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Epac1 and PDZ-GEF cooperate in Rap1 mediated endothelial junction control

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ABSTRACT

Epac1 and its effector Rap1 are important mediators of cAMP induced tightening of endothelial junctions and consequential increased barrier function. We have investigated the involvement of Rap1 signalling in basal, unstimulated, barrier function of a confluent monolayer of HUVEC using real time Electric Cell-substrate Impedance Sensing. Depletion of Rap1, but not Epac1, results in a strong decrease in barrier function. This decrease is also observed when cells are depleted of the cAMP independent Rap exchange factors PDZ-GEF1 and 2, showing that PDZ-GEFs are responsible for Rap1 activity in control of basal barrier function. Monolayers of cells depleted of PDZ-GEF or Rap1 show an irregular, zipper-like organization of VE-cadherin and live imaging of VE-cadherin-GFP reveals enhanced junction motility upon depletion of PDZ-GEF or Rap1. Importantly, activation of Epac1 increases the formation of cortical actin bundles at the cell–cell junctions, inhibits junction motility and restores barrier function of PDZ-GEFs depleted, but not Rap1 depleted cells. We conclude that PDZ-GEF activates Rap1 under resting conditions to stabilize cell–cell junctions and maintain basal integrity. Activation of Rap1 by cAMP/Epac1 induces junctional actin to further tighten cell–cell contacts.
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1. Introduction

Endothelial cells are crucial cellular components of the human vasculature. These cells tightly regulate the passage of fluid, solutes, macromolecules and activated immune cells from the blood to the surrounding tissue by specialized transcellular systems of transport vesicles and coordinated stabilization and destabilization of cell–cell contacts. The importance of vascular integrity is stressed by the fact that vascular hyperpermeability is involved in diseases like chronic inflammation disorders, atherosclerosis and diabetes. The presence, composition, strength and organization of cell–cell contacts are crucial for the integrity of the vascular barrier function. Cell-cell contact is provided by several specialized adhesion complexes, the major ones being Tight Junctions and Adherens Junctions. Human umbilical vein endothelial cells (HUVECs) mainly rely on the latter for their cell–cell interactions, with Tight Junctions thought to have a fine-tuning function [1]. The core of the Adherens Junction is the homophilic interaction of cadherin proteins that form the actual physical contact between cells. Endothelial cells express several members of the cadherin family but mainly VE-cadherin. The cytosolic tail of VE-cadherin interacts with catenin family members that allow dynamic linkage of the Adherens Junction to the actin cytoskeleton, thereby allowing regulation of junction integrity [2].

Assembly and disassembly of endothelial cell–cell contacts are regulated by various hormones and agonists. Elevation of the second messenger cAMP increases endothelial barrier function [3–6]. The dynamic control of endothelial cell–cell contacts by cAMP is mediated by both PKA and Epac1 via independent pathways [7,8]. Epac1 and Epac2 proteins are cAMP responsive Guanine nucleotide Exchange Factors (GEFs) for the Rap family of small G-proteins [9]. In endothelial cells Epac1 is known to be the predominant form, which can be specifically activated by the cAMP analogue 8-pCPT-2′-O-Me-cAMP (hereafter named 007) [10]. 007 treatment of an endothelial monolayer inhibits basal and thrombin-induced permeability [3,11–13]. Furthermore, 007 inhibits transendothelial migration of leukocytes and monocytes, but not neutrophils [11,13], and *in vivo* inhibits vessel leakage induced by VEGF or mechanical ventilation induced lung injury [3,14]. A number of effects of Epac1 activation on cell permeability have been described. Several groups reported that Epac1, most likely through Rap, induces a reorganization of the actin cytoskeleton from stress fibers that span the cell body towards cortical bundles along cell–cell contacts, hereafter referred to as junctional actin [3,11,12,14,15]. These junctional actin bundles have been proposed to mediate the stabilization of VE-cadherin at the junction [8,16]. In addition, Epac1 activation results in the recruitment of the Adherens Junction protein β-catenin through the Rap1 effector KRIT1 [17,18]. Interestingly, KRIT1 has been shown to suppress the

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Rho/contractility pathway and thereby enhance barrier function [19]. Finally, Epac1 was shown to control microtubule dynamics and barrier function in a Rap1 independent manner [15]. These observations suggest that Epac1 regulates different aspects of barrier function. Dependent on the cell type or the cellular environment one of these mechanisms may be prevalent.

Whereas Rap1 signalling has been solidly shown to mediate barrier function upon cAMP induction, the effect of Rap1 on endothelial barrier under basal, unstimulated conditions has been less investigated. Overexpression of RapGAP to inhibit endogenous Rap1 signalling increased permeability of monolayers for mannitol or HRP [13,17]. Furthermore, RapGAP delays the accumulation of junction proteins to nascent junctions [13]. Basal activity of Rap1 could be caused by steady state cAMP levels, as increased basal permeability upon depletion of Epac1 has been reported [15]. Alternatively, other RapGEFs could function to activate Rap1 in endothelial cells [20]. The PDZ-GEFs are of particular interest, as the PDZ-GEF1 knockout mice die at 9.5 days post coitum due to defects in blood vessel formation [21]. This phenotype was later attributed to decreased VE-cadherin accumulation in allantois endothelial cells [22]. Indeed, PDZ-GEF1 localizes to cell–cell junctions via β-catenin and MAGI proteins, the latter being required for activation of Rap1 at cell–cell contacts [23–25]. Furthermore, depletion of PDZ-GEF2 causes endothelial cell–cell junctions to appear irregular and zipper-like [26].

Here, we have further explored the role of the Rap1 signalling pathway in endothelial barrier function. We used Electric Cell-substrate Impedance Sensing (ECIS) to measure the impedance that is opposed to an alternating electrical current by HUVEC monolayers. This assay monitors the permeability of junctions in a monolayer as a measure of barrier function [8,13,15,27]. We found that depletion of Rap1(A/B) or depletion of PDZ-GEF(1/2), but not Epac1 results in a strong reduction in basal impedance, indicating the critical role of Rap1 and PDZ-GEF in the maintenance of basal barrier function. Furthermore, live cell imaging reveals highly motile junctions upon depletion of Rap1 or depletion of PDZ-GEF, suggesting a link between increased permeability and increased junction motility. Finally, we provide evidence that Epac1/Rap1 activation enhances barrier function independently of Rho inactivation. We conclude that Rap1 is activated by PDZ-GEFs and Epac1 to control both basal and cAMP induced barrier function, respectively.

2. Materials and methods

2.1. Cells and reagents

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics. Human umbilical vein endothelial cells (HUVECs) (Lonza) were cultured in EBM-2 culture medium supplemented with EGM-2 SingleQuots (hEGF, hydrocortisone, fetal bovine serum, VEGF, hFGF-B, R3-IGF-1, ascorbic acid, GA-1000, heparin) (Lonza) on dishes coated with 0.5% gelatin. HUVECs were cultured maximally 14 days before experiments, which were performed on 5 μg/ml home purified human Fibronectin. All siRNA transfections were performed with 50 nM ON-TARGETplus SMARTpools (Dharmacon Inc.) using Dharmafect-1 (Dharmacon Inc.), which were confirmed with Dharmacon single siRNAs J-003623-06 (siRap1A), J-010364-07 (siRap1B), J-009742-05 (siPDZ-GEF1) and J-008593-06 (siPDZ-GEF2). It should be noted that the ON-TARGETplus SMARTpool targeting Epac1 did reduce basal impedance much more than three out of four single siRNAs that also completely inhibited the 007-AM effect. Therefore, the ON-TARGETplus single oligo J-007676-07 (Dharmacon Inc.) was used for subsequent experiments. A modified version of 007 with increased membrane permeability was used, 007-AM (8-pCPT-2′-O-Me-cAMP-AM) (Biolog Life Sciences) at 1 μM [28]. C3 transferase CT04 (Cytoskeleton, Inc) was used at 1 μg/ml and Blebbistatin (EMD Millipore) at 100 μM. The Rap1 antibody was from Santa Cruz. Antibodies against VE-cadherin and β-catenin were from BD Bioscience and the α-Tubulin antibody was from Calbiochem. CCM1 and CCM2 antibodies were from Covance. The PDZ-GEF1 antibody was from Abnova. The PDZ-GEF2 antibody has been described before [26]. Fluorescently labeled Phalloidin and secondary antibodies were from Invitrogen.

2.2. ECIS measurements

1×10^5 HUVEC cells were plated onto L-cysteine reduced, Fibronectin coated 8W10E electrodes (Applied Biophysics) either directly (for untransfected cells) or 48 h after transfection and grown to confluency for another 24 h. Electrical impedance was measured in real time at 37 °C and 6% CO_2 using a 1600R Electrical Cell Impedance Sensing (ECIS) system (Applied Biophysics) at 4000 Hz. Shown are time lapse impedance graphs of representative experiments. It should be noted that in some publications impedance, which is measured by alternating current, is erroneously referred to as Trans Endothelial Electrical Resistance, which is measured by direct current.

2.3. Rap1 activation assay

HUVECs were plated onto Fibronectin coated dishes either directly (for untransfected cells) or 48 h after transfection and grown to confluency for another 24 h. Rap1 activity was assayed as described previously [29]. Briefly, HUVECs were stimulated for 10 min with 1 μM 007-AM and subsequently lysed in buffer containing 1% NP-40, 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 10% glycerol, 2 mM $MgCl_2$, and protease and phosphatase inhibitors. Lysates were cleared by centrifugation, and Rap1-GTP was precipitated with a glutathione *S*-transferase (GST) fusion protein of the RA-domain of RalGDS precoupled to glutathione-Sepharose beads. Bound proteins were eluted in Laemmli buffer and analyzed by SDS-PAGE followed by Western blotting.

2.4. Immunofluorescence

HUVECs were plated onto Fibronectin coated glass coverslips either directly (for untransfected cells) or 48 h after transfection and grown to confluency for another 24 h. After 10 min stimulation with 1 μM 007-AM cells were fixed with 4% formaldehyde for 20 min, permeabilized with 0.2% TX-100 for 3 min and blocked with 1% BSA for at least 2 h. Next, cells were incubated with indicated primary antibodies for 1 h, secondary antibody for 30 min and mounted onto glass slides, which were subsequently examined on an Axioskop 2 mot plus microscope (Zeiss) with a 40× or 100× immersion oil objective and Axiocam camera.

2.5. Live cell imaging

Live imaging of VE-cadherin-GFP was performed with either adenovirus or lentivirus infected HUVECs. For adenoviral infections, human VE-cadherin fused to GFP [30] was expressed by adenoviral transductions using the Virapower Adenoviral Expression system (Invitrogen). The virus was a gift from Dr. Jaap van Buul, Sanquin, Amsterdam. For lentiviral infections, human VE-cadherin-GFP was cut out of a pEGFP-VE-cadherin vector (also kindly provided by Dr. Jaap van Buul, Sanquin, Amsterdam) using NdeI and XbaI restriction enzymes and cloned into a sin-activating lentiviral pLV-CMV-ires-puro vector using its NdeI and NheI sites. Lentiviral particles were isolated from the supernatant of HEK293 cells transiently transfected with third-generation packaging constructs and the pLV-VE-cadherin-GFP vector. HUVECs were infected 24 h after siRNA transfection with undiluted supernatant containing lentiviral particles in the presence of 8 μg/ml polybrene overnight. Then, VE-cadherin-GFP expressing HUVECs were plated on Fibronectin coated Lab-Tek dishes. Imaging

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