

# Proline-rich Tyrosine Kinase 2 (Pyk2) Mediates Vascular Endothelial-Cadherin-based Cell-Cell Adhesion by Regulating $\beta$ -Catenin Tyrosine Phosphorylation\*<sup>§</sup>

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Jaap D. van Buul<sup>‡§¶</sup>, Eloise C. Anthony<sup>‡¶</sup>, Mar Fernandez-Borja<sup>‡\*\*</sup>, Keith Burridge<sup>§</sup>,  
and Peter L. Hordijk<sup>‡‡</sup>

From the <sup>‡</sup>Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, 1066 CX Amsterdam, The Netherlands and <sup>§</sup>Department of Cell and Developmental Biology, Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599

**Vascular endothelial-cadherin (VE-cadherin) controls endothelial cell-cell adhesion and preserves endothelial integrity. In order to maintain endothelial barrier function, VE-cadherin function is tightly regulated through mechanisms that involve protein phosphorylation and cytoskeletal dynamics. Here, we show that loss of VE-cadherin function results in intercellular gap formation and a drop in electrical resistance of monolayers of primary human endothelial cells. Detailed analysis revealed that loss of endothelial cell-cell adhesion, induced by VE-cadherin-blocking antibodies, is preceded by and dependent on a rapid activation of Rac1 and increased production of reactive oxygen species. Moreover, VE-cadherin-associated  $\beta$ -catenin is tyrosine-phosphorylated upon loss of cell-cell contact. Finally, the redox-sensitive proline-rich tyrosine kinase 2 (Pyk2) is activated and recruited to cell-cell junctions following the loss of VE-cadherin homotypic adhesion. Conversely, the inhibition of Pyk2 activity in endothelial cells by the expression of CRNK (CADTK/CAK $\beta$ -related non-kinase), an N-terminal deletion mutant that acts in a dominant negative fashion, not only abolishes the increase in  $\beta$ -catenin tyrosine phosphorylation but also prevents the loss of endothelial cell-cell contact. These results implicate Pyk2 in the reduced cell-cell adhesion induced by the Rac-mediated production of ROS through the tyrosine phosphorylation of  $\beta$ -catenin. This signaling is initiated upon loss of VE-cadherin function and is important for our insight in the modulation of endothelial integrity.**

Vascular endothelial-cadherin (VE-cadherin,<sup>1</sup> Cadherin-5) is a transmembrane, calcium-dependent homophilic adhesion

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\*\* Supported by Grant 1249 from the Land Steiner Foundation for Blood Transfusion Research.

<sup>‡‡</sup> Fellow of the Landsteiner Foundation for Blood Transfusion Research (Grant 9910). To whom correspondence should be addressed: Dept. of Molecular Cell Biology, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Plesmanlaan 125, 1066 CX, Amsterdam, The Netherlands. Tel.: 31205123263; Fax: 31205123474; E-mail: p.hordijk@sanquin.nl.  
<sup>1</sup> The abbreviations used are: VE-cadherin, vascular endothelial-cad-

molecule that connects adjacent endothelial cells. A loss of VE-cadherin function results in unstable endothelial junctions and a decrease in endothelial monolayer electrical resistance, despite the fact that several other adhesion proteins such as claudin, occludin, and platelet-endothelial cell adhesion molecule 1 are also concentrated at sites of endothelial cell-cell contact (1, 2). Thus, VE-cadherin function is indispensable for the maintenance of the endothelial barrier function.

VE-cadherin is linked to the actin cytoskeleton via the armadillo family members  $\beta$ - and  $\gamma$ -catenin that bind the actin-binding protein  $\alpha$ -catenin (3, 4). VE-cadherin function is controlled by cytoskeletal dynamics and by protein phosphorylation events. Lampugnani *et al.* (5) showed that tyrosine phosphorylation of VE-cadherin and associated catenins is increased in loosely confluent endothelial monolayers, whereas tyrosine phosphorylation is reduced in confluent cells. Recently, a novel VE-protein tyrosine phosphatase was shown to interact with VE-cadherin and to increase VE-cadherin-mediated barrier function (6). In addition, Ukropec *et al.* (7) reported that the phosphatase SHP-2 (SH2-containing tyrosine phosphatase Shp2) interacts with  $\beta$ -catenin and thereby regulates thrombin-induced changes in the endothelial barrier function (7). The specific association of VE-protein tyrosine phosphatase with VE-cadherin and SHP-2 with  $\beta$ -catenin provides further evidence that tyrosine phosphorylation of the VE-cadherin-catenin complex is important for the regulation of endothelial cell-cell adhesion.

Recently, it became clear that Rac1-induced reactive oxygen species (ROS, *e.g.* H<sub>2</sub>O<sub>2</sub>) disrupt VE-cadherin-based cell-cell adhesion (8, 9). Moreover, Rac1-induced ROS regulate protein tyrosine phosphorylation by inhibiting tyrosine phosphatase activity (10–12). In addition, Tai *et al.* (12) reported recently that shear stress induces activation of the tyrosine kinase Pyk2 in a ROS-dependent manner in endothelial cells (12). Pyk2, also known as CAK $\beta$ /RAF<sup>TK</sup>/CADTK and FAK2, is a redox-sensitive tyrosine kinase that can be dephosphorylated by SHP-2 (13, 14).

In this study, the role of Pyk2 in VE-cadherin function was explored. To induce the loss of VE-cadherin-mediated cell-cell adhesion, we made use of antibodies that block interactions between the extracellular regions of the VE-cadherin protein

herin; ROS, reactive oxygen species; CRNK, CADTK/CAK $\beta$ -related non-kinase; Pyk2, proline-rich tyrosine kinase 2; p-Pyk2, phospho-Pyk2; wt, wild type; pY, phosphotyrosine; mAb, monoclonal antibody; Ab, antibody; HUVEC, human umbilical vein endothelial cells; FITC, fluorescein isothiocyanate; FN, fibronectin; DHR, dihydrorodamine-1,2,3; N-AC, N-acetylcysteine; PBS, phosphate-buffered saline; SH2, Src homology 2.

(15–17). This strategy mimics the loss of endothelial integrity induced by pro-inflammatory cytokines or by leukocyte transendothelial migration. We found that the loss of VE-cadherin function activates the small GTPase Rac1 and increases production of ROS, which subsequently leads to the loss of cell-cell adhesion. This reduced cell-cell adhesion is accompanied by increased tyrosine phosphorylation of  $\beta$ -catenin, which depends on the activation of Pyk2. Together, these data provide novel information on the role of Pyk2 in the regulation of VE-cadherin-based endothelial cell-cell adhesion and endothelial integrity.

#### MATERIALS AND METHODS

**Reagents and Antibodies**—Monoclonal antibodies (mAbs) to VE-cadherin (c175),  $\beta$ -catenin,  $\alpha$ -catenin, Pyk2, and phosphotyrosine (PY-20) were from BD Transduction Laboratories. VE-cadherin mAb 7H1 was from Pharmingen (San Diego, CA).  $\beta$ -Catenin polyclonal Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal Ab to phosphotyrosine was from Zymed Laboratories Inc.. The polyclonal Ab to Pyk2 was a kind gift of Dr. L. Graves (University of North Carolina, Chapel Hill, NC).  $\alpha$ -Myc monoclonal antibodies were purchased from Invitrogen. Texas Red phalloidin, Alexa 633 phalloidin, FITC-labeled 3000 Dextran, Alexa 488-labeled goat- $\alpha$ -mouse-Ig, Alexa 568-labeled goat- $\alpha$ -mouse-Ig, and Alexa 488-labeled goat- $\alpha$ -rabbit-Ig secondary Abs were from Molecular Probes (Leiden, The Netherlands). Horseradish peroxidase-labeled goat- $\alpha$ -mouse-Ig or goat- $\alpha$ -rabbit-Ig was from DAKO (Glostrup, Denmark). Fibronectin (FN) was obtained from the Sanguin Research (Amsterdam, The Netherlands). Fetal calf serum was from Invitrogen. Basic fibroblast growth factor was from Roche Applied Science. Phospho-pyk2 (pY402) was purchased from BIOSOURCE (Camarillo, CA). EDTA, EGTA, and  $\alpha$ -FLAG monoclonal Ab (clone M2) were from Sigma.

**Cell Cultures and Treatments**—Immortalized human umbilical vein endothelial cells (HUVEC) (16) and primary HUVEC isolated from umbilical cord or purchased from Cambrex (Baltimore, MD) were cultured in FN-coated culture flasks (Nunc, Invitrogen) in Medium 199 (Invitrogen) supplemented with 20% (v/v) pooled heat-inactivated fetal calf serum, 1 ng/ml basic fibroblast growth factor, 5 units/ml heparin, 300  $\mu$ g/ml glutamine, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. After reaching confluency, the endothelial cells were passaged by treatment with trypsin/EDTA (Invitrogen). To disrupt VE-cadherin-based cell-cell adhesion, cells were treated with either 12.5 or 25  $\mu$ g/ml anti-VE-cadherin c175 antibody, as indicated in the figure legends. To study signaling effects during the induced loss of VE-cadherin-based cell-cell adhesion, cells were stimulated for only 10 min with c175, as indicated in figure legends. All of the cell lines were cultured or incubated at 37 °C at 5% CO<sub>2</sub>.

**Peptide Synthesis**—The Rac17-32 peptide, which inhibits Rac1 function (18), was designed in conjunction with the protein transduction domain of the human immunodeficiency virus Tat protein (19). The resulting peptide (YGRKKRRQRRRGTCLLISYTTNAPFGEY) was synthesized at the Netherlands Cancer Institute (Amsterdam, The Netherlands).

**Adenoviral Infection of CRNK Construct**—The recombinant adenoviral vector CRNK was a kind gift of Dr. L. Graves (University of North Carolina) (20). Endothelial cells were serum-starved for 30 min and subsequently infected with adenovirus containing the CRNK-construct in serum-free culture medium. After 3 h, medium was replaced by normal culture medium, and following 16–24 h of infection, cells were washed three times with phosphate-buffered saline and used for assays.

**Immunocytochemistry**—HUVEC were cultured on FN-coated glass coverslips, fixed, and immunostained as described previously (16) with a mAb to VE-cadherin (7H1, 10  $\mu$ g/ml) or to PY-20 (10  $\mu$ g/ml). Polyclonal anti-phosphotyrosine (10  $\mu$ g/ml), anti- $\alpha$ -catenin (10  $\mu$ g/ml), and anti- $\beta$ -catenin (10  $\mu$ g/ml) were used when endothelial cells were pretreated with mAbs to VE-cadherin. Subsequent visualization was performed with fluorescently labeled secondary Abs (10  $\mu$ g/ml). F-actin was visualized with Texas Red phalloidin (1 unit/ml). In some experiments, cells were pretreated for 30 min at 37 °C with 20  $\mu$ g/ml Rac17-32 peptide followed by washing. Images were recorded with a ZEISS LSM510 confocal microscope with appropriate filter settings. Cross-talk between the green and red channel was avoided by use of sequential scanning.

**Electric Cell Substrate Impedance Sensing**—Endothelial cells were seeded at 100,000 cells/well (0.8 cm<sup>2</sup>) on FN-coated electrode arrays and grown to confluency. After the electrode check of the array and when the basal electrical resistance of the endothelial monolayer reached a

plateau, Abs to VE-cadherin were added and electrical resistance was monitored on-line at 37 °C and at 5% CO<sub>2</sub> with the electric cell substrate impedance sensing Model-100 Controller from BioPhysics, Inc. (Troy, NY). After 8 h, the data were collected and changes in resistance of endothelial monolayer were analyzed.

**Permeability**—Permeability of HUVEC monolayers cultured on 5- $\mu$ m pore 6.5-cm Transwell filters (Costar, Cambridge, MA) was determined using FITC-labeled 3000 Dextran as described previously (8). The permeability response to thrombin (1 unit/ml) after 30 min was used as a control. After the assay, filters were washed with ice-cold Ca<sup>2+</sup>- and Mg<sup>2+</sup>-containing PBS and then fixed with 2% paraformaldehyde and 1% Triton X-100-containing PBS and stained with Texas Red phalloidin to inspect the HUVEC monolayer by confocal laser-scanning microscopy.

**Immunoprecipitation and Western Blot Analysis**—Cells were grown to confluency on FN-coated dishes (50 cm<sup>2</sup>), washed twice gently with ice-cold Ca<sup>2+</sup>- and Mg<sup>2+</sup>-containing PBS, and lysed in 1 ml of lysis buffer (25 mM Tris, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 2 mM EDTA, 0.02% (w/v) SDS, 0.2% (w/v) deoxycholate, 1% Nonidet P-40, 0.5 mM orthovanadate with the addition of fresh protease-inhibitor-mixture tablets (Roche Applied Science), pH 7.4). After 10 min on ice, cell lysates were collected and pre-cleared for 30 min at 4 °C with protein G-Sepharose (15  $\mu$ l for each sample, Amersham Biosciences). The supernatant separated by centrifugation (14,000  $\times$  g, 15 s at 4 °C) was incubated with 15  $\mu$ l of protein G-Sepharose that had been coated with 5  $\mu$ g/ml  $\beta$ -catenin mAb for 1 h at 4 °C under continuous mixing. The beads were washed three times in lysis buffer, and proteins were eluted by boiling in SDS-sample buffer containing 4% 2-mercaptoethanol (Bio-Rad). The samples were analyzed by SDS-PAGE. Proteins were transferred to 0.45- $\mu$ m nitrocellulose (Schleicher & Schüll), and the blots were blocked with blocking buffer (1% (w/v) low fat milk in Tris-buffered saline with Tween 20) for 1 h and subsequently incubated at room temperature with the appropriate Abs for 1 h followed by incubation with rabbit- $\alpha$ -mouse-Ig-horseradish peroxidase for 1 h at room temperature. Between the various incubation steps, the blots were washed three times with Tris-buffered saline with Tween 20 and finally developed with an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

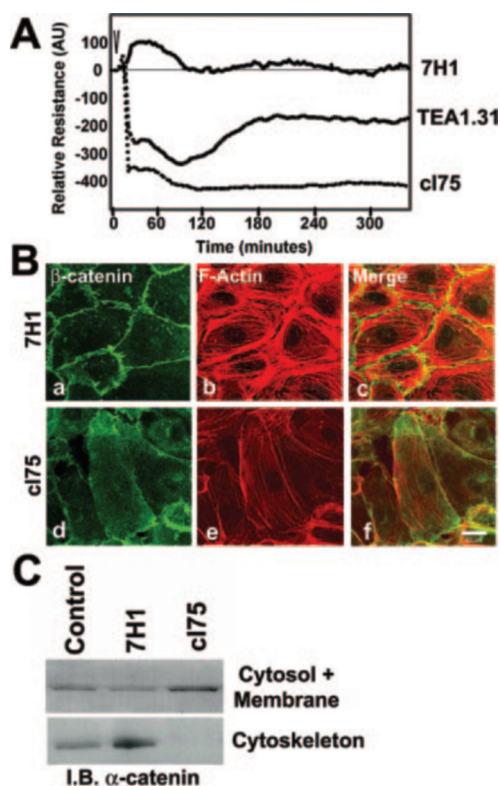
**Rac1 Activity Assays**—Cells were stimulated for the indicated times with c175 (25  $\mu$ g/ml) or 5 mM EDTA. Cells were kept on ice and washed with ice-cold PBS, lysed for 10 min in lysis buffer, and assayed for Rac activation with glutathione S-transferase-p21-activating kinase, as described by Sander *et al.* (21). Finally, in both assays, the beads were washed four times with lysis buffer. After the fourth wash, the beads were put into new tubes and subsequently suspended in 2 $\times$  sample buffer containing 4% 2-mercaptoethanol. Samples were analyzed by SDS-PAGE as described above.

**Cell Fractionation**—Cells were grown to confluency on FN-coated dishes (50 cm<sup>2</sup>), washed twice gently with ice-cold Ca<sup>2+</sup>- and Mg<sup>2+</sup>-containing PBS, and lysed in 1 ml of lysis buffer, as described above, containing 1% Triton X-100 instead of 1% Nonidet P-40. After 10 min on ice, cell lysates were collected and separated by centrifugation (14,000  $\times$  g, 1 min at 4 °C). The pellet fraction contained Triton X-100-insoluble proteins associated to the actin cytoskeleton, and the supernatant contained Triton X-100-soluble cytosolic proteins. Samples were boiled in SDS sample buffer containing 4% 2-mercaptoethanol and were immediately analyzed by SDS-PAGE and continued as described above.

**Measurement of ROS**—To measure generation of reactive oxygen species (ROS) in endothelial cells, primary HUVEC cultured on fibronectin-coated glass coverslips were loaded with dihydrorodamine-1,2,3 (DHR, 30  $\mu$ M; Molecular Probes) for 30 min, washed, and subsequently treated with the VE-cadherin Ab c175, control Ab IgG, or medium. Fluorescence of DHR was quantified by time lapse confocal microscopy. Intensity values are shown as the percentage increase relative to the basal DHR values at the start of the experiment.

#### RESULTS

VE-cadherin is an essential homotypic adhesion molecule that specifically localizes to adherens junctions and controls endothelial integrity. Using Abs that recognize distinct epitopes in the extracellular domain of VE-cadherin, we and others (15, 17, 22) have described differential effects on the permeability of endothelial cells. However, these studies were in part based on diffusion of a fluorescently labeled high molecular weight marker over the endothelial monolayer to measure the endothelial integrity. We now used a more sensitive approach, which is based on real-time analysis of the electrical



**FIG. 1. Blocking antibodies to VE-cadherin decrease endothelial electrical resistance and alter the distribution of the cadherin-catenin complex.** *A*, the electrical resistance over a cultured endothelial monolayer of pHUVEC (pHUVEC) was measured as described under “Materials and Methods.” At the start of the experiment, the indicated antibodies to VE-cadherin (25  $\mu$ g/ml) were added to the wells. The control anti-VE-cadherin Ab 7H1 did not affect the electrical resistance (triangles), whereas cl75 Abs rapidly decreased electrical resistance of the endothelial monolayers (squares). Data are a mean of duplicate measurements. A representative of four experiments is shown. AU, arbitrary units. *B*, endothelial cells were cultured and grown to confluency on FN-coated glass coverslips and subsequently incubated with the non-blocking 7H1 antibody (*a–c*) or the blocking cl75 (*d–f*) for 30 min. Treatment of the cells with cl75 induced the loss of cell-cell contacts and redistribution of  $\beta$ -catenin over the endothelial cell surface.  $\beta$ -Catenin is shown in green (*a* and *d*), F-actin is shown in red (*b* and *e*), and yellow shows co-localization between  $\beta$ -catenin and F-actin (*c* and *f*). Bar, 20  $\mu$ m. *C*, cells were cultured as described under “Materials and Methods” and treated with antibodies as indicated for 30 min. Cells were lysed in Triton X-100 buffer, and fractions were separated into a cytosolic and membrane fraction (Triton X-100-soluble fraction) and a cytoskeletal fraction (Triton X-100-insoluble fraction) and analyzed by SDS-PAGE. Immunoblots were probed for  $\alpha$ -catenin and show that cl75 treatment induced a shift of  $\alpha$ -catenin from the cytoskeleton to the cytosolic fraction. A representative of three independent experiments is shown.

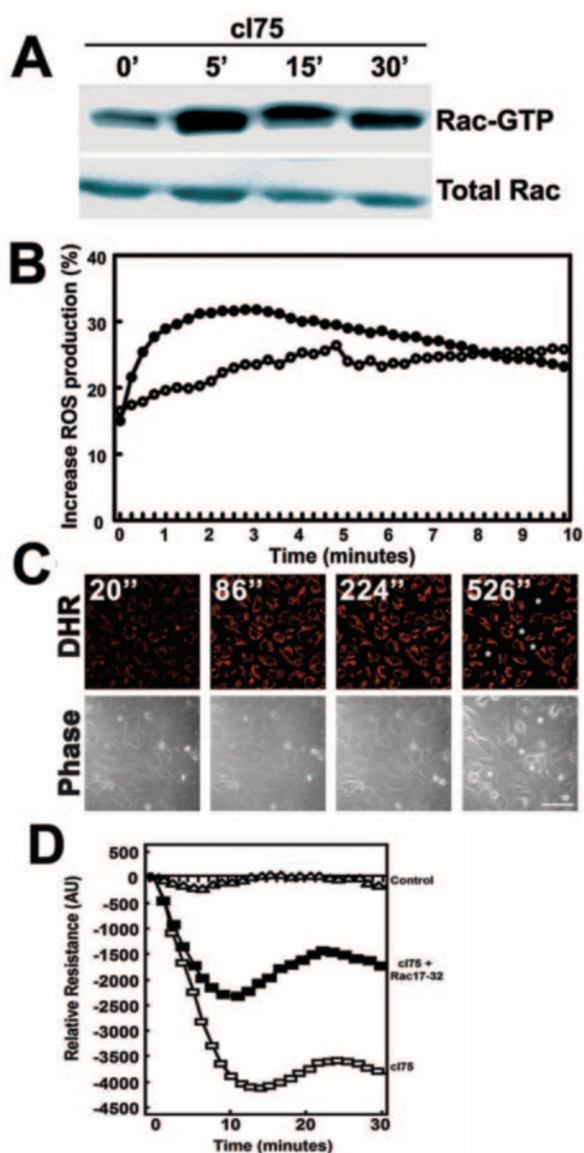
resistance of endothelial monolayers. This analysis shows that the VE-cadherin-blocking Ab cl75 rapidly reduces the transendothelial resistance, whereas the non-blocking Ab 7H1 did not have any effect on the resistance (Fig. 1A). Previous reports from our group and from others (15, 17, 22) have shown that blocking VE-cadherin Abs are able to disrupt endothelial junctions and induce a redistribution of VE-cadherin over the endothelial cell surface. We also found that  $\beta$ -catenin became diffusely distributed when the endothelial cells were treated with the cl75 Ab (Fig. 1B). To study whether a loss of VE-cadherin-mediated cell-cell adhesion induced the dissociation of the VE-cadherin complex from the actin cytoskeleton, we fractionated the cl75-treated endothelial cells and analyzed the cytoskeletal and the cytosol/membrane fractions for the presence of  $\alpha$ -catenin, which links VE-cadherin and  $\beta$ -catenin to the actin cytoskeleton. These experiments showed that

$\alpha$ -catenin shifted from the cytoskeleton to the membrane and cytosol fraction when VE-cadherin-mediated cell-cell contacts were disrupted (Fig. 1C). Also,  $\beta$ -catenin translocated to the same fraction as  $\alpha$ -catenin upon the loss of cell-cell contact (see Fig. 8B). These data indicate that “outside-in” signaling induced by the loss of VE-cadherin-mediated homotypic interaction dissociates the entire VE-cadherin complex from the cytoskeleton.

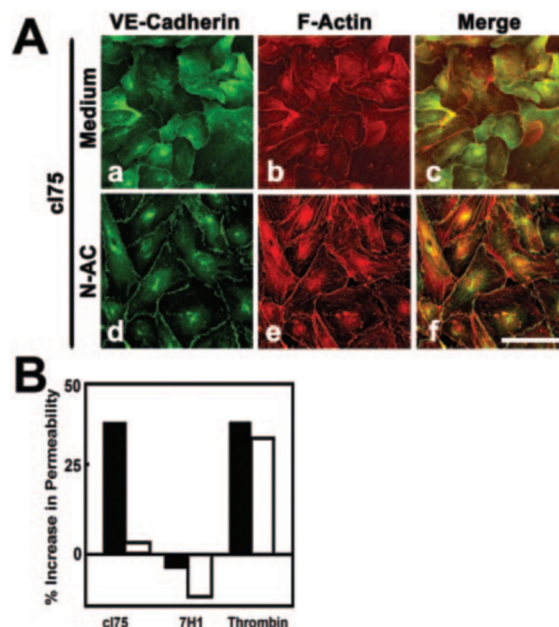
Previously, we have shown by protein transduction that an active mutant of the small GTPase Rac, RacV12, induced the loss of cell-cell contacts of confluent endothelial cells (8), in agreement with findings by others (24–26). In addition, Waschke *et al.* (27) showed recently that Rac1 inhibition leads to a loss of VE-cadherin-adhesive capacity. To test whether “outside-in” signaling induced by loss of VE-cadherin function involves Rac1, Rac1-GTP loading was measured by pull-down assays with glutathione *S*-transferase-p21-activating kinase. These experiments showed that cl75 indeed induces Rac1 activation (Fig. 2A). The response was highest at 5 min, after which it declined somewhat, although Rac1 activation remained elevated up to 30 min (Fig. 2A). Rac1 activation results in increased ROS production in neutrophils as well as in endothelial cells (8, 28, 29). Therefore, we tested the possibility that Rac activation, effected by the loss of VE-cadherin function, induces ROS production. Consistently, cl75-induced loss of VE-cadherin-mediated cell-cell contacts promoted a rapid increase in ROS production (Fig. 2B). Additional experiments showed that irrelevant antibodies such as isotype control IgG1 or medium changes did not mimic the induction of ROS. Moreover, the ROS production is followed by loss of cell-cell contacts, as is observed with real-time phase-contrast microscopy imaging (Fig. 2C, and supplemental movies). These observations indicate that activation of Rac1 and the production of ROS are rapidly induced following the loss of VE-cadherin function.

To define whether the cellular response to VE-cadherin-mediated outside-in signaling, *i.e.* the formation of intercellular gaps, in fact depends on Rac1 signaling, we used a cell-permeable peptide inhibitor of Rac1, Tat-Rac17-32. This peptide represents part of the effector loop of Rac1 and competes with Rac1-effector interactions, thus preventing downstream signaling (18, 30). After 30 min, cl75 had maximally reduced the endothelial resistance of untreated control monolayers (Fig. 2D). Tat-Rac17-32-incubated monolayers showed a reduced response to the cl75 treatment (~50% reduction in the loss of resistance, Fig. 2D). This indicates that the loss of cell-cell contact depends, at least in part, on Rac1 activity. Interestingly, under control conditions, we observed a small decrease in the electrical resistance of the endothelial monolayer when the confluent endothelial monolayers were incubated with Tat-Rac17-32 (data not shown). This finding suggests that, to maintain stable endothelial junctions, a low level of active Rac1 is required (27). In conclusion, these findings indicate that VE-cadherin-mediated outside-in signaling, resulting from a loss of cadherin function, requires Rac1 to induce loss of cell-cell adhesion.

Scavenging ROS by incubating the cells with *N*-acetylcysteine (N-AC) prevented the cl75-induced redistribution of VE-cadherin (Fig. 3A). In line with this result, the Ab-induced increase in endothelial monolayer permeability was prevented by N-AC (Fig. 3B). Interestingly, we found that N-AC decreased the permeability of 7H1-treated endothelial monolayers (Fig. 3B) and medium-treated monolayers (data not shown). The effect of thrombin on the permeability seemed to act independently from ROS, because N-AC was not able to prevent thrombin-induced permeability. These findings indicate that Rac-mediated production of ROS regulates the endothelial bar-



**FIG. 2. Loss of VE-cadherin-mediated cell-cell contact induces Rac1 activation and rapid generation of ROS.** *A*, cells were cultured and treated as indicated and as described under "Materials and Methods." Rac1 activation assay (*Rac.GTP*) shows that Rac1 was activated after 5 min of cl75 treatment (*Rac.GTP*). *Lower panel* shows immunoblot for Rac in the cell lysates (*Total Rac*). *B*, endothelial cells were cultured and grown to confluency on FN-coated glass coverslips and loaded with DHR as described under "Materials and Methods." Time-lapse recording of the increase in fluorescent signal quantified by confocal laser-scanning microscopy reflects the increased ROS production. Values at  $t = 0$  represent the basal levels of ROS in resting endothelial cells. *Closed circles* show ROS production in endothelial cells after cl75 treatment, *i.e.* loss of VE-cadherin function. *Open circles* show ROS production in untreated endothelial cells. A representative of three independent experiments is shown. *C*, endothelial cells were cultured and grown to confluency on FN-coated glass coverslips loaded with DHR, as described under "Materials and Methods," and treated with cl75 to induce the loss of cell-cell adhesion. Panels are fluorescence images taken from a time lapse video and show an increase in DHR signal in endothelial cells. Time in seconds is indicated in *upper left corner*. *Asterisks* represent sites of reduced cell-cell adhesion. A representative of two independent experiments is shown. *Bar*, 100  $\mu\text{m}$ . Videos of this experiment are available as Quick Time movies. Supplemental Movie 1 shows the time lapse series of the DHR signal. *C*, supplemental movie 2 shows the corresponding phase-contrast time lapse series. *D*, cells were treated for 30 min with either the Rac-inhibiting Tat-Rac17-32 peptide or left untreated and further processed as described under "Materials and Methods" and legend of Fig. 1*A*. *Open squares* represent the cl75-induced decrease in resistance. The reduction in resistance by cl75 of the Tat-Rac17-32-treated monolayers is indicated by the *closed squares*. *Triangles* show control levels of endothelial monolayer resistance. A representative of three independent experiments is shown.

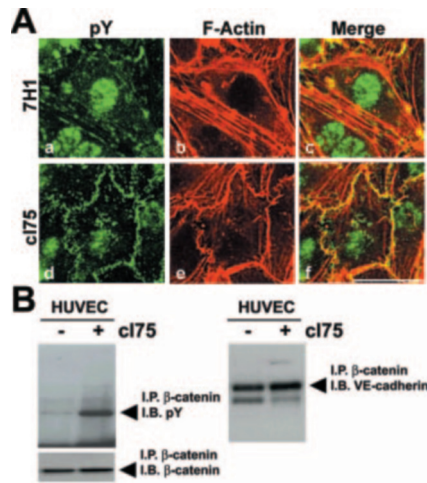


**FIG. 3. VE-cadherin-mediated loss of cell-cell contacts requires ROS and active Rac1.** *A*, endothelial cells were cultured and grown to confluency on FN-coated glass coverslips and were pre-treated with 5 mM oxygen radical scavenger N-AC overnight or left untreated. Subsequently, the cells were incubated with 12.5  $\mu\text{g/ml}$  cl75 (*a-f*) for 30 min. Scavenging of ROS prevented cl75-induced loss of cell-cell contacts. VE-cadherin is shown in *green* (*a* and *d*), F-actin is shown in *red* (*b* and *e*), and *yellow* shows co-localization between VE-cadherin and F-actin (*c* and *f*). Experiment was repeated twice with similar results. *Bar*, 50  $\mu\text{m}$ . *B*, scavenging ROS preserves cell-cell contacts. HUVEC were cultured on Transwell filters and incubated overnight with medium (*filled bars*) or N-AC (*open bars*) at 37  $^{\circ}\text{C}$  as indicated. Subsequently, cells were treated with cl75 or the non-blocking 7H1 or with 1 unit/ml thrombin as a positive control as described under "Materials and Methods" and FITC-labeled 3000 Dextran was added to the upper compartment of the Transwell. After 3 h, the level of fluorescence in the lower compartment was measured and calculated as the percentage increase compared with control levels. Control levels represent basal leakage of the endothelial monolayer incubated with medium alone. A representative of two independent experiments is shown.

rier function and that ROS are important mediators of VE-cadherin outside-in signaling.

Low levels of cell-cell contact are associated with tyrosine phosphorylation of the VE-cadherin complex (5). Since cl75 induces the loss of endothelial cell-cell contacts, we studied its effects on the tyrosine phosphorylation of junctional proteins in confluent monolayers. Immunocytochemical analysis showed that, in control situations, most tyrosine-phosphorylated proteins appear to reside at the end of actin stress fibers (Fig. 4*A*). Inhibiting VE-cadherin function for 10 min induced increased tyrosine phosphorylation at endothelial cell-cell junctions and a relative decrease in the amount of stress fibers (Fig. 4*A*). Biochemical analysis showed that  $\beta$ -catenin was the prime substrate for tyrosine phosphorylation within the cadherin complex (Fig. 4*B*). Additional analysis revealed that the loss of cell-cell contact did not result in the dissociation of VE-cadherin from  $\beta$ -catenin (Fig. 4*B*). These findings show that the loss of VE-cadherin function increased tyrosine phosphorylation of VE-cadherin-associated  $\beta$ -catenin.

Previous reports already suggested that changes in tyrosine phosphorylation of junctional proteins regulates cadherin-based cell-cell adhesion, although the mechanism is still unclear (5, 31, 32).  $\text{H}_2\text{O}_2$ , one of the ROS produced in endothelial cells, inhibits tyrosine phosphatase activity, resulting in increased tyrosine kinase activity, and is involved in many cell signaling pathways (10, 11). Pyk2, also known as RAFTK/

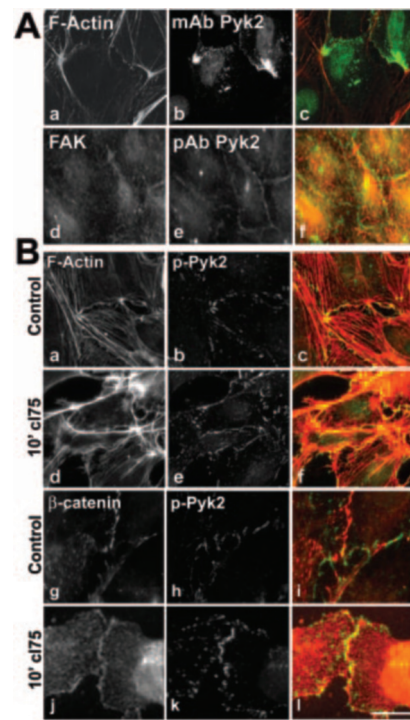


**FIG. 4. Loss of cell-cell contact induces tyrosine phosphorylation of junctional proteins.** *A*, endothelial cells were cultured and grown on FN-coated glass coverslips and treated with 7H1 (*a–c*) or cl75 (*d–f*) antibodies for 15 min. Cells were fixed, permeabilized, and stained for phosphotyrosine in green (pY; *a* and *d*) and F-actin in red (*b* and *e*). Merged images are shown in panels *c* and *f*. cl75 increased the levels of phosphotyrosine, in particular at the cell-cell junctions. Bar, 50  $\mu$ m. The nuclear staining is due to aspecificity of the polyclonal antibody. *B*, cells were cultured as described under “Materials and Methods” and treated for 30 min with cl75 followed by  $\beta$ -catenin immunoprecipitation (I.P.). Samples were analyzed by SDS-PAGE and subsequently analyzed by immunoblot (I.B.) with a specific mAb to pY. Left upper panel shows increased phosphotyrosine levels of  $\beta$ -catenin after 30 min of cl75-induced loss of cell-cell contacts in primary HUVEC. The right upper panel shows that VE-cadherin and  $\beta$ -catenin remain associated. The immunoprecipitation (I.P.) for  $\beta$ -catenin and immunoblots (I.B.) for VE-cadherin (7H1) show no changes in their association. The lower left panel shows equal amounts of  $\beta$ -catenin in the I.P. A representative of two independent experiments is shown.

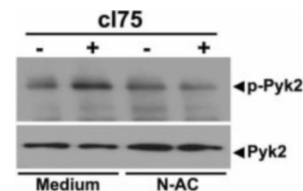
CAK $\beta$ /CADTK/FAK2, is activated through ROS in endothelial cells (33, 34). Using different antibodies against Pyk2, we found cytosolic as well as junctional localization of Pyk2 in endothelial cells, whereas FAK staining showed diffuse punctate localization (Fig. 5A). Using a phosphospecific antibody directed against the autophosphorylation site Tyr<sup>402</sup> of Pyk2, we found that activated Pyk2 translocates to the cell-cell junctions upon the loss of VE-cadherin-based adhesion and co-localizes with  $\beta$ -catenin (Fig. 5B). These findings implicate Pyk2 in the tyrosine phosphorylation of junctional proteins that drives the loss of VE-cadherin-based endothelial cell-cell adhesion.

Biochemical analysis showed that Pyk2 autophosphorylation was increased upon the loss of VE-cadherin function (Fig. 6). Because Pyk2 is redox-sensitive, we tested the role of ROS in its activation. Scavenging ROS with N-AC resulted not only in preventing cl75-induced loss of cell-cell adhesion and subsequent increased permeability (Fig. 3, A and B) but also prevented Pyk2 phosphorylation induced by the loss of cell-cell contact (Fig. 6). These data indicate that ROS that are rapidly generated upon loss of VE-cadherin cell-cell adhesion mediate Pyk2 activation.

To study the role of Pyk2 in the regulation of endothelial cell-cell adhesion in more detail, we used a Pyk2 deletion mutant (Fig. 7A, CRNK) that lacks the kinase domain and therefore acts as a dominant negative (20). Adenoviral transduction of  $10 \times 108$  plaque-forming units of Pyk2wt or  $1 \times 108$  pfu of CRNK constructs resulted in the efficient protein expression of these constructs in endothelial cells (Fig. 7B). Immunofluorescent imaging of endothelial monolayers showed that  $\sim 95\%$  endothelial cells expressed the transfected construct (Fig. 7C and data not shown). Pyk2wt showed cytosolic as well as junctional localization, whereas CRNK was more diffusely localized



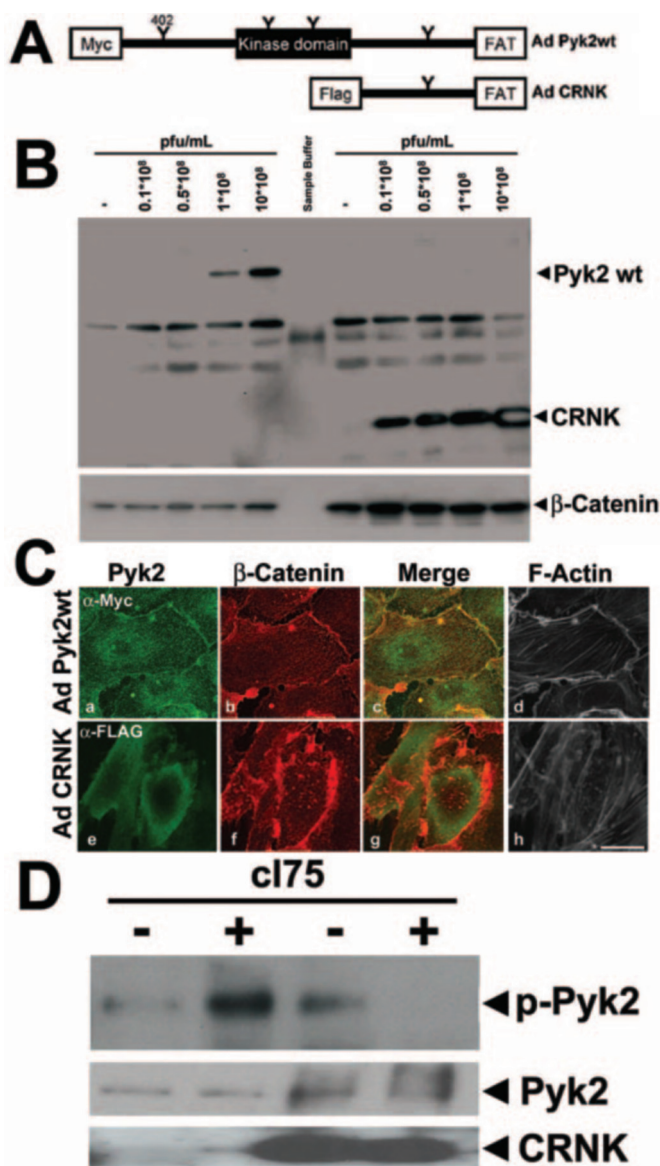
**FIG. 5. Phosphorylated Pyk2 localizes at cell-cell adhesion.** *A*, endothelial cells were cultured and grown on glass coverslips, left untreated and fixed, permeabilized, and stained for F-actin in red (*a*) or for Pyk2 with a monoclonal Ab in green (*b*). Merged image shows colocalization in yellow (*c*). FAK staining is shown in *d*. Pyk2 staining using polyclonal Ab is shown in *e*, and merge is shown in *f*. *B*, to study localization of p-Pyk2 upon the loss of VE-cadherin-based cell-cell adhesion, HUVEC were treated with cl75 for 10 min (cl75: *d–f*, *j*, *k*, and *l*) or left untreated (Control: *a–c* and *g–i*). Cells were fixed, permeabilized, and stained for pY402-Pyk2 (*a*, *d*, *g*, and *h*) or F-actin (*b* and *e*) or  $\beta$ -catenin (*h* and *k*). Merged images show colocalization between either F-actin and p-Pyk2 (*c* and *f*) or  $\beta$ -catenin and p-Pyk2 (*i* and *l*) in yellow. Bars, 50  $\mu$ m.



**FIG. 6. Loss of VE-cadherin-based cell-cell adhesion induces ROS-mediated tyrosine phosphorylation of Pyk2.** Cells were cultured as described under “Materials and Methods,” pretreated overnight with 5 mM ROS scavenger N-AC or left untreated, and subsequently treated with cl75 for 30 min. Cells were lysed in sample buffer and analyzed by SDS-PAGE. Results show that N-AC blocked the cl75-induced phosphorylation of Pyk2. Immunoblots were probed for p-Pyk2 (pY402; upper panel) or stripped and reprobed with Pyk2 to confirm equal protein loading (lower panel). A representative of three independent experiments is shown.

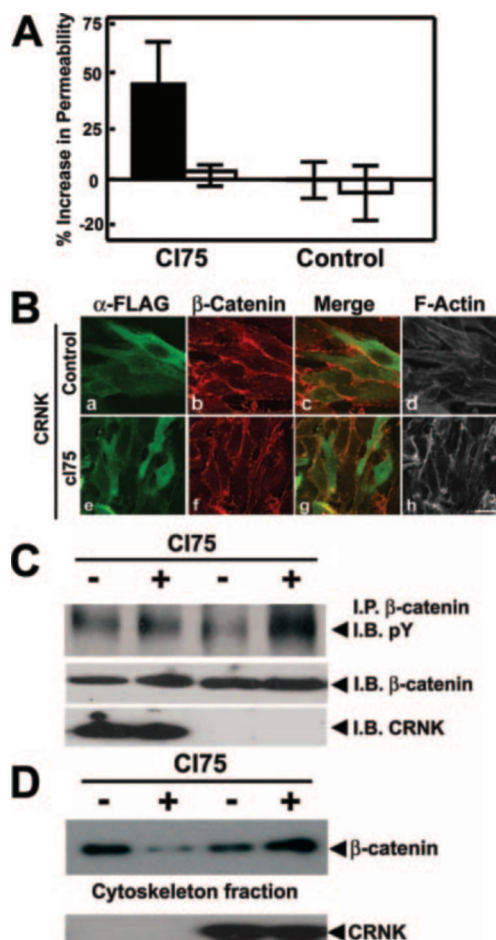
in the cytosol and largely absent at cell-cell junctions (Fig. 7C). Moreover, the expression of CRNK blocked Pyk2 phosphorylation upon the loss of VE-cadherin function (Fig. 7D). In line with this finding, the expression of CRNK also reduced the cl75-induced permeability of endothelial monolayers (Fig. 8A). Immunofluorescent images showed that CRNK-overexpressing endothelial monolayers remain intact after cl75 treatment, as deduced from the observation that  $\beta$ -catenin is still localized at sites of cell-cell junctions (Fig. 8B). Together, these data demonstrate that Pyk2 is an important regulator of endothelial junction stability.

In a final set of experiments, we focused on tyrosine phosphorylation events known to occur upon the loss of cell-cell



**FIG. 7. Expression, localization, and activation of Pyk2.** *A*, schematic overview of the adenoviral (*Ad*) Pyk2wt or CRNK constructs that were used to study Pyk2 regulation in endothelial cells. *B*, cells were cultured and grown to confluency as described under “Materials and Methods” and transduced overnight with different concentrations of plaque-forming units/ml Ad Pyk2wt or Ad CRNK. Subsequently, the cells were lysed in sample buffer and analyzed by SDS-PAGE. Immunoblots were probed with a polyclonal antibody directed against CRNK and showed increased expression of Pyk2wt (110 kDa) and CRNK (40 kDa) (upper panel).  $\beta$ -Catenin expression was shown to confirm equal protein levels (lower panel). A representative of two independent experiments is shown. *C*, cells were cultured on glass coverslips and transduced with either Ad Pyk2wt or Ad CRNK. Fixed and permeabilized cells were stained for the Myc or FLAG tag (*a* and *e*) and for  $\beta$ -catenin (*b* and *f*). Merge (*c* and *g*) shows co-localization in yellow, and actin is shown in grayscale (*d* and *h*). Bar, 20  $\mu$ m. *D*, cells were cultured as described under “Materials and Methods” and transduced overnight with Ad CRNK (right lanes). Subsequently, the cells were treated as indicated for 30 min, lysed in sample buffer, and analyzed by SDS-PAGE. Immunoblots were probed for p-Pyk2 (pY402; upper panel) or stripped and reprobed for total Pyk2 to confirm equal Pyk2 levels (middle panel). The lower panel shows the expression of the truncated Pyk2 mutant, CRNK, at 40 kDa. A representative of two independent experiments is shown.

adhesion (35). Because inhibition of Pyk2 by expressing CRNK prevented cl75-induced loss of cell-cell adhesion, we studied whether Pyk2 is involved in the tyrosine phosphorylation of  $\beta$ -catenin. Expression of CRNK prevented cl75-mediated tyro-



**FIG. 8. Pyk2 is involved in endothelial permeability and phosphorylation of  $\beta$ -catenin upon the loss of VE-cadherin function.** *A*, expression of CRNK prevents cl75-induced permeability. HUVEC were cultured and grown to confluency on Transwell filters and infected with Ad CRNK as described under “Materials and Methods” (open bars) or left untreated (closed bars). Subsequently, cells were treated with cl75 or left untreated as described under “Materials and Methods” and FITC-labeled 3000 Dextran was added to the upper compartment of the Transwell. After 3 h, the level of fluorescence in the lower compartment was measured and calculated as the percentage increase compared with control levels. Control levels represent basal leakage of the endothelial monolayer incubated with medium alone. The experiment is performed twice in triplicate. CRNK significantly reduces cl75-induced increase of endothelial monolayer permeability ( $p < 0.01$ ). *B*, expression of CRNK prevents cl75-induced loss of endothelial cell-cell adhesion. Cells were cultured on glass covers, infected with Ad CRNK, and treated with cl75. Fixed and permeabilized cells were stained for CRNK expression in green ( $\alpha$ -FLAG; *a* and *e*),  $\beta$ -catenin in red (*b* and *f*), and F-actin in grayscale (*d* and *h*). Merge shows CRNK and  $\beta$ -catenin localization (*c* and *g*). Bar, 20  $\mu$ m. *C*, Pyk2 mediates  $\beta$ -catenin phosphorylation. Cells were treated as described in the legend of Fig. 4*B* with the exception of the expression of CRNK (lower panel). Upper panel shows increased tyrosine phosphorylation of  $\beta$ -catenin after 30 min of incubation with cl75, which is inhibited by CRNK. The middle panel shows equal amounts of  $\beta$ -catenin per immunoprecipitation (I.P.). The experiment was done three times with similar results. *D*, Pyk2 mediates  $\beta$ -catenin association with the cytoskeleton. Cells were cultured as described in the legend of Fig. 1*C*. Immunoblotting for  $\beta$ -catenin shows that cl75 treatment induced a loss of  $\beta$ -catenin from the cytoskeletal fraction (upper panel), which is prevented by CRNK. The lower panel shows CRNK expression. A representative of three independent experiments is shown.

sine phosphorylation of  $\beta$ -catenin (Fig. 8*C*). Moreover, blocking Pyk2 signaling by expression of CRNK inhibited the cl75-induced dissociation of  $\beta$ -catenin from the actin cytoskeleton (Fig. 8*D*). This shows that Pyk2 regulates the interaction of the VE-cadherin complex with the actin cytoskeleton and that Pyk2 is involved in the regulation of VE-cadherin complex

stability and cell-cell adhesion by regulating the tyrosine phosphorylation of junctional  $\beta$ -catenin.

#### DISCUSSION

VE-cadherin is a key regulator of endothelial integrity. Given the role of the endothelium in a variety of physiological processes and disorders (*e.g.* coagulation, chronic inflammation, angiogenesis), insight into the mechanisms that control proper endothelial cell-cell adhesion is of great (clinical) importance. Reduced endothelial integrity is brought about by receptor-mediated signaling, *e.g.* by tumor necrosis factor- $\alpha$  or thrombin (25, 36), but also upon the migration of activated leukocytes across the endothelial lining of post-capillary venules (17, 37).

The current study focuses on signaling that controls endothelial integrity and is initiated upon the loss of VE-cadherin-mediated homotypic adhesion. We show that blocking VE-cadherin function with interfering antibodies results in changes in the cellular redox state in the actin cytoskeleton and in the phosphorylation of junctional proteins, leading to reduced endothelial barrier function. These antibodies are able to inhibit tumor growth, most probably by preventing the formation of new blood vessels (38–40). In addition, we and others (15, 17, 22) have reported that blocking VE-cadherin function with these antibodies results in increased permeability of endothelial monolayers and increased transendothelial migration of leukocytes *in vitro* as well as *in vivo*.

The observation that the antibody-mediated loss of VE-cadherin-based cell-cell adhesion can be prevented either by scavenging ROS, inhibiting Rac1, or Pyk2 signaling suggests that the disruption of the cell-cell adhesion is not simply due to the antibody physically inhibiting adhesion but due to the signaling pathways induced. The antibody will block homotypic interactions of the extracellular domains of VE-cadherin, which will probably lead to a conformational change within the cadherin molecules. This will trigger intracellular signaling, leading to Rac1 activation, ROS production, and increased tyrosine phosphorylation, resulting in impaired cell-cell adhesion.

Cadherin-induced signaling has recently received much interest. Several reports describe the use of cadherin engagement to induce intracellular signaling, thereby mimicking the formation of cadherin-based junctions. These studies showed that E-cadherin engagement promotes Rac1 activation (41–43). Interestingly, endothelial and epithelial cell-cell junctions are regulated differently by Rac1, as has been observed by our group as well as by others (8, 24, 25, 44). Active Rac1 promotes strong cell-cell adhesion in many epithelial cells but reduces cell-cell adhesion in endothelial cells, although membrane ruffling and cell spreading require Rac1 activity in both cell types. Whereas several studies have described the effects of active Rac1 on cell-cell junctions by introducing Rac1 mutants into the cells, in this paper we show that the initial loss of VE-cadherin-mediated cell-cell contacts induces rapid and prolonged activation of endogenous Rac1, which is also necessary for the loss of endothelial integrity. Thus, these data show that both the induction of cadherin function (41, 42) and the loss of cadherin function are more than just mechanical events and are associated with and even require intracellular signaling.

Active Rac1 elevates the production of ROS in several cell types (23, 45). One of these ROS,  $H_2O_2$ , mediates the oxidation of critical cysteine residues on tyrosine phosphatases, thereby deactivating these enzymes, which results in increased tyrosine kinase activity (11). In line with this finding, we discovered that the loss of VE-cadherin function increases the phosphotyrosine levels of the redox-sensitive kinase Pyk2 in a ROS-dependent manner (12, 13, 33, 34). The hypothesis that Pyk2 is involved in the regulation of cell-cell adhesion is

supported by the fact that Pyk2 co-localizes with  $\beta$ -catenin and our finding that Pyk2 is responsible for the increased tyrosine phosphorylation of  $\beta$ -catenin after the loss of VE-cadherin-based cell-cell adhesion. Ozawa and Kemler (23) reported that pervanadate treatment, leading to tyrosine kinase activation and phosphorylation of junctional proteins, results in the loss of E-cadherin function and cell-cell adhesion. These studies proposed that the dissociation of the cadherin complex from the actin cytoskeleton underlies the dysfunction of E-cadherin. In agreement with this notion, we found that the loss of VE-cadherin function by a blocking antibody induces the dissociation of the VE-cadherin-catenin complex from the actin cytoskeleton, which is prevented by a dominant-negative Pyk2 construct. These results support the idea that tyrosine phosphorylation of  $\beta$ -catenin and the interaction between the VE-cadherin-catenin complex and the actin cytoskeleton are critically regulated by Pyk2 (31). Thus, Pyk2 controls the strength of VE-cadherin-mediated cell-cell adhesion.

In conclusion, this paper provides new evidence concerning the molecular events that accompany endothelial damage, specifically triggered by the loss of VE-cadherin homotypic interactions. Our data implicate the Pyk2 tyrosine kinase in the induction of  $\beta$ -catenin phosphorylation and the resulting loss of cell-cell adhesion mediated by the Rac-ROS signaling pathway. Understanding the factors that regulate endothelial cell-cell junctions is important for a wide range of (patho)physiological processes in which vascular integrity is compromised, such as leukocyte extravasation, angiogenesis, ischemia-reperfusion injury, and chronic inflammatory disorders.

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