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Rap1 GTPase Inhibits Leukocyte Transmigration by Promoting Endothelial Barrier Function*[S]

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The passage of leukocytes out of the blood circulation and into tissues is necessary for the normal inflammatory response, but it also occurs inappropriately in many pathological situations. This process is limited by the barrier presented by the junctions between adjacent endothelial cells that line blood vessels. Here we show that activation of the Rap1 GTPase in endothelial cells accelerated *de novo* assembly of endothelial cell-cell junctions and increased the barrier function of endothelial monolayers. In contrast, depressing Rap1 activity by expressing Rap1GAP led to disassembly of these junctions and increased their permeability. We also demonstrate that endogenous Rap1 was rapidly activated at early stages of junctional assembly, confirming the involvement of Rap1 during junctional assembly. Intriguingly, elevating Rap1 activity selectively within endothelial cells decreased leukocyte transendothelial migration, whereas inhibiting Rap1 activity by expression of Rap1GAP increased leukocyte transendothelial migration, providing physiological relevance to our hypothesis that Rap1 augments barrier function of inter-endothelial cell junctions. Furthermore, these results suggest that Rap1 may be a novel therapeutic target for clinical conditions in which an inappropriate inflammatory response leads to disease.

The movement of molecules and cells out of the blood circulation and into tissues is governed by both the permeability properties of the endothelial cells lining blood vessels and the junctions between them (1). Thus, during inflammation and edema, the permeability of endothelial junctions is increased, allowing passage of leukocytes and fluid, respectively. Many factors affect vascular permeability, and generally this occurs by regulating endothelial cell junctions (2). The regulation of cell junctions is clearly important during the process of leukocyte transendothelial migration because this barrier must first be selectively opened to allow passage of the migrating leukocyte, followed by re-sealing. In general, permeability properties of the junctional barrier parallel the ability of leukocytes to complete transendothelial migration (3). Furthermore, it is thought that leukocyte binding to endothelial cell ligands prior to and during transendothelial migration leads to downstream signaling events within the endothelial cell, which are required for the appropriate regulation of junctional disassembly and reassembly following leukocyte transmigration (4).

Signaling molecules such as the small GTPases RhoA and Rac1 have been implicated in this junctional assembly, disassembly, and maintenance (5). More recently, work on another small GTPase, Rap1, has provided several lines of evidence suggesting a similar role for Rap1 in junctional regulation. Rap1 binds to the junctional protein AF-6/afadin (6) and Rap1 GTPase-activating proteins (GAPs),[1] and guanine nucleotide exchange factors are also recruited to tight junctions (7, 8); furthermore, GFP-Rap1 expressed in epithelial cells co-localizes with E-cadherin at cell junctions (9). Taken together, these data place Rap1 and its regulatory proteins in the right location to exert an effect on cell junctions. During *Drosophila* development, the positioning of adherens junctions between epithelial cells is disrupted if Rap1 is absent (10). In addition, an activator of Rap1, DOCK4, induces formation of adherens junctions when transfected into an osteosarcoma cell line that normally lacks junctions (11).

It is also known that elevated cAMP enhances the barrier function of endothelial cells (5). The mechanism has been interpreted as due to the activation of protein kinase A and its inhibition of myosin-generated tension on the junctions between endothelial cells (12). However, there are additional effects of cAMP on vascular permeability beyond the inhibition of cellular contractility (12). The synthesis of a novel cAMP analog, 8CPT-2′OMe-cAMP (13), has provided a tool to study cAMP-responsive pathways that are independent of protein kinase A; this analog cannot bind to protein kinase A, but it specifically binds to and activates the cAMP-responsive Rap1 guanine nucleotide exchange factor, Epac (13, 14). In this study, we ask whether endothelial junctional integrity (and therefore leukocyte transmigration) is affected by endothelial Rap1 activity.

EXPERIMENTAL PROCEDURES

Cell Culture—HUVECs and primary monocytes were obtained from Cambrex (East Rutherford, NJ) and cultured as described previously

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[1] The abbreviations used are: GAP, GTPase-activating protein; HBMEC, human brain microvascular endothelial cell; HUVEC, human umbilical cord endothelial cell; TER, transendothelial electrical resistance; GFP, green fluorescent protein; 8CPT-2′OMe-cAMP, 8-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate.

(15). HBMECs isolated from brain cortex were obtained from Applied Cell Biology Research Institute (Kirkland, WA) and cultured with the recommended media. The HL-60 promyelocytic cell line was obtained from the University of North Carolina Lineberger Comprehensive Cancer Center Tissue Culture Facility and grown in Optimem plus 5% fetal bovine serum. Differentiation to a neutrophil-like lineage was achieved by adding 1.3% Me_2SO for 3–5 days (16).

Transient Transfection and Immunofluorescence—GFP-Rap was transiently transfected into HUVECs using Amaxa Nucleofector Technology™ (Amaxa, Koeln, Germany) according to the manufacturer's protocol. After 24 h, cells were stimulated with 8CPT-2′OMe-cAMP for 10 min prior to fixation and co-staining.

HUVECs cultured on Matrigel (BD Biosciences)-coated coverslips were fixed and permeabilized as described elsewhere (17). The following primary antibodies were used: polyclonal anti-β-catenin antibody (Sigma), monoclonal anti-PECAM clone 9G11 (R&D Systems), monoclonal anti-VE-cadherin clone BV6 (Chemicon), and monoclonal anti-ZO-1 (Zymed Laboratories Inc.). Secondary antibodies used were Alexa 594-conjugated anti-rabbit IgG and Alexa 488-conjugated anti-mouse IgG (Molecular Probes). Coverslips were viewed with a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Thornwood, NY). Digital images were obtained with a MicroMAX 5-MHz charge-coupled device camera (Princeton Instruments), utilizing Metamorph imaging software (Universal Imaging Corp).

Calcium Switch Experiments—For junctional assembly, HUVEC monolayers were switched to Dulbecco's modified Eagle's medium plus 5% fetal bovine serum containing 4 mM EGTA for 30 min to disrupt Ca^{2+}-dependent cell-cell adhesion. EGTA was washed out by rinsing, and synchronous junctional reassembly was stimulated by re-addition of Dulbecco's modified Eagle's medium containing 1.8 mM $CaCl_2$ for the indicated lengths of time. For experiments monitoring junctional disassembly, EGTA-containing medium was added for various times. 8CPT-2′OMe-cAMP was obtained from Biolog Life Science Institute (Bremen, Germany) and used at a concentration of 50 or 250 μM (Fig. 5 only) to activate Rap1.

TER Measurements—Endothelial cells were plated at confluent density (3×10^5 cells/cm^2) on 12-mm-diameter, 0.4-μm-pore size Transwell filters and cultured for 48 h, with media replaced daily. TER was measured using an Endohm-12 Transwell chamber connected to an EVOM voltohmmeter according to the manufacturer's instructions.

Mannitol Flux Experiments—The flux of [^{3}H]mannitol was measured in the apical to basolateral direction with confluent HUVEC monolayers grown on Transwell filters as described for TER measurements. Cells were allowed to equilibrate in media containing 1 mM cold mannitol prior to adding 4 μCi/ml [^{3}H]mannitol to the apical chamber. After 30 min, a sample was removed from both the apical (upper) and basolateral (lower) compartment and then read in a liquid scintillation counter. The ratio of ^{3}H counts in the lower compartment divided by the counts in the upper compartment was plotted to represent paracellular permeability.

Adenoviral Infection—Recombinant adenoviruses engineered for expression of GFP and Rap1GAP were constructed by subcloning into the Adtrack-CMV vector (a gift of Robert Weinberg, Johns Hopkins University Medical Center) and then recombined with pAdEasy-1 in BJ5183 *Escherichia coli* (Stratagene.) The resulting DNA was transfected into HEK 293 cells with Lipofectamine (Invitrogen), and then the viruses were serially amplified and purified using Adeno-X™ Virus Purification Kits (BD Biosciences). HUVECs were infected at a multiplicity of infection of 5 for 3 h at 37 °C. Medium containing unabsorbed virus was then removed, and cells were cultured for 24 h in EGM-2. Infection efficiencies ranged from ~75% to 100%, as determined by immunofluorescence.

Rap1 Activity Assays—We performed Rap1 activity assays as described previously (18), with minor modifications. HUVECs were lysed in modified radioimmune precipitation assay buffer (50 mM Tris, pH 7.4, 75 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors, 1 mM NaF, and 1 mM sodium vanadate. Lysates were clarified by centrifugation and incubated with 50 μg of GST-RalGDS-RBD (provided by Dr. Leslie Parise, University of North Carolina at Chapel Hill) immobilized on glutathione beads to pull out active Rap1. The amount of active Rap1 and total Rap1 (reserved total cell lysate) was determined by Western blotting with a polyclonal anti-Rap1 antibody (Santa Cruz Biotechnology) that recognizes both Rap1A and Rap1B, followed by a horseradish peroxidase-conjugated secondary antibody (Chemicon). Where indicated, HUVECs were incubated with 1 unit/ml thrombin (Sigma) for 30 min in EGM-2. As a positive control for Rap activation, HUVECs were pre-treated with 50 μM 8CPT-2′OMe-cAMP for 10 min. Quantification of Rap1 activity was performed by densitometric analysis of Western blots using ImageJ software. The relative amount of active Rap1 was calculated by dividing the amount of active Rap1 by the total amount from cell lysates.

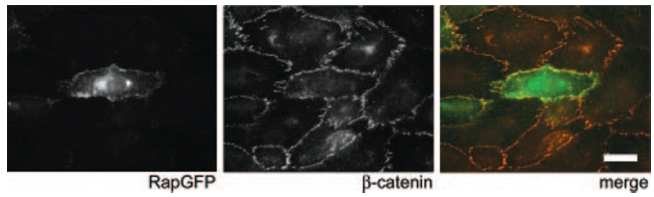

FIG. 1. **GFP-Rap1 co-localizes with β-catenin at endothelial cell junctions.** HUVECs were transiently transfected with a GFP-Rap1 construct, followed by co-staining for the adherens junction marker β-catenin. The *merged image* shows co-localization of GFP-Rap1 and β-catenin as *yellow*. *Bar* = 25 μm.

Leukocyte Transendothelial Migration Experiments—Quantitative transendothelial migration experiments were performed using HUVECs plated at confluent density (7.5×10^5 cells/cm^2) on Transwell filter inserts (6.5-mm diameter, 8-μm pore size). After 2 days, cells were activated with 5 ng/ml interleukin-1β overnight (R&D Systems). Where indicated, HUVECs were pre-treated with 8CPT-2′OMe-cAMP for 15 min, and then it was removed by washing so that it was absent during the incubation with leukocytes. Differentiated HL-60 cells were added to the top well (1×10^6 cells) and allowed to transmigrate for 30 min, after which time the medium from the bottom well was collected, and transmigrated cells were counted using a hemacytometer.

For video microscopy, HUVECs plated on glass-bottomed MatTek plates (MatTek Corp., Ashland, MA) were activated with tumor necrosis factor α (30 ng/ml) for 4 h. Primary monocytes were added to the HUVEC monolayer for 10 min to allow adhesion, and then non-adherent cells were washed away with fresh media. Cells were incubated in an environmentally controlled chamber at 37 °C with 10% CO_2. Transmigration was monitored by phase video microscopy (Olympus 1X81) at ×20 magnification. Images were collected every 15 s for 15 min with a Hamamatsu C4880 charge-coupled device camera driven by Metamorph imaging software.

RESULTS

The subcellular localization of Rap1 has been described variously as perinuclear (19), endosomal (20), plasma membrane (21), or even nuclear (22), depending on cell type and culture conditions. In epithelial cells, recent work has demonstrated that Rap1 can be found at sites of cell-cell contact (9). We examined the localization of Rap1 in endothelial cells by expressing GFP-Rap1 in HUVECs. As shown in Fig. 1, GFP-Rap1 is concentrated at the cell periphery, co-localizing with the adherens junction protein β-catenin, confirming that Rap1 is present at cell junctions in endothelia as it is in epithelia (9, 23).

To study the role Rap1 plays in junctional regulation in endothelial cells, we took advantage of a newly developed drug to specifically activate Rap1. 8CPT-2′OMe-cAMP binds to and activates the Rap1 exchange factors Epac1 and Epac2 but does not activate other cAMP-responsive pathways involving protein kinase A and other downstream pathways, *e.g.* Ras and extracellular signal-regulated kinase (13). We first determined whether our endothelial cell system was responsive to this compound by treating HUVEC monolayers with 8CPT-2′OMe-cAMP and found a robust increase in the amount of active Rap1 compared with untreated cells (Fig. 2). In addition, no effect was seen on the activity of the Rho-family GTPases RhoA, Rac1, or Cdc42 in HUVECs with this treatment (data not shown).

Because this compound gave us a specific means to manipulate Rap1 activity in endothelial cells, we looked at the assembly of intercellular junctions when Rap1 is thus activated by using the calcium switch experimental paradigm. This system is based on the observation that both adherens junctions and tight junctions are disrupted when cells are switched to medium containing EGTA to chelate extracellular calcium (24,

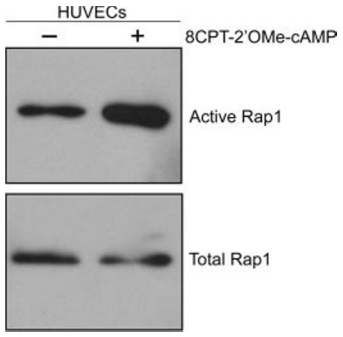

FIG. 2. **HUVECs treated with 8CPT-2′OMe-cAMP have increased Rap1 activity.** Confluent monolayers of HUVECs were treated with or without 50 μM 8CPT-cAMP for 10 min. The amount of active GTP-bound Rap1 was determined by a Rap1 activity assay, detected by Western blotting, and compared with total Rap1 from cell lysates as a control for equal protein concentration. Cells treated with 8CPT-2′OMe-cAMP have significantly higher levels of active Rap1 than untreated HUVECs.

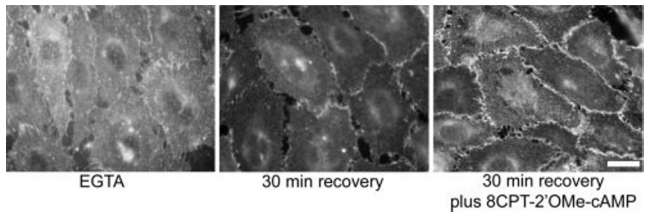

FIG. 3. **HUVECs treated with 8CPT-2′OMe-cAMP to elevate Rap1 activity reassemble cell junctions faster.** β-Catenin staining of HUVEC monolayers after complete junctional disassembly (*left panel*) or after re-addition of calcium-containing media for 30 min to induce junctional reassembly in the absence or presence of 50 μM 8CPT-2′OMe-cAMP (*middle* and *right panels*, respectively). Cells treated with this compound reassemble junctions more rapidly than untreated controls. *Bar* = 20 μm.

25), due to the loss of calcium-dependent cadherin-mediated adhesion. Subsequent washout of EGTA and restoration of physiological levels of calcium result in the synchronous *de novo* assembly of both junction types. HUVEC monolayers were subjected to EGTA treatment, followed by washout and readdition of calcium-containing media with or without 8CPT-2′OMe-cAMP for 30 min. β-Catenin is more quickly recruited to sites of cell contact in the presence of 8CPT-2′OMe-cAMP compared with HUVECs recovering in the absence of 8CPT-2′OMe-cAMP (Fig. 3). This suggests that Rap1 activity enhances endothelial cell junctional assembly.

To confirm this, we used another measure of junctional integrity, TER. When cultured in the presence of the Rap1-activating compound for various times, HUVEC monolayers demonstrate a slight but statistically significant increase (5–10%) in TER compared with untreated monolayers (data not shown). The low basal TER of HUVECs may make it difficult to see junction enhancing effects of Rap1 activation. Therefore, we also used HBMECs, which make up the more electrically impermeable blood-brain barrier (26). When we performed this TER experiment using HBMECs, we observed a clear increase in TER (~40% increase) when cells were treated with 8CPT-2′OMe-cAMP (Fig. 4*A*). These experiments demonstrate that activating Rap1 not only enhances the assembly of cell junctions (Fig. 3) but also promotes a more electrically impermeable monolayer (Fig. 4*A*). To confirm the TER result, we also utilized mannitol flux assays as a second means to measure paracellular permeability of HUVECs. As shown in Fig. 4*B*, monolayers treated with the Rap-activating compound have decreased permeability to mannitol, further confirming that endothelial barrier function is enhanced when Rap1 is activated.

What happens in the converse situation, when Rap1 activity is inhibited? We inhibited Rap1 activity in endothelial cells by utilizing an adenovirus expression system to express Rap1GAP. Rap1GAP exhibits specific GAP activity toward Rap1, but not toward other related GTPases such as Rap2, Ras, and the Rab family (27). We repeated the mannitol flux permeability assay on HUVECs expressing either GFP or Rap1GAP, treating them with or without 8CPT-2′OMe-cAMP (Fig. 4*C*). Activating Rap1 with 8CPT-2′OMe-cAMP in GFP-expressing HUVECs significantly reduced permeability to mannitol, as was observed in uninfected HUVECs (Fig. 4*B*). However, expression of Rap1GAP to inhibit Rap1 activity enhanced the monolayer permeability to mannitol, and this could not be reversed by co-treatment with 8CPT-2′OMe-cAMP (Fig. 4*C*).

Because Rap1 activity seemed to exert positive effects on junctional assembly, we hypothesized that junctions would be resistant to disassembly under conditions of high Rap1 activity. Because the endothelial barrier is composed of both adherens junctions and tight junctions, we treated HUVECs for 1, 15, or 30 min with EGTA in the presence or absence of 250 μM 8CPT-2′OMe-cAMP and then stained for β-catenin (Fig. 5*A*) and the tight junction protein ZO-1 (data not shown). After 1 min of EGTA treatment, HUVECs without 8CPT-2′OMe-cAMP already exhibit disrupted β-catenin and ZO-1 localization and loss of cell adhesion, whereas HUVECs treated with 8CPT-2′OMe-cAMP to activate Rap1 retained a significant degree of junctional integrity. This resistance to disassembly when Rap1 is activated was also observed after 15 and 30 min of EGTA treatment relative to controls. We also utilized thrombin treatment for various time points as a more physiological means to disrupt endothelial cell junctions. Fig. 5*B* demonstrates that 30 min of thrombin treatment (5-, 15-, and 60-min treatments; data not shown) induces junctional disassembly (loss of continuous β-catenin membrane staining; ZO-1, data not shown) in untreated cells (*minus 8CPT-2′OMe-cAMP*). However, when Rap1 is specifically activated by 8CPT-2′OMe-cAMP co-treatment, there is a resistance to junctional disassembly similar to that observed with the calcium switch in Fig. 5*A*. In certain cell types, particularly platelets, thrombin induces robust activation of Rap1 (18). We compared Rap1 activity in HUVECs that were untreated or treated with 1 unit/ml thrombin for 30 min (as in Fig. 5*B*) and/or 50 μM 8CPT-2′OMe-cAMP. The representative blot in Fig. 5*C* shows that in HUVECs, Rap1 is not activated by thrombin under these conditions. Quantification of blots from three independent experiments confirmed this result (Fig. 5*C*, *graph*).

The preceding experiments reveal that in endothelial cells, increasing Rap1 activity promotes junctional assembly and barrier function. How is the localization of junctional proteins affected when Rap1 activity is inhibited by expression of Rap1GAP during junctional assembly induced by a calcium switch? We first looked at the effect on β-catenin localization in untreated HUVEC monolayers expressing GFP alone or co-expressing Rap1GAP and GFP as a marker for infected cells. Expression of Rap1GAP but not GFP alone significantly disrupted the junctional localization of β-catenin in the monolayer (Fig. 6*A*). The typical continuous plasma membrane localization of other endothelial cell junction proteins such as VE-cadherin and ZO-1 (Fig. 6*B*), as well as PECAM and α-catenin (data not shown), was also impaired when Rap1GAP but not GFP alone was expressed. When we subjected these same cells to a calcium switch (Fig. 6*C*), the monolayer of GFP-expressing cells completely reassembled its cell junctions within 1 h of recovery, whereas the monolayer composed of Rap1GAP-expressing cells did not. The Rap1GAP-expressing cells remained more rounded and often exhibited delayed or absent recruit-

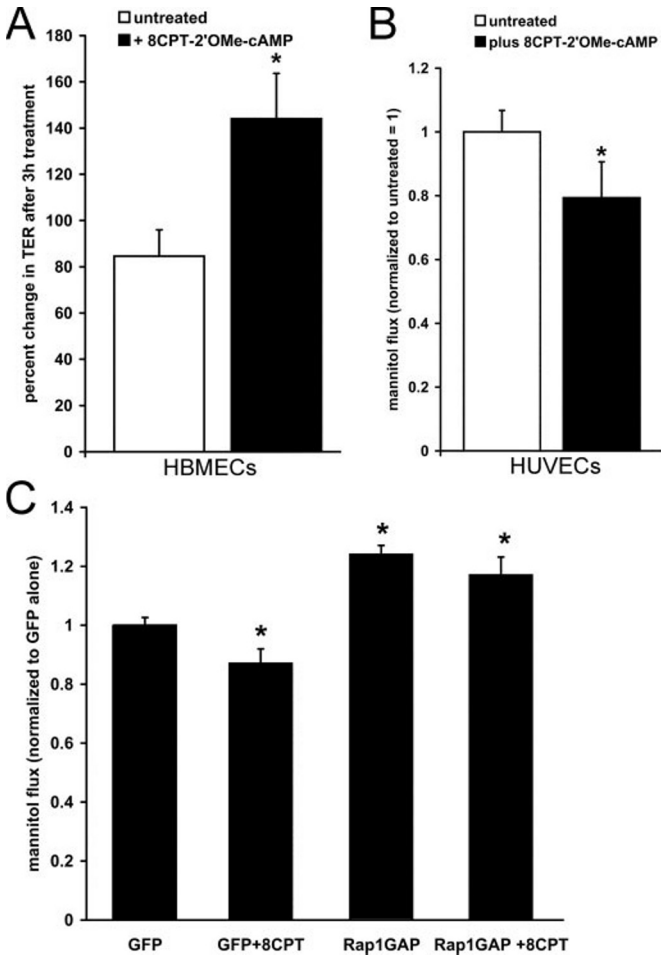

FIG. 4. **Activating Rap1 with 8CPT-2′OMe-cAMP increases endothelial cell electrical resistance and decreases paracellular permeability; inhibition of Rap1 increases permeability.** *A*, TER of HBMECs was measured after 3 h in the presence or absence of 50 μM 8CPT-2′OMe-cAMP. The results from independent experiments are plotted as the percentage change after 3 h treatment ± S.D. ($n = 6$ filters for each condition; *, $p = 0.000077$). *B*, permeability to mannitol was measured on HUVEC monolayers treated in the presence or absence of 8CPT-2′OMe-cAMP. The results are plotted as mean permeability after 30 min of incubation ± S.D., normalized to untreated = 1 ($n = 5$ filters for each condition; *, $p = 0.008$). *C*, adenovirus-mediated expression of GFP alone or Rap1GAP in HUVECs, followed by mannitol permeability experiments done in the presence or absence of 50 μM 8CPT-2′OMe-cAMP. Rap1GAP inhibition of Rap1 activity increases the permeability of mannitol across a HUVEC monolayer. Expression of Rap1GAP was confirmed visually by immunofluorescence. The results are plotted as mean mannitol flux after 30 min of incubation ± S.D., all normalized to GFP-expressing HUVECs = 1 ($n = 3$ filters for each condition; *, $p < 0.05$).

ment of β-catenin to nascent cell junctions. Note the cells in the center of the field (Fig. 6*C*, *asterisk*) that are not expressing Rap1GAP; these cells do form β-catenin-positive cell junctions after 1 h. Collectively, these experiments indicate that Rap1 activity is involved in the formation and maintenance of both adherens junctions and tight junctions in endothelial cells and furthermore, that these processes are impaired when Rap1 activation is inhibited.

The previous experiments utilize an artificial means of stimulating Rap1 activity by treatment with the Rap-activating compound 8CPT-2′OMe-cAMP. We wanted to know whether activation of endogenous Rap really occurs in the cell during dynamic junctional rearrangements. To this end, we performed Rap1 activity assays (18) on HUVECs that were subjected to a calcium switch to induce synchronous junctional reassembly for various lengths of time. As shown in Fig. 7, there is an increase in Rap1 activity within 1–5 min of EGTA washout. Therefore, endogenous Rap1 is, in fact, rapidly activated at the early stages of junctional assembly in endothelial cells.

Dynamic modulation of endothelial cell-cell junctions is a requirement for the physiological process of leukocyte transmigration. We performed quantitative transmigration assays using differentiated HL-60 cells as a model leukocyte-like cell line. HUVECs grown on Transwell filter inserts were pre-treated with 8CPT-2′OMe-cAMP or media alone for 15 min, which was then washed out before the addition of the HL-60 cells to the apical side of the monolayer. Rap1 activity remained elevated in HUVECs for at least an hour after washout of 8CPT-2′OMe-cAMP (data not shown), so transmigration was allowed to proceed for 30 min to stay within that time frame. Fewer HL-60 cells (~60%) were able to migrate across an endothelial monolayer that was pre-treated with the Rap-activating compound than across an untreated monolayer (Fig. 8*A*). We confirmed this result using primary monocytes by analysis of video microscopy (see the supplemental data), scoring individual monocytes on the basis of whether they were adherent to the apical surface of the HUVECs yet stayed rounded up, whether they flattened and moved around on the apical surface, or whether they completed diapedesis. Fig. 8*B* shows that whereas 40% of the counted monocytes completed transmigration across the untreated monolayer, only 12% were able to cross the monolayer that was pre-treated with 8CPT-2′OMe-cAMP. The inhibition of transmigration was not due to an effect on the ability of the monocytes to adhere and migrate across the surface of the monolayer because the number of monocytes that were scored as being flattened and migratory under these conditions was not decreased. In fact, analysis of the video indicates a greater number of monocytes migrating around on the surface of 8CPT-2′OMe-cAMP-treated HUVECs, and often these cells seem to use membrane protrusions to probe at the intercellular regions between adjacent HUVECs. Finally, to directly confirm that Rap1 plays a role in the inhibition of leukocyte transendothelial migration, we used our adenovirus expression system to express either GFP alone or Rap1GAP in HUVECs and then performed Transwell transmigration assays. Expression of Rap1GAP to inhibit Rap1 activity in HUVECs significantly increased leukocyte transendothelial migration by almost 2-fold (Fig. 8*C*). Together, these experiments show that Rap1 enhancement of endothelial cell junction integrity has implications for leukocyte transendothelial migration.

DISCUSSION

The results of this study reveal that the small G protein Rap1 plays a key role in the regulation of endothelial cell junctions and that the activation of Rap1 enhances endothelial cell barrier function. Activating Rap1 with 8CPT-2′OMe-cAMP exerts a positive effect on endothelial cell junctions, as determined by the recruitment of junction-specific proteins to sites of cell contact, by an increase in endothelial cell TER with corresponding decrease in paracellular permeability of mannitol, and by the observation that HUVECs become more resistant to junctional disruption when Rap1 is activated. Conversely, inhibiting Rap1 activity by expression of Rap1GAP induces widespread loss of cell-cell adhesion, disruption of both adherens junction and tight junction protein components, and decreased endothelial barrier function (TER, mannitol permeability). We also show that activation of endogenous Rap1 occurs during junctional remodeling, with a rapid increase in activity during very early stages of junctional assembly. Collectively, these data are consistent with the existence of two synergistic mechanisms of endothelial barrier enhancement involving cAMP: the previously described protein kinase A-dependent inhibition of myosin-generated tension on the junctions (28), and the protein kinase A-independent mechanism

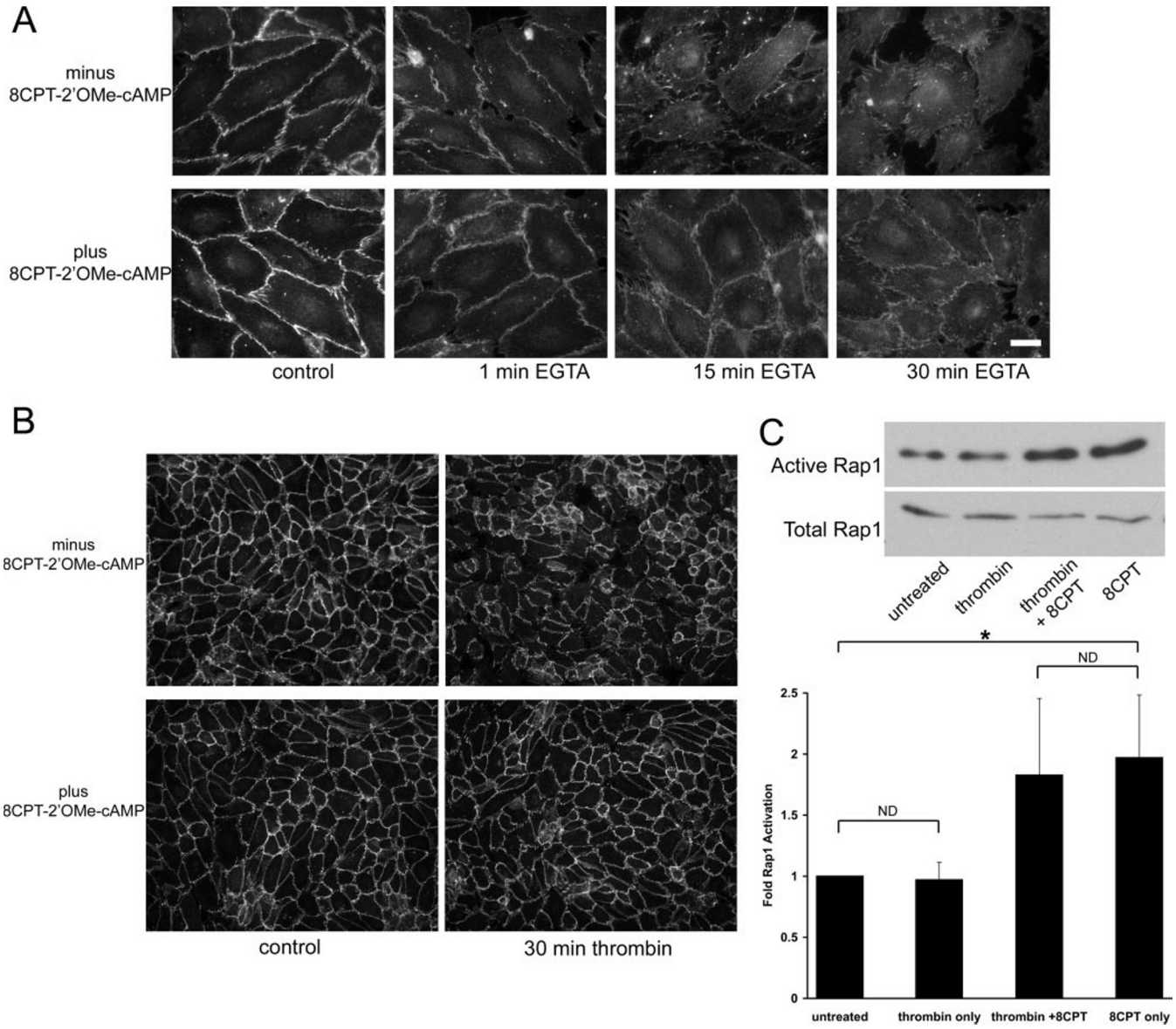

FIG. 5. **Activating Rap1 makes HUVECs more resistant to junctional disassembly.** *A*, HUVEC monolayers were pre-treated with or without 8CPT-2′OMe-cAMP for 10 min and then incubated for 0, 1, 15, or 30 min with EGTA to disrupt intercellular junctions, in the presence or absence of 250 μM 8CPT-2′OMe-cAMP. In the absence of 8CPT-2′OMe-cAMP, mislocalization of β-catenin and disruption of intercellular contacts are evident beginning at 1 min of EGTA treatment. When Rap1 is activated by 8CPT-2′OMe-cAMP, β-catenin-positive cell junctions are relatively resistant to disassembly, even after 30 min in EGTA. *Bar* = 20 μm. *B*, HUVECs were treated with or without 8CPT-2′OMe-cAMP as described above and incubated with 1 unit/ml thrombin for 0 or 30 min to disrupt intercellular junctions (visualized by β-catenin staining). Disruption of continuous β-catenin membrane staining occurs after 30 min of thrombin treatment in the absence of 8CPT-2′OMe-cAMP, whereas the localization remains indistinguishable from controls when Rap1 is activated in the presence of 8CPT-2′OMe-cAMP. *C*, thrombin treatment under these same conditions does not activate Rap1 in HUVECs. Rap1 activity assays were performed on HUVECs incubated with 1 unit/ml thrombin and/or 50 μM 8CPT-2′OMe-cAMP for 30 min. A representative blot is shown, and the graph shows quantification of Rap1 activity from three independent experiments combined ($n = 3$; *, $p < 0.05$; *ND*, not significantly different).

involving Epac and Rap1 described here. Finally, and most interestingly in a physiological context, is the observation that Rap1 enhancement of junctional barrier function in HUVECs results in the inhibition of transendothelial migration of leukocytes.

Our observation that activating Rap increases TER in HUVECs (data not shown) was observed consistently, and although the differences were quite subtle (5–10% increase), they were statistically significant. We believe that the small effect on TER is due to the fact that HUVECs have very low resistance (25–30 ohms/cm^2) compared with epithelial cell lines such as MDCK strain II (reported values range from 50 to 300 ohms/cm^2) (29). We addressed this issue in two ways; first, we performed the same experiment with human brain microvascular endothelial cells, which comprise the tightly sealed blood-brain barrier (26), and saw a much more robust (~40%) TER increase when Rap1 was activated (Fig. 4*A*); and second, we utilized mannitol flux as a more reliable method of assessing barrier properties of low electrical resistance cell types such as HUVECs (Fig. 4*B*). Our permeability assays are corroborated by a study (23) published during the review of our manuscript, showing decreased permeability to a dextran marker when endothelial cells were treated with forskolin or 8CPT-2′OMe-cAMP; our results additionally show an affect on TER.

Our results suggest that Rap1 modulation is particularly critical when junctions are undergoing dynamic changes such as assembly and disassembly. We do not see the consistent or dramatic changes in the pattern of junctional protein staining

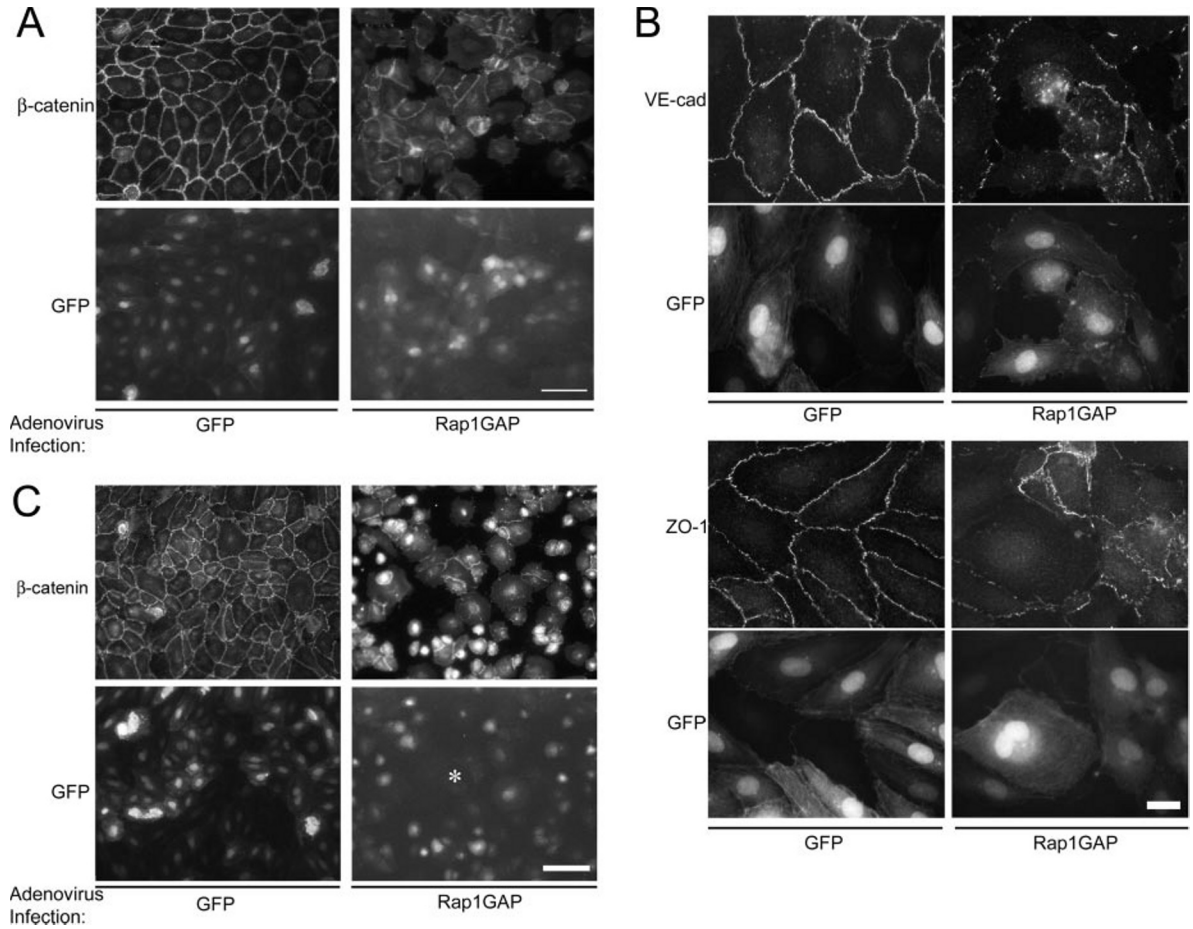

FIG. 6. **Rap1GAP expression in HUVECs causes junctional disassembly.** *A*, confluent HUVECs expressing either GFP alone or co-expressing Rap1GAP and GFP (as a marker for infected cells) were stained for β-catenin. HUVECs infected with GFP alone retain a β-catenin-positive cell junction staining pattern, whereas cells expressing Rap1GAP show significantly disrupted β-catenin localization. *Bar* = 100 μm. *B*, expression of Rap1GAP in HUVEC monolayers disrupts the localization of other adherens junctional proteins such as VE-cadherin (*top panel*) and the tight junction protein ZO-1 (*bottom panel*). *Bar* = 20 μm. *C*, cells subjected to a calcium switch followed by 1 h of junctional reassembly. β-Catenin recruitment to junctions is complete after 1 h in GFP cells (*top left panel*) but is delayed or absent in Rap1GAP cells (*top right panel*). *Asterisk* indicates an area of uninfected cells. *Bar* = 100 μm.

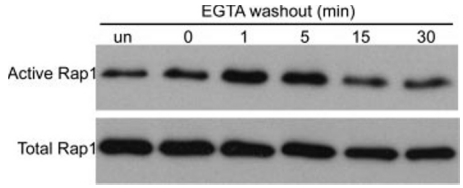

FIG. 7. **Endogenous Rap1 activity is increased at early stages of junctional reassembly.** Dishes of confluent HUVECs were serum-starved in Dulbecco's modified Eagle's medium plus 0.1% fetal bovine serum for 30 min before the start of the experiment and then treated with EGTA for 30 min to disrupt cell junctions. EGTA was washed out, and then Rap1 activity assays were performed after 0, 1, 5, 15, and 30 min of recovery. Rap1 activity is rapidly elevated from 1 to 5 min of EGTA washout.

when HUVECs at steady state are treated with 8CPT-2′OMe-cAMP (Fig. 5, *left panels*) that others have reported (23). However, we do see an obvious effect when cell junctions are actively assembling or disassembling junctions in response to a calcium switch (Figs. 3 and 4, *EGTA-treated panels*). It is also important to note that in addition to using EGTA treatment as a means of studying junctional disassembly, we have also used thrombin treatment to disrupt endothelial cell junctions and observed the same result (Fig. 5*B*); increasing Rap1 activity provides a degree of protection from agents that cause junctional disassembly. It is interesting that in platelets, thrombin activates Rap1 (18), whereas we have not detected any elevation in Rap1 activity in HUVECs treated with thrombin; this is consistent with thrombin promoting endothelial junctional disassembly during inflammation.

Importantly, we have shown that during junctional assembly following EGTA washout, there is a detectable increase in endogenous Rap activity (Fig. 7), and as in platelet activation (18), it is a rapid, transient, and early signal. We hypothesize that engagement of VE-cadherin during junctional reassembly is a possible mechanism for inducing Rap1 activation. We and others have previously demonstrated that E-cadherin engagement can directly induce Rac activation (30, 31). This phenomenon has now been demonstrated for E-cadherin and Rap1 in epithelia. Using an E-cadherin extracellular domain construct, Hogan *et al.* (9) observed that adhesion to this chimeric construct increases Rap1 activity. Similarly, Price *et al.* (32) show that E-cadherin-mediated adhesion is inhibited if Rap1 activity is suppressed. Our data advance these observations by verifying that endogenous Rap1 can be activated during actual junctional reassembly processes in endothelial cells.

One cytoplasmic protein that likely plays a key role in linking Rap1 signaling to junctional assembly is AF-6/afadin. AF-6 is present at both tight junctions and adherens junctions (33, 34), as well as nectin-based cell adhesion sites (35), and can be detected in HUVECs by Western blot (data not shown). AF-6 can interact with tight junction proteins, ZO-1 (34), JAM (36), and ZO-3 (17) via its Ras-binding domain, and active GTP-bound Ras competes with ZO-1 for binding to AF-6 (37). More

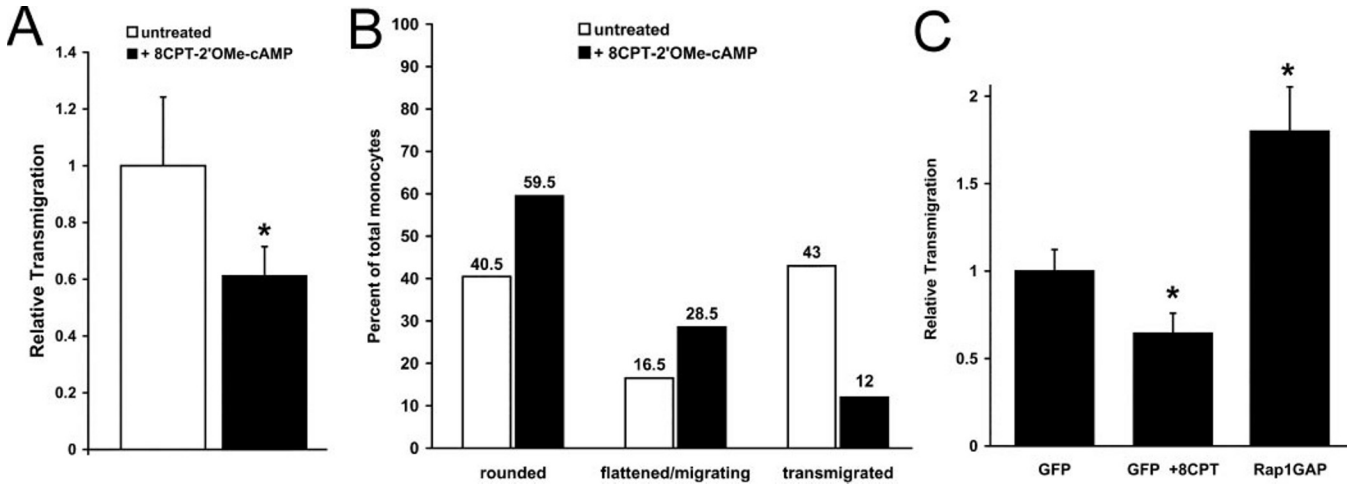

FIG. 8. **Activation of endothelial Rap1 inhibits transendothelial migration of leukocytes, whereas inhibition of Rap1 enhances leukocyte transendothelial migration.** *A*, Transwell assays were used to quantify the ability of differentiated HL-60 cells to transmigrate across HUVEC monolayer pre-treated for 15 min with 8CPT-2′OMe-cAMP or media alone. Transendothelial migration is reduced by ~40% when HUVECs are pre-treated with 8CPT-2′OMe-cAMP. Data are from two combined representative experiments ($n = 6$ Transwells; $p < 0.05$). *B*, quantification of primary monocyte transmigration using video microscopy. Video analysis allowed for scoring individual monocytes on the basis of whether they remained rounded and immobile, became flattened and motile, or completed transmigration. Fewer monocytes were able to transmigrate across the HUVEC monolayer pre-treated with 8CPT-2′OMe-cAMP ($n = 79–84$ cells). *C*, transendothelial migration assays were performed as described in *A*, with HUVECs expressing either GFP alone or Rap1GAP. Rap1GAP-expressing cells allowed a significantly higher number of differentiated HL-60 cells to transmigrate. The graph shows a representative experiment ($n = 3$ Transwells for each condition, $p < 0.05$).

recently, AF-6 was also found to interact with Rap1 and also with the Rap GAP SPA-1 through a domain that is conserved among Rap GAPs (7). In epithelial cells, Ras activation disrupts cell-cell junctions and induces a mesenchymal, migratory phenotype (38–40); in contrast, Price *et al.* (32) observed that Rap1 activation in Ras-transformed cells can counteract this mesenchymal transformation. Thus, this result, combined with our observation that Rap activation enhances junctional assembly in endothelial cells, shows that Ras and Rap1 appear to regulate junctions in opposite ways, and the putative effector AF-6 is in a position to play a key role in this process. Future experiments will explore the role of AF-6 at junctions and address which cell adhesion molecules mediate the outside-in signaling to Rap1 during junctional assembly and during events such as leukocyte transmigration.

Rap1 GTPase plays a prominent signaling role in leukocytes, platelets, and T cells, particularly relating to motility and adhesion (41). Furthermore, engagement of PECAM (CD31) on the surface of T cells increases Rap1 activation, and this increases adhesion via β_1 VLA-4) and β_2 (LFA-1) integrins to adhesion molecules present on vascular endothelium (42). The observation that Rap1 plays a key role in these physiological processes is highlighted by the discovery that a newly characterized leukocyte adhesion deficiency disease (LAD-III) is caused by impaired Rap1 activation (43). Despite the attention Rap1 signaling in immune cells has received, relatively little is known about the function of Rap1 in other cell types. Recent data have implicated Rap1 in the formation of cadherin-based cell-cell adhesions in epithelia, and this occurs independently of Rap1 effects on integrin-mediated adhesion (9, 32).

Our work in endothelia extends this idea, identifying an important role for Rap1 signaling events within the vascular endothelium, affecting junctional integrity and, most interestingly, impacting the ability of leukocytes to cross the barrier presented by an endothelial cell monolayer. Based on our results, one could predict that localized decreases in Rap activity would allow transmigrating cells to more easily penetrate the endothelial cell barrier due to a weakening of the intercellular junctions. Our observation that increasing Rap activity with specific drugs such as 8CPT-2′OMe-cAMP can increase the barrier function of endothelial cells may have broad clinical implications for conditions in which an inappropriate inflammatory response leads to disease. For example, it will be important to determine whether misregulation of Rap1 activity in endothelial cells contributes to such auto-immune diseases as arthritis or vascular diseases such as atherosclerosis, in which leukocyte exit from the blood circulation is critical to the pathology.

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