VASP is involved in cAMP-mediated Rac 1 activation in microvascular endothelial cells

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Schlegel N, Waschke J. VASP is involved in cAMP-mediated Rac 1 activation in microvascular endothelial cells. Am J Physiol Cell Physiol 296: C453–C462, 2009. First published December 31, 2008; doi:10.1152/ajpcell.00360.2008.—Accumulating evidence points to a significant role of vasodilator-stimulated phosphoprotein (VASP) in the maintenance of endothelial barrier functions. We have recently shown that impaired barrier functions in VASP-deficient microvascular myocardial endothelial cell monolayers (MyEnd VASP−/−) correlated with decreased Rac 1 activity. To further test the hypothesis that VASP is required in regulation of Rac 1 activity, we studied cAMP-dependent Rac 1 activation. Both inhibition of Rac 1 activation by NSC-23766 and inhibition of PKA by PKI completely blunted the efficacy of forskolin/rolipram (F/R)-mediated cAMP increase to stabilize barrier functions as revealed by measurements of transendothelial resistance (TER). Because these results indicate that PKA/Rac 1 activation is important for barrier stabilization, we tested this signaling pathway in VASP−/− cells. We found that F/R and isoproterenol reduced permeability measured as FITC-dextran flux across VASP−/− monolayers, but not below baseline levels of wild-type cells (WT). Moreover, cAMP-mediated Rac 1 activation was reduced to ~50% of WT levels, and both PKA inhibition by PKI and PKA anchoring via A kinase anchoring peptides (AKAPs) by HT31 almost completely abolished Rac 1 activation in VASP−/− and WT endothelium. Accordingly, HT31 significantly reduced F/R-mediated TER increase in WT cells and completely blocked the protective effect of cAMP on endothelial barrier properties. Together, our data underline the significant role of cAMP-mediated Rac 1 activation for endothelial barrier stabilization and demonstrate that both AKAP-mediated PKA anchoring and VASP are required for this process.

endothelial barrier functions; vasodilator-stimulated phosphoprotein; adenosine 3′,5′-cyclic monophosphate

THE ENDOTHELIUM PROVIDES a selective barrier between blood vessels and the interstitium. Inflammation leads to breakdown of endothelial barrier functions by the formation of intercellular gaps, which causes vascular leakage, edema, and impairment of the microcirculation (37). The maintenance of endothelial barrier properties requires the appropriate assembly of intercellular adhesion proteins such as VE-cadherin or claudin 5 to form adherens and tight junctions (26). All types of cell junctions are directly or indirectly linked to the actin cytoskeleton. Therefore, regulation of endothelial barrier functions is dependent on actin dynamics, which in turn are modulated by Rho GTPases (37, 45). There is increasing evidence for a crucial role of the Rho GTPase Rac 1 to maintain endothelial barrier functions (11, 32, 38, 42, 46). Moreover, it has been demonstrated that the well-established barrier-protective effects of cAMP are at least in part mediated via Rac 1 activation (7, 11).

Previously, it was reported that vasodilator-stimulated phosphoprotein (VASP), a member of the Ena/VASP family, is required for endothelial barrier functions in vivo and in vitro (9, 19, 30, 32). The Ena/VASP family of molecular scaffolds have been implicated in the control of actin dynamics by regulating multiple processes such as nucleation, bundling, and capping of actin filaments (5, 8, 25, 31). However, the mechanism by which VASP contributes to the strengthening of the endothelial barrier remains unclear.

In our previous study, we demonstrated that impaired barrier properties in VASP−/− myocardial microvascular endothelial (MyEnd) monolayers correlated with decreased activity of Rac 1. Furthermore, direct activation of Rac 1 by cytotoxic necrotizing factor (CNF-1) was equally effective in VASP−/− and wild-type (wt) monolayers to stabilize the endothelial barrier, indicating that VASP is not a downstream effector of Rac 1. Moreover, active Rac 1 at cell borders in MyEnd cells transfected with constitutively active Rac 1 (pEGFP-Rac 1 CA)-induced translocation of VASP to cell borders, which in summary suggested a role for VASP in the regulation of Rac 1 activity (32, 39). The present study was carried out to further test this hypothesis and thereby extend our investigations on the role of Rac 1 and cAMP in endothelial barrier regulation. We have demonstrated in the present study that Rac 1 activation is a crucial signaling pathway for cAMP-mediated endothelial barrier stabilization. Moreover, we have provided evidence that both VASP and anchoring of protein kinase A (PKA) at A-kinase anchoring peptides (AKAPs) are required to enable cAMP-mediated Rac 1 activation.

MATERIALS AND METHODS

Cell culture. The immortalized mouse microvascular endothelial cell lines from myocardium (MyEnd VASP wt and MyEnd VASP−/−) were grown in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Karlsruhe, Germany) supplemented with 50 U/ml penicillin-G, 50 μg of streptomycin, and 10% fetal calf serum (FCS; Biochrom, Berlin, Germany) in a humidified atmosphere (95% air-5% CO2) at 37°C. MyEnd cells were prepared from mouse myocardial tissue of wt and VASP−/− mice (24). Preparation, immortalization, and characterization of MyEnd VASP wt and MyEnd VASP−/− cell lines was carried out as described in detail previously (32). Cultures were used for experiments when grown to confluent monolayers (day 3 up to day 7).

Test reagents. Forskolin and rolipram (F/R) to increase cAMP levels (both from Sigma-Aldrich, Taukirchen, Germany) were used for 60 min at 5 and 10 μM, respectively. Isoproterenol (Sigma-

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Aldrich) to increase cAMP levels was used at 10 μM according to preliminary experiments. NSC-23766 (Calbiochem/Merck, Darmstadt, Germany), which inhibits Rac 1 GDP/GTP exchange activity by interfering in Rac 1 interaction with the Rac 1-specific guanine nucleotide exchange factors Trio and Tiam1, was used at 200 μM for 30 min as described in the literature (10). cAMP analog 8-(4-chlorophenylthio)-2′-O-methyl-cAMP (O-Me-cAMP; Biolog, Bremen, Germany), which preferentially stimulates PKA-independent signaling via Epac/Rap 1, was used at 200 μM for 1 h. In a previous study we showed that this dose was effective to activate Epac/Rap 1 but not PKA signaling (7). The cell-permeable PKA-inhibitor myristoylated PKI 14 – 22 amide (PKI) (Toxicis/Biozol, Eching, Germany) was used at 20 μM. The cell-permeable inhibitor peptide St-Ht31 (HT31; Promega, Madison, WI), which inhibits interaction of PKA RIi subunits with AKAPs, was used at a concentration of 1 μM according to the literature (28).

CNF-1 from Escherichia coli was supplied by Gudula Schmidt (Department of Pharmacology and Toxicology, University of Freiburg, Germany) and has been prepared and described previously (35). Similar to previous studies, we used CNF-1 at 300 ng/ml for 120 min for all experiments (39). In the present study we have demonstrated that CNF-1 specifically activates Rac 1 and Cdc 42, but not Rho A, in the MyEnd cell line.

cAMP and cGMP activation assay. For quantification of intracellular cAMP or cGMP levels, the cAMP enzyme immunoassay or cGMP enzyme immunoassay (both from Sigma-Aldrich) were used according to the manufacturer’s recommendations as described previously (7). Concentrations of cAMP or cGMP (in pmol/ml) from each control experiment were set at 100% and compared with cAMP levels of cell lysates treated as indicated below.

Rac 1 activation assay. For measurement of Rac 1 activation, the Rac G-Lisa Activation Assay Biochem kit (Cytoskeleton, Denver, CO) was used according to the manufacturer’s recommendations as described previously (32). In brief, MyEnd VASP wt and MyEnd VASP−/− were grown to confluence in 35-mm dishes and incubated in the presence or absence of different mediators. Finally, the signal was measured at 490 nm using a microplate spectrophotometer (Sunrise; Tecan, Crailsheim Germany). The results were analyzed using Microsoft Excel software.

Rac 1 pull-down assay. To test for interaction of active Rac 1 with VASP in MyEnd cells, we performed pull-down assays of GFP-bound (activated) Rac 1 under control conditions and after treatment with CNF-1 (300 ng/ml, 120 min) using the Rac 1 activation assay kit (Upstate Biotechnology, Lake Placid, NY) following the manufacturer’s instructions as described previously (6). MyEnd cells were grown to confluence in T75 culture flasks for 3 days and then incubated in the absence (control) or presence of CNF-1 (300 ng/ml, 120 min) in DMEM containing 10% FCS. After 15% SDS-PAGE, Western blotting and immunodetection with a mouse monoclonal IgG directed against Rac 1 (Upstate) using horseradish peroxidase-labeled goat anti-rabbit IgG (Dianova; diluted 1:600 in PBS). MyEnd VASP−/− cells were transfected with pCMV-VASP (VASP) or empty pEGFP-vector (MOCK) as negative control 1 day after plating using TurboFect in vitro transfection reagent (Merck, Darmstadt, Germany) according to the manufacturer’s instructions. The pCMV-VASP plasmid was kindly provided by Ulrich Walter (University of Würzburg, Würzburg, Germany) and constructed as described elsewhere (23).

Cytochemistry and quantification of cell areas. Immunostaining has been described in detail previously (32). In brief, endothelial cells were grown to confluence on cover slips for 7–10 days. After incubation with mediators as indicated below, fixation with 2% formaldehyde, and treatment with 0.1% Triton X-100, monolayers were incubated with rat monoclonal antibody 11D4.1 (undiluted hybridoma supernatant) directed to the ectodomain of mouse VE-cadherin (22). We used Cy3-labeled goat anti-rat IgG (Dianova; diluted 1:600 in PBS) as secondary antibody. For visualization of filamentous actin (F-actin), monolayers were incubated with Alexa-phalloidin (Molecular Probes, Göttingen, Germany; diluted 1:60 in PBS, 1 h at RT). Coverslips were mounted on glass slides with 60% glycerol in PBS containing 1.5% n-propyl gallate (Serva, Heidelberg, Germany) as anti-fading compound. Monolayers were photographed with a confocal microscope (LSM 510; Carl Zeiss Microimaging, Göttingen, Germany), and cell areas were quantified using ImageJ morphometric software. For each condition, all cell areas of three randomly chosen fields of endothelial monolayers from four independent experiments were measured.

Measurement of FITC-dextran flux across monolayers of cultured endothelial cells. As described previously (32), endothelial cells were seeded on top of Transwell chambers for six-well plates (0.4-μm pore size) (Falcon, Heidelberg, Germany) and grown to confluence. After being rinsed with PBS, cells were incubated with fresh DMEM, without phenol red (Sigma), containing 10 mg/ml FITC-dextran (4 kDa; Sigma) in the presence or absence of F/R. Paracellular flux was assessed by taking 100-μl aliquots from the outer chamber over 2 h of incubation. Fluorescence was measured using a Wallac Victor 2 fluorescence spectrophotometer (Perkin-Elmer, Überlingen, Germany) with excitation and emission at 485 and 535 nm, respectively. For transendothelial flux under the different experimental conditions, the permeability coefficient (Pm) was calculated as described in detail previously (32).

Measurement of transendothelial resistance. ECIS 1600R (Applied BioPhysics) was used to measure transendothelial resistance (TER) of endothelial monolayers to assess endothelial barrier functions as described previously (7). Endothelial cells were seeded in the wells of the electrode array and grown to confluence for 8–10 days. Medium was then exchanged (200 μl), and baseline TER was measured for 10 min to equilibrate monolayers. Afterward, 200 μl of medium containing different mediators were applied to the wells.

Statistics. Values are means ± SE. Possible differences in FITC-dextran flux, TER, or Rac 1 activation between groups were assessed using unpaired Student’s t-test and the nonparametric Mann-Whitney statistic. Statistical significance was assumed for P < 0.05.

RESULTS

Requirement of Rac 1 activity for cAMP-mediated endothelial barrier stabilization. The well-established barrier-protective effects of increased cAMP are known to be at least in part mediated via Rac 1 activation (7). To further characterize the role of Rac 1 in cAMP-mediated endothelial barrier stabilization, we tested whether inhibition of Rac 1 activation by NSC-23766 would affect cAMP-mediated endothelial barrier stabilization. Mean baseline TER of MyEnd VASP wt cells was 20.71 ± 0.91 Ω·cm² (Table 1). Incubation of MyEnd VASP wt cells with F/R was effective to significantly increase
Table 1. Cell area and barrier properties of MyEnd VASP wt and MyEnd VASP−/− cells

<table>
<thead>
<tr>
<th></th>
<th>Mean Cell Area, μm²</th>
<th>TER, Ω·cm²</th>
<th>P&lt;sub&gt;0&lt;/sub&gt;, cm²·s⁻¹ × 10⁻⁶</th>
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</thead>
<tbody>
<tr>
<td>MyEnd VASP wt</td>
<td>487±21</td>
<td>20.71±0.91</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>MyEnd VASP−/−</td>
<td>612±24*</td>
<td>18.84±0.89</td>
<td>4.1±0.9*</td>
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Values are means ± SE. Mean cell areas were significantly larger in vasodilator-stimulated phosphoprotein (VASP)-deficient microvascular myocardial endothelial cells (MyEnd VASP−/−) than in wild-type (wt) cells. Transendothelial resistance (TER) under baseline conditions displayed no differences in both cell lines (n = 15 each), although permeability coefficient (P<sub>0</sub>) of FITC-dextran flux was significantly higher in MyEnd VASP−/− (n = 23) compared with MyEnd VASP−/− wt cells (n = 28). *P < 0.05 compared with wt cells.

TER to 124 ± 1% after 1 h compared with untreated controls (Fig. 1A). Preincubation of MyEnd VASP wt cells with NSC-23766 for 0.5 h before F/R was applied completely blocked cAMP-induced increase of TER (Fig. 1A). After addition of F/R to NSC-23766, TER significantly dropped below controls.

Measurements of Rac 1 activity demonstrated that F/R treatment of MyEnd VASP wt cells led to Rac 1 activation to 333 ± 55% within 1 h (Fig. 1B). After preincubation of endothelial monolayers with NSC-23766 for 0.5 h before F/R was applied for 1 h, cAMP-mediated activation of Rac 1 was blunted. Under these conditions, Rac 1 activity was significantly decreased to 86 ± 4% of controls.

In the following experiment, we tested whether cAMP-induced endothelial barrier stabilization under our experimental conditions was mediated via PKA or Epac/Rap 1, which are both known to be relevant pathways for cAMP-signaling (14). Both direct activation of Epac/Rap 1 using O-Me-cAMP and treatment with F/R to increase intracellular cAMP significantly increased TER to 117 ± 2 and 128 ± 1% of controls, respectively (Fig. 2). Therefore, to test whether F/R-induced increase of TER was mediated via PKA, Epac/Rap 1, or both, we preincubated monolayers with the cell-permeable PKA-inhibitor PKI for 1 h before F/R was applied. Under these conditions, F/R-mediated increase of TER was completely blocked and dropped to 83 ± 2% of controls. In contrast, preincubation with PKI alone had no effect on TER (102 ± 2%). In summary, these data demonstrate that endothelial barrier stabilization by F/R was primarily mediated by PKA, rather than by Epac/Rap 1, under the experimental conditions used for this study, but direct activation of Epac/Rap 1 in principal was also effective to enhance endothelial barrier properties.

Reconstitution of VASP increases Rac 1 activity and endothelial barrier properties in MyEnd VASP−/− monolayers. In our previous study we demonstrated that impaired endothelial barrier properties in MyEnd VASP−/− monolayers closely correlated with decreased Rac 1 activity (32). In the present study we have confirmed that Rac 1 activity was significantly decreased in MyEnd VASP−/− monolayers to 0.58 ± 0.21 of wt levels compared with MyEnd VASP wt monolayers under resting conditions (Fig. 3A). To support our hypothesis that VASP plays an important role for regulation of Rac 1 activity and thereby for stabilization of endothelial barrier functions, we transfected MyEnd VASP−/− cells with VASP (MyEnd VASP−/−/VASP; Fig. 3B) or with vehicle (MyEnd VASP−/−/MOCK) and measured TER and Rac 1 activity. Rac 1 activity was significantly increased 48 h after transfection in MyEnd VASP−/−/VASP to 146 ± 9% compared with MyEnd VASP−/−/MOCK (Fig. 3C). Under these conditions, TER was significantly increased to 130 ± 5% in MyEnd VASP−/−/VASP compared with MyEnd VASP−/−/MOCK (Fig. 3d). In summary, these data demonstrate that VASP is required for Rac 1 activation and thereby for stabilization of endothelial barrier properties.

cAMP-mediated endothelial barrier stabilization is impaired in MyEnd VASP−/− monolayers. Mean baseline TER of MyEnd VASP wt cells was 20.71 ± 0.91 Ω·cm² (Table 1), and mean baseline TER of MyEnd VASP−/− cells was 18.84 ± 0.89 Ω·cm², which was not significantly different between both cell lines, although our previous data demonstrated that barrier properties of MyEnd VASP−/− monolayers were significantly impaired (32). Interestingly, measurement of cell areas revealed that in MyEnd VASP−/− monolayers, cell area was significantly larger in MyEnd VASP−/−/VASP compared with MyEnd VASP−/−/MOCK (333 ± 55% compared with 233 ± 50%), but permeability coefficient (P<sub>0</sub>) of FITC-dextran flux was significantly higher in MyEnd VASP−/−/VASP compared with MyEnd VASP−/−/MOCK (333 ± 55% compared with 233 ± 50%).

Fig. 1. cAMP-mediated endothelial barrier stabilization requires Rac 1 activity. A: measurements of transendothelial resistance (TER) were performed to determine endothelial barrier alterations in microvascular myocardial endothelial cell (MyEnd) vasodilator-stimulated phosphoprotein (VASP) wild-type (wt) monolayers. Incubation with forskolin/rolipram (F/R) for 1 h (n = 6) was effective to significantly increase TER to 124 ± 1% compared with untreated controls (n = 7). Preincubation of endothelial monolayers with NSC-23766 to inhibit Rac 1 activation for 0.5 h before F/R was applied for 1 h (NSC-23766 + F/R; n = 4) completely blocked cAMP-mediated increase of TER to 70 ± 13% at that time. In the time course of measurements, TER dropped continuously and remained significantly reduced compared with controls. B: Rac 1 activation analyses by G-Lisa demonstrated that increased cAMP by F/R treatment for 1 h strongly activated Rac 1 in MyEnd VASP wt to 333 ± 55% of controls (n = 6). NSC-23766 preincubation for 0.5 h followed by F/R application for 1 h (n = 4) was effective to completely block F/R-mediated Rac 1 activation. Rac 1 activity was significantly lower compared with controls. Values are means ± SE. *P < 0.05 compared with controls. #P < 0.05 compared with F/R alone.
Fig. 2. F/R-induced endothelial barrier stabilization is mediated via PKA. Incubation of MyEnd VASP wt cells with (4-chlorophenylthio)-2-O-methyl-cAMP (O-Me-cAMP; n = 5) and F/R (n = 6) for 1 h significantly increased TER to 117 ± 2 and 128 ± 1% of controls, respectively. Treatment of endothelial monolayers with the PKA inhibitor PKI (n = 8) did not alter TER within 1 h (102 ± 2%). However, preincubation with PKI before application of F/R for 1 h (PKI + F/R; n = 8) completely blocked cAMP-induced increase of TER (83 ± 2%); TER was even significantly reduced compared with controls and F/R treatment alone. Values are means ± SE. *P ≤ 0.05 compared with controls. #P ≤ 0.05 compared with F/R alone.

surface area was significantly larger (mean: 612 ± 24 μm²) compared with MyEnd VASP wt monolayers (mean: 487 ± 21 μm²). Because it is conceivable that larger cell areas may result in increased TER levels, this finding explains why absolute TER values in both cell lines were comparable whereas barrier properties in MyEnd VASP wt/− cells were impaired (21). To rule out the possibility that F/R-mediated effects on TER were due to cAMP-induced alterations of cell areas rather than alterations of endothelial barrier properties, we measured cell areas after F/R treatment in both endothelial cell lines. However, cell areas were not altered by F/R treatment in both MyEnd VASP wt (490 ± 27 μm² after F/R) and MyEnd VASP wt/− monolayers (613 ± 33 μm² after F/R).

To directly compare barrier properties of MyEnd VASP wt and MyEnd VASP wt/− monolayers, we performed measurements of 4-kDa FITC-dextran flux under control conditions and after F/R and isoproterenol treatment and calculated the $P_E$ for each condition (Fig. 4). In MyEnd VASP wt/− monolayers, $P_E$ was significantly increased ~4-fold (4.1 ± 0.9 cm/s × 10⁻⁶) under control conditions compared with MyEnd VASP wt monolayers, where $P_E$ was 1.3 ± 0.3 cm/s × 10⁻⁶ (Table 1). Incubation of endothelial monolayers with F/R was equally effective in both cell lines to decrease $P_E$ to ~50% of control values. In MyEnd VASP wt monolayers, F/R treatment significantly decreased $P_E$ to 0.6 ± 0.4 cm/s × 10⁻⁶. In contrast, in MyEnd VASP wt/− F/R treatment was only effective to enhance barrier functions to $P_E$ values of 1.9 ± 0.5 cm/s × 10⁻⁶, i.e., to the range of baseline levels of MyEnd VASP wt controls. Similarly, incubation of endothelial cells with the G protein-coupled receptor (GPCR) agonist isoproterenol decreased endothelial permeability in both cell lines but did not reduce permeability of MyEnd VASP wt/− monolayers below control levels of MyEnd VASP wt monolayers. In summary, this demonstrated that impaired endothelial barrier functions of MyEnd VASP wt/− monolayers were only partially restored by increased cAMP. Moreover, our TER data show that TER measurements are suitable to detect alterations of endothelial barrier properties within one cell line but that differences of cell areas have to be taken into account when different cell lines are compared.

Next, we characterized whether both F/R and isoproterenol were effective to significantly increase cAMP levels in MyEnd VASP wt and MyEnd VASP wt/− monolayers (Fig. 5A). Under control conditions, cAMP levels of 5.4 ± 1.5 and 6.4 ± 0.2 pmol/ml were detected in wt and VASP wt/− monolayers, respectively, but were not significantly different in both cell lines. F/R increased cAMP levels to 340 ± 17% compared with controls in wt and to 357 ± 73% in MyEnd VASP wt/− monolayers. Isoproterenol was equally effective in both endothelial cell lines to increase cAMP levels to 124 ± 6% of controls in wt and to 126 ± 7% of controls in VASP wt/− monolayers. In contrast, cGMP levels were not affected by F/R and isoproterenol treatment in both endothelial cell lines (Fig. 5B).

Rac 1 activation by cAMP requires VASP and PKA anchoring. Because Rac 1 activity under resting conditions was reduced and endothelial barrier properties under resting conditions as well as in response to cAMP were impaired in MyEnd VASP wt/− monolayers, we tested whether cAMP-mediated Rac 1 activation was affected in MyEnd VASP wt/− cells. Therefore, we performed time-course studies of Rac 1 activation after F/R was applied (Fig. 6A). After 5 min of F/R treatment, Rac 1 activity was significantly increased in MyEnd VASP wt cells to 344 ± 27% compared with controls. Increased Rac 1 activity was maintained throughout 2 h of measurements. In contrast, Rac 1 activation after F/R treatment in MyEnd VASP wt/− cells was significantly reduced at all time points and was ~40–50% lower compared with wt cells. Moreover, when reduced baseline levels of Rac 1 activity in VASP wt/− cells were taken into account, cAMP-mediated Rac 1 activation in VASP wt/− cells by F/R was in the range of Rac 1 activity of wt controls. This correlates with measurements of FITC-dextran flux, where F/R was not effective to increase $P_E$ levels in VASP wt/− monolayers beyond $P_E$ levels of wt controls. Similar results were obtained when we incubated cells with isoproterenol for 1 h, which resulted in strongly increased Rac 1 activity to 400 ± 66% in wt cells but was significantly lower in MyEnd VASP wt/− cells (204 ± 47%; Fig. 6B). Measurement of Rac 1 activity using O-Me-cAMP revealed strong Rac 1 activation to 339 ± 17% in MyEnd VASP wt cells but was significantly lower in MyEnd VASP wt/− cells (173 ± 27%). In summary, these data demonstrate that VASP is required for cAMP-mediated Rac 1 activation.

Next, we characterized the role of PKA signaling for Rac 1 activation. After inhibition of PKA by preincubation with PKI for 1 h before addition of F/R for 1 h, Rac 1 activation was abolished in both MyEnd VASP wt (111 ± 11%) and My End VASP wt/− cells (101 ± 1%), demonstrating that Rac 1 activation by F/R was mediated by PKA (Fig. 6C). Similarly, preincubation of both cell lines with HT31 to block binding of PKA to AKAPs before F/R was applied significantly reduced Rac 1 activation to 156 ± 11% in MyEnd VASP wt cells. Moreover, in MyEnd VASP wt/− cells, preincubation with HT31 completely blocked cAMP-mediated Rac 1 activation (111 ± 14%).

We performed measurements of TER to test whether impaired Rac 1 activation was relevant for endothelial barrier functions. Application of F/R was effective to significantly
increase TER in MyEnd VASP wt cells to 134 ± 1% and in MyEnd VASP−/− cells to 149 ± 11% of baseline values within 15 min (Fig. 7, A and B). Because preincubation of HT31 was effective to completely prevent Rac 1 activation in MyEnd VASP−/− but not in wt cells, we tested whether this would affect F/R-mediated endothelial barrier stabilization. Preincubation of both cell lines with HT31 for 1 h did not lead to alterations of TER compared with controls. After F/R was added to MyEnd VASP wt cells, TER increased significantly within 15 min to 115 ± 1% but was significantly lower than TER after F/R treatment alone. In contrast, addition of F/R to MyEnd VASP−/− cells after preincubation with HT31 did not significantly alter TER (110 ± 2%) in the time course of measurements. Together, our results demonstrate that both VASP and PKA anchoring are required for cAMP-mediated Rac 1 activation and endothelial barrier stabilization.

VASP and active Rac 1 interact as revealed by pull-down assays. We transfected MyEnd VASP wt cells with pEGFP-Rac 1 CA to demonstrate that activated Rac 1 and VASP colocalize at cell borders. In transfected cells, pEGFP-Rac 1 CA was located at cell borders (arrows in Fig. 8A). Immunostaining for VASP showed a punctuated staining pattern reminiscent of focal adhesions in MyEnd VASP wt cells (Fig. 8B). Transfection of VASP resulted in increased Rac 1 activity to 146 ± 9% (n = 3). Under the same conditions, TER was increased to 130 ± 5% in MyEnd VASP−/− cells (n = 7) compared with MOCK transfection (n = 4). *P ≤ 0.05 compared with MOCK transfection.

Fig. 3. Reduced Rac 1 activity in VASP−/− cells is increased by transfection with VASP. A: Rac 1 activation analyses by G-Lisa demonstrated that Rac 1 activity was significantly reduced 0.58 ± 0.21-fold in VASP−/− cells (n = 15) compared with MyEnd VASP wt cells (n = 19) under resting conditions. #P ≤ 0.05 compared with wt. B: Western blotting served to document the positive transfection of VASP in MyEnd VASP−/− cells. The VASP-specific band was detected at 46 kDa in MyEnd VASP wt cell lysates and was absent in MyEnd VASP−/− cells but was clearly visible in transfected VASP−/− cells (VASP−/−/VASP). Western blot shown is representative of n = 3 experiments. C: Rac 1 activity was measured in MyEnd VASP−/− cells transfected with either vehicle (MyEnd VASP−/−/MOCK) or VASP (MyEnd VASP−/−/VASP). Transfection of VASP resulted in increased Rac 1 activity to 146 ± 9% (n = 3). D: under the same conditions, TER was increased to 130 ± 5% in MyEnd VASP−/−/VASP cells (n = 7) compared with MOCK transfection (n = 4). *P ≤ 0.05 compared with MOCK transfection.
stabilization in both cell lines. After F/R treatment, 1 h with F/R to increase cAMP resulted in significant endothelial barrier activity compared with VASP

Similarly, inhibition of PKA anchorage at AKAPs using HT31 measured as FITC-dextran flux across VASP

Although both F/R and isoproterenol reduced permeability layers was used to assess barrier functions in MyEnd VASP wt and VASP−/− cells under resting conditions and after F/R treatment. Under control conditions, permeability coefficient (Pf) of MyEnd VASP−/− cells was significantly increased ~4-fold (4.1 ± 0.9 cm/s × 10−6; n = 23) compared with MyEnd VASP wt cells (1.3 ± 0.3 cm/s × 10−6; n = 28). Incubation of monolayers for 1 h with F/R to increase cAMP resulted in significant endothelial barrier stabilization in both cell lines. After F/R treatment, Pf of MyEnd VASP−/− cells (1.9 ± 0.5 cm/s × 10−6; n = 10) was only reduced to the range of MyEnd VASP wt controls and was thus significantly higher compared with MyEnd VASP wt cells treated with F/R (0.6 ± 0.4 cm/s × 10−6; n = 14). Similarly, incubation of both endothelial cell lines significantly reduced Pf to 0.9 ± 0.02 cm/s × 10−6 in wt (n = 8) and to 1.9 ± 0.7 cm/s × 10−6 in VASP−/− cells (n = 13). Values are means ± SE. *P ≤ 0.05 compared with controls.

DISCUSSION

In the present study we tested our hypothesis that VASP is involved in the regulation of Rac 1 in microvascular endothelial cells. In a first line of evidence, we demonstrate that in MyEnd VASP wt monolayers, cAMP-mediated Rac 1 activation is an important signaling pathway for endothelial barrier stabilization, because Rac 1 inhibitor NSC-23766 completely blocked F/R-mediated increase of TER. Similarly, inhibition of PKA by PKI blunted cAMP-mediated stabilization of endothelial barrier functions, demonstrating that this signaling pathway was PKA dependent under our experimental conditions. In a second step, we transfected MyEnd VASP−/− cells with VASP, which resulted in increased TER and increased Rac 1 activity compared with VASP−/− cells transfected with vehicle. These data demonstrated the involvement of Rac 1 in VASP-mediated endothelial barrier stabilization. To investigate whether this mechanism is important for physiological signaling pathways, we tested cAMP-mediated Rac 1 activation in wt and VASP−/− endothelial cells. Time-course studies of Rac 1 activation showed that cAMP-mediated Rac 1 activation was significantly impaired in MyEnd VASP−/− cells. Although both F/R and isoproterenol reduced permeability measured as FITC-dextran flux across VASP−/− monolayers, endothelial barrier stabilization was not effective to reduce FITC-dextran flux below baseline levels of MyEnd VASP wt monolayers. PKA inhibition by PKI completely abolished Rac 1 activation in VASP−/− and MyEnd VASP wt endothelium. Similarly, inhibition of PKA anchorage at AKAPs using HT31 significantly reduced cAMP-mediated Rac 1 activation in MyEnd VASP wt cells, and activation by F/R was completely blocked in MyEnd VASP−/− cells Rac 1. Accordingly, HT31 significantly attenuated F/R-mediated TER increase in MyEnd wt cells and completely blunted the protective effect of cAMP on endothelial barrier properties in VASP−/− cells. Finally, colocalization of constitutively active Rac 1 and VASP as well as pull-down of active Rac 1 revealed interaction of Rac 1 with VASP. Together, our data underline the significant role of cAMP-mediated Rac 1 activation for endothelial barrier stabilization and demonstrate that the recruitment of VASP and PKA anchoring are required for this process.

Rac 1 activation is required for cAMP-mediated endothelial barrier stabilization. Small GTPases of the Rho family are known to be important for the regulation of endothelial barrier functions (37, 45). Activity of Rac 1 plays a key role for the maintenance and stabilization of endothelial barrier functions in vivo and in vitro (10, 11, 16, 32, 39, 42). Moreover, increased cAMP protects the endothelial barrier during inflammation and is required to maintain baseline barrier functions under resting conditions in vivo and in vitro (1–4, 32). In the present study we have demonstrated that increased cAMP by

the VASP-specific band at 46 kDa in whole cell lysates as well as in the pull-down fraction, demonstrating that VASP interacts with active Rac 1 (Fig. 9B).

Fig. 4. cAMP-induced endothelial barrier stabilization is impaired in VASP−/− cells. FITC-dextran (4 kDa) flux across untreated endothelial monolayers was used to assess barrier functions in MyEnd VASP wt and VASP−/− cells under resting conditions and after F/R treatment. Under control conditions, permeability coefficient (Pf) of MyEnd VASP−/− cells was significantly increased ~4-fold (4.1 ± 0.9 cm/s × 10−6; n = 23) compared with MyEnd VASP wt cells (1.3 ± 0.3 cm/s × 10−6; n = 28). Incubation of monolayers for 1 h with F/R to increase cAMP resulted in significant endothelial barrier stabilization in both cell lines. After F/R treatment, Pf of MyEnd VASP−/− cells (1.9 ± 0.5 cm/s × 10−6; n = 10) was only reduced to the range of MyEnd VASP wt controls and was thus significantly higher compared with MyEnd VASP wt cells treated with F/R (0.6 ± 0.4 cm/s × 10−6; n = 14). Similarly, incubation of both endothelial cell lines significantly reduced Pf to 0.9 ± 0.02 cm/s × 10−6 in wt (n = 8) and to 1.9 ± 0.7 cm/s × 10−6 in VASP−/− cells (n = 13). Values are means ± SE. *P ≤ 0.05 compared with controls.

Fig. 5. cAMP levels but not cGMP levels are increased by F/R and isoproterenol. ELISA-based measurements were used to determine the effects of F/R and isoproterenol on cAMP (A) and cGMP levels (B). Incubation of both endothelial cell lines with F/R for 1 h resulted in significantly increased cAMP levels to 340 ± 17% in wt (n = 4) and to 357 ± 73% (n = 6) in MyEnd VASP−/− cells. Isoproterenol incubation for 1 h significantly increased cAMP levels to 124 ± 6% of controls in wt (n = 8) and to 126 ± 7% of controls in VASP−/− cells (n = 6). cGMP levels were unaltered in both endothelial cell lines after F/R and isoproterenol treatment (n = 5 for each condition). Values are means ± SE. *P ≤ 0.05 compared with controls.
F/R treatment leads to strong Rac 1 activation in microvascular endothelial cells. TER measurements using Rac 1 inhibitor NSC-23766 showed that Rac 1 activation by cAMP is essential to mediate barrier stabilizing effects. Moreover, incubation of MyEnd wt cells with NSC-23766 not only completely abolished F/R-mediated increase of TER and Rac 1 activation but even decreased Rac 1 activity and endothelial barrier properties. Thus our present data underline the importance of Rac 1 activation by increased cAMP for endothelial barrier stabilization. This is supported by previous studies demonstrating that decreased Rac 1 activity due to inactivation by bacterial toxins or in inflammation leads to endothelial barrier breakdown that can be restored by increased cAMP levels (7, 41). Moreover, both prostaglandins and atrial natriuretic peptide (ANP) have been shown to mediate their barrier-protective effects via cAMP-mediated Rac 1 activation (11, 12).

cAMP-mediated Rac 1 activation by F/R is mediated via PKA. In principle, both PKA and Epac/Rap 1 signaling pathways could account for cAMP-mediated Rac 1 activation (7,
This is underlined by our data showing that direct Epac/Rap 1 activation using O-Me-cAMP was effective to increase Rac 1 activity. However, because cAMP-mediated Rac 1 activation by F/R was blocked by inhibition of PKA using PKI, it can be concluded that in our study F/R-induced increase of TER and Rac 1 activity was primarily mediated by PKA rather than by Epac/Rap 1 signaling. This may be explained by the fact that PKA responds to nanomolar concentrations of cAMP, whereas Epac/Rap 1 is only activated when cAMP concentration reaches micromolar levels (18). In our experiments, total cAMP levels were in the range of 5–6 pmol/ml in both MyEnd VASP wt and MyEnd VASP−/− cells under control conditions. F/R treatment in endothelial cells increased cAMP to 300–400% of controls, i.e., to nanomolar cAMP concentrations. Thus it may be assumed that cAMP levels in our experiments were not high enough to induce Epac/Rap 1 activation. On the other hand, it must be emphasized that localized concentrations of cAMP levels in specific subcellular compartments may vary considerably (44). This could explain the fact that the GPCR agonist isoproterenol was equally effective to increase Rac 1 activity like F/R, although total cAMP levels were considerably lower in isoproterenol-treated monolayers. Therefore, local activation of Epac/Rap 1 signaling may also occur.

It is interesting to note that Rac 1 activation via Epac/Rap 1 was also impaired in MyEnd VASP−/− cells, underlining the requirement of VASP to activate Rac 1. However, because inhibition of PKA signaling completely blunted F/R-induced Rac 1 activation in our study, the contribution of Epac/Rap 1 to stabilize barrier functions seems not to be significant when cAMP is increased by F/R treatment.

Because TER was even significantly decreased after incubation with PKI and F/R, it can be speculated that maintenance of endothelial barrier functions requires permanent activity of PKA. This hypothesis is supported by studies showing that inflammatory stimuli decrease intracellular cAMP levels, which leads to disruption of endothelial barrier functions (7, 15, 16, 35).

cAMP-mediated Rac 1 activation requires VASP and AKAPs. In the present study, we have demonstrated for the first time that VASP is required for cAMP-mediated Rac 1 activation and thereby contributes to stabilization of endothelial barrier functions. Furthermore, we have provided evidence that decreased Rac 1 activity in VASP−/− cells can be augmented by reconstitution of VASP, which demonstrates for the first time the crucial role for VASP in the regulation of Rac 1 activity. This study extends the investigations of our previous study in which we finally hypothesized that VASP plays a role in the regulation of Rac 1 activity in microvascular endothelium (32). Although it has been established in several studies that VASP is required for maintenance of endothelial barrier functions in vivo and in vitro, the mechanisms remain largely unclear (9, 19, 30, 32). The involvement of VASP in the regulation of Rac 1 may explain the mechanism by which downregulation of VASP in response to hypoxia (30) induces breakdown of endothelial barrier functions (46).

Alternatively, it has been proposed that VASP phosphorylation at S157 by PKA may stabilize endothelial barrier functions by relaxation of cytoskeletal tension (17). However, because VASP in the latter study was shown to reduce endothelial barrier functions, the relevance of this mechanism is yet to be defined. Benz et al. (9) recently reported that the interaction of VASP with αII-spectrin plays a crucial role for the maintenance of endothelial barrier functions. However, in that study it was also reported that VASP upon PKA-dependent phosphorylation negatively regulates the interaction between αII-spectrin and VASP (9), which in turn would lead to a barrier-destabilizing effect of PKA. Thus it appears unlikely...
that this interaction is crucial for the well-established barrier-stabilizing effects of PKA.

Because it is well known that VASP is involved in the regulation of actin dynamics (29), differences in barrier properties in wt and VASP<sup>−/−</sup> cells in our study could be caused by alterations of cell shape and surface area. It has indeed been reported that TER is affected by alterations of cell areas or cell spreading, respectively (21). However, this seems not to account for cAMP-induced barrier stabilization, because cell surface areas were not affected by increased cAMP. Furthermore, because measurements of FITC-dextran flux correlated with TER measurements, it can be assumed that paracellular permeability rather than cell spreading was tested in our study. Therefore, barrier enhancement by cAMP is likely caused by stabilization of cell junctions.

The mechanism by which VASP participates in the regulation of Rac 1 is not clear at present. It is known that VASP is a substrate for both cAMP- and cGMP-dependent signaling pathways (13, 36). Therefore, cGMP measurements in the present study were used to rule out the possibility that F/R or isoproterenol affected cGMP levels and thus cGMP was involved in VASP-mediated Rac 1 activation. In principle, VASP could interfere with intrinsic GTP hydrolysis and thereby inactivate Rac 1, because VASP can interact with p120Ras GAP as well as GTP exchange factors for Rac 1 (20). According to our previous study (32), active Rac 1 and VASP colocalize in MyEnd cells. Moreover, VASP was detected in the pull-down fraction of active Rac 1, which indicates interaction of both proteins. In view of our findings that anchorage of PKA via AKAPs is required to fully activate Rac 1, we suggest that AKAPs and VASP may build a scaffolding multitransmembrane complex. The structural integrity of such a complex may be the prerequisite for PKA-mediated Rac 1 activation. This hypothesis conforms to the idea that VASP is part of a multiprotein complex including junctional proteins, spectrin, and other unidentified proteins that stabilizes endothelial barrier functions (9). In this respect, it is known that Wiskott-Aldrich syndrome protein family verprolin homology 1 (WAVE 1) is able to bind PKA via its RII subunit, and this interaction is crucial for the well-established barrier-enhancing effects in wt and VASP<sup>−/−</sup> cells in our study. Because the F/R-mediated Rac 1 activation was completely blocked by HT31 in VASP<sup>−/−</sup> cells, the involvement of VASP in this complex is reasonable. In summary, from our present data we conclude that VASP is involved in cAMP-mediated Rac 1 activity and thereby regulates barrier functions in microvascular endothelial cells.

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