Cdc42 Regulates Adherens Junction Stability and Endothelial Permeability by Inducing α-Catenin Interaction With the Vascular Endothelial Cadherin Complex

Michael T. Broman, Panos Kouklis, Xiaopei Gao, Ramaswamy Ramchandran, Radu F. Neamu, Richard D. Minshall, Asrar B. Malik

Abstract—The adherens junctions (AJs) consist of trans-oligomers of membrane spanning vascular endothelial (VE)-cadherin proteins, which bind β-catenin through their cytoplasmic domain. β-Catenin in turn binds α-catenin and connects the AJ complex with the actin cytoskeleton. We addressed the in vivo effects of loss of VE-cadherin interactions on lung vascular endothelial permeability and the role of specific Rho GTPase effectors in regulating the increase in permeability induced by AJ destabilization. We used cationic liposomes encapsulating the mutant of VE-cadherin lacking the extracellular domain (ΔEXD) to interfere with AJ assembly in mouse lung endothelial cells. We observed that lung vascular permeability (quantified as microvessel filtration coefficient $K_f$) was increased 5-fold in lungs expressing ΔEXD. This did not occur to the same degree on expression of the VE-cadherin mutant, ΔEXDΔβ, lacking the β-catenin–binding site. The increased vascular permeability was the result of destabilization of VE-cadherin homotypic interaction induced by a shift in the binding of β-catenin from wild-type VE-cadherin to the expressed ΔEXD mutant. Because ΔEXD expression in endothelial cells activated the Rho GTPase Cdc42, we addressed its role in the mechanism of increased endothelial permeability induced by AJ destabilization. Coexpression of dominant-negative Cdc42 (N17Cdc42) prevented the increase in $K_f$ induced by ΔEXD. This was attributed to inhibition of the association of α-catenin with the ΔEXD–β-catenin complex. The results demonstrate that Cdc42 regulates AJ permeability by controlling the binding of α-catenin with β-catenin and the consequent interaction of the VE-cadherin/catenin complex with the actin cytoskeleton. (Circ Res. 2006;98:0-0.)

Key Words: adhesion molecules ■ gene transfer ■ catenins ■ Cdc42 ■ VE-cadherin

A dherens junctions (AJs) are dynamic structures mediating endothelial cell–cell adhesion and thereby regulate endothelial barrier function and tissue fluid homeostasis. Endothelial cell adhesion to contiguous cells, forming the endothelial monolayer, is determined in part by the transmembrane protein vascular endothelial (VE)-cadherin, which forms homotypic adhesive interactions and binds 2 members of the catenin family of cytosolic proteins ($\beta$ and $\gamma$ catenins). $\beta$-Catenin binds $\alpha$-catenin and connects junctional cadherins with the actin cytoskeleton. Permeability-increasing mediators such as histamine and thrombin increase functional permeability in part by inducing the disassembly of AJs. The end result is a shift in fluid and plasma proteins to the extravascular space and development of protein-rich tissue edema.

Phosphorylation of AJ proteins contributes to the mechanism of destabilization of AJs. Studies showed that histamine induced the phosphorylation of VE-cadherin and $\beta$- and $\gamma$-catenins within 60 sec consistent with the rapidly increased microvascular permeability secondary to the formation of 100 to 400 nm-wide interendothelial gaps. It was also shown that thrombin-induced activation of PKC$\alpha$ regulated the phosphorylation of VE-cadherin and catenins and thereby contributed to disruption of AJs and the increase endothelial permeability. Phosphatases such as protein tyrosine phosphatase VE-PTP may also regulate the phosphorylation of VE-cadherin/catenin components and thus AJ integrity.

In addition, Rho GTPases RhoA, Rac1, and Cdc42 are important in regulating AJ assembly. Inhibition of RhoA prevented both thrombin- and histamine-induced disassembly of AJs and the increase in endothelial permeability. RhoA is also directly linked to AJs through p120 catenin, a Src substrate that binds to the juxta-membrane domain of VE-cadherin. Overexpression of p120 catenin led to inhibition of RhoA in a GDP dissociation inhibitor–like manner. Up-or downregulation of p120 catenin in endothelial cells influenced VE-cadherin levels in endothelial cells. In addition, activation of Cdc42 and Rac1 regulated the post-Golgi transport of endothelial cadherin to the AJs. Other studies showed that Cdc42 can signal the activation of actin polymerization at the plasma membrane leading to formation of filopodia and thus contributing to assembly of AJs. 

Original received May 16, 2005; revision received October 17, 2005; accepted November 10, 2005.
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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/01.RES.0000198387.44395.e9
and Rac1 may also affect AJ function through their interactions with the scaffold protein IQGAP1. Although the role of IQGAP1 in regulating AJ assembly has not been studied in endothelial cells, it was shown to colocalize with members of the AJ complex in other cell types and thus may be an important determinant of junctional permeability downstream of Cdc42 and Rac1.

In the present study, we addressed the role of Cdc42 in the mechanism of increased endothelial permeability. Studies were made in the mouse lung microcirculation, so that inferences could be drawn concerning the in vivo regulation of endothelial permeability. We used the VE-cadherin mutant lacking the extracellular domain (ΔEXD) and another mutant lacking both extracellular domain and cytosolic distal β-catenin–binding domain (ΔEXDΔβ). We tested the hypothesis that expression of ΔEXD promotes a shift in the VE-cadherin–β-catenin toward the juxta-membrane domain; DBD, distal β-catenin–binding domain. The first mutant, ΔEXD, lacks the extracellular domain and contains a FLAG epitope. The second mutant, ΔEXDΔβ, lacks both the extracellular domain and DBD. B, Expression of ΔEXD and N17Cdc42 in mouse lung endothelial cells. Endothelial cell–enriched lung lysates were analyzed for expression of constructs. The second and third lanes show the expression of FLAG-ΔEXD (36 kDa). The third and fourth lanes show the coexpression of FLAG-ΔEXD with myc-N17Cdc42 and myc-N19Rho, respectively (25 kDa). Expression of these constructs did not affect expression of total VE-cadherin (140 kDa) and Cdc42 (23 kDa). C, Expression of ΔEXD was also determined by immunofluorescence in lung endothelial cells isolated from liposome–ΔEXD cDNA-injected mice. Lung endothelial cells were obtained 24 hours after IV injection of constructs and stained for FLAG-ΔEXD with anti-FLAG (green) and anti-VE-cadherin (red) antibodies. Maximum expression was seen at this time period (bottom) (scale = 10 μmol/L). Top, Control endothelial cells showing only VE-cadherin staining (scale = 20 μmol/L). Results are representative of 4 experiments.

### Materials and Methods

**VE-Cadherin and Rho GTPase Mutants**

The plasmid vector pcDNA3 was cleaved at its multicloning site and specific cDNAs corresponding to truncated sequences of VE-cadherin were inserted downstream of cytomegalovirus promoter. We constructed, as described, a VE-cadherin mutant lacking the extracellular domain (ΔEXD) and a mutant lacking both the extracellular domain and cytosolic distal β-catenin–binding domain (ΔEXDΔβ). Expression of these constructs did not affect expression of total VE-cadherin (140 kDa) and Cdc42 (23 kDa). C, Expression of ΔEXD was also determined by immunofluorescence in lung endothelial cells isolated from liposome–ΔEXD cDNA-injected mice. Lung endothelial cells were obtained 24 hours after IV injection of constructs and stained for FLAG-ΔEXD with anti-FLAG (green) and anti-VE-cadherin (red) antibodies. Maximum expression was seen at this time period (bottom) (scale = 10 μmol/L). Top, Control endothelial cells showing only VE-cadherin staining (scale = 20 μmol/L). Results are representative of 4 experiments.

**Figure 1.** Expression of ΔEXD mutant in junctions of lung endothelial cells in situ. A, Wild-type VE-cadherin protein is shown. S indicates signal peptide; P, pre-peptide; CR, cadherin repeat; JMD, juxta-membrane domain; DBD, distal β-catenin–binding domain. The first mutant, ΔEXD, lacks the extracellular domain and contains a FLAG epitope. The second mutant, ΔEXDΔβ, lacks both the extracellular domain and DBD. B, Expression of ΔEXD and N17Cdc42 in mouse lung endothelial cells. Endothelial cell–enriched lung lysates were analyzed for expression of constructs. The second and third lanes show the expression of FLAG-ΔEXD (36 kDa). The third and fourth lanes show the coexpression of FLAG-ΔEXD with myc-N17Cdc42 and myc-N19Rho, respectively (25 kDa). Expression of these constructs did not affect expression of total VE-cadherin (140 kDa) and Cdc42 (23 kDa). C, Expression of ΔEXD was also determined by immunofluorescence in lung endothelial cells isolated from liposome–ΔEXD cDNA-injected mice. Lung endothelial cells were obtained 24 hours after IV injection of constructs and stained for FLAG-ΔEXD with anti-FLAG (green) and anti-VE-cadherin (red) antibodies. Maximum expression was seen at this time period (bottom) (scale = 10 μmol/L). Top, Control endothelial cells showing only VE-cadherin staining (scale = 20 μmol/L). Results are representative of 4 experiments.

**Cell Culture and Transfection**

HMEC-1 (Human Microvascular Endothelial Cells) and HPAEC (Human Pulmonary Artery Endothelial Cells) were grown...
transfected by electroporation as described in the online data supplement, available at http://circres.ahajournals.org.

Immunofluorescence and Confocal Microscopy
Endothelial cells were either seeded onto glass coverslips posttransfection and allowed to grow for 24 hours in complete medium or freshly obtained from collagenase-digested lungs and plated onto coverslips for 1.5 hour (described below). Cells were immunostained and visualized as described in the online data supplement.

Preparation of Cationic Liposomes for In Vivo Studies
Liposomes composed of dimethyldioctadecyl ammonium bromide (Sigma) in a 1:1 molar ratio with cholesterol (Sigma) were prepared as described, except that the dried lipid film was resuspended in 5% dextrose in water and then sonicated for 20 minutes, followed by incubation at 42°C for 20 minutes and 0.45-μm filtration. Liposomes were extruded through a 50-nm pore polycarbonate filter (Avestin). CD1 mice (Charles River, Wilmington, Mass.), weighing 20 to 25 g, were injected IV with 50 μg of plasmid DNA, which was mixed with 100 μL of liposome suspension and allowed to equilibrate for 20 minutes before injection. Protein expression constructs was assayed 24 hours thereafter.

Lung Endothelial Fractionization
CD1 mice were anesthetized and excised lungs were perfused with Hanks’ balanced salt solution through the pulmonary artery for 5 minutes, then with endothelial fractionation buffer (0.2% Triton X-100, 50 mmol/L Tris-Cl pH 7.9, 1× Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktails 1 and 2, Sigma) at 0.5 mL/min for 30 sec. Eluate was collected from the left atrial cannula (200 μL) for Western blotting, and remaining lung tissue was homogenized in tissue lysis buffer (1.5% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% dioctylsulfate, 100 mmol/L phenylmethylsulfonyl fluoride, 1× Protease Inhibitor Cocktail in PBS), followed by centrifugation at 3000g at 4°C for 10 minutes to remove insoluble material. Remaining lung tissue was compared with endothelial-rich lysate by Western blotting using endothelial cell markers (eg, angiotensin-converting enzyme, VE-cadherin). Endothelial-rich lysate stained strongly positive for endothelial antigens (data not shown); the purity of this lysate was 90%.

Isolation of Endothelial Cells by Collagenase Digestion of Mouse Lungs
To assess the expression of transfected proteins in endothelial cells, mice were euthanized 20 hours postinjection of liposome–DNA mixture. Lungs were perfused as above, diced into 2-mm cubes and transferred to 1% collagenase A (Roche/Hanks’ balanced salt solution (Gibco) and mixed at 37°C for 1 hour. Lung tissue was aspirated through a serological pipette and allowed to settle at room temperature. The supernatant was removed and centrifuged at 3000g for 1 minute. The supernatant was discarded, and cells were resuspended in modified EGM-2 (penicillin/streptomycin instead of gentamicin/amphotericin B; Clonetics), and allowed to adhere to gelatinized coverslips for 1.5 hour. Cells were subsequently analyzed by immunofluorescence.

Pulmonary Microvascular Filtration Coefficient and Isogravimetric Lung Water Determinations
CD1 mice were anesthetized and lungs were removed, ventilated, and perfused ex vivo using our methods to obtain stable pulmonary artery pressure (7 cm H2O) and lung wet weight over a minimum of a 90-minute period. Microvasseles filtration coefficient (Kf) was measured by applying a 10 cm H2O brief step increase in left atrial pressure at 20 minutes postextraction (during the isogravimetric period).

Immunoprecipitation and Western Blotting
Cell and tissue lysates were subjected to immunoprecipitation and Western blotting as described in the online data supplement.

Cdc42 Activation Assay
Cdc42 activity was measured using the Cdc42 activation assay Biochem Kit (BK034, Cytoskeleton, Denver, Colo). Whole cell lysates were also analyzed for Cdc42 expression and total protein.

Data Analysis
Data were analyzed using the 2-tailed Student’s t test as well as ANOVA. Values are reported as mean±SEM. Values were considered significant at P<0.05. Densitometry measurements of Western blots were performed using the ImageJ program (NIH).

Results
Expression of ΔEXD in Junctions of Lung Endothelial Cells In Situ
Figure 1B shows the expression of FLAG-ΔEXD as well as myc-N17Cdc42 in mouse lung endothelial cells. Endothelial cell-enriched lysates from mouse lungs were analyzed for expression of the constructs. The second and third lanes show the expression of FLAG-ΔEXD (36 kDa), and the third lane shows the coexpression of myc-N17Cdc42 (25 kDa) (Figure 1B). Expression of FLAG-ΔEXD construct did not affect the expression of wild-type VE-cadherin and Cdc42. Figure 1C shows the expression of ΔEXD in lung endothelial cells isolated from the liposome–DNA–injected mice. Lung endothelial cells were obtained 24 hours postinjection of construct and stained with anti-FLAG (green) and anti–VE-cadherin (red) antibodies. Maximum expression of ΔEXD at the junctions seen at 24 hours was colocalized with the endogenous VE-cadherin (bottom) in contrast to control endothelial cells (top) (Figure 1C).

Expression of ΔEXD in Confluent Endothelial Cells Induces Junctional Destabilization
Figure 2A shows results of confocal microscopy using anti-FLAG and anti–β-catenin antibodies in the ΔEXD-expressing (top) and ΔEXDΔβ-expressing (bottom) confluent HPAEC monolayers. β-Catenin was localized to the cell junctions. Expression of ΔEXD caused the formation of numerous intercellular gaps and filopodia, whereas this did not occur with expression of ΔEXDΔβ (Figure 2A). Similar studies were performed in HMEC-1 cells (supplemental Figure 1). ΔEXD, but not ΔEXDΔβ, colocalized with β-catenin. Figure 2B shows the results of anti–β-catenin immunoprecipitation of HMEC lysates expressing ΔEXD or ΔEXDΔβ followed by immunoblotting. A decrease of total β-catenin–bound VE-cadherin was observed on expression of ΔEXD (25±5% decrease, second lane), but not with ΔEXDΔβ (top, second and third lanes). β-Catenin interacted with ΔEXD but not with ΔEXDΔβ (third panel). The VE-cadherin truncation mutants showed equal expression (bottom) and did not alter the endogenous VE-cadherin expression (fourth panel). Figure 2C shows inhibition of interaction of VE-cadherin with β-catenin following different levels of ΔEXD expression. HMECs were transfected with 12 μg or 6 μg ΔEXD cDNA (third and fourth lanes), and lysates were immunoprecipitated with anti–VE-cadherin antibody. Decrease in binding between VE-cadherin and β-catenin (bottom) in the
The presence of ΔEXD was proportional to the level of expression of ΔEXD (middle). The first lane shows immunoprecipitation with an isotype-matched control goat antibody.

**Cdc42 Regulates the Association of α-Catenin With VE-Cadherin**

The top 2 panels of Figure 3A show results of immunoprecipitation of HMEC lysates with anti-FLAG antibody followed by immunoblotting with anti-α-catenin or β-catenin monoclonal antibody (mAb). In ΔEXD-expressing cells, both α- and β-catenin were associated with ΔEXD (second lane), but not with ΔEXDΔβ (third lane) (Figure 3A). Coexpression of N17Cdc42 interfered with the association of α-catenin with ΔEXD, whereas the association of β-catenin with ΔEXD was unaffected (fourth lane) (Figure 3A). In contrast, coexpression of N19RhoA had no effect on the association of

**Figure 2.** Expression of ΔEXD in confluent endothelial cells disrupts adherens junctions. A, Confocal microscopy using anti-FLAG and anti-β-catenin antibodies in ΔEXD-transfected (top) and ΔEXDΔβ-transfected (bottom) confluent HPAEC monolayers at 24 hours post-transfection. β-Catenin was prominently localized at the plasma membrane. Expression of ΔEXD caused widespread formation of intercellular gaps and filopodia (arrows) (top, middle), whereas this did not occur with the expression of ΔEXDΔβ (bottom, middle). Also ΔEXD colocalized with β-catenin (yellow in merged image, top), and this did not occur to the same degree following expression of ΔEXDΔβ (bottom). Results are representative of 4 experiments (scale = 10 μmol/L). B, Interactions of VE-cadherin mutants and β-catenin. Lysates from HMECs expressing ΔEXD and ΔEXDΔβ were immunoprecipitated with anti–β-catenin antibody and immunostained for endogenous VE-cadherin, β-catenin, and FLAG-ΔEXD or -ΔEXDΔβ. In the first panel of B, ΔEXD-expressing cells (second lane) showed decreased interaction between β-catenin and endogenous VE-cadherin compared with control cells (first lane). In contrast, ΔEXDΔβ failed to coimmunoprecipitate with β-catenin (third lane). Quantification of results indicated 25±5% decrease in association from control between β-catenin and endogenous VE-cadherin following ΔEXD expression and no change following ΔEXDΔβ expression. In the second panel of B, immunoblotting for β-catenin showed similar levels of β-catenin expression in the ΔEXD- or ΔEXDΔβ-expressing endothelial cells. In the third panel of B, immunoblotting for FLAG showed association with β-catenin in the ΔEXD-but not in ΔEXDΔβ-expressing endothelial cells. The fourth and fifth panels of B show immunoblots of whole cell lysates with VE-cadherin and expressed FLAG-tagged ΔEXD or ΔEXDΔβ mutants. All results are representative of 4 separate experiments. C, β-Catenin binding to ΔEXD is dependent on level of ΔEXD expression. HMECs were transfected with 0, 6, or 12 μg of ΔEXD cDNA (second, third, and fourth lanes) and immunoprecipitated with control antibody (first lane) or anti-VE-cadherin antibody (second, third, and fourth lanes). Equal loading was measured by anti-VE-cadherin immunoblot (top). Middle, The two different levels of ΔEXD expression (third and fourth lanes). VE-cadherin/β-catenin interaction was reduced to a greater extent with the higher level of ΔEXD expression (decrease of 34% vs 17%) (bottom, third lane vs fourth lane).
α-catenin or β-catenin with ΔEXD (fifth lane). The bottom 3 panels of Figure 3A show expression of similar amounts of FLAG-tagged ΔEXD or ΔEXDΔβ (in second through fifth lanes) as well as similar expression of myc-tagged dnRhoA, dnCdc42, and endogenous VE-cadherin. Probing these immunoblots for the transfected myc-Cdc42 and myc-RhoA showed no interaction with the FLAG-ΔEXD construct (data not shown).

Figure 3B shows the results of immunoprecipitation of lysates with β-catenin mAb followed by immunoblotting with anti-α-catenin antibody. In ΔEXD-expressing cells, both α- and β-catenin were immunoprecipitated (second lane); however, this failed to occur after expression of ΔEXDΔβ (third lane). Coexpression of N17Cdc42 prevented the association of α-catenin, but not β-catenin, with ΔEXD (fourth lane). In contrast, coexpression of N19RhoA had no effect on the association of α-catenin or β-catenin with ΔEXD (fifth lane). The third and fourth panels of A show similar levels of expression of ΔEXD and ΔEXDΔβ as well as wild-type VE-cadherin in all lanes. The fifth panel of A shows similar levels of myc expression in cells coexpressing N17Cdc42 or N19RhoA. B, Results of immunoprecipitation of lysates with β-catenin mAb followed by immunoblotting with anti-α-catenin Ab. First panel of B shows increased association of β-catenin with β-catenin in the ΔEXD-expressing cells (3-fold increase over control, second lane) but not in ΔEXDΔβ-expressing cells (third lane). N17Cdc42 prevented the ΔEXD-mediated increase (1.3-fold, fourth lane); in contrast, expression of N19RhoA had no effect (3-fold, fifth lane). The second panel of B shows results of immunoblotting for β-catenin following β-catenin immunoprecipitation. The third panel of B shows β-catenin immunoblot in whole-cell lysates.

β-catenin (fourth lane), whereas coexpression of N19RhoA had no effect on α-catenin association with β-catenin (fifth lane). Expression of ΔEXD and Rho GTPases also had no effect on endogenous β-catenin expression (bottom 2 panels) (Figure 3B).

Figure 4A shows results of Cdc42 GTPase activity. Expression of ΔEXD and ΔEXDΔβ increased the amount of activated Cdc42 in HMECs, but a reduced response was observed following ΔEXDΔβ expression (second lane vs third lane). Coexpression of N17Cdc42 abrogated this response (fourth lane), whereas N19RhoA had no effect (fifth lane). Total Cdc42 levels were assessed by Western blotting (middle); the upper band in Lane 4 represents myc-tagged N17Cdc42. The expression levels of myc-tagged Cdc42 and RhoA were comparable (bottom). B. Dominant-active Cdc42 mutant promotes increased binding of α-catenin and β-catenin. Results of immunoprecipitation of HMEC lysates with anti-β-catenin mAb followed by immunoblotting with anti-α-catenin mAb. The first panel shows transfection with dominant-active (V12) Cdc42 caused an increase in the affinity of α-catenin for β-catenin (third lane), whereas dominant-negative (N17) Cdc42 decreased this interaction by 75% (fourth lane). Expression levels of immunoprecipitated β-catenin (second panel), lysate α-catenin and myc-tagged Cdc42 were comparable (second and third panels). All results shown above are representative of 3 experiments.
this interaction (top, third lane), whereas N17 dnCdc42 decreased it by 75% (top, fourth lane). Immunoprecipitation with mouse nonspecific IgG did not immunoprecipitate \( \beta \)-catenin (first lane), and lysate levels of \( \beta \)-catenin and myc-Cdc42 constructs were comparable (second and third lanes, Figure 4B).

**Cdc42-Dependent Increase in Lung Vascular Permeability Following \( \alpha \)EXD Expression**

Figure 5A shows the gravimetric analysis of lungs obtained from mice expressing \( \alpha \)EXD or \( \alpha \)EXD\( \Delta \beta \) mutant in endothelia 24 hours postinjection of construct. The \( \Delta \)EXD-expressing lungs became markedly edematous in contrast to control vector-transfected lungs (\( P<0.05 \)). \( \Delta \)EXD\( \Delta \beta \)-expressing lungs were less edematous than \( \Delta \)EXD-expressing lungs at 70 to 90 minutes after initiation of perfusion (\( \alpha \)P<0.05, \( n=4 \)). B, Coexpression of N17Cdc42, in contrast to N19RhoA, reduced edema formation in \( \Delta \)EXD-expressing lungs between 80 and 90 minutes (\( P<0.05 \)). Coexpression of N17Rac1 also failed to inhibit pulmonary edema at these time points (data not shown). C, Analysis of lung wet weight gain from A and B during the 60- to 90-minute perfusion period. Lungs transfected with \( \Delta \)EXD and \( \Delta \)EXD+\( \alpha \)EXD\( \Delta \beta \) showed differences in rate of weight increase compared with \( \Delta \)EXD and \( \Delta \)EXD+\( \alpha \)EXD\( \Delta \beta \) groups (\( **P<0.05 \)). All values were different from control vector group (\( P<0.05 \)). D, Lung microvessel filtration coefficient (\( K_{f,c} \)). Expression of \( \Delta \)EXD and \( \Delta \)EXD\( \Delta \beta \) increased \( K_{f,c} \), compared with control lungs (\( P<0.05 \); \( n=4 \) in each group). The increase in \( K_{f,c} \) was less in \( \Delta \)EXD\( \Delta \beta \)-expressing lungs than in \( \Delta \)EXD-expressing lungs (\( **P<0.05 \)). Coexpression of N17Cdc42, in contrast to N19RhoA or N17Rac1 (\( \alpha \)P<0.05), reduced the increase in \( K_{f,c} \) seen in \( \Delta \)EXD-expressing lungs (\( **P<0.05 \); \( n=3 \) in each group). Expression of N17Cdc42 alone did not increase \( K_{f,c} \). All values are reported as mean±SEM.

**Discussion**

We performed these studies to address the effects of destabilizing the VE-cadherin homotypic interaction by the in vivo
expression of the VE-cadherin cytoplasmic domain on lung vascular permeability and to determine the role of Rho GTPases in mediating the alterations in permeability. We showed that destabilization of VE-cadherin junctions induced by the expression of the VE-cadherin mutant, ΔEXD, increased pulmonary vascular endothelial permeability in mice. Studies in confluent endothelial cells showed that expression of ΔEXD resulted in the formation of intercellular gaps, indicating that the ΔEXD competitively disrupted the normal VE-cadherin homotypic interactions, and thus increased vascular permeability. Previous studies have shown that cadherin proteins display a strong homophilic interaction in the presence of catenins bound to the cytoplasmic domain of cadherin.30 We show herein that the ΔEXD-expressing cells disrupted normal AJ assembly because of the redistribution of α- and β-catenins away from the endogenous VE-cadherins localized at the AJs. This notion is supported by the finding that the expression of ΔEXD promoted its association with α- and β-catenins, whereas these effects were not observed after transfection of the ΔEXDΔβ mutant lacking the β-catenin–binding domain.

Our mouse lung studies were performed using cationic liposomes to transduce the expression of the ΔEXD mutant in lung vascular endothelial cells in vivo. We have shown that proteins are encoded in lung endothelial cells following the IV injection of the liposome–DNA complex.25,31 Using endothelial-rich fractions and endothelial cells obtained by collagenase digestion of mouse lungs, we demonstrated the expression of the FLAG-tagged ΔEXD construct in endothelial cells. The expression of the ΔEXD mutant was maximal at 24 hour after the liposome–DNA construct injection when all of the physiological measurements were made.

In studies using confluent endothelial cells, we observed that ΔEXD colocalized with β-catenin at the plasma membrane. Expression of ΔEXD caused widespread formation of intercellular gaps and filopodia, whereas these changes did not occur with the expression of ΔEXDΔβ. The impairment of normal VE-cadherin/catenin interactions induced by ΔEXD likely interfered with AJ formation because of the loss of connection to the actin cytoskeleton through the β- and α-catenins.32 We observed a marked shift of β-catenin from native VE-cadherin to the expressed ΔEXD mutant in support of this concept. In contrast, the expression of ΔEXDΔβ had a less pronounced effect on lung vascular permeability compared with ΔEXD. We noted that the transient transfection of ΔEXD in the present study did not reduce endogenous VE-cadherin levels, an effect seen previously in studies using adenoviral transfection of a VE-cadherin mutant similar to ΔEXD.16 On stable transfection of ΔEXD in HMEC-1 using a retroviral vector; however, we have also observed a decrease in endogenous VE-cadherin expression (data not shown). Taken together, these findings support the hypothesis that translocation of β-catenin from endogenous VE-cadherin to the expressed ΔEXD mutant resulted in junctional instability and increased vascular permeability.

We have shown that expression of the ΔEXD mutant in endothelial cells induces the specific activation of Cdc42 as compared with RhoA and Rac1;22 thus, we addressed the possibility that Cdc42 may be involved in regulating AJ disassembly induced by ΔEXD expression. These experiments were made by cotransfecting dominant-negative mutants of Cdc42, RhoA, and Rac1 along with ΔEXD. Our goal in using these constructs was to minimize the potential nonspecific effects of Rho GTPase-modifying reagents33 and ensure the simultaneous delivery of both VE-cadherin and Rho GTPase constructs to assess protein–protein interactions and the role of Rho GTPases in mediating AJ disassembly. We identified an important role of Cdc42 in regulating the association of α-catenin with β-catenin following the binding of β-catenin to the expressed ΔEXD. Strikingly, the coexpression of dnCdc42 (N17Cdc42) prevented the association of α-catenin with the ΔEXD–β-catenin complex, whereas dnRhoA or dnRac had no effect. In a study involving the immunoprecipitation of endothelial cell lysates with β-catenin mAb followed by immunoblotting with anti–α-catenin antibody, we observed that ΔEXD expression induced the association of α- with β-catenin, whereas this did not occur on expression of ΔEXDΔβ. The coexpression of dnCdc42 with ΔEXD prevented the association of α-catenin with β-catenin; in contrast, coexpression of dnRhoA or dnRac had no effect on this association. Our findings demonstrate the key role of Cdc42 in regulating the binding of α-catenin to ΔEXD–β-catenin complex without altering the interaction of β-catenin to ΔEXD. Thus, the mechanism of AJ instability induced by the Cdc42-mediated α-catenin association with the ΔEXD–β-catenin complex involves a shift of actin filaments from the endogenous VE-cadherin/catenin complex to the expressed ΔEXD.

The mutant ΔEXDΔβ served as a useful control for ΔEXD expression in these studies because it did not significantly disrupt AJs in endothelial cells. Additionally, the level of Cdc42 activation following ΔEXDΔβ expression was significantly lower than ΔEXD. We observed that ΔEXDΔβ expression in the mouse lung increased vascular permeability, although the response was significantly attenuated compared with ΔEXD. The basis of this diminished but persistent effect of ΔEXDΔβ in increasing endothelial permeability is not clear, but it may be attributed to the reported interaction of ΔEXDΔβ with p120 catenin,23,34 which could result in actin cytoskeletal rearrangement at the level of AJs.32,35 and promote AJ alterations, albeit to a lesser degree than ΔEXD.

We observed that the increase in vascular permeability following expression of ΔEXD in mouse lung endothelial cells was attributed to Cdc42. Gravimetric analysis of lungs obtained from mice expressing the VE-cadherin mutants in lung endothelia showed that the ΔEXD-expressing lungs became markedly edematous and coexpression of dnCdc42 significantly reduced both edema formation and increase in \( K_e \) in these lungs. However, expression of dnCdc42 did not completely inhibit these effects of ΔEXD, suggesting there may also be a Cdc42-independent effect on AJs. Another explanation for the incomplete inhibition of permeability is that dnCdc42 expression may not have fully blocked Cdc42 activity.

Although our results show that Cdc42 is involved in the mechanism of increased vascular permeability induced by the loss of homotypic VE-cadherin interactions, we cannot rule out the possibility that Cdc42 has additional other regulatory
effects on endothelial barrier function. Our previous studies have shown that the reannealing of endothelial AJ's occurring 1 to 2 hours following the thrombin-induced increase in permeability depends on Cdc42 activation.23 These dual actions of Cdc42 (ie, promoting the binding of α-catenin to β-catenin shown in the present study and previously described reannealing of AJ's)33,36 suggest that Cdc42 activation regulates both AJ disassembly as well as reassembly. This concept is consistent with the versatility of Rho GTPases as effectors,7,38 thus, the Cdc42-GTP “switch” may integrate both events depending on its spatial and temporal activation in the endothelial cell membrane. An important pathophysiologic implication of our studies is that the Cdc42-mediated binding of α-catenin with β-catenin may serve to dampen the increase in endothelial permeability in response to inflammatory mediators.

References

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Circ Res. published online December 1, 2005;

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Materials and Methods

**Immunofluorescence and Confocal Microscopy** Cells were fixed in 3.7% Formaldehyde/PBS, permeabilized, exposed to primary antibodies [anti-FLAG (rabbit, Sigma), anti-myc (9E10, Santa Cruz Biotech, Santa Cruz, CA), anti-VE-cadherin (C19, Santa Cruz)] at 4ºC for 1 hr. Cells were then washed, blocked for 30 min, and exposed to Alexa-Fluor 488 and 594 secondary antibodies (Molecular Probes, Eugene, OR) in blocking buffer for 1 hr, washed and mounted. Confocal microscopy was carried out using the Zeiss LSM 510 microscope. DAPI staining was visualized through non-confocal excitation using an Hg-lamp. Fluorescence emission was detected separately for each fluorophore using a multi-track configuration.

**Immunoprecipitation and Western Blotting** Cell lysates were re-suspended in IP buffers (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1% NP-40, 0.1% SDS) and equal protein (300 µg) were subjected to IP. Lysates were pre-cleared using Protein A/G agarose beads (Santa Cruz) for 1 hr at 4ºC. Lysates were immunoprecipitated with antibodies [anti-FLAG (M2, mouse, Sigma), anti-β-catenin (mouse, BD Biosciences), anti-VE-cadherin (C19, Santa Cruz)], and Protein A/G agarose beads for 3 hr at 4ºC, followed by centrifugation at 3000xg at 4ºC and 3x washing with ice-cold IP Buffer. Remaining bead fractions were analyzed by Western blotting. Primary antibodies were anti-FLAG Rabbit (Sigma), anti-VE-cadherin (C-19, Santa Cruz), anti-Cdc42 (Cytoskeleton), anti-α-catenin (H-297, Santa Cruz), anti-myc (9E10 and A-14, Santa Cruz) and anti-β-catenin (C18, Santa Cruz). Endothelial-rich lung
lysates were also analyzed by Western blotting using above antibodies. Western membranes were blocked using 3% nonfat dry milk in TBS containing 0.3% Tween-20.

**Electroporation** HMEC-1 and HPAEC were electroporated using 180mV voltage and 0.950mF capacitance (Gene Pulser II, Bio-Rad, Hercules, CA). Cells (1 x 10^6) were seeded in a 100 mm² dish the day before transfection. After 20-24 hr, cells were trypsinized for 5 min and re-suspended in EGM-2 medium containing plasmid DNA (12µg) mixed with 14 µg salmon sperm carrier DNA (Stratagene) in 0.4 cm cuvettes (Gene Pulser II, Biorad). Cells were allowed to proliferate for 24 hr, and lysed or fixed as described¹.
Online Figure 1

Confocal microscopy using anti-FLAG and anti-β-catenin antibodies in ∆EXD (upper panel) and ∆EXDΔβ (lower panel) -transfected confluent HPAEC monolayers at 24 hr post-transfection. β-catenin was localized at the plasma membrane. Expression of ∆EXD caused widespread formation of inter-cellular gaps and filopodia (arrows, upper panel), this did not occur with expression of ∆EXDΔβ (lower panel). Also ∆EXD co-localized with β-catenin (yellow in merged image, upper panel), and this did not occur to the same degree following expression of ∆EXDΔβ (lower panel). Results are representative of 2 experiments (scale =10 µM).
References