VE-cadherin-induced Cdc42 Signaling Regulates Formation of Membrane Protrusions in Endothelial Cells*

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The cytoplasmic domain of cadherins and the associated catenins link the cytoskeleton with signal transduction pathways. To study the signaling function of non-junctional VE-cadherin, which can form during the loss VE-cadherin homotypic adhesion, wild type VE-cadherin or VE-cadherin cytoplasmic domain (ΔEXD) was expressed in sub-confluent endothelial cells. We observed that Cdc42 was activated in transfected cells and that these cells also developed Cdc42-dependent >70-μm-long plasma membrane protrusions. The formation of these structures required actin polymerization, and they developed specifically in endothelial cells as compared with epithelial cells. Expression of the VE-cadherin cytoplasmic domain lacking the β-catenin binding site also induced Cdc42 activation; thus, its activation cannot be ascribed to β-catenin binding. However, these cells were not able to form the protrusions. These results suggest that the cytoplasmic domain of non-junctional VE-cadherin can serve as a scaffold involved in Cdc42 activation at the endothelial plasma membrane. β-Catenin and the associated α-catenin may serve as support sites for actin polymerization, leading to formation of long plasma membrane protrusions. Thus, non-junctional VE-cadherin actively participates in inside-out signaling at the plasma membrane, leading to the development of endothelial membrane protrusions.

Cadherin molecules are membrane receptors involved in cell-cell adhesion (1, 2). They form a large family of proteins with tissue specificity that associates in a homophilic manner. They are indispensable in tissue formation during development, and in addition to adhesion, they serve morphogenetic functions (3–6). Classical cadherins are anchored to actin cytoskeleton by α-catenin, which binds to β-catenin (7). Catenins have multiple functions in signal transduction and transcription regulation (8) and, thus, they are directly involved in the transduction of proliferation signals (6, 9, 10).

tk;2Endothelium forms a semi-permeable barrier that regulates the flux of liquid and solutes (11, 12) as well as leukocyte transmigration from the microvasculature to sites of injury and infection (13–15). Adherens junctions formed between endothelial cells contain VE-cadherin, which is specifically expressed in the endothelium (16). Direct involvement of VE-cadherin to endothelial barrier function was demonstrated by VE-cadherin blocking antibodies in cell-culture and mouse models (17). In a

different situation, VE-cadherin/catenins reorganize during polymorphonuclear or monocyte transmigration through the endothelial monolayer (18). VE-cadherin targeting revealed that in addition to its role in cell-cell adhesion, it is required for transduction of the vascular endothelial growth factor proliferation pathway through β-catenin association to phosphatidylinositol 3-kinase, which is downstream of vascular endothelial growth factor receptor 2. Ablation of VE-cadherin interferes with endothelial proliferation and causes apoptosis (6). However, it is remarkable that the VE-cadherin function was not compensated despite the presence of N-cadherin, which also associates with β-catenin and is expressed in endothelial cells. Thus, it is possible that the cytoplasmic domain of VE-cadherin serves functions that are not completely understood. Although VE-cadherin is classified as a "classical" cadherin in respect to its primary structure, the cytoplasmic tail is unique among the members of this subgroup (19). To study the role of VE-cadherin in signaling mechanisms in endothelial cells, wild type VE-cadherin or the cytoplasmic domain of VE-cadherin mutant was expressed in sub-confluent endothelial cells. The cytoplasmic domain of cadherins has been characterized in a variety of studies as the "dominant negative mutant" for cell-cell adhesion, since its expression blocked cell adhesion in cells of epithelial, neuronal, muscle, and endothelial origin (20–24).[1] In endothelial cells a chimera between interleukin 2 receptor extracellular domain and VE-cadherin cytoplasmic domain interfered with proliferation by titrating-out β-catenin from its downstream effectors, and its stable expression in tissue culture cells led to down-regulation of endogenous VE-cadherin and defects in proliferation (25).

In the present study, we observed that non-junctional VE-cadherin induced through its cytoplasmic domain the activation of Cdc42 and the formation of >70-μm-long plasma membrane protrusions. Cdc42 activation was shown to be necessary for the formation of these protrusions. We further demonstrated that Cdc42 activation was not dependent upon β-catenin association to VE-cadherin. Two critical factors were required for the formation of the protrusions, (*a*) membrane localization of VE-cadherin and (*b*) association of β-catenin with its cytoplasmic domain. Furthermore, the formation of membrane protrusions was endothelial-specific since it did not occur in epithelial cells. These results suggest a novel role of VE-cadherin cytoplasmic domain in the formation of membrane protrusions, which may be involved in the restoration of endothelial junctional barrier function after the loss of homotypic VE-cadherin adhesion.

MATERIALS AND METHODS

Cell Culture—Human microvascular endothelial cells (HMECs)[2] were gifts from Dr. E. W. Ades (26) (NIAID, National Institutes of

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[1] P. Kouklis and A. B. Malik, unpublished data.

[2] The abbreviations used are: HMEC, human microvascular endothelial cell; wt, wild type; GST, glutathione *S*-transferase.

Health, Atlanta, GA). HMECs were cultured in MCDB 131 (Invitrogen) supplemented with 5% fetal bovine serum (Hyclone Laboratories, Logan, UT). Primary human pulmonary arterial endothelial cells were cultured in EBM 2 (endothelial basal medium) complete medium (Bio-Whittaker, Walkerville, MD). Bovine lung microvascular endothelial cells, purchased were from VEC Technologies (Renssalaer, NY), were grown in MCDB 131 medium supplemented with 5% fetal bovine serum and endothelial cell growth supplement (Sigma-Aldrich). Alveolar epithelial cells A549 were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum.

Antibodies—A monoclonal antibody against VE-cadherin was from Chemicon (Temecula, CA), and a goat polyclonal antibody was from Research Diagnostics (Flanders, NJ). Rabbit polyclonal anti-FLAG and anti-Myc-tag monoclonal were from Zymed Laboratories Inc. (San Franscisco, CA). Mouse monoclonal anti-FLAG (M2) was from Sigma-Aldrich. Monoclonal antibodies for β-catenin, p120, and Rac were from Transduction Laboratories (Lexington, KY). Rabbit anti-Cdc42 was from Santa Cruz (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies were from Jackson Laboratories (West Grove, PA). For immunofluorescence experiments, we used either fluorescein isothiocyanate and Texas Red (Jackson Laboratories) or Alexa 488 and Alexa 568 (Molecular Probes, Eugene, OR)-conjugated antibodies. Actin was visualized with Alexa 488-conjugated phalloidin (Molecular Probes).

Drugs—Latrunculin A was purchased from Molecular Probes.

VE-cadherin Constructs and Transfections—VE-cadherin-ΔEXD mutants with and without FLAG tag (DYKDDDDK) were constructed by PCR (plasmid pcDNA3-VEC-ΔEXD). The octapeptide sequence encoding for the FLAG-tag sequence replaced the VE-cadherin extracellular domain by PCR. The human wt VE-cadherin cDNA inserted in the *Eco*RI site of the pcDNA3 vector was used (plasmid pcDNA3-VEC) as the template. We used either primer D-FL containing the FLAG encoding sequence as sense primers (GAGTCGCAAGAATGCCGACTA-CAAGGACGACGATGACAAGACCTTCTGCGAGGATATGG) or primer D that lacks the FLAG encoding sequence (GTGAGTCGCAAGAATGC-CACCTTCTGCGAGGATATGG). Antisense primer 2002 (ACCTTCT-GCGAGGATATGG) was used in both cases. PCR products were digested with *Bsm*I/*Bst*EII, and these fragments were inserted in the *Bsm*I (partial)/*Bst*EII site of pcDNA3-VEC. The mutant version with FLAG (termed ΔEXD) was used generally except where indicated ΔEXD without FLAG.

To construct VE-cadherin-ΔEXD-Δβcat mutant, the plasmid pcDNA3-VEC-ΔEXD was digested with *Hin*dIII and *Bpu*10I (partial digestion and Klenow-blunt). This fragment was inserted in the *Hin*dIII/*Eco*RV site of pcDNA3 (plasmid pcDNA3-VEC-ΔEXD-Δβcat). To make the VE-cadherin-ΔEXD-Δp120 construct, residues 645–654 were deleted by PCR. We used as the sense primer TGGTCACCATGGACAC-CACCAGCTACGATG. To make the VE-cadherin-ΔEXD-G/A construct we substituted residues GGG (649–651) with AAA by PCR using as sense primer TGGTCACCTACGACGAGGAGGCAGCAGCAGAGATG. For both PCR reactions, we used as the antisense primer TACAGCT-CAGCCAGCATCTTAAAC. PCR products containing the deletion or substitution were cloned in the TA cloning vector (Invitrogen) and digested with *Bst*EII/*Blp*I fragments to replace the wild type *Bst*EII/*Blp*I sequence from the VE-cadherin-ΔEXD construct. All PCR products were verified by sequencing.

Transfection—Endothelial cells were transfected by electroporation or liposome-mediated DNA transfer (Qiagen, Valencia, CA). For electroporation, 10^6 cells were seeded in a 100-mm² dish the day before transfection. After 20–24 h, cells were trypsinized and re-suspended in 300 μl of medium. Plasmid DNA (10 μg) mixed with 14 μg of salmon sperm DNA was added, and cells were electroporated in 0.4-cm cuvettes (Gene Pulser II, Bio-Rad). The conditions were set at 180-mV voltage and 950-millifarad capacitance. To obtain confluent monolayers, 50 μl of transfected cells were applied directly on a gelatinized coverslip and left for 1 h to adhere. Medium was added, and cells were fixed after 48 h. We typically observed 30% transfection efficiency with liposomes and 40% efficiency with electroporation.

Immunofluorescence Microscopy—Cells were grown on 1% gelatin (Sigma-Aldrich)-coated coverslips. They were fixed and stained as described previously (Kouklis *et al.* (29)). The stained cells were visualized either with Nikon Diaphot 200 or Zeiss Pascal confocal microscopes.

Protein Assay—Protein concentrations of cell extracts were quantified using the BCA protein assay from Pierce.

Immunoprecipitation and Western Blotting—Endothelial cells were washed, scraped with phosphate-buffered saline, and pelleted in 3×10^3 rpm. Total cell extracts were made using immunoprecipitation buffer containing 20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 0.5% Nonidet P-40 supplemented with Complete protease inhibitor mixture (Roche Molecular Biochemicals). Cells were incubated for 10 min in 4 °C in a shaker and centrifuged in 14×10^3 rpm for 5 min to remove insoluble material. The supernatant was used for immunoprecipitation and Western-blotting experiments (thereafter called "extract"). Extracts were pre-cleared with 2 μg of mouse IgG (Jackson Laboratories) and incubated with 2–3 μg of the first antibody for 2 h in 4 °C before the addition of 20 μl of protein A/G-agarose (incubation for 1 h in 4 °C). Immunoprecipitates were subjected to SDS-PAGE and Western blot analysis. Nitrocellulose membranes were blocked with TBST buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 1% gelatin (from cold water skin fish, Sigma-Aldrich), incubated for 1 h with primary antibodies, washed with TBST, and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies. For anti-Cdc42 antibodies, membranes were blocked with TBST containing 5% nonfat milk and incubated at room temperature for 3 h. The horseradish peroxidase signal was developed using Super Signal (Pierce).

p21-activated Kinase Binding Assay—BL21 bacterial cultures (20 ml) transformed with GST-PBD expression construct (a gift from Dr. G. Bokoch, Scripps) were induced with 2 mM isopropyl-1-thio-β-D-galacto-pyranoside for 3 h, lysed with 0.2 mg/ml lysozyme, and extracted in 1% Triton X-100, phosphate-buffered saline with protease inhibitors (Complete, Roche Molecular Biochemicals). The supernatant was incubated for 45 min at room temperature with 120 μl of glutathione-Sepharose beads (Amersham Biosciences). The beads were washed 3× with phosphate-buffered saline and 2× with MLB buffer (25 mM HEPES, pH 7.5, 250 mM NaCl, 5% IGEPAL CA-630, 10 mM MgCl₂, 1 mM EDTA, 2% glycerol, and protease inhibitors). GST-bound beads (20 μl) were incubated with HMEC extracts (200–300 μg of total protein) in 400 μl of MLB buffer O/N at 4 °C. The beads were washed thoroughly with MLB buffer and boiled in 50 μl of SDS-PAGE loading buffer for subsequent Western blot analysis.

RESULTS

Expression of VE-cadherin Cytoplasmic Domain or wt VE-cadherin Induces Cdc42 Activation—Human VE-cadherin cDNA (a gift of Dr. E. Dejana, Mario Negri Institute, Milan, Italy) was used to generate the VE-cadherin extracellular domain deletion mutant (ΔEXD) (Fig. 1A). Sequences encoding the signal peptide (*SP*), pre-peptide (*PP*), and the transmembrane domain (*TM*) were not deleted so that the mutant could be inserted in membrane. We also constructed mutants identical to ΔEXD with the exception that the residues involved in the binding of β-catenin (ΔEXD-Δβcat) or p120 (ΔEXD-G/A and ΔEXD-Δp120) were deleted (Fig. 1A). In ΔEXD-Δβcat, residues 743–784 were deleted including SLSS, a highly conserved motif in all cadherin molecules involved in association of β-catenin with cadherins (27) (Fig. 1A). Substitution of GGG residues to AAA (AA 649–651) in ΔEXD-G/A and deletion of residues 645–654 in ΔEXD-Δp120 at the juxtamembrane domain removed the p120 binding site (28) (Fig. 1A). To determine expression and correct size of the mutants, HMEC were transfected with ΔEXD, ΔEXD-G/A, ΔEXD-Δp120, or ΔEXD-Δβcat, and Triton X-100 extracts from transfected cells were analyzed by Western blotting with anti-FLAG monoclonal antibody. This antibody reacted specifically with 34–36-kDa bands in the ΔEXD-, ΔEXD-Δp120-, and ΔEXD-G/A-transfected cells and a 29-kDa band in ΔEXD-Δβcat-transfected cells (Fig. 1B). No reaction was detected in mock-transfected control cells (Fig. 1B).

HMEC were transiently transfected with wt VE-cadherin, ΔEXD, or ΔEXD-Δβcat mutants. Cells were harvested 24 h after transfection, and extracts were tested for Cdc42 activation using the GST-PBD pull-down assay. HMEC cell extracts were incubated with GST-PBD coupled to glutathione-Sepharose beads. Beads were washed and analyzed by Western blotting for Cdc42 binding. We observed that active Cdc42 was bound to PBD in extracts from HMECs transfected with wt VE-cadherin, -ΔEXD, or -ΔEXD-Δβcat (Fig. 2). Cdc42 activation remained at the basal level in mock-transfected cells (Fig. 2, *GST-PBD*). In control experiments, GST beads did not asso-

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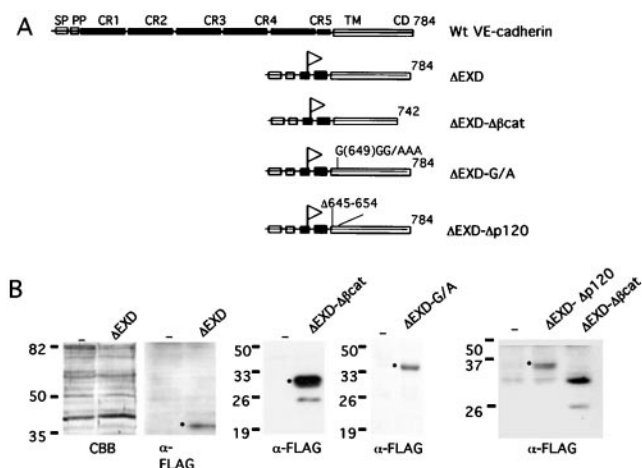
FIG. 1. **Construction of VE-cadherin mutants and their expression in endothelial cells.** *A*, ΔEXD, extracellular domain of VE-cadherin was deleted, and the sequence encoding for the FLAG epitope was inserted in its position. *ΔEXD-Δβcat*, 42 C-terminal residues of VE-cadherin, which includes the β-catenin binding domain, were removed from the ΔEXD construct. *ΔEXD-G/A*, AAA (residues 649–651) were replaced with GGG in the ΔEXD construct. *ΔEXD-Δp120*, residues 645–654 were removed from the ΔEXD construct. VE-cadherin domains: *SP*, signal peptide sequence; *PP*, pre-peptide sequence; *CR*, cadherin repeat; *TM*, transmembrane domain; *CD*, cytoplasmic domain. *B*, immunoblots of protein extracts from HMEC transfected with VEC-ΔEXD, VEC-ΔEXD-Δβcat, VEC-ΔEXD-G/A, and VEC-ΔEXD-Δp120. At 24 h after transfection, HMEC were extracted using 1% TX-100 in phosphate-buffered saline buffer, and the soluble fraction was separated by SDS-PAGE. Polyacrylamide gels were either stained with Coomassie Brilliant Blue (*CBB*) or transferred on nitrocellulose for immunoblotting analysis using a FLAG antibody. Extracts from control (−) and ΔEXD-, ΔEXD-Δβcat (Δβcat)-, ΔEXD-G/A-, and ΔEXD–Δp120-transfected HMECs were analyzed. In these extracts 34–36-kDa bands in ΔEXD-, ΔEXD-G/A-, and ΔEXD-Δp120- and ~29-kDa bands in ΔEXD-Δβcat-transfected cells reacted specifically with anti-FLAG antibody.
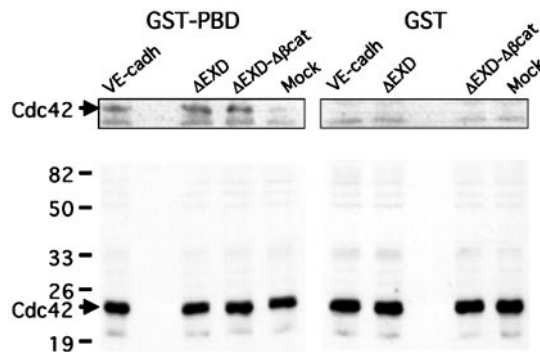
FIG. 2. **Cdc42 activation in HMECs transfected with wt VE-cadherin or VE-cadherin cytoplasmic domain mutants.** GST-PBD or GST control beads were incubated with extracts from subconfluent HMEC transfected with VE-cadherin, VE-cadherin-ΔEXD, or VE-cadherin-ΔEXD-Δβcat as well as mock-transfected cells. Complexes were separated in PAGE and immunoblotted with anti-Cdc42 antibodies. GST-PBD beads precipitate Cdc42 in extracts from VE-cadherin, ΔEXD, and ΔEXD-Δβcat but not from mock-transfected cells. Control GST beads showed specificity of PBD binding to activated Cdc42. Immunoblots of corresponding extracts were analyzed by anti-Cdc42 to ensure equal amounts of proteins in the extracts (*lower panel*). The experiments were repeated three times with similar results.

ciate with Cdc42 under the same conditions (Fig. 2, *GST*).

Formation of Membrane Protrusions in Endothelial Cells Expressing VE-cadherin Cytoplasmic Domain (ΔEXD) or wt VE-cadherin—At 18–24 h post-transfection of HMECs, the expressed proteins localized at the plasma membrane and the cytoplasm in vesicular structures. We observed that an unusually high percentage of transfected endothelial cells developed
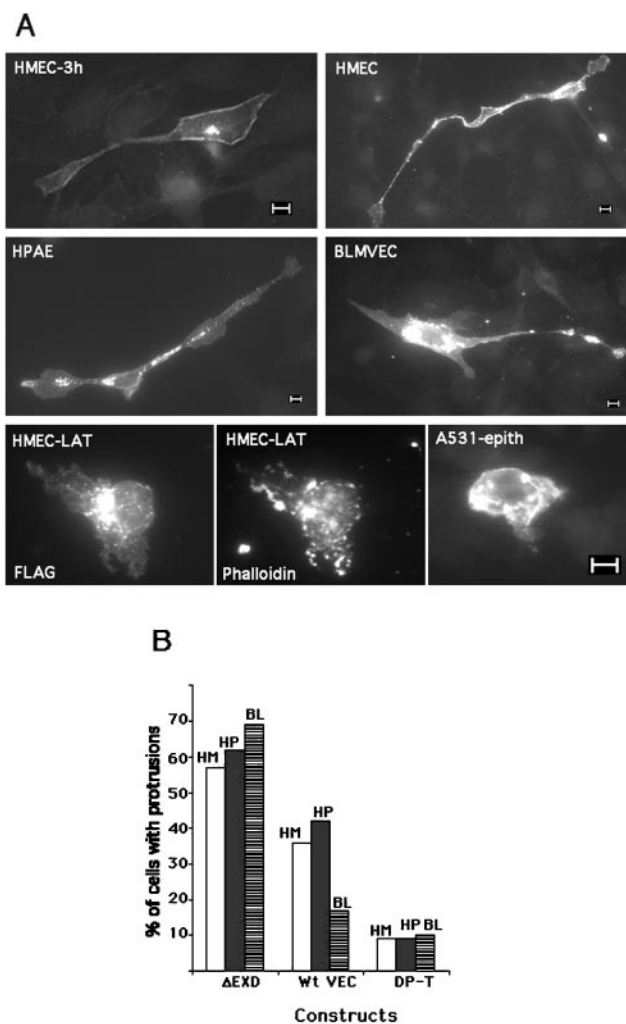
FIG. 3. **Expression of VE-cadherin cytoplasmic domain induces membrane protrusions in endothelial cells.** *A*, endothelial (HMECs, bovine lung microvascular endothelial cells (*BLMVEC*), human pulmonary arterial endothelial cells (*HPAE*)) and epithelial (A531) cells were transfected with VE-cadherin ΔEXD mutant, fixed at 18–24 h post-transfection (except *top left panel*, where they were fixed at 3 h) and stained with anti-FLAG. Endothelial cells displayed morphological changes in response to expression of ΔEXD mutant. Note that protrusions are evident just 3 h post-transfection, when the amount of expressed protein is barely above background level. Under the same conditions transfected epithelial cells showed no such changes. ΔEXD-transfected HMEC treated with 200 nM latrunculin (*LAT*) failed to form membrane protrusions. Cells were double-stained with anti-FLAG and phalloidin. Epithelial (*epith*) and latrunculin-treated endothelial cells are the same magnification. The *bar* represents 10 μm. Experiments were repeated 3–8 times with similar results. *B*, percentage of endothelial cells forming membrane protrusions after expression of VE-cadherin (*wt VEC*) or VE-cadherin cytoplasmic domain (*ΔEXD*). Three different endothelial cell types (HMECs (*HM*), human pulmonary (*HP*) arterial endothelial cells, and bovine lung (*BL*) microvascular endothelial cells) were transfected with plasmids expressing ΔEXD, wt VE-cadherin (*wt VEC*), and desmoplakin tail domain (*DP-T*) as the negative control. Between 50–80 transfected cells per experiment were captured on-screen, and the size of the membrane protrusions was measured for each individual transfected cell. Cells with membrane protrusions greater than 70 μm (3 times longer than average cell body) were scored as positive. *Numbers* represent the results of 3–8 transfection experiments.

very long extensions with a striking cell shape change (Fig. 3*A*). These extensions typically showed branching patterns. All cells from randomly selected fields (a total of 50–100 cells/experiment) were photographed to measure protrusion lengths. Cells with extensions greater than 70 μm were scored as positive. Long extensions were evident in ~60% of cells transfected with

ΔEXD and ~30% of cells transfected with wild type VE-cadherin (Fig. 3*B*). As controls, we transfected HMEC with the cytoplasmic domain of another endothelial adherens junction protein, desmoplakin, known to associate with intermediate filaments (29); only 9% of these cells showed protrusions. Moreover, only 4% of mock-transfected HMEC showed such protrusions (data not shown). To rule out the possibility that cell shape changes were the result of a transfection artifact, we determined the earliest point that the protrusions were seen in relation to ΔEXD expression. Protrusions were observed as early as 3 h post-transfection at a time when expression of transfected ΔEXD was barely detectable (Fig. 3*A*). Mutant molecules localized at the plasma membrane, and protrusions reached maximum length within 6 h (as in Fig. 7*C*). We also expressed ΔEXD in HMEC using retrovirus infection with identical results (data not shown), indicating the development of the protrusion was not secondary to the method used.

To extend these observations to other endothelial cells, we repeated the same transfection experiments in primary endothelial cells, human pulmonary arterial endothelial cells, and BMVEC (Fig. 3*A*); in both cases, membrane extensions formed 24 h after transfection of wt VE-cadherin and -ΔEXD. Endothelial cells transfected with ΔEXD mutant were treated with the actin polymerization inhibitor, latrunculin A (30), and cells were fixed 24 h post-transfection. Latrunculin A in all cases prevented the formation of membrane protrusions (Fig. 3*A*), indicating the requirement of actin polymerization in the response.

We also transfected alveolar epithelial cells (A531, a type II alveolar epithelial cell line) with ΔEXD to study protrusion formation in non-endothelial cells. No cell shape change or membrane extensions were observed in these cells (Fig. 3*A*), demonstrating the importance of cellular background in the mechanism of the response.

Membrane Protrusions in Endothelial Cells Expressing VE-cadherin Cytoplasmic Domain Require Cdc42—In HMEC co-transfected with dominant-negative mutant N17Cdc42 and ΔEXD mutant we observed that co-expression of N17Cdc42 inhibited the formation of protrusions (Fig. 4). We also co-transfected HMECs with dominant-negative mutants of Rac1 (N17Rac) or RhoA (N14RhoA) and the ΔEXD mutant. N17Rac co-expression resulted in shorter and thinner protrusions in contrast to the inhibition seen with Cdc42 dominant negative mutant (Fig. 4). N14RhoA co-expression had no effect on the ΔEXD-induced protrusion formation (Fig. 4).

To quantify the degree of inhibition caused by co-expression of dominant negative mutants of Rho, Rac, and Cdc42 along with ΔEXD we measured protrusion lengths in a large number (90–120) of double-transfected cells (Fig. 5). Expression of N17Cdc42 blocked protrusion formation in 100% of double-transfected cells, whereas ~56% of N19Rho-expressing cells developed protrusions, a percentage very similar to ΔEXD-alone transfected cells. N17Rac blocked protrusion formation to a certain degree but not entirely, *i.e.* ~20% of N17Rac-expressing cells formed protrusions of >70 μm. In addition, a significant percentage of double-transfected cells (~15%) formed slightly shorter protrusions (60–70 μm) in these cells.

Differential Roles of β-Catenin and p120 in Mediating Endothelial Cell Shape Change—Expression of wt VE-cadherin and VE-cadherin cytoplasmic domain had profound effects on endothelial cell shape as shown above. Because the cadherin cytoplasmic domain associates with β- and p120-catenins, we investigated the possible roles of these interactions in cell shape change. We generated VE-cadherin cytoplasmic domain mutants ΔEXD-Δβcat lacking the binding site for β-catenin and ΔEXD-G/A and ΔEXD-Δp120 lacking the binding site for
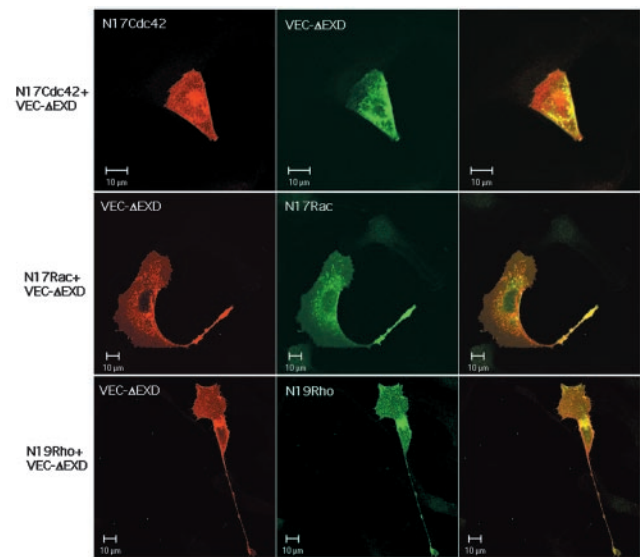

FIG. 4. **Cdc42 dominant negative mutant inhibits protrusion formation induced by VE-cadherin cytoplasmic domain (ΔEXD) in endothelial cells.** HMEC were double-transfected with FLAG-tagged ΔEXD and a plasmid expressing Myc-tagged N17Cdc42 (*upper panel*). Transfected cells were visualized in double immunofluorescence with anti-Myc recognizing N17Cdc42 and anti-FLAG recognizing ΔEXD. VEC-ΔEXD without the FLAG tag was co-transfected with N17Rac (*middle panel*) or N19Rho (*lower panel*), both FLAG-tagged. Transfected cells were double-stained with anti-FLAG recognizing Rho and Rac mutants and anti-VE-cadherin recognizing VE-cadherin cytoplasmic domain. ΔEXD-transfected cells were easily identified due to high expression levels of ΔEXD mutant in comparison to endogenous VE-cadherin. Note that expression of the Cdc42 dominant negative mutant inhibited membrane protrusion formation, whereas the effect of Rac dominant negative mutant was less dramatic; Rho dominant negative mutant had no effect. The experiments were repeated at least six times with similar results.
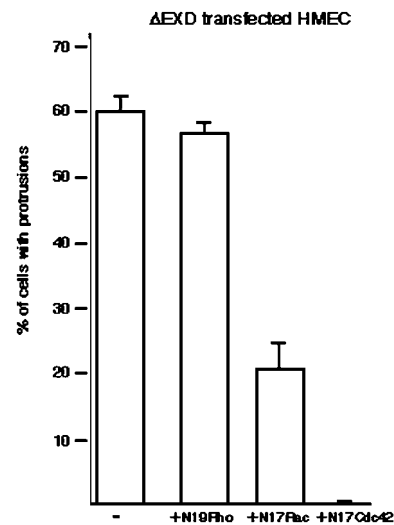
FIG. 5. **Quantification of membrane protrusions formed in HMEC co-expressing ΔEXD and either Rho or Rac or Cdc42 dominant negative mutants.** HMEC were processed as in Fig. 4. All double-transfected cells from randomly selected areas were captured, and the length of the protrusions was calculated using NIH Image 1.63 software. Cells with membrane protrusions >70 μm were scored as positive. The experiments were repeated 3 times, and 30–40 cells were evaluated in each experiment.

p120. wt VE-cadherin, ΔEXD, or ΔEXD-Δβcat were transfected in HMEC, and their association with β-catenin was determined by immunoprecipitation and Western blotting. We showed that β-catenin associated with VE-cadherin and -ΔEXD and did not associate with the ΔEXD-Δβcat mutant (Fig. 6*A*). Also, extracts
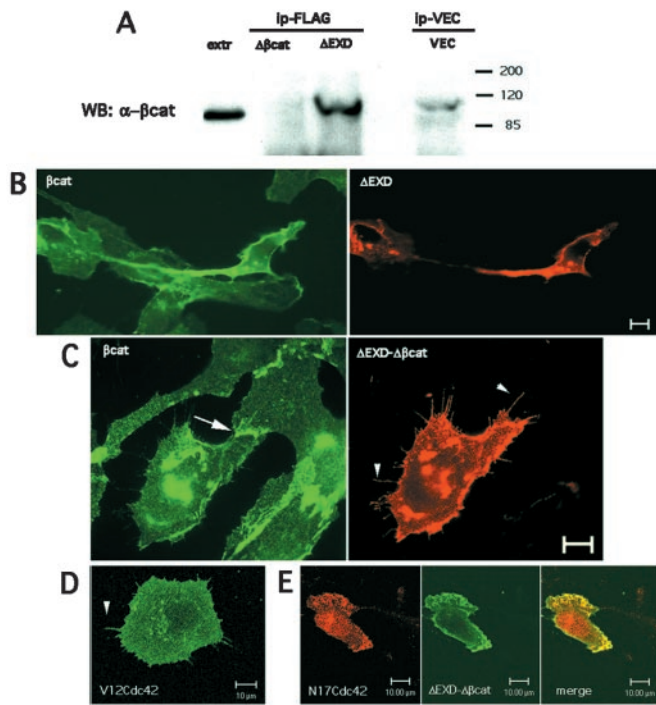
FIG. 6. **Role of β-catenin in formation of endothelial membrane protrusions.** *A*, association of β-catenin with VE-cadherin cytoplasmic mutants. HMECs were transfected with plasmids expressing ΔEXD-Δβcat or ΔEXD (*lanes Δβcat* and *ΔEXD*). Cells were lysed, and the mutants were immunoprecipitated (*ip*) with anti-FLAG monoclonal antibody (M2). Immunoprecipitates were subjected to Western blot (*WB*) analysis using anti-β-catenin antibody. *Lane extr*, HMEC extract; *lane VEC*, mock-transfected HMEC immunoprecipitated with anti-VE-cadherin antibody. Note that ΔEXD-Δβcat did not associate with β-catenin. *B*, HMECs were transfected with ΔEXD mutant, fixed 24 h post-transfection, and double-stained with anti-FLAG and anti-β-catenin antibodies. Transfected cells formed membrane protrusions, and ΔEXD co-localized in the plasma membrane and in vesicular structures with endogenous β-catenin (the *bar* is 10 μm). *C*, HMECs transfected with ΔEXD-Δβcat, fixed 24 h post-transfection, and double-stained with anti-FLAG and anti-β-catenin antibodies. ΔEXD-Δβcat induced the formation of thin filopodia-like structures (*arrowheads*) that were morphologically different from ΔEXD-induced membrane protrusions (compare with ΔEXD-expressing cells). Note the formation of adherens junctions in ΔEXD-Δβcat-expressing cells (*arrow*) (the *bar* is 10 μm). *D*, expression V12Cdc42 mutant in HMEC induced formation of filopodia with a similar morphology as ΔEXD-Δβcat-expressing cells (*arrowhead*). *E*, HMECs were double-transfected with FLAG-tagged ΔEXD-Δβcat and Myc-tagged N17Cdc42. Co-expression of N17Cdc42 blocked cell shape changes in double-transfected cells. All experiments above were repeated at least three times.

from HMEC transfected with ΔEXD, ΔEXD-Δβcat, ΔEXD-G/A, or ΔEXD-Δp120 were immunoprecipitated with anti-FLAG monoclonal antibody, and p120 association with VE-cadherin mutants was determined by Western blot analysis. p120 associated specifically with ΔEXD and ΔEXD-Δβcat but not with either ΔEXD-G/A or ΔEXD-Δp120 mutants (Fig. 7*A*). Double immunofluorescence showed that ΔEXD co-localized with endogenous β-catenin at the plasma membrane and cytoplasmic vesicular structures in transfected cells (Fig. 6*B*). ΔEXD-transfected cells also appeared morphologically different from the ΔEXD-Δβcat-transfected cells. The latter formed random, spike-like projections over the entire cell membrane surface (similar to filopodia) that were clearly thinner than the membrane protrusions induced by the ΔEXD mutant (Fig. 6*C*). Interestingly, the ΔEXD-Δβcat mutant did not block adherens junction formation (Fig. 6*C*, *βcat*; *arrow*).

Because Cdc42 was activated in ΔEXD-Δβcat-transfected endothelial cells (Fig. 2), we compared cell shape changes induced
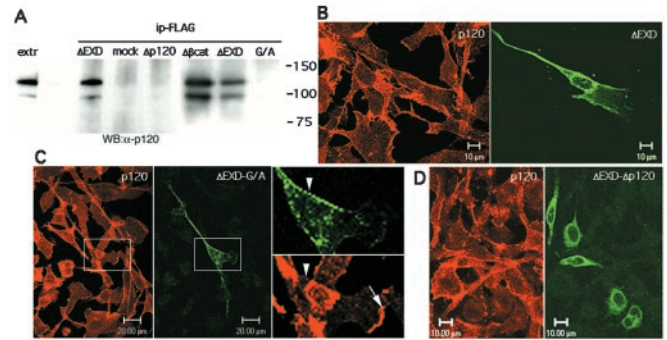
FIG. 7. **Plasma membrane localization of cadherin cytoplasmic domain but not p120 association is required for membrane protrusion formation.** *A*, HMEC were transfected with ΔEXD-Δβcat, ΔEXD, ΔEXD-G/A, or ΔEXD-Δp120 (*lanes Δβcat*, *ΔEXD*, *G/A*, and *Δp120*). Cells were lysed, and the mutants were immunoprecipitated (*ip*) with M2 monoclonal antibody. Immunoprecipitates were subjected to Western blot analysis using anti-p120 antibody. Note that ΔEXD-G/A and ΔEXD-Δp120 did not associate with p120. *B*, HMEC were transfected with ΔEXD, fixed 24 h post-transfection, and double-stained with anti-FLAG and anti-p120 antibodies. *C*, HMECs transfected with ΔEXD-G/A, fixed 6 h post-transfection, and double-stained with anti-FLAG and anti-p120 antibodies. ΔEXD-G/A induced protrusions similar to ΔEXD. *Arrowheads* indicate ΔEXD-G/A, and the *arrow* shows p120 localization at the membrane. *D*, HMECs transfected with ΔEXD-Δp120 fixed after 24 h and double-stained with anti-FLAG and anti-p120 antibodies. ΔEXD-Δp120 localized exclusively in the cytoplasm with no apparent cell shape changes in the transfected cells. All of the above experiments were repeated at least three times.

by expression of ΔEXD-Δβcat and the activated mutant of Cdc42. Transfection of HMEC with the constitutively active Cdc42 mutant (V12Cdc42) induced a large number of filopodia (Fig. 6*D*). Expression of ΔEXD-Δβcat and V12Cdc42 induced similar filopodia structures secondary to the activation of Cdc42. No striking cell shape changes were observed when ΔEXD-Δβcat was co-transfected with N17Cdc42 (Fig. 6*E*), indicating that Cdc42 is involved in filopodia formation induced by ΔEXD-Δβcat. N17Cdc42 was also shown to co-localize with ΔEXD-Δβcat at distinct plasma membrane sites as shown by confocal microscopy (Fig. 6*E*).

Mutant ΔEXD-G/A did not associate with p120 (Fig. 7*A*). To study the localization of ΔEXD-G/A with endogenous p120, cells were fixed at 6 h post-transfection when mutant expression level was low and examined by confocal microscopy. The mutant localized at membrane patches distinct from p120 and cytoplasmic vesicular structures similar to ΔEXD (Fig. 7, *B* and *C*). Importantly, at this time point, ΔEXD-G/A-transfected cells also developed long protrusions similar to those after expression of ΔEXD; thus, formation of these protrusions is independent of p120 binding to the VE-cadherin cytoplasmic domain. Quantification of the effect of ΔEXD-G/A expression in protrusion formation showed that ~55% of ΔEXD-G/A-transfected cells developed protrusions (data not shown).

We generated an additional deletion mutant (ΔEXD-Δp120) of VE-cadherin cytoplasmic domain lacking the entire domain for p120 binding (residues 645–654) (28). We observed that ΔEXD-Δp120 localized exclusively in the cytoplasm and that membrane protrusions did not develop in these cells (Fig. 7*D*). This finding suggests the importance of membrane localization in the mechanism of protrusion formation.

DISCUSSION

Activation of Cdc42 by VE-cadherin Cytoplasmic Domain Signal Formation of Endothelial Membrane Protrusions—We expressed wt VE-cadherin or VE-cadherin cytoplasmic domain mutant in subconfluent endothelial cells. We showed that expression of either of these constructs induced Cdc42 activation and that the expressed cadherins localized at the plasma mem-

brane. These findings suggest that the non-junctional VE-cadherin and especially its cytoplasmic domain was involved in Cdc42 activation. Rho GTPases have been implicated in formation of actin-driven membrane rearrangements (31). Cdc42 in particular is considered as an important GTPase, signaling the formation of filopodia (32, 33). We showed that expression of wt VE-cadherin and VE-cadherin cytoplasmic domain induced the formation of membrane protrusions. Co-expression experiments using dominant negative mutants of Rho, Rac, or Cdc42 along with ΔEXD mutant demonstrated that formation of these protrusions was predominantly the result of Cdc42 activation. It is not clear how the cytoplasmic domain of VE-cadherin activates Cdc42. The guanine exchange factor Tiam1 is localized at the cell-cell junctions of epithelial cells and promotes invasiveness of T lymphoma cells across the epithelial barrier (34). It is possible that a similar guanine exchange factor may be localized at the VE-cadherin cytoplasmic domain that enables activation of Cdc42 in close proximity to the plasma membrane.

To rule out the possibility that formation of membrane protrusions induced by the VE-cadherin cytoplasmic domain is not restricted to a transformed endothelial cell line, we also studied two primary endothelial cells with similar results. Development of long membrane protrusions was clearly evident within 3 h after transfection of the ΔEXD mutant; that is, at a time when mutant expression was barely detected. Thus, it is unlikely that the protrusions were the result of massive overexpression of the transfected mutant. In a control experiment, the expression of desmoplakin, a vimentin binding protein (35), did not affect endothelial cell morphology. In another control experiment expression of VE-cadherin cytoplasmic domain in type II alveolar epithelial cells (A531 cells) also failed to induce membrane protrusions, implying that the effect is endothelial cell-specific. The latter finding is consistent with observations in cultured epithelial cells that expression of E- or N-cadherin ΔEXD mutant failed to induce a cell shape change (23, 36). It is well established that cadherin and associated catenins mediate outside-in signaling (6, 9, 10). In the present study, we show that non-junctional VE-cadherin induces signaling initiated inside endothelial cells, which in turn induces a cell shape change involving formation of membrane protrusion that may restore junctional integrity after the loss of homotypic VE-cadherin adhesion.

Different Roles of β-Catenin and p120 in Induction of Membrane Protrusions in Endothelial Cells—β- and p120-catenins associate directly with cadherins at well conserved cytoplasmic domains (37, 38). Because their association with VE-cadherin may be important in the formation of membrane protrusions, we studied their role by expressing the two VE-cadherin cytoplasmic domain mutants lacking either β-catenin or p120 binding sites. An extended domain of 100 residues in cadherin cytoplasmic domain was identified in mediating association with β-catenin (39), but a short conserved motif, SLSS, is required for this association (27, 39). Expression of a construct (ΔEXD-Δβcat) in which this motif was deleted abolished the binding of this mutant to β-catenin without affecting the p120 association; thus, it is likely that ΔEXD-Δβcat mutant was inserted in the membrane in its proper conformation. We showed that the expression of ΔEXD-Δβcat induced the activation of Cdc42 in endothelial cells, but interestingly, this mutant resulted in the formation of thin, needle-like structures resembling filopodia, similar to those induced by expression of the constitutively active Cdc42 mutant. Therefore, VE-cadherin lacking the β-catenin binding site is unable to induce formation of the characteristic membrane protrusions seen with the expression of cytoplasmic domain of VE-cadherin. It is known

that β-catenin mediates the association of actin cytoskeleton with cadherin through the actin binding proteins α-catenin (38), α-actinin, and vinculin (40); thus, β-catenin plays a crucial role as a linker regulating interaction of cadherin with actin. The differences in cell shape change induced by ΔEXD *versus* ΔEXD-Δβcat can be explained by the absence of β-catenin/α-catenin association in the latter case. Thus actin may fail to bind to VE-cadherin-catenin complex in an appropriate manner to form the characteristic long membrane protrusions.

Because it is possible that p120 may also contribute to the formation of membrane protrusions, we expressed a VE-cadherin mutant that was unable to associate with p120. Substitution of GGG to AAA (residues 649–651) abolished p120 binding to VE-cadherin (mutant ΔEXD-G/A), consistent with studies using E-cadherin (28). Interestingly, the expression of ΔEXD-G/A mutant resulted in a similar phenotype as ΔEXD, indicating that the p120-VE-cadherin association is not essential for protrusion formation. In other studies we compared the results of the ΔEXD-G/A mutant with another mutant, ΔEXD-Δp120, lacking 10 residues at the p120 binding domain. This deletion mutant did not localize at the membrane, and it also failed to induce the cell shape change. Thus, these results suggest that sorting of VE-cadherin at the membrane is required for the formation of membrane protrusions.

In previous studies, expression of p120 was shown to induce membrane protrusions in fibroblasts and epithelial cells (a "dendritic" or "branching" phenotype) (41, 42). The expression of cytosolic p120 inhibited RhoA activity in both studies. Noren *et al.* (42) show that p120 expression resulted in Rac1 and Cdc42 activation and that p120 co-precipitated with the guanine exchange factor vav2, suggesting a direct role of cytosolic p120 in Rac1 and Cdc42 activation. Anastasiadis *et al.* (41) propose that the branching phenotype could be the result of RhoA inhibition and the resultant loss of stress fibers and re-organization of ERM family proteins in the cortical cytoskeleton. Both studies suggest that p120-activated signaling was initiated in the cytoplasm. Our results in endothelial cells show that VE-cadherin is involved in Cdc42 activation at the plasma membrane, but they do not rule out a parallel regulation of GTPases in the cytoplasm induced by p120.

In the present study, we provide evidence for the an important role of VE-cadherin in the formation of long protrusions in endothelial cells. Our data show that membrane protrusions form as a result of actin polymerization through Cdc42 activation and the anchorage of the newly synthesized actin filaments to the VE-cadherin cytoplasmic domain. Actin-binding proteins associated with VE-cadherin-β-catenin complex are necessary for the extension of actin cytoskeleton after Cdc42 activation. The dual role of VE-cadherin cytoplasmic domain in Cdc42 activation and formation of actin cytoskeleton extensions may be important in the reassembly of adherens junctions and restoration of endothelial barrier function upon the loss of adherens junctional integrity.

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