that plakoglobin plays a specific role in the attachment of an intermediate filament binding protein to VE-cadherin. These findings raise the possibility that VE-cadherin in endothelial junctions may be coupled to both the actin microfilament system and the intermediate filament cytoskeleton.
failed to associate with DP-NTP. Similarly, plakoglobin mediates the interaction between the desmosomal cadherins and DP-NTP (Kowalczyk et al., 1997) (E. A. Bornslaeger and K. J. Green, unpublished), suggesting that plakoglobin plays a critical role in linking desmoplakin to both the desmosomal cadherins and VE-cadherin. Unlike the desmosomal cadherins, VE-cadherin also binds to \( \beta \)-catenin. Although \( \beta \)-catenin and plakoglobin are closely related, \( \beta \)-catenin could not substitute for plakoglobin in the recruitment of DP-NTP to cell junctions with VE-cadherin. Furthermore, DP-NTP binds directly to plakoglobin in the yeast two hybrid system (Fig. 9) and forms complexes with plakoglobin that can be co-immunoprecipitated from L-cell lysates (Fig. 8). However, we were unable to detect complex formation between DP-NTP and \( \beta \)-catenin or direct binding between these proteins using the two hybrid analysis. These results suggest that one function of plakoglobin in endothelial junctions is to promote association with the vimentin network, and this function appears to be specific to plakoglobin and not \( \beta \)-catenin.

In keratinocytes and other epithelia, associations between the keratin intermediate filament cytoskeleton and desmosomes is thought to play an important role in maintaining the structural integrity of epithelial sheets (Fuchs, 1994; Coulombe and Fuchs, 1994; Steinitz and Bale, 1993; Fuchs and Cleveland, 1998). The ability of plakoglobin to couple VE-cadherin to desmoplakin suggests that these complexes may play an important role in the structural integrity of endothelial monolayers. The kinetics of endothelial junction assembly after switching the cells from low to normal calcium levels to induce junction formation indicates that \( \beta \)-catenin is rapidly assembled into the junctions, whereas plakoglobin (Lampugnani et al., 1995) and desmoplakin (Valiron et al., 1996) are associated preferentially with mature endothelial junctions. Experiments in which antiserum oligonucleotides were used to inhibit plakoglobin expression indicated that plakoglobin is important for endothelial cells to resist the forces of shear stress (Schmittler et al., 1997). One possible explanation for these observations is that initial endothelial junction assembly is driven by VE-cadherin and actin associated proteins, whereas mature junctions also incorporate plakoglobin and vimentin binding proteins such as desmoplakin, thereby promoting the mechanical strength of the junctions. This remains to be demonstrated directly, and it is likely that other intermediate filament associated proteins in addition to desmoplakin are assembled into endothelial intercellular junctions.

The observation that VE-cadherin associates with both actin and intermediate filament binding proteins raises the intriguing possibility that endothelial cells assemble junctions that are anchored to both the actin and intermediate filament networks. In epithelial cells, actin associated adherens junctions and intermediate filament associated desmosomes occupy different plasma membrane domains. For example, in normal epithelial cells, desmoplakin and \( \alpha \)-catenin do not colocalize. However, in HDMEC, we observed significant co-localization of desmoplakin and \( \alpha \)-catenin (Fig. 1) and in L-cells, VE-cadherin recruited both DP-NTP and \( \alpha \)-catenin to the same regions of the plasma membrane (Fig. 3). These observations lead us to propose that mature endothelial intercellular junctions might be organized as shown in Fig. 10. In this model, VE-cadherin could associate with either plakoglobin or \( \beta \)-catenin, with \( \beta \)-catenin mediating linkage primarily to the actin cytoskeleton and plakoglobin mediating interactions primarily with the vimentin cytoskeletal network. Homophilic or lateral (Norvell and Green, 1998) interactions between cadherin molecules on adjacent cells could recruit both actin and vimentin associated proteins to the same domains of the plasma membrane, thereby leading to the assembly of a mixed junction, containing both actin and vimentin binding proteins. The data presented in the present study as well as in previous papers (Schmelz and Franke, 1993; Schmelz et al., 1994; Valiron et al., 1996) are consistent with this model. It should be noted that some membrane domains could be pure, rather than mixed, and might consist of only actin or vimentin associated proteins. At present, this model represents an hypothesis, and it will be important to define further the nature of these junctions using both ultrastructural and biochemical approaches to understand precisely how both actin and vimentin associated proteins might co-assemble at a common adhesive interface.

The presence of mixed junctions containing both actin and intermediate filament binding proteins was reported previously in A431 epithelial cells expressing the desmoplakin amino-terminal polypeptide DP-NTP (Bornslaeger et al., 1996). Expression of this protein uncoupled keratin filament attachment to desmosomes and caused mixing of adherens junction and desmosomal components. Interestingly, co-expression of DP-NTP with plakoglobin and the desmosomal cadherins in L-cells caused the cadherin-plakoglobin complex to cluster into punctate regions of staining (Kowalczyk et al., 1997). In contrast, staining for VE-cadherin and plakoglobin was not punctate in L-cells co-expressing DP-NTP (Fig. 2). In these cells, VE-cadherin, plakoglobin and DP-NTP were present at cell-cell borders in a somewhat continuous staining pattern. Unlike desmosomes, which are highly punctate and densely organized structures, endothelial junctions are more similar to adherens junctions, and tend to be much more continuous along the plasma membrane. This suggests that the associations between desmoplakin and plakoglobin might be influenced by the type of cadherin to which plakoglobin is bound. These interactions might influence both the degree of clustering that occurs as well as whether the cadherin associates with actin, intermediate filaments, or with both types of cytoskeletal networks.

In addition to co-expressing DP-NTP with VE-cadherin and plakoglobin, we also co-expressed full-length desmoplakin with VE-cadherin and plakoglobin. While the amino-terminal domain of desmoplakin binds to plakoglobin, the carboxyl terminal domain of desmoplakin binds to intermediate filament polypeptides, including keratins, vimentin, and desmin (Kouklis et al., 1994; Meng et al., 1997; Stappenbeck and Green, 1992). Although VE-cadherin, plakoglobin, and DP-NTP were expressed homogeneously in the L-cells, we were unable to isolate stable L-cell lines that expressed full-length desmoplakin in a homogeneous manner. It was possible to clearly demonstrate that full-length desmoplakin was recruited to cell-cell borders with VE-cadherin, but it was not possible to do biochemical or functional analyses on these cell lines. Interestingly, forskolin treatment of L-cells expressing full-length desmoplakin increased desmoplakin accumulation at cell-cell borders and decreased the degree of filamentous staining. Activation of protein kinase A causes
phosphorylation of a serine residue near the desmoplakin carboxyl terminus and decreased interaction between desmoplakin and intermediate filament networks (Stappenbeck et al., 1994) (Fig. 4). Upregulation of cAMP in endothelial cells appears to enhance endothelial barrier function in cells treated with inflammatory mediators or growth factors (Minnear et al., 1989). These results are consistent with the hypothesis that a balance between kinases and phosphatases regulates desmosplakin assembly into junctions and may thereby influence junctional integrity.

It is currently unclear why the vimentin and actin networks might be co-assembled around a common adhesive interface in endothelial cells. It is possible that this type of architectural arrangement facilitates the ability of endothelial cells to rapidly alter the state of intercellular junctions in response to inflammatory mediators that modulate endothelial barrier function. In events such as angiogenesis and leukocyte diapedesis, alterations in cell adhesion and cytoskeletal organization would need to be tightly coupled, and the assembly of a common adhesive site for both types of cytoskeletal networks might serve to integrate changes in these networks with alterations in cell adhesion and motility. Thus, it will be important to determine what regulates the incorporation of plakoglobin into endothelial junctions and how these complexes with intermediate filament binding proteins might contribute to endothelial barrier function, angiogenesis, and leukocyte diapedesis.

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