p120 regulates endothelial permeability independently of its NH2 terminus and Rho binding

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Herron CR, Lowery AM, Hollister PR, Reynolds AB, Vincent PA. p120 regulates endothelial permeability independently of its NH2 terminus and Rho binding. Am J Physiol Heart Circ Physiol 300: H36–H48, 2011. First published October 22, 2010; doi:10.1152/ajpheart.00812.2010.—The association of p120-catenin (p120) with the juxtamembrane domain (JMD) of vascular endothelial (VE)-cadherin is required to maintain VE-cadherin levels and transendothelial resistance (TEER) of endothelial cell monolayers. To distinguish whether decreased TEER was due to a loss of p120 and not to the decrease in VE-cadherin, we established a system in which p120 was depleted by short hairpin RNA delivered by lentivirus and not to the decrease in VE-cadherin, we established a system in which p120 was depleted by short hairpin RNA delivered by lentivirus and VE-cadherin was restored via expression of VE-cadherin fused to green fluorescent protein (GFP). Loss of p120 resulted in decreased TEER, which was associated with decreased expression of VE-cadherin, β-catenin, plakoglobin, and α-catenin. Decreased TEER was rescued by restoration of p120 but not by the expression of VE-cadherin-GFP, despite localization of VE-cadherin-GFP at cell-cell borders. Expression of VE-cadherin-GFP restored levels of β-catenin and α-catenin but not plakoglobin, indicating that p120 may be important for recruitment of plakoglobin to the VE-cadherin complex. To evaluate the role of p120 interaction with Rho GTPase in regulating endothelial permeability, we expressed a recombinant form of p120, lacking the NH2 terminus and containing alanine substitutions, that eliminates binding of Rho to p120. Expression of this isoform restored expression of the adherens junction complex and rescued permeability as measured by TEER. These results demonstrate that p120 is required for maintaining VE-cadherin expression and TEER independently of its NH2 terminus and its role in regulating Rho.

Endothelial cells line the inner intimal surface of the vasculature, where they provide both a structural and metabolic role in regulating vascular function. One such function is the regulation of the transvascular movement of fluid, macromolecules, and cells across the endothelial monolayer into the interstitial space of tissues. The integrity of the endothelial barrier is regulated, in part, by the adherens junction, a cell-cell adhesion complex composed of cadherins and catenins. Specific to endothelial cells is vascular endothelial (VE)-cadherin, a membrane protein found to form homotypic associations between endothelial cells (reviewed in Ref. 15). The cytoplasmic domain of VE-cadherin is bound by β-catenin and plakoglobin, both of which associate with α-catenin, a protein suggested to support the interaction of the VE-cadherin-catenin complex with the actin cytoskeleton. Phosphorylation of the adherens junction proteins has been implicated in the decrease of endothelial barrier function during a number of disease states. Indeed, recent studies (29, 34) have demonstrated that VEGF-induced phosphorylation of adherens junction proteins plays an important role in edema following stroke or myocardial infarction and that Src plays an important role in this process. Although phosphorylation of adherens junction proteins has been implicated in increased permeability, the mechanisms responsible for these effects have not been elucidated.

Plakoglobin, β-catenin, and p120 all bind to VE-cadherin through a central domain consisting of a series of armadillo repeats (ARM domain) (reviewed in Ref. 15). Both β-catenin and plakoglobin bind to the cadherin cytoplasmic tail at the cadherin binding domain (CBD) in a mutually exclusive fashion (23, 33). Association of the cadherin-catenin complex to the actin cytoskeleton is achieved by the binding of α-catenin to the NH2 terminus and first ARM repeat of plakoglobin or β-catenin; however, this association is not direct, since α-catenin association with the cadherin-catenin complex and the actin cytoskeleton is mutually exclusive (12, 40). The ARM domain of p120 binds to the juxtamembrane domain (JMD) of cadherins. Binding of p120 to the JMD of VE-cadherin in endothelial cells is required for the stabilization of VE-cadherin at the plasma membrane. Using chimeric molecules of the cytoplasmic domain of VE-cadherin fused to the extracellular domain of the IL-2 receptor, investigators in the Kowalczyk laboratory have demonstrated that in the absence of p120, VE-cadherin is endocytosed via a clathrin-dependent pathway. They further demonstrated that VE-cadherin endocytosis requires AP-2 binding to the JMD region of VE-cadherin but that this process is independent of changes in RhoA activity (8).

Cadherin endocytosis has been suggested to mediate changes in barrier function, since VEGF-induced decreases in endothelial monolayer permeability were found to be dependent on the regulation of VE-cadherin endocytosis (16). The interaction of p120 with VE-cadherin may be important to the regulation of endothelial monolayer integrity, because a number of inflammatory agents known to increase endothelial permeability also have been shown to change the phosphorylation state of p120. The addition of phorbol 12,13-dibutyrate (31), histamine (31), and VEGF (35) all cause a dephosphorylation of serine/threonine residues in p120 that can be detected by a shift in the migration pattern on SDS-PAGE. Histamine and VEGF also increase the tyrosine phosphorylation of p120, in addition to other proteins in the adherens junction complex (5, 13). Although changes in the phosphorylation of p120 have been correlated with mediator-induced increases in endothelial permeability, a direct link between

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p120 phosphorylation and endothelial monolayer permeability has not been elucidated.

Our laboratory began studying the role of p120 in regulating endothelial monolayer integrity by forced expression of the JMD region of bovine VE-cadherin as a means to compete with intact VE-cadherin for binding to p120. Expression of the JMD resulted in reduced VE-cadherin levels and a decrease in endothelial monolayer integrity, an effect associated with an increase in stress fibers and enhanced phosphorylation of myosin light chain (19). This suggested that displacement of p120 from the cell junction may play a role in regulating barrier function through the modulation of Rho GTPase activity. Overexpression of p120 has been shown to result in the formation of dendritic processes that require an inhibition of Rho and activation of Rac (4, 28). We found that overexpression of p120 in bovine endothelial cells resulted in an increase in endothelial permeability that was also correlated with an inhibition of Rho activity (19). Of note is that in both of these experimental models, the overexpression of p120 or the JMD region of VE-cadherin no longer solely located p120 at the plasma membrane but also resulted in the accumulation of p120 in the cytoplasm. This altered localization of p120 may result in changes in the activity of small GTPases and increased endothelial permeability.

To further understand the role of p120 in regulating endothelial barrier function, we have developed a model in which endogenous p120 is depleted from human endothelial cells by using lentivirus to deliver short hairpin RNA (shRNA) targeted to p120, and then either p120 or recombinant isoforms of p120 are expressed back to endogenous levels via adenoviral delivery. This model allows us to study the role of different domains of p120 in regulating endothelial permeability independently of endogenous protein and to keep the recombinant protein near endogenous levels. The NH2 terminus of p120 has been implicated as a scaffolding domain that is important to the function of the adherens junction. Indeed, this domain has been shown to scaffold a number of proteins, including Src family kinases (SFKs) (21, 30) and phosphatases (20, 42), each of which has been demonstrated to participate in the regulation of cell–cell adhesion. The association of p120 with these signaling molecules identifies p120 as an ideal candidate to participate in the regulation of transvascular flux across the endothelial cell monolayer.

In this study, a recombinant form of p120 was designed to determine whether the NH2 terminus or Rho binding were critical to rescue VE-cadherin levels and the formation of a restrictive barrier. In addition, we were able to restore expression of VE-cadherin independently of p120 to determine whether p120 was required for maintaining endothelial permeability. Using this model, we demonstrated that decreased expression of p120 resulted in an increase in endothelial permeability, as measured by transendothelial electrical resistance (TEER), that could not be rescued by restoration of VE-cadherin expression alone, an effect correlated with a loss of plakoglobin expression. We also demonstrated that both VE-cadherin and plakoglobin expression, as well as TEER, were restored by reexpression of full-length p120 and also by a recombinant that lacks the NH2 terminus and contains an alanine substitution that eliminates binding of Rho to p120. Together, our data suggest that p120 maintenance of TEER does not require the NH2 terminus of p120 and is independent of its role in regulating Rho.

MATERIALS AND METHODS

Cell culture. Human dermal microvascular endothelial cells (HDMECs) were isolated from neonatal foreskin obtained from the maternity ward of the Albany Medical Center Hospital with Institutional Review Board approval as previously described (14). Briefly, following cell dispersion by dispase treatment for 16 h, endothelial cells were separated from other cell types by incubation with magnetic beads coated with an antibody to CD31 (Invitrogen, Carlsbad, CA). When cells reached confluence, endothelial origin was assessed by evaluation of VE-cadherin and lectin binding, and then cells were frozen and stored in liquid nitrogen. Cells were cultured in EGM2-MV endothelial growth medium (Lonza, Walkersville, MD) supplemented with 5% FBS and used between passages 5 and 12. Confluent monolayers were seeded at 1 × 104 cells/cm2.

RNA interference. Small interfering RNA (siRNA) was purchased from either Dharmaco (human p120 catenin OnTarget Plus chemically modified individual sequences, negative control siRNA no. 3; Lafayette, CO) or Ambion (predisigned siRNA sequences for human p120, siRNA ID no. 16104 (sip120-1, GCCAGAGGGUGGUGCG-AUA); Austin, TX). siRNA was delivered into cells at 0.7 or 1.4 μg of siRNA per 375,000 cells via electroporation using Ambion siPORT electroporation buffer and the Bio-Rad GenePulser Xcell electroporation system. Knockdown was evaluated by Western blotting.

Lentiviral constructs. Nontargeting siRNA control (shNTS) was designed from a nontargeting sequence from Dharmaco (negative control siRNA no. 3) and ligated into pSUPER.puro (OligoEngine, Seattle, WA). The H1 promoter and shRNA sequences of shNTS and shRNA to human p120 in psUPER.puro (11) were then PCR amplified using primers with a PacI restriction site and ligated into either pFUWG-green (GFP) or -red fluorescent protein (RFP) lentiviral vectors using these PacI sites (pFUWG was obtained from Dr. Kevin Pumiglia, Albany Medical College, Albany, NY; Ref. 24). Lentivirus was produced by transfection of HEK-293FT cells (Invitrogen) with pFUWG containing shRNA, pCMV-dR8.2 dvpr, and pCMV-VSV-G (both generous gifts from Dr. Kevin Pumiglia). Medium was collected between 24 and 48 h after transfection, and lentivirus was then concentrated using an Amicon Ultra centrifugal filter (Millipore, Billerica, MA) and stored at −80°C. HDMECs were infected at 50% confluence in EGM2-MV supplemented with 5% heat-inactivated FBS. Medium was changed to growth medium, and the infected cells were allowed to grow to confluence before trypsinization and reseeding for experiments (Supplementary Fig. 1A). (Supplemental data for this article is available online at The American Journal of Physiology-Heart and Circulatory Physiology website.) Infection efficiency was evaluated by visualization of GFP or FP expression in live cells, and p120 knockdown was evaluated by Western blotting.

Adenoviral constructs. To generate murine p120 constructs, we performed PCR using p120 1A-GFP (11) as a template and forward primers consisting of the start site of isoform p120-4A with an AgeI restriction site and Kozak (5′-GAATTCGCCACCATGGAC-GAATTCGCCACCATGGAC-GAATTCGCCACCATGGAC-3′) along with the reverse primer containing an AgeI site and the COOH terminus of p120 (5′-GACCGCTTTGAGGCAGTTGAG-TGTTGAAGAGGTCCTCG-3′). For the p120-AK→ΔA construct, PCR was performed using 120-1A K022.623 construct (4) by using primers consisting of the start site of isoform p120-4A with an EcoRI restriction site and Kozak (5′-GAATTCGCCACCATGGAC-GAATTCGCCACCATGGAC-GAATTCGCCACCATGGAC-3′) and the COOH-terminal primer described above. Recombinant p120 fragments were placed in pCR 2.1 TOPO (Invitrogen) and then fused to TagGFP (Evrogen, Moscow, Russia) in a Gateway entry clone (Evrogen). Recombinase was then used to transfer p120-GFP into pAdCMV/V5/DEST (Invitrogen). GFP control was generated by transferring TagGFP in a Gateway entry clone (Evrogen) into pAdCMV/V5/DEST as described previ-
ously. VE-cadherin-GFP was a generous gift from Dr. Keith Burridge (University of North Carolina, Chapel Hill, NC). Adenoviruses were amplified in QB1-HEK-293A cells (Qbiogene, Carlsbad, CA) and purified using cesium chloride gradients. All infections were performed 72 h after seeding HDMECs stably infected with lentivirus containing shRNA and were accompanied by a control GFP infection with multiplicity of infection (MOI) at or above the greatest MOI used for experimental groups (Supplementary Fig. 1B). Infection rates were determined and plotted.

### Treatments
HDMECs stably infected with lentivirus expressing shRNA were seeded at confluence and infected with adenovirus 72 h later. For thrombin treatments, cells were serum starved for 24 h in 0.3% FBS (Atlanta Biologicals, Lawrenceville, GA) in EBM-2 medium before treatment with vehicle control [PBS containing calcium and magnesium (PBS+)] or 5 U/ml thrombin (Sigma, St. Louis, MO).

### Gel electrophoresis and immunoblotting

At indicated time points following experimental procedures, cells were lysed in Laemmli buffer alone or containing Complete protease inhibitor cocktail (Roche, Indianapolis, IN), Phostop phosphatase inhibitor cocktail (Roche), and 0.1 mM pepsin and were then boiled. Lysates were loaded on SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were performed by blocking membranes with 3% skim milk in PBS+ or 5% bovine serum albumin in PBS+ supplemented with 0.01% Tween 20 (Sigma) followed by an overnight incubation with the following antibodies: p120 (Santa Cruz Biotechnology, Santa Cruz, CA), VE-cadherin (Santa Cruz Biotechnology; Strategic Diagnostics, Newark, DE), β-catenin (BD Transduction, Rockville, MD), plakoglobin (BD Transduction), α-catenin (BD Transduction), nectin-2 (R&D Systems, Minneapolis, MN), ZO-1 (Invitrogen), and β-actin (Sigma). Secondary mouse, rabbit, or goat antibodies conjugated with peroxidase (Jackson ImmunoResearch, West Grove, PA) were incubated for 2 h at room temperature (RT), and membranes were developed using SuperSignal West Pico or Femto chemiluminescent substrate (Pierce, Rockford, IL) and the Fujifilm LAS-3000 imaging system. Densitometric analysis was performed using FujiFilm Multigauge 3.0 software.

### Immunofluorescence microscopy

Cells seeded (1 × 10⁵ cells/cm²) on Ibidi µ-Slide eight-well culture plates (Applied Biophysics, Troy, NY) were fixed with 4% paraformaldehyde (USB, Cleveland, OH) in PBS+ for 20 min at 4°C, washed three times with Tris-buffered saline (TBS), and processed for immunofluorescence. Briefly, cells were permeabлизed with 0.1% Triton X-100 (Sigma) in TBS for 15 min, treated with Image-It FX signal enhancer (Invitrogen) for 30 min, and then blocked with 5.5% bovine serum in TBS. Antibodies (listed above) were incubated for 2 h at 4°C. Secondary anti-goat, -rabbit, or -mouse antibodies conjugated with Alexa Fluor 488, Alexa Fluor 594, Alexa Fluor 647 (Invitrogen), or Cy2 (Jackson Immunoresearch) were incubated for 1 h at RT in the presence or absence of Alexa Fluor 488-conjugated phallolidin (Invitrogen). Images were obtained using a Zeiss Axio Observer Z1 inverted microscope and Zeiss AxioVision software. For ZO-1 and nectin-2 immunofluorescence images, profile lines were drawn perpendicular to individual junctions, and each cell was counted only once. For quantification, the peak intensity values were determined for 15 x-values on either side of the highest peak and plotted using GraphPad Prism software. The area under each curve was then determined and plotted.

### Permeability assays

Monolayer permeability was determined by measuring changes in electrical resistance using an electric cell-substrate impedance sensor (ECIS; Applied BioPhysics) (18) as previously described (1, 19, 39). Briefly, a confluent monolayer of HDMECs was seeded onto ECIS 8W1E or 8W10E cultureware precoated with 0.1% gelatin. Electrical impedance across the monolayer was measured as previously described (1) such that a decrease in resistance is indicative of an increase in permeability. Endothelial permeability to albumin was determined by seeding cells on Costar 0.4-µm Transwell filters (Corning, Corning, NY) at confluence. Seventy-two hours later, FITC-labeled albumin (0.5 mg/ml; Sigma) was added to the upper chamber, and fluorescence readings were taken in duplicate from the lower chamber after 20 min. Clearance was calculated as described previously (9, 19), and values are expressed as normalized to that of empty filter.

### Statistical analysis

Western blots and normalized ECIS experiments were analyzed by single-sample t-test. Permeability to albumin was analyzed by a one-way ANOVA followed by a post hoc Dunnett’s test. Error bars indicate standard error measurement, and P < 0.05 was considered statistically significant.

### RESULTS

To determine whether p120 expression is necessary for the regulation of endothelial barrier integrity, HDMECs electroporated with siRNA targeted to p120 were plated at confluence. Seventy-two hours after seeding, multiple siRNA sequences targeted to p120 markedly decreased p120 expression compared with luciferase controls (Fig. 1A). The two bands observed by Western blot analysis correspond to the isoforms 1A and 3A, as determined by RT-PCR (data not shown). We observed a decrease in the level of VE-cadherin in association with p120 knockdown (Fig. 1A) consistent with previous studies that found VE-cadherin expression is dependent on p120 levels (19, 37, 38). We next measured changes in TEER of HDMEC monolayers with decreased p120 expression to determine whether the loss of p120 affected endothelial permeability. As shown in Fig. 1A, cells were electroporated with siRNA targeted to p120 and plated at confluence, and electrical resistance was monitored immediately after seeding and for the next 96 h. Both the control cells and the cells expressing siRNA to p120 displayed similar changes in electrical resistance for the first 24 h; however, p120 knockdown resulted in a decrease in endothelial resistance across the monolayer between 48 and 96 h after plating (Fig. 1B). This effect was paralleled by a decrease in p120 and VE-cadherin expression at these time points (Supplementary Fig. 2). Using multiple siRNA sequences to different target sites in p120, we observed a similar increase in monolayer permeability as measured by a decrease in TEER 72 h after seeding (Fig. 1C), providing support for the notion that the increase in permeability was not due to off-target effects. Interestingly, we did not observe changes in monolayer permeability to albumin at this same time (Fig. 1D). Thrombin was used in this assay as a positive control to show that changes in monolayer permeability could be assessed.

To generate a system that would promote the stable production of short hairpins to p120 (shp120), we utilized a lentiviral vector for delivery of shRNA into endothelial cells. In this model, endothelial cells at 50% confluence were infected with lentivirus containing shRNA to p120 driven by an H1 promoter and also RFP driven by a ubiquitin promoter. Cells were allowed to form a confluent monolayer over the next 72 h, and assessment of RFP showed that infection efficiency was typically 98–100%. HDMECs stably expressing either shNts or shp120 were then trypsinized and seeded at a cell number to achieve confluence (1 × 10⁵ cells/cm²). Using this system, we found that p120 expression was decreased by up to 98% at 72 h after seeding (Fig. 2A), which, as expected, was accompanied by a reduction in VE-cadherin expression as measured by Western blotting and immunofluorescence (Fig. 3A). Evaluation of endothelial monolayer resistance confirmed that a loss of p120 expression resulted in an increase in permeability as
measured by a lower TEER (Fig. 2B). Similar to studies shown in Fig. 1, TEER was similar for both control and p120-depleted cells immediately after plating; however, p120-deficient cells maintained a lower TEER than control cells receiving shRNA to a nontargeting sequence beyond 24 h. Similar findings were observed using shRNA targeted to a separate site in p120 (Supplementary Fig. 3), providing further support for the idea that decreased TEER was not a result of an off-target effect.

The lack of a change in monolayer permeability to albumin suggested that the morphology of the endothelial cell monolayer may not be drastically altered by the knockdown of p120. Immunofluorescence microscopy showed a decrease in the localization of VE-cadherin at cell borders (Fig. 3E), an effect paralleled by a decrease in β-catenin, plakoglobin, and α-catenin levels (Fig. 3), revealing that the loss of p120 expression results in a loss of the VE-cadherin-mediated adherens junction.

Fig. 1. Loss of p120 expression results in decreased transendothelial electrical resistance (TEER). Luciferase control (Luc) and chemically modified (sip120-2, sip120-4) and unmodified (sip120-1) small interfering RNA (siRNA) targeted to p120 were electroporated into human dermal microvascular endothelial cells (HDMECs) at 0.7 or 1.4 μg and seeded at confluence. A: cells were lysed at 72 h and immunoblotted for p120 and vascular endothelial (VE)-cadherin, as well as β-actin to ensure equal loading. Values are means ± SE (n = 4 experiments). *P < 0.05 compared with Luc (as determined by single-sample t-test). B: changes in electrical resistance for sip120-1 were measured over 96 h on an electric cell-substrate impedance sensor (ECIS) in an 8W1E plate. Values are means ± SE (n = 8; 4 experiments in duplicate). C: changes in electrical resistance were measured 48–96 h after electroporation and averaged over 4 h relative to 72 h on an ECIS in a 8W10E plate. Values are means ± SE (n = 6; 3 experiments in duplicate). *P < 0.05 compared with Luc (as determined by single-sample t-test). D: changes in permeability to FITC-albumin were measured at 72 h after seeding. Values are means ± SE expressed as normalized relative to empty filter (n = 6; 3 experiments in duplicate). *P < 0.05 compared with control (Con) (as determined by 1-way ANOVA followed by post hoc Dunnett's test to control).

Fig. 2. Short hairpin RNA (shRNA) targeted to p120 decreases TEER. HDMECs stably infected with lentivirus containing nontargeting shRNA (shNTS) or shRNA to p120 (shp120) were seeded at confluence. A: cells were lysed 72 h after seeding and immunoblotted for p120 and red fluorescent protein (RFP), as well as β-actin to ensure equal loading. Values are means ± SE (n = 4 experiments). *P < 0.0001 compared with shNTS (as determined by single-sample t-test). B: changes in electrical resistance were measured over 96 h on an ECIS in a 8W10E plate. Values are means ± SE (n = 8; 4 experiments in duplicate).
complex. This loss did not disrupt the junctional localization of other proteins known to be associated with endothelial cell-cell junctions. Indeed, actin, nectin-2, and ZO-1 could all be observed at the cell-cell junction at levels comparable to that of control cells (Fig. 4). These results suggest that although the VE-cadherin adherens junction complex does play a role in regulating endothelial permeability, as demonstrated by the decrease in TEER, the complex is not required for all aspects...
of endothelial barrier regulation, such as permeability to albumin.

We reconstituted expression of a p120 1A-GFP fusion protein as a means to rescue the loss of TEER produced by decreased expression of p120. Cells expressing shp120 or shNTS were seeded on ECIS plates and allowed to reach a stable TEER by 72 h after seeding. Adenovirus was then added to the monolayer, and TEER was assessed for 48 h after infection. We found that restoration of p120 to endogenous levels resulted in membrane localization of p120 (Fig. 5, A and B; Supplementary Fig. 4) and restored expression of the adherens junction components VE-cadherin, β-catenin, plakoglobin, and α-catenin (see Figs. 5A and 7A). In these cells, decreased TEER was restored to control levels within 48 h after infection, whereas GFP alone did not restore TEER in shp120-expressing cells (Fig. 5C). In addition, we overexpressed p120 1A-GFP in p120-deficient cells to levels well above endogenous, which resulted in cytoplasmic localization of p120 (Fig. 5B; Supplementary Fig. 4) and increased expression of adherens junction proteins (see Figs. 5A and 7A). Interestingly, expression of p120 at these levels resulted in restoration of TEER by 24 h after infection (Fig. 5C). This result suggests that higher levels of p120 expression increase resistance more rapidly and, furthermore, support the idea that p120 expression participates in regulating TEER.

Depletion of p120 resulted in a loss of VE-cadherin and its associated catenins (Figs. 2 and 3), raising the question as to which of these proteins is responsible for the loss in TEER. To evaluate whether the effects of p120 depletion on endothelial permeability are a direct consequence of the loss of p120 expression or, rather, are indirectly due to the loss of the adherens junction complex as a whole, we restored VE-cad-

Fig. 4. p120 depletion does not alter the structure of the actin cytoskeleton or expression of other cell junction proteins. HDMECs stably infected with lentivirus containing shNTS or shp120 were seeded at confluence. Cells were fixed 72 h after seeding and immunostained for p120 and actin (A), nectin-2 (B), and ZO-1 (E). For image analysis, profiles were drawn perpendicular to cell junctions and fluorescence intensity was determined at each horizontal position (C). Mean intensity plots were evaluated for each region, and bar graphs represent the area under the curve for each region for nectin-2 (D) and ZO-1 (F). Values are means ± SE (n = 4 experiments).
herin protein levels using forced expression of VE-cadherin with GFP fused at its COOH terminus (VE-cadherin-GFP) (3) in cells depleted of p120. As shown in Fig. 6, restoration of VE-cadherin expression to levels comparable to endogenous did not rescue TEER in p120-deficient cells. Live cell imaging showed that VE-cadherin-GFP localized at cell-cell junctions (Supplementary Fig. 5); however, some cells showed an accumulation of vesicular VE-cadherin-GFP, consistent with the known increase in endocytosis of VE-cadherin not associated with p120 (37, 38). To confirm VE-cadherin expression levels at the membrane, we performed immunofluorescence and evaluated expression at a higher magnification. We found that VE-cadherin was indeed expressed at the membrane throughout the monolayer (Fig. 6C; Supplementary Fig. 6). To ensure that we were overwhelming the endocytic process, we increased the forced expression of VE-cadherin-GFP to levels well above those found in control monolayers (Fig. 6; Supplementary Fig. 5). However, even expression of VE-cadherin at these high levels was not sufficient to rescue TEER in cells deficient of p120 (Fig. 6B). Immunofluorescence microscopy and immunoblot analysis of p120-depleted cells expressing VE-cadherin-GFP revealed that restoring VE-cadherin also rescued the levels and cell junction localization of the adherens junction components β-catenin and α-catenin (Fig. 7B; Supplementary Fig. 6). Interestingly, forced expression of VE-cadherin-GFP did not rescue the level of plakoglobin at either low or high levels of VE-cadherin expression (Fig. 7B; Supplementary Fig. 6). These results suggest that VE-cadherin is not sufficient to restore TEER and plakoglobin expression in endothelial monolayers depleted of p120.

Robust overexpression of p120 has been demonstrated to result in the cytoplasmic localization of the protein, leading to changes in the activity of RhoA (4, 28), Rac (28), and Cdc42 (28). To determine whether p120 regulation of TEER was dependent on p120 regulation of Rho, we expressed a p120 recombinant (p120 4AKΔGFP) that has been shown not to inhibit Rho activity. This construct contains an alanine substitution of two lysines (K622,623A) and also an NH2-terminal truncation, both of which have been demonstrated to prevent the inhibition of Rho produced by overexpression of intact p120-1A (4, 7) (Fig. 8A). The NH2-terminal truncation of p120 also removes a phosphorylation domain containing a cluster of

Fig. 5. Restoration of p120 1A-green fluorescent protein (GFP) in p120-depleted cells rescues TEER. HDMECs stably infected with lentivirus containing shNTS or shp120 were seeded at confluence. Seventy-two hours later, monolayers were infected with adenovirus containing GFP or p120 1A-GFP at low and high doses. A: cells were lysed 48 h postinfection and immunoblotted for p120 and VE-cadherin, as well as β-actin as loading control. Values are means ± SE (n = 3 experiments). *P < 0.05 compared with shNTS + GFP (as determined by single-sample t-test). B: live fluorescence images of p120 1A-GFP expression were taken 48 h after adenoviral infection (n = 3 experiments). C: changes in electrical resistance were measured from 2 h before infection to 48 h postinfection on an ECIS in a 8W10E plate. Values are means ± SE (n = 6; 3 experiments in duplicate). Adeno, adenovirus.
Serine/threonine and tyrosine phosphorylation sites found to be important for p120 function in other cell types (7, 25, 30, 36). Thus the use of this construct promotes the investigation of the role of the NH2-terminal domain and p120 scaffolding of Rho in regulating endothelial permeability. Fluorescence microscopy under live conditions showed that the p120 4AK-A-GFP recombinant localized to the cell-cell junction when expressed at levels comparable to endogenous in p120-depleted cells (Fig. 8B; Supplementary Fig. 7). In addition, expression of this construct in cells deficient of p120 restored levels of VE-cadherin, /H9252-catenin, plakoglobin, and /H9251-catenin (Fig. 8C; Supplementary Fig. 8).

Expression of the p120 4AK-A-GFP recombinant in p120-depleted cells restored the TEER back to control levels similar to expression of the p120 1A isoform (Fig. 9A). Treatment of endothelial monolayers with inflammatory mediators has been demonstrated to increase endothelial permeability, which has been correlated with changes in p120 phosphorylation (6, 16, 22, 35) and Rho activity (19). Interestingly, thrombin treatment of p120-depleted cells expressing either p120 1A- or 4AK-A-GFP resulted in a comparable increase in permeability (Fig. 9B), supporting the idea that p120 mediates TEER independently of its regulation of Rho. These results demonstrate that NH2-terminal and Rho scaffolding by p120 are not required for the formation of monolayers with maximal TEER.

**DISCUSSION**

We have successfully established a model in which p120 is knocked down in endothelial cells and then replaced with recombinant forms of the molecule. On knockdown of p120, we observed the expected decrease in expression of VE-cadherin and the catenins that are associated with the VE-cadherin-mediated adherens junction complex (α-, β-, and γ-catenin/plakoglobin) (Fig. 3). The knockdown of p120 resulted in an increase in endothelial permeability as measured by TEER (Figs. 1 and 2). This was specific to a loss of p120, since reexpression of p120-1A rescued the decrease in resistance, whereas restoration of VE-cadherin levels did not (Figs. 5 and 6).

Interestingly, although we observed a decrease in TEER in cells expressing siRNA to p120, we did not detect changes in the permeability of the monolayer to albumin (Fig. 1). This suggests that the interaction of p120 and maintenance of...
VE-cadherin are more important for restricting the flux of small solutes and possibly water through a small pore pathway rather than a large pore pathway. As reviewed by Michel and Curry (26), the permeability of the endothelial monolayer to macromolecules can be described in terms of convection and diffusion through two populations of pores, a small pore that would allow the flux of fluid and small solutes and a large pore for molecules such as albumin. In our studies, changes in TEER would be consistent with increases in the small pore pathway, whereas the lack of changes in albumin clearance would suggest no change in the large pore pathway. If applied to an in vivo condition, this would suggest that the VE-

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Fig. 7. Restoration of VE-cadherin-GFP in p120-depleted cells does not restore expression of plakoglobin. HDMECs stably infected with lentivirus containing shNTS or shp120 were seeded at confluence. Seventy-two hours later, monolayers were infected with adenovirus containing GFP or p120 1A-GFP (A) or VE-cadherin-GFP (B) at low and high doses. Cells were lysed 48 h postinfection and immunoblotted for β-catenin, plakoglobin, and α-catenin, as well as β-actin as loading control. Values are means ± SE (n = 3 experiments). *P < 0.05 compared with shNTS + GFP (as determined by single-sample t-test).
cadherin mediated junction would have a regulatory role in fluid flux ($J_v$) by maintaining the hydraulic conductivity ($L_p$) of the endothelial monolayer while having little effect on the reflection coefficient ($\sigma$). We were surprised to find that the loss of p120 and the corresponding loss of the adherens junction complex did not result in changes in monolayer permeability to larger molecules, given that Gavard et al. (16) previously demonstrated that expression of siRNA to VE-cadherin in human umbilical vein endothelial cells (HUVECs) resulted in increased permeability to dextran. This discrepancy, however, may be explained by the heterogeneity of endothelial cells, since we have found that baseline TEER of HUVECs is much lower than that of HDMECs (data not shown). Nonetheless, our findings suggest that p120 is an important regulator of TEER and may play an important role in mediating endothelial permeability to small ions and water.

As observed in Fig. 4, cortical actin was present in p120-depleted monolayers, suggesting that expression of the VE-cadherin adherens junction complex is not necessary for stabilization of cortical actin. This finding is consistent with a recent study showing that the expression of siRNA to VE-cadherin in HUVECs did not affect the organization of actin (27). In addition, our data also show that there was no change in the localization of nectin-2 or ZO-1 at cell-cell borders (Fig. 4). Thus structures that can support the formation of cortical actin at cell-cell junctions were still present despite the loss of p120 and the associated decrease in VE-cadherin levels. The lack of a change in the actin cytoskeleton is consistent with no observed change in the albumin permeability. Indeed, inflammatory mediators that cause an increase in albumin permeability across endothelial monolayers, such as tumor necrosis factor or thrombin, produce rearrangement of the actin cytoskeleton from a peripheral band to stress fibers. The present data suggest that a loss of the cortical actin found following treatment with inflammatory mediators may not be driven by disruption of the VE-cadherin complex, but rather is a result of the loss of the cortical actin band through some other mechanism. Further experiments are needed to determine the role of the

**Fig. 8.** Expression of a Rho-independent recombinant p120 in p120-depleted cells rescues expression of adherens junction components. A: schematic of p120-GFP recombinants expressed in p120-depleted cells. B and C: HDMECs stably infected with lentivirus containing shNTS or shp120 were seeded at confluence. Seventy-two hours later, monolayers were infected with adenovirus containing GFP, p120 1A-GFP, or p120 4AK→A-GFP. Live fluorescence images of p120 recombinant expression (B) were taken 48 h after infection ($n = 4$ experiments). Cells were lysed 48 h postinfection and immunoblotted (C) for p120 and VE-cadherin, $\beta$-catenin, plakoglobin, and $\alpha$-catenin, as well as $\beta$-actin as loading control. Values are means ± SE ($n = 3$ experiments). *$P < 0.05$ compared with shNTS + GFP (as determined by single-sample $t$-test).
VE-cadherin complex in the regulation of the cortical actin cytoskeleton.

We have previously demonstrated that the displacement of p120 from VE-cadherin by expression of a JMD peptide results in decreased expression of VE-cadherin and also increased monolayer permeability of albumin in bovine pulmonary artery endothelial cells (19). At first glance this would seem to be at odds with the findings of this study, which showed no change in albumin permeability with a loss of p120 and decreased VE-cadherin levels. However, we have found that bovine cells possess a higher amount of N-cadherin compared with VE-cadherin (14), and a recent study by Gentil-dit-Maurin et al. (17) suggested that these two cadherins participate in different signaling pathways due to differential association with lipid rafts. Because HDMECs express higher levels of VE-cadherin compared with N-cadherin, signaling differences may exist between the bovine and human endothelial cells, thereby explaining the difference in albumin permeability found between cell types following a decrease in p120 and VE-cadherin. A second difference between these two studies is the level of free p120 that is found in cytoplasm of each model. In the JMD study, p120 is still being generated and is broken down after synthesis due to binding of p120 to the expressed JMD peptide (19). The p120-JMD complex may induce signaling through activation of small GTPases, as has been implicated for free p120, that leads to disruption of the actin cytoskeleton and an increase in monolayer permeability. In the present studies, p120 is never produced due to the inhibition of translation by the siRNA generated against p120, and thus free p120 is not found in the cytoplasm. Hence, the method used to decrease p120-VE-cadherin interaction, as well as the differences between species, may explain the differences found between this study and that of Iyer et al. (19).

The decrease in TEER upon p120 depletion was associated with a decrease in the level of VE-cadherin, β-catenin, plakoglobin, and α-catenin (Figs. 2 and 3), demonstrating that decreased expression of p120 results in a loss of the adherens junction complex. To determine whether the loss of p120 expression or the loss of the adherens junction complex as a whole is responsible for the decrease in TEER, we restored the level of VE-cadherin in p120-depleted cells. Although restoration of VE-cadherin expression rescued protein levels of β-catenin and α-catenin at the cell-cell junction (Figs. 6 and 7; Supplementary Figs. 5 and 6), TEER was not restored (Fig. 6B). Interestingly, plakoglobin levels were not rescued by restoration of VE-cadherin expression in p120-depleted cells, even though other adherens junction proteins were restored (Fig. 7; Supplementary Fig. 6). Restoration of full-length p120 rescued expression of plakoglobin and decreased TEER in p120-depleted cells (Figs. 5C and 7A; Supplementary Fig. 8), suggesting that the inability of VE-cadherin to rescue TEER may be due to a lack of plakoglobin restoration at the junction. Previous studies have demonstrated that “mature” endothelial monolayers, monolayers that are confluent for 48–72 h, displayed increased plakoglobin expression and association with VE-cadherin compared with monolayers confluent for only 18 h (23). Because plakoglobin and β-catenin bind to the same region of VE-cadherin, the increase in plakoglobin results in the displacement of β-catenin at the cell junction (33). In collaboration with the Kowalczyk laboratory (33), we found that forced expression of plakoglobin in HMEC-1 cells resulted in an increase in TEER and a decrease in albumin flux. In addition, plakoglobin has also been suggested to strengthen endothelial cell-cell junctions, since loss of plakoglobin is associated with decreased monolayer integrity in response to shear stress (32). These findings suggest that plakoglobin may be important to junctional integrity; however, further research is needed to delineate the role of plakoglobin in VE-cadherin-mediated adhesion and regulation of endothelial cell-cell junctions.

To begin evaluating the role of the different functional domains of p120 in regulating endothelial permeability, we reconstituted a recombinant isoform of p120 that would not bind Rho GTPase. Previous studies have evaluated functional domains of p120 by forced expression of p120 recombinant proteins. Robust overexpression of p120 results in an increase in the cytoplasmic localization of p120 that has been demonstrated to inhibit Rho and increase Rac activity, resulting in a dendritic phenotype in a number of cell types (4, 8, 10, 28). The model we have established alleviates the cytoplasmic accumulation of p120 by first depleting cells of p120 and then reconstituting expression to levels comparable to endogenous p120 (Figs. 5 and 8). The recombinant form of p120 used for the studies in Figs. 5–8 (p120 4AK→A) was made by deleting the NH₂ terminus of p120 and making substitutions of lysines 622 and 623 to alanines. Expression of a recombinant p120 protein containing either a lysine-to-alanine mutation within the ARM domain (K622,623A) or a truncation of the NH₂...
terminus was demonstrated to prevent p120 inhibition of Rho and also blocked the formation of dendrites (2, 4, 7, 41). In collaboration with the Kowalczyk laboratory (8), we found that expression of p120 4AK-A prevented VE-cadherin endocytosis, suggesting that p120 regulation of Rho and the NH2-terminal domain of p120 are not necessary for regulating VE-cadherin endocytosis. In the present study, we found that this recombinant restored expression of the adherens junction components, including plakoglobin, and also restored TEER similar to that of full-length p120 (Figs. 8 and 9; Supplementary Fig. 8). These findings demonstrate that the NH2 terminus of p120 is not required for the expression of the adherens junction and maintenance of endothelial permeability and that p120 regulates these functions through a Rho-independent mechanism.

The NH2 terminus of p120 also has been demonstrated to scaffold a number of kinases, phosphatases, and other regulatory proteins such as SFK and SHP-1 (20, 21, 30, 42). This domain of p120 also contains a large number of phosphorylation sites that have been implicated in the regulation of permeability in response to inflammatory mediators. For example, changes in p120 phosphorylation have been correlated with thrombin-induced changes in endothelial barrier function (22). Our data, however, suggest that the NH2 terminus of p120 does not participate in the thrombin-induced change in permeability, but that p120 interaction with VE-cadherin is required for the recovery following thrombin-induced decreases in TEER (Fig. 9).

In summary, we have found that the interaction of p120 with VE-cadherin is required for the formation of a mature cell-cell junction with maximal TEER. The decrease in TEER following p120 depletion is not due to the associated decrease in VE-cadherin, because restoration of VE-cadherin levels is not sufficient to restore TEER. Although reexpression of VE-cadherin will rescue the level of β-catenin and α-catenin, plakoglobin expression is not restored in the absence of p120. In addition, the formation of a junction with maximal TEER does not require the NH2 terminus of p120 and is independent of Rho binding to p120. Ultimately, this demonstrates a critical role for p120 in the maintenance of endothelial monolayer permeability that is independent of its NH2 terminus and its ability to regulate Rho. Further research is needed to completely elucidate the mechanisms by which p120 regulates endothelial permeability.

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DISCLOSURES
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REFERENCES