The role of Rap1 in cell-cell junction formation

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The role of Rap1 in cell-cell junction formation

De rol van Rap1 in de formatie van cel-cel juncties
(met een samenvatting in het Nederlands)

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 12 juni 2008 des middags te 2.30 uur

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Rap1: a key regulator of cell-cell junctions

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Rap1: a key regulator in cell-cell junction formation

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Summary

Rap1 is a Ras-like small GTPase that is activated by many extracellular stimuli and strongly implicated in the control of integrin-mediated cell adhesion. Recent evidence indicates that Rap1 also plays a key role in formation of cadherin-based cell-cell junctions. Indeed, inhibition of Rap1 generates immature adherens junctions, whereas activation of Rap1 tightens cell-cell junctions. Interestingly, Rap1 guanine nucleotide exchange factors, such as C3G and PDZ-GEF, are directly linked to E-cadherin or to other junction proteins. Furthermore, several junction proteins, such as afadin/AF6 and proteins controlling the actin cytoskeleton function as effectors of Rap1. These findings point to a role of Rap1 in spatial and temporal control of cell-cell junction formation.

Introduction

Rap1 is a small GTPase that was identified as a clone in a revertant screen for cell transformation by oncogenic Ras and independently as a very close relative of Ras (Bourne et al., 1990; Kitayama et al., 1989). Initial studies mainly focused on the possibility that Rap1 interferes with Ras signalling by directly interacting with Ras effectors, but more recent results indicate that Rap1 functions in independent signalling pathways that control diverse processes, such as cell adhesion, cell-cell junction formation, and cell polarity (Bos, 2005; Knox and Brown, 2002; Schwamborn and Puschel, 2004). Rap1 is activated by various extra cellular stimuli, which induce the conversion of the inactive, GDP-bound form into the active, GTP-bound form, by stimulating different guanine nucleotide exchange factors (GEFs). Many of these GEFs are regulated by common second messengers, such as calcium, diacylglycerol, and cyclic AMP (cAMP) (Fig. 1). Inactivation of Rap1 is mediated by a number of specific GTPase-activating proteins (GAPs). Downstream of Rap1, a variety of proteins that interact with its GTP-bound form have been identified and these may serve as effectors in Rap1-regulated processes, such as integrin activation, vesicle trafficking, neuronal polarity and phagocytosis (reviewed in (Bos, 2005; Caron, 2003)). Furthermore, Rap1 is involved in the regulation of cadherin-based cell-cell junctions.

Cadherins comprise a large group of cell-cell adhesion molecules that mediate intercellular adhesion by engaging in Ca^{2+}-dependent, homophilic trans-interactions. E-cadherin and VE-cadherin are two of the most studied members of the family. These proteins are major components of adherens junctions, in epithelial and endothelial cells respectively. Adherens junctions, together with tight junctions, are assembled at cell-cell contacts when cells form a monolayer (Fig. 2). Cell surface proteins such as nectins may be involved in the initial contacts (Takai et al., 2003). Subsequently junctional proteins, such as E/VE-cadherin and junctional adhesion molecules (JAMs), are recruited to the contact sites. Clustering of adhesion molecules and recruitment of intracellular components connecting the junctional complex to the actin cytoskeleton induce further maturation of the junction and subsequent formation of tight junctions.

The adhesive activity of classical cadherins is mediated by their N-terminal extracellular domain and requires extracellular Ca^{2+}. Upon Ca^{2+} binding, they form cis and trans dimers; removal of Ca^{2+} by EGTA leads to loss of cadherin-mediated cell-cell adhesion. (Adams and Nelson, 1998; Bazzoni, 2003; Reynolds and Roczniak-Ferguson, 2004; Takeichi, 1995). The cytoplasmic tail comprises two domains: the C-terminal, distal β-catenin-binding domain, which regulates adhesion to the actin cytoskeleton through a complex of adaptor proteins including β-catenin, α-catenin, vinculin and α-actinin; and the juxtamembrane domain, which contains the p120-catenin-binding site that regulates clustering, transport, and endocytosis of cadherins (Davis et al., 2003; Kowalczyk and Reynolds, 2004; Nieset et al., 1997; Yap et al., 1998).

Cell-cell junctions are dynamic structures that display high levels of turnover of E/VE-cadherin. For instance, endothelial cell permeability is largely regulated at the level of VE-cadherin. Tyrosine phosphorylation of β-catenin (Brunton et al., 2004), degradation of E-cadherin through the binding of the E3 ligase Hakai (Fujita et al., 2002), and recycling through the

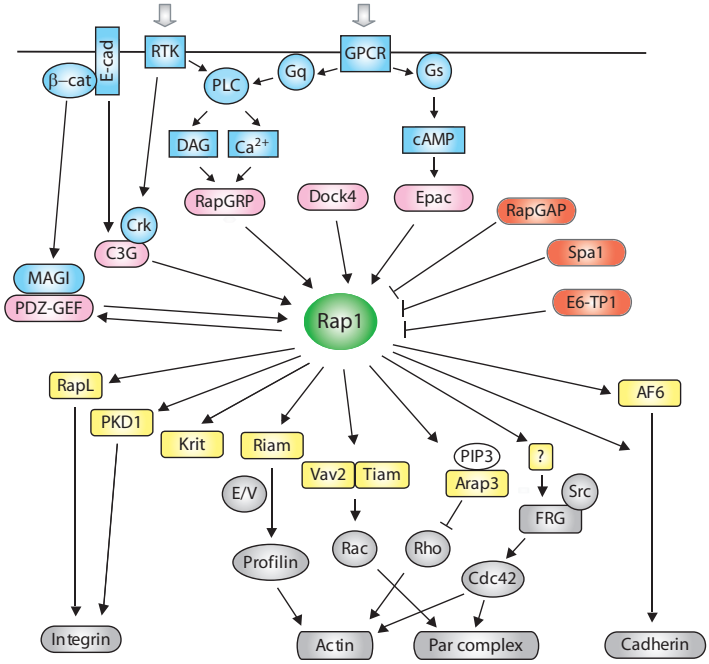

Fig. 1. Overview of the Rap1 signalling network.
Adapted from (Bos, 2005). DAG, Diacylglycerol; E/N, Ena/Vasp; GPCR, G-protein coupled receptor; PIP3, Phosphoinositol-tri-phosphate; PLC, phospholipase C; RTK, Receptor Tyrosine Kinase.

endocytic/exocytic compartments (Davis et al., 2003; Xiao et al., 2005) are among the processes that regulate these junctions. Noteworthy, disruption of the integrity of cell-cell junctions has serious pathological consequences, including unwanted vessel leakage and, in cancer, the induction of metastasis. Recently, the Rap1 signalling pathway has been found to play a crucial role in the regulation and maintenance of cell-cell contacts. The present review focuses on the evidence that led to the appreciation of Rap1 as a master regulator of cell-cell junctions.

Rap1 and E-cadherin regulation

The first evidence that Rap1 is involved in the regulation of cell-cell junctions came from genetic studies in *Drosophila melanogaster* (Knox and Brown, 2002). *rap1*-mutant cell clones in the wing have a different shape and disperse into the surrounding normal tissue, which indicates a defect in cell-cell junction formation. Indeed, whereas Drosophila E-cadherin (DE-cadherin) is evenly distributed along the lateral side of the wild-type (WT) wing cells, the distribution is not uniform in *rap1*-mutant cells: DE-cadherin is present in clusters and frequently localizes at one side of the cell. These clusters contain other junction proteins including α-catenin, β-catenin, ZO-1, and the Afadin/AF-6 ortholog Canoe. Importantly, apical-basolateral polarity and the localisation of α-catenin, β-catenin and DE-cadherin along the apicobasal axis are not affected in *rap1*-mutant cells. Moreover, septate junctions, located at the basal side of adherens junctions, form normally. The uneven distribution of DE-cadherin in rap1-mutant cells is generated during or after cell division, which suggests that Rap1 helps maintaining the circumferential distribution of adherens junctions. This is compatible with the observation that GFP-tagged Rap1 concentrates at sites of adherens junctions in imaginal disk cells and is enriched at the junction of newly formed sister cells. Interestingly, at sites where mutant cells attach to WT cells, normal adherens junctions are formed, which suggests that correct distribution of proteins at one side of the cell-cell junction is sufficient for proper junction formation. A role for Rap1 in cell-cell junction regulation in mammalian cells was highlighted by the identification of DOCK4 as an atypical RapGEF. This protein was identified in a screen for tumour suppressor genes in mice, and inactivating mutations in DOCK4 are also found in human tumour cell lines (Yajnik et al., 2003). Cell-cell junctions are not present in an osteosarcoma cell line lacking DOCK4 but readily form after the introduction of either WT DOCK4 or an active form of Rap1. In addition, knocking down DOCK4 in primary mouse osteoblasts results in the disruption of cell-cell

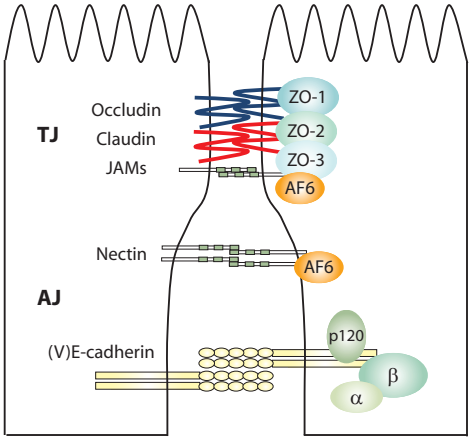

Fig. 2. Schematic overview of adherens and tight junctions. AJ, adherens junctions; TJ, tight junctions; JAMs, ZO, Zona occludens; junctional adhesion molecules; AF-6, Afadin/AF-6; p120, p120catenin; β, β-catenin; α, α-catenin.

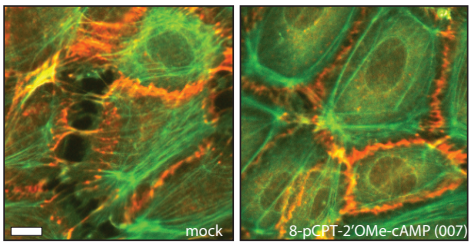

Fig. 3. Activation of Rap1 in endothelial cells induces cell-cell junction maturation. Anti-VE-cadherin and F-actin staining of HUVE cells treated for 30 minutes with vehicle or the Epac-specific cAMP analog 007. Stimulation with 007 clearly increased cell-cell junction maturation as the adherens junctions form a smoother line between the cells. Endothelial cell permeability is reduced as a biological consequence of the junction tightening (bar = 10µm).

adhesion. This emphasizes the importance of DOCK4 in cell-cell junction formation and clearly reveals that activation of Rap1 stimulates this process. Importantly, the re-introduction of DOCK4 into the mutant osteosarcoma cell line reduced its metastatic property; Rap1 activation may therefore have anti-metastatic effects.

In Madine-Darby Canine Kidney (MDCK) cells, oncogenic Ras induces the disruption of cell-cell junctions, resulting in the conversion of an epithelial phenotype into a spindle-cell-like phenotype. This effect can be rescued completely by the introduction of active Rap1. Furthermore, activation of endogenous Rap1 blocks hepatocyte growth factor (HGF)-induced junction breakdown and cell scattering, which indicates that Rap1 activity regulates cell-cell junction stability. Furthermore, adhesion of ovarian carcinoma cells (OVCAR) to Fc-E-cadherin is inhibited by expression of dominant negative Rap1, which means that Rap1 regulates E-cadherin directly. (Price et al., 2004). Importantly, Hogan et al found that introduction of RapGAP into MCF7 cells does not disrupt mature E-cadherin based cell-cell junctions, but strongly reduces the reformation of adherens junctions upon re-addition of Ca^{2+} (so-called Ca^{2+} switch), which supports a role for Rap1 in junction maturation, not maintenance (Hogan et al., 2004). Remarkably, the tight junction marker ZO-1 is present at cell-cell contacts after Ca^{2+} chelation. Rap1 might therefore only affect adherens junctions regulation and not tight junctions directly. However, Hogan et al. did not discuss whether the tight junctions were disrupted in any way under these experimental conditions (Hogan et al., 2004).

Rap1 and the regulation of VE-cadherin

Endothelial cell-cell junctions are highly dynamic structures, allowing the regulation of endothelial integrity and permeability as well as diapedesis of leukocytes (Dejana, 2004). Junctional integrity is regulated by cAMP, and stimuli such as prostacyclins that elevate the level of cAMP inhibit cell permeability (Moy et al., 1998). It was anticipated that protein kinase A (PKA) mediates this effect, but the findings that Rap1 is involved in formation of E-cadherin-based junctions and that cAMP directly activates the RapGEF Epac prompted several groups to examine whether Epac and Rap1 mediate the cAMP-induced effect. Indeed, endothelial cells treated with the Epac-specific cAMP analogue 8-pCPT-2'OMe-cAMP (007) (Enserink et al., 2002) show increased junction maturation (Fig. 3) (Cullere et al., 2005; Fukuhara et al., 2005; Wittchen et al., 2005), and this effect is completely abolished by knocking down Epac1 (Kooistra et al., 2005). Consequently, 007 also decreases permeability of the endothelial monolayer and inhibits thrombin-induced increase in permeability. One study also reported that endothelial transmigration of differentiated HL-60 cells is inhibited (Wittchen et al., 2005), whereas others observed no effect of Rap1-activation on endothelial transmigration of human neutrophils (Cullere et al., 2005).

Although the mechanism of regulation is still elusive, VE-cadherin is considered to play a key role in the integrity of endothelial cell-cell junctions and the

regulation of permeability. Indeed, adhesion of cells to Fc-VE-cadherin coated plates is inhibited by expression of RapGAP, which indicates that Rap1 activity is required for VE-cadherin-mediated adhesion (Fukuhara et al., 2005). Furthermore, inhibition of endothelial cell permeability by 007 is lost in VE-cadherin-null cells (Kooistra et al., 2005). In contrast with the observation in MCF7 cells that junction disassembly is not initiated by RapGAP expression, it causes junction disassembly, including loss of the tight junction protein ZO-1 in endothelial cells (Hogan et al., 2004; Wittchen et al., 2005). In spite of this, Rap1-induced regulation of E- and VE-cadherin may not be a general mechanism for all classical cadherins since overexpression of RapGAP in HEK293 cells does not affect N-cadherin mediated cell-cell junction formation (Hogan et al., 2004).

Activation of Rap1 by cell-cell contacts

Having established that Rap1 is involved in the control of cell-cell adhesion probably through E/VE-cadherin, two questions arise. First, how is Rap1 is activated and, second, how does Rap1 regulate cell-cell junctions? The accumulation of Rap1 at the cell-cell contacts suggests that Rap1 functions locally, rather than being part of a signalling cascade that ultimately targets the junction. Also, Rap1 is activated after restoration of the cell-cell junctions following a Ca^{2+} switch and after interaction with Fc-VE-cadherin in endothelial cells (Sakurai et al., 2006; Wittchen et al., 2005). As mentioned above, in Drosophila imaginal disk cells, Rap1 accumulates at adherens junctions. In addition, GFP-tagged Rap1 translocates specifically to relatively mature cell-cell junctions after calcium restoration in MCF7 cells and to the interaction site with Fc-E-cadherin-coated beads, where it becomes activated. Indeed, the RapGEF C3G interacts with E-cadherin and competes with β-catenin for binding to E-cadherin. The interaction is most prominent when cell-cell adhesion is induced after a Ca^{2+} switch, and C3G disappears as the junctions mature (Hogan et al., 2004). C3G is also involved in nectin-induced activation of Rap1 (Fukuyama et al., 2005). Nectins are Ca^{2+}-independent Ig-like cell-cell adhesion molecules that form adherens junctions cooperatively with cadherins (Sakisaka and Takai, 2004). When nectins are engaged, the tyrosine kinase Src is activated and through Crk recruits C3G to nectins, resulting in the activation of Rap1 (Fukuyama et al., 2005).

Another exchange factor for Rap1 that might be involved in cell-cell adhesion is PDZ-GEF1. PDZ-GEF1 binds to β-catenin and to the scaffold proteins MAGI-1 and MAGI-2, which both interact with β-catenin as well (Dobrosotskaya and James, 2000; Kawajiri et al., 2000; Mino et al., 2000; Ohtsuka et al., 1999; Sakurai et al., 2006). The presence of MAGI-1 at the endothelial cell-cell junctions and its β-catenin binding site are required for cell-cell-contact-induced Rap1 activation (Sakurai et al., 2006). Whether Epac1 is also located at the endothelial cell junctions is unclear. Interestingly, in epithelial cells, Epac1 is predominantly on the apical side of polarized cells (J. Zhao and J.L. Bos, unpublished observations); yet its activation can block HGF-induced scattering and junction breakdown (Price et al., 2004). Thus, it seems that localized GEFs are involved in the activation of Rap1 after initial cell-cell adhesion. In addition, external stimuli can induce activation of Rap1 that impinges on its regulation of cell-cell junctions, as shown for prostacyclins in endothelial cells (Fukuhara et al., 2005). Overall, it appears that Rap1 is activated and required during the initial phase of cell-cell junction formation and is down-regulated when the junctions have matured. Interestingly, when junctions are disassembled, Rap1 is activated again and induces formation of focal adhesions. This involves Src and is triggered by internalisation of E-cadherin. Since Rap1 also regulates integrins, these observations indicate the complex role of Rap1 signalling in cell adhesion (Balzac et al., 2005).

Mechanism of action of Rap1

Rap1 thus seems to be locally activated during the initial phase of cell-cell junction formation and required for the maturation of junctions. How this maturation occurs is still elusive and so is the role of Rap1 in this process. Rap1 has been implicated in two different processes: the regulation of the actin cytoskeleton and the recruitment of E-cadherin.

Hogan and colleagues have reported that Rap1 activity is required for the activation of the Rho family GTPase Cdc42 during cell-cell junction formation. They showed that introduction of a ‘fast-cycling’ active mutant of Cdc42 can rescue the effects of RapGAP on cell-cell junction formation, placing Cdc42 downstream of Rap1 (Hogan et al., 2004). Others have shown that activation of Rap1 by nectins is required for the activation of Cdc42 and another Rho family member, Rac. Fukuyama et al. have proposed a model in which activation of Vav, a GEF for Rac, and FRG, a GEF for Cdc42, requires both active Src and

active Rap1 (Fukuyama et al., 2005; Fukuyama et al., 2006). Interestingly, Vav2 interacts with Rap1-GTP and Rap1 is required for Vav2-induced cell spreading (Arthur et al., 2004). Moreover, the RacGEF Tiam1 interacts with Rap1 and is required for cell-cell junction maturation (Arthur et al., 2004; Malliri et al., 2004; Mertens et al., 2005). Thus, one of the functions of Rap1 activation may be the recruitment of Rac and Cdc42 GEFs to the site of initial cell-cell contact to provide a link with the actin cytoskeleton. The effect of Rap1 on the actin cytoskeleton is also apparent in endothelial cells, in which stimulation of Rap1 results in the formation of cortical actin. This effect is independent of cell-cell adhesion and could be the driving force for the decreased permeability of the cell monolayer (Cullere et al., 2005; Kooistra et al., 2005). Interestingly, in these cells, Rap1 activation reduces thrombin-induced RhoA activity (Cullere et al., 2005). Rap1 may also be involved in the recruitment of junctional proteins. One protein heavily involved in this process is afadin/AF6, an adaptor protein that binds to various junctional proteins, such as nectins, ZO-1, and JAM-A (Boettner et al., 2000; Ebnet et al., 2000; Takahashi et al., 1999). Afadin/AF6 has two Ras-association (RA) –domains, which can bind to both Ras and Rap1. *In vitro* studies show that afadin/AF6, in the presence of Rap1, inhibits endocytosis of E-cadherin that is not engaged in a homophilic trans-interaction. A mutant of afadin/AF6 lacking the RA domain, but not WT afadin/AF6, can inhibit endocytosis in the absence of Rap1. Furthermore, afadin/AF6 forms a complex with p120-catenin which increases the interaction between p120-catenin and E-cadherin (Hoshino et al., 2005). In MDCK cells, knocking down afadin/AF6 disrupts the recruitment of the tight junction proteins claudin-1, occludin, JAM-A and ZO-1 to cell-cell contact sites, but not E-cadherin, β-catenin, α-catenin, and p120-catenin. However, E-cadherin appears to be modified under these conditions, since common antibodies fail to recognize the protein in AF6-knockdown cells. Interestingly, the effect of knocking down afadin/AF6 on E-cadherin can be rescued by a mutant p120-catenin (ΔN-p120-catenin) that is proposed to be constitutively active, but not by full length p120-catenin (Sato et al., 2006). These results point to a model in which nectins activate Rap1, which then binds to and activates afadin/AF6. Active afadin/AF6 interacts indirectly with p120-catenin, which inhibits endocytosis of E-cadherin and induces trans-interactions between E-cadherin molecules. This model is an interesting starting point for further investigation.

Conclusions

It is clear that Rap1 plays a key role in the control of adherens junctions at different levels in the process. First, when cell-cell junctions are formed, initial cell-cell contact results in the activation of Rap1 by various means, including the engagement of nectins and the interaction of C3G with E-cadherin. This activation of Rap1 is required for the maturation of cell-cell junctions through the inhibition of endocytosis of E-cadherin, activation of E-cadherin or remodelling of the actin cytoskeleton. Further maturation may be mediated by PDZ-GEF, which interacts with β-catenin, or by extracellular stimuli that activate Rap1 (Fig. 4). As a Rap1 effector and a regulator of p120-catenin, afadin/AF6 is a good candidate as a mediator of Rap1-dependent effects on E-cadherin (Boettner et al., 2000; Davis et al., 2003; Sato et al., 2006). In addition,

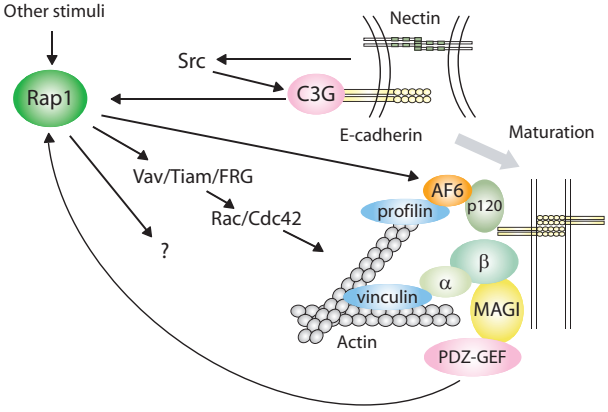

Fig. 4. Model for the involvement of Rap1 in cell-cell junction formation.
At initial cell-cell contacts, C3G bound to E-cadherin is activated, resulting in Rap1 activation. Upon maturation, C3G is displaced by β-catenin, which interacts with PDZ-GEF to further activate Rap1. Also, extracellular stimuli, including stimuli that stimulate production of cAMP which activates Epac, induce Rap1 activation.

Rap1 plays an important role in the regulation of the cytoskeleton by activating Rac and Cdc42 through the binding and recruitment of the GEFs Tiam1 and Vav2 (Arthur et al., 2004). Nevertheless, additional mechanisms of Rap1 function in cell-cell junction regulation are likely to exist. For instance, Rap1 regulates axon-fate in neural development through the Cdc42-Par3-Par6-aPKC complex (Schwamborn and Puschel, 2004), which is also recruited to the initial contact sites in junctions and is essential for junction maturation (Suzuki and Ohno, 2006).

Furthermore, Rap1 has a role in the maintenance of cell-cell adhesion. Particularly in endothelial cells, activation of Rap1 by cAMP results in an inhibition of cell permeability. This process requires VE-cadherin, but how Rap1 induces the tightening of junctions remains elusive (Kooistra et al., 2005). It may involve the induction of cortical actin, perhaps through the inhibition of Rho (Cullere et al., 2005). Currently, it is unclear whether the regulation of cadherins and the regulation of the actin cytoskeleton by Rap1 are independent or interconnected processes.

Finally, Rap1 may regulate cell-cell adhesion through tight junctions, although the precise role of Rap1 in this is process is not clear. For instance, inhibition of Rap1 in MCF7 cells does not affect localisation of the tight junction marker ZO-1 (Hogan et al., 2004), whereas it is affected in endothelial cells (Wittchen et al., 2005). Note that activation of Rap1 in cardiomyocytes induces the formation of gap junctions, a process that is preceded by the formation of adherens junctions (Somekawa et al., 2005).

The function of Rap1 is not restricted to the regulation of cell-cell junctions and also includes the regulation of integrin-mediated cell adhesion and secretion. A common theme appears to be that Rap1 is involved in processes at the plasma membrane that require a link to the actin cytoskeleton. At these sites, Rap1 responds to spatial cues, such as the initial contact site in junctions, or to second messengers. In this respect, the analogy with yeast is striking; the yeast ortholog of Rap1, Rsr1, in response to positional cues is responsible for guiding the position of the future bud. Activation of Rsr1 results in the association with a GEF for Cdc42 and the subsequent activation of Cdc42 as a regulator of the actin cytoskeleton (Chant and Herskowitz, 1991; Park et al., 2002). Despite this analogy, the function of Rap1 in mammalian cells seems to be more complex and not restricted to the regulation of the actin cytoskeleton. We have only begun our journey towards understanding the function of Rap1 in the regulation of cell-cell adhesion, but it is already clear that it plays an essential role.

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References

Adams, C. L. and Nelson, W. J. (1998). Cytomechanics of cadherin-mediated cell-cell adhesion. Curr Opin Cell Biol 10, 572-7.

Arthur, W. T., Quilliam, L. A. and Cooper, J. A. (2004). Rap1 promotes cell spreading by localizing Rac guanine nucleotide exchange factors. J. Cell. Biol. 167, 111-122.

Balzac, F., Avolio, M., Degani, S., Kaverina, I., Torti, M., Silengo, L., Small, J. V. and Retta, S. F. (2005). E-cadherin endocytosis regulates the activity of Rap1: a traffic light GTPase at the crossroads between cadherin and integrin function. J Cell Sci 118, 4765-83.

Bazzoni, G. (2003). The JAM family of junctional adhesion molecules. Curr Opin Cell Biol 15, 525-30.

Boettner, B., Govek, E. E., Cross, J. and Van Aelst, L. (2000). The junctional multidomain protein AF-6 is a binding partner of the Rap1A GTPase and associates with the actin cytoskeletal regulator profilin. Proc Natl Acad Sci U S A 97, 9064-9.

Bos, J. L. (2005). Linking Rap to cell adhesion. Curr Opin Cell Biol 17, 123-8.

Bourne, H. R., Sanders, D. A. and McCormick, F. (1990). The GTPase superfamily: a conserved switch for diverse cell functions. Nature 348, 125-32.

Brunton, V. G., MacPherson, I. R. and Frame, M. C. (2004). Cell adhesion receptors, tyrosine kinases and actin modulators: a complex three-way circuitry. Biochim Biophys Acta 1692, 121-44.

Caron, E. (2003). Cellular functions of the Rap1 GTP-binding protein: a pattern emerges. J Cell Sci 116, 435-440.

Chant, J. and Herskowitz, I. (1991). Genetic control of bud site selection in yeast by a set of gene products that constitute a morphogenetic pathway. Cell 65, 1203-12.

Cullere, X., Shaw, S. K., Andersson, L., Hirahashi, J., Luscinskas, F. W. and Mayadas, T. N. (2005). Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. Blood 105, 1950-1955.

Davis, M. A., Ireton, R. C. and Reynolds, A. B. (2003). A core function for p120-catenin in cadherin turnover. J Cell Biol 163, 525-34.

Dejana, E. (2004). Endothelial cell-cell junctions: happy together. Nat. Rev. Mol. Cell. Biol. 5, 261-270.

Dobrosotskaya, I. Y. and James, G. L. (2000). MAGI-1 interacts with beta-catenin and is associated with cell-cell adhesion structures. Biochem Biophys Res Commun 270, 903-9.

Ebnet, K., Schulz, C. U., Meyer Zu Brickwedde, M. K., Pendl, G. G. and Vestweber, D. (2000). Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1. J Biol Chem 275, 27979-88.

Enserink, J. M., Christensen, A. E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H. G., Doskeland, S. O., Blank, J. L. and Bos, J. L. (2002). A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat. Cell. Biol. 4, 901-906.

Fujita, Y., Krause, G., Scheffner, M., Zechner, D., Leddy, H. E., Behrens, J., Sommer, T. and Birchmeier, W. (2002). Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. Nat Cell Biol 4, 222-31.

Fukuhara, S., Sakurai, A., Sano, H., Yamagishi, A., Somekawa, S.,

Takakura, N., Saito, Y., Kangawa, K. and Mochizuki, N. (2005). Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling. pathway. Mol. Cell. Biol. 25, 136-146.

Fukuyama, T., Ogita, H., Kawakatsu, T., Fukuhara, T., Yamada, T., Sato, T., Shimizu, K., Nakamura, T., Matsuda, M. and Takai, Y. (2005). Involvement of the c-Src-Crk-C3G-Rap1 signaling in the nectin-induced activation of Cdc42 and formation of adherens junctions. J. Biol. Chem. 280, 815-825.

Fukuyama, T., Ogita, H., Kawakatsu, T., Inagaki, M. and Takai, Y. (2006). Activation of Rac by cadherin through the c-Src-Rap1-phosphatidylinositol 3-kinase-Vav2 pathway. Oncogene 25, 8-19.

Hogan, C., Serpente, N., Cogram, P., Hosking, C. R., Bialucha, C. U., Feller, S. M., Braga, V. M., Birchmeier, W. and Fujita, Y. (2004). Rap1 regulates the formation of E-cadherin-based cell-cell contacts. Mol. Cell. Biol. 24, 6690-6700.

Hoshino, T., Sakisaka, T., Baba, T., Yamada, T., Kimura, T. and Takai, Y. (2005). Regulation of E-cadherin endocytosis by nectin through afadin, Rap1, and p120ctn. J. Biol. Chem. 280, 24095-24103.

Kawajiri, A., Itoh, N., Fukata, M., Nakagawa, M., Yamaga, M., Iwamatsu, A. and Kaibuchi, K. (2000). Identification of a novel beta-catenin-interacting protein. Biochem Biophys Res Commun 273, 712-7.

Kitayama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y. and Noda, M. (1989). A ras-related gene with transformation suppressor activity. Cell 56, 77-84.

Knox, A. L. and Brown, N. H. (2002). Rap1 GTPase regulation of adherens junction positioning and cell adhesion. Science 295, 1285-1288.

Kooistra, M. R., Corada, M., Dejana, E. and Bos, J. L. (2005). Epac1 regulates integrity of endothelial cell junctions through VE-cadherin. FEBS Lett 579, 4966-72.

Kowalczyk, A. P. and Reynolds, A. B. (2004). Protecting your tail: regulation of cadherin degradation by p120-catenin. Curr Opin Cell Biol 16, 522-7.

Malliri, A., van Es, S., Huveneers, S. and Collard, J. G. (2004). The Rac exchange factor Tiam1 is required for the establishment and maintenance of cadherin-based adhesions. J Biol Chem 279, 30092-8.

Mertens, A. E., Rygiel, T. P., Olivo, C., van der Kammen, R. and Collard, J. G. (2005). The Rac activator Tiam1 controls tight junction biogenesis in keratinocytes through binding to and activation of the Par polarity complex. J Cell Biol 170, 1029-37.

Mino, A., Ohtsuka, T., Inoue, E. and Takai, Y. (2000). Membrane-associated guanylate kinase with inverted orientation (MAGI)-1/brain angiogenesis inhibitor 1-associated protein (BAP1) as a scaffolding molecule for Rap small G protein GDP/GTP exchange protein at tight junctions. Genes Cells 5, 1009-16.

Moy, A. B., Bodmer, J. E., Blackwell, K., Shasby, S. and Shasby, D. M. (1998). cAMP protects endothelial barrier function independent of inhibiting MLC20-dependent tension development. Am. J. Physiol. 274, L1024-1029.

Nieset, J. E., Redfield, A. R., Jin, F., Knudsen, K. A., Johnson, K. R. and Wheelock, M. J. (1997). Characterization of the interactions of alpha-catenin with alpha-actinin and beta-catenin/plakoglobin. J Cell Sci 110 (Pt 8), 1013-22.

Ohtsuka, T., Hata, Y., Ide, N., Yasuda, T., Inoue, E., Inoue, T., Mizoguchi, A. and Takai, Y. (1999). nRap GEP: a novel neural GDP/GTP exchange protein for rap1 small G protein that interacts with synaptic scaffolding molecule (S-SCAM). Biochem Biophys Res Commun 265, 38-44.

Park, H. O., Kang, P. J. and Rachfal, A. W. (2002). Localization of the Rsr1/Bud1 GTPase involved in selection of a proper growth site in yeast. J Biol Chem 277, 26721-4.

Price, L. S., Hajdo-Milasinovic, A., Zhao, J., Zwartkruis, F. J., Collard, J. G. and Bos, J. L. (2004). Rap1 regulates E-cadherin-mediated cell-cell adhesion. J. Biol. Chem. 279, 35127-35132.

Reynolds, A. B. and Roczniak-Ferguson, A. (2004). Emerging roles for p120-catenin in cell adhesion and cancer. Oncogene 23, 7947-56.

Sakisaka, T. and Takai, Y. (2004). Biology and pathology of nectins and nectin-like molecules. Curr Opin Cell Biol 16, 513-21.

Sakurai, A., Fukuhara, S., Yamagishi, A., Sako, K., Kamioka, Y., Masuda, M., Nakaoka, Y. and Mochizuki, N. (2006). MAGI-1 is required for Rap1 activation upon cell-cell contact and for enhancement of vascular endothelial cadherin-mediated cell adhesion. Mol Biol Cell 17, 966-76.

Sato, T., Fujita, N., Yamada, A., Ooshio, T., Okamoto, R., Irie, K. and Takai, Y. (2006). Regulation of the assembly and adhesion activity of E-cadherin by nectin and afadin for the formation of adherens junctions in Madin-Darby canine kidney cells. J Biol Chem 281, 5288-99.

Schwamborn, J. C. and Puschel, A. W. (2004). The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity. Nat. Neurosci. 7, 923-929.

Somekawa, S., Fukuhara, S., Nakaoka, Y., Fujita, H., Saito, Y. and Mochizuki, N. (2005). Enhanced functional gap junction neoformation by protein kinase A-dependent and Epac-dependent signals downstream of cAMP in cardiac myocytes. Circ Res 97, 655-62.

Suzuki, A. and Ohno, S. (2006). The PAR-aPKC system: lessons in polarity. J Cell Sci 119, 979-87.

Takahashi, K., Nakanishi, H., Miyahara, M., Mandai, K., Satoh, K., Satoh, A., Nishioka, H., Aoki, J., Nomoto, A., Mizoguchi, A. et al. (1999). Nectin/PRR: an immunoglobulin-like cell adhesion molecule recruited to cadherin-based adherens junctions through interaction with Afadin, a PDZ domain-containing protein. J Cell Biol 145, 539-49.

Takai, Y., Irie, K., Shimizu, K., Sakisaka, T. and Ikeda, W. (2003). Nectins and nectin-like molecules: roles in cell adhesion, migration, and polarization. Cancer Sci 94, 655-67.

Takeichi, M. (1995). Morphogenetic roles of classic cadherins. Curr Opin Cell Biol 7, 619-27.

Wittchen, E. S., Worthylake, R. A., Kelly, P., Casey, P. J., Quilliam, L. A. and Burridge, K. (2005). Rap1 GTPase inhibits leukocyte transmigration by promoting endothelial barrier function. J. Biol. Chem. 280, 11675-11682.

Xiao, K., Garner, J., Buckley, K. M., Vincent, P. A., Chiasson, C. M., Dejana, E., Faundez, V. and Kowalczyk, A. P. (2005). p120-Catenin regulates clathrin-dependent endocytosis of VE-cadherin. Mol Biol Cell 16, 5141-51.

Yajnik, V., Paulding, C., Sordella, R., McClatchey, A. I., Saito, M., Wahrer, D. C., Reynolds, P., Bell, D. W., Lake, R., van den Heuvel, S. et al. (2003). DOCK4, a GTPase activator, is disrupted during tumorigenesis. Cell 112, 673-684.

Yap, A. S., Niessen, C. M. and Gumbiner, B. M. (1998). The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. J Cell Biol 141, 779-89.

Chapter 2

General introduction

Abstract

The inner wall of the vasculature is comprised of endothelial cells. The main function of the endothelial cells is their involvement in angiogenesis and, in the adult vessels, the formation of a barrier to regulate the passage of macromolecules, leukocytes and fluids from the blood to the underlying tissue. Pathological conditions such as oedema, chronic inflammation and sepsis show aberrant barrier function regulation. The endothelial integrity depends on the intercellular junctions as well as actomyosin based cell contractility. Here we discuss the endothelial cell-cell junctions and how the endothelial barrier is regulated by various extra-cellular stimuli.

Endothelial cell-cell junctions

Cell-cell junctions are most thoroughly studied in MDCK epithelial cells, where they consist of two separate compartments, adherens junctions and tight junctions. During the formation of cell-cell junctions in these cells we can identify multiple steps leading from initial cell-cell contacts to a fully polarised and impermeable monolayer. First, initial cell-cell contact results in the accumulation of E-cadherin at the cell-cell interface and the formation of an adherens junction. The next step involves the induction of polarity by the cadherins through the PAR-complex which results in the formation of tight junctions. Although the endothelial cell-cell junctions also consist of adherens and tight junctions, in contrast with epithelial cells these are often overlapping. The establishment of the endothelial cell-cell junctions is similar to epithelial cell-cell junctions, although endothelial cells express specific adhesion molecules such as VE-cadherin and PECAM (reviewed in (Dejana, 2004)). Whereas the adherens junctions are present throughout the vasculature, the organisation of the endothelial tight junctions varies in composition in concordance with their location in the vascular tree. For instance, tight junctions are poorly organised in capillaries where fluids are traversing over the endothelium rapidly, whereas these junctions in the brain vasculature are highly organised to achieve high stringency in the barrier function.

VE-Cadherin

The main adhesion molecule of endothelial adherens junctions is VE-cadherin. VE-cadherin is specifically expressed in the endothelium and forms calcium-dependent homophilic interactions (Lampugnani et al., 1992). Through its cytoplasmic tail VE-cadherin binds to β-catenin and p120-catenin, which anchor cadherin proteins to the actin cytoskeleton, but also induce signalling pathways. Despite the fact that VE-cadherin null cells still form PECAM- and JAM-based cell-cell junctions, a monolayer of these cells is more permeable for solutes than wild-type cells (Lampugnani et al., 2002). Furthermore, inhibition of homophilic interactions of VE-cadherin with specific antibodies decreases the endothelial barrier (Corada et al., 2001). Whereas in epithelial cells E-cadherin is only required for the formation of cell-cell junctions, this indicates that in endothelial cells VE-cadherin is also required for the maintenance of cell-cell junctions. However, VE-cadherin is not only involved in the formation and maintenance of endothelial integrity, it also plays a crucial role in survival signalling and angiogenesis as VE-cadherin null mice die at e9.5 due to their inability to remodel and mature the vascular tree (Carmeliet et al., 1999). Furthermore, VE-cadherin interacts with the Rac-GEF Tiam, resulting in the activation of Rac and the reorganisation of the actin cytoskeleton (Lampugnani et al., 2002). Interestingly, Tiam is involved in the signalling from adherens junctions towards tight junction formation in epithelial cells (Mertens et al., 2005), but whether it has a similar role in endothelial cells is still unknown. In addition, a recent report shows that VE-cadherin interacts with the polarity proteins PAR-3 and PAR-6 but no atypical PKC, the classical interaction partner for PAR-3 and PAR-6, suggesting a new role for VE-cadherin in the establishment of endothelial polarity. Interestingly, PAR-3 co-localizes very rapidly with VE-cadherin after reaching confluence, whereas PAR-6 is only found in cell-cell contacts 3 days after reaching confluency, suggesting distinct functions for these polarity proteins in endothelial cells (Iden et al., 2006).

Nectin/AF6

Besides VE-cadherin, the adherens junctions comprise of the Nectin adhesion complex. Although their function in endothelial cells is poorly studied, Nectins form homophilic interactions independent of calcium. Their main interaction partner is Afadin/AF-6. AF-6 localises to cell-cell contacts, both in epithelial and endothelial cells and functions as a linker between Nectins and the actin cytoskeleton (reviewed in (Takai and Nakanishi, 2003)). Indeed, evidence is accumulating that AF6 plays an important role in the formation of cell-cell junctions. For instance, although E-cadherin and ZO-1 localize normally to the junctions of epithelial cells in the intestine of AF6 knock-out mice, the integrity

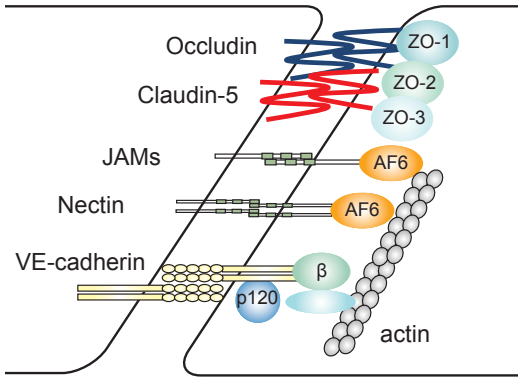

Fig. 1. Endothelial cell-cell junctions.

of the intestinal epithelium is affected (Zhadanov et al., 1999). Similarly, knock-down of AF6 in epithelial cells results in loss of tight junctions. It is suggested that this is dependent on the involvement of AF-6 in changing the conformation of E-cadherin by nectins through p120-catenin (Sato et al., 2006).

Junctional adhesion molecules (JAMs)

Besides Nectins, AF-6 also interacts with the junctional proteins JAMs and ZO1. JAMs are trans-membrane adhesion molecules localised in the tight junctions of both epithelial and endothelial cells, although localisation in adherens junctions is also reported. The three isoforms of JAM, JAM-A/B/C, hetero-dimerise and localise to intercellular contacts, where they participate in the assembly and maintenance of junctions, signalling to cytoskeleton-associated proteins and recruitment of polarity proteins, such as PAR3 (Ebnet et al., 2003; Ebnet et al., 2001; Weber et al., 2007). Although JAMs are involved in junction formation through the establishment of polarity, their role in endothelial permeability is rather ambiguous. JAM-A null cells have similar permeability as wild-type cells (Bazzoni et al., 2005), whereas knock-down of JAM-C decreases permeability presumably by increasing VE-cadherin dependent adhesion. Interestingly, also Rap1 activity is increased upon depletion from JAM-C (Orlova et al., 2006). Moreover, JAMs are more and more implicated in other processes linked to the endothelial barrier function, such as leukocyte adhesion and transmigration to the endothelium. As JAMs are also expressed on circulating blood cells, it is suggested that JAMs act as docking molecules for leukocyte adhesion and transmigration (reviewed in (Weber et al., 2007)). Furthermore, JAM-A regulates the activity of β1-integrin through the GTPase Rap1 in epithelial cells, possibly through AF-6, which can bind to both JAM-A and Rap1 (Mandell et al., 2005).

Tight junctions

Together with JAMs, the main constituents of endothelial tight junctions are occludin and claudin-5. Although down regulation of these proteins reduces the barrier function of the endothelium, occludin-null embryo's bear no vascular defects (Saitou et al., 1998). Adult mice suffer, amongst other abnormalities, from chronic inflammation, suggesting a more complex role for occludin. Ablation of the endothelial tight-junction protein Claudin-5 in mice results in death shortly after birth due to leakage specifically in the blood-brain-barrier, despite normal development of the vasculature (Nitta et al., 2003). In conclusion, endothelial adherens junctions are present throughout the vasculature and its proteins are involved in barrier function, as well as angiogenesis and leukopedesis, whereas tight junction proteins are mainly expressed in the brain vascularity where they seal the blood-brain barrier for leukocytes and large molecules.

Down-regulation of the endothelial barrier function

Under normal conditions, where leukocytes do not pass and junctions are normally present, the endothelium is in a quiescent state. However, during injury of the vascular wall, which also includes deposition of atherosclerotic plaques, or when the underlying tissue is inflamed, the endothelium is activated by various extra-cellular stimuli allowing migration of endothelial cells into the wound and passage of leukocytes towards the inflamed region. Obviously, under such conditions, the endothelial cell-cell junctions are disrupted and the endothelial barrier is reduced. In contrast, upon platelet activation or

hormonal regulation, endothelial cells are stimulated to enhance the endothelial barrier function.

Thrombin

Decreased barrier function is caused by impaired cell-cell contacts or actomyosin contraction. Loss of cell-cell junctions can be induced by phosphorylation and internalisation of junctional components. Signalling pathways regulating cell-cell junction components will therefore likely affect endothelial barrier function. The mechanisms underlying thrombin-induced endothelial permeability has been studied most intensively and it is suggested that the effects of thrombin-induced breakdown of endothelial barrier is due to increased actomyosin contractility (Wojciak-Stothard and Ridley, 2002). Increased actomyosin contractility is reflected by the formation of stress fibres associated with non-muscle myosin II. The best characterized signal triggering formation of stress fibres is phosphorylation of the regulatory part of the myosin molecule to change conformation, interact with actin and slide along actin filaments causing contractility. Thrombin-induced actomyosin contractility can be inhibited by Myosin-light-chain kinase (MLCK) inhibitors (Moy et al., 1996) and scavenging of intracellular calcium inhibits thrombin-induced stress fibre formation (van Nieuw Amerongen et al., 1998). Also, the RhoA GTPase is activated upon thrombin stimulation and inhibition of RhoA as well as inhibition of ROCK with Y-27632 prevents thrombin induced permeability (Carbajal and Schaeffer, 1999; van Nieuw Amerongen et al., 2007; van Nieuw Amerongen et al., 2000). Other vaso-active substances, such as lysophosphatidic acid (LPA), histamine and bacterial lipopolysaccaride (LPS) also induce actomyosin contraction. Although the underlying mechanisms are not completely clear, RhoA activation is suggested to be required for most permeability-increasing agents (reviewed in (Wojciak-Stothard and Ridley, 2002)). It should be noted though that RhoA activation with the E. coli toxin CNF-1 induces stress fibres, it is not sufficient to decrease the endothelial barrier function, indicating that other signalling pathways besides RhoA are involved (Vouret-Craviari et al., 1999).

VEGF

Vascular endothelial growth factor (VEGF) stimulates angiogenesis, proliferation and migration of endothelial cells in vivo and therefore is targeted for therapeutic interventions in cancer. Furthermore, VEGF induces endothelial permeability both in vitro and in vivo and similar to other permeability-inducing factors, VEGF induces stress-fibres and focal contact formation in endothelial cells (Soga et al., 2001). Although RhoA, Rac and Cdc42 have all been linked to VEGF induced permeability through CNF1, only Rac-signalling was shown to be required for VEGF-Receptor mediated permeability (Soga et al., 2001). Also, inhibiting the translocation of the Rac-effector PAK with a peptide, blocks, amongst others, VEGF-induced permeability (Stockton et al., 2004). More recently, data were presented that showed that PAK activation upon VEGF stimulation is required for the activation of ERK, which is also required for VEGF-dependent permeability, probably through phosphorylation of MLC. Finally, the complex between β-PIX, GIT1 and PAK was implicated in this process, leading towards a model that suggests that VEGF-induced permeability requires cooperative signalling between Rac, via PAK/bPIX/GIT1 and Ras via ERK (Stockton et al., 2007).

Enhancement of the endothelial barrier

Beside its role in VEGF signalling, the Rho-like GTPase Rac is also required for assembly and maturation of endothelial junctions and Rac is activated during junction formation. Although the mechanisms employed by Rac are not clear, one possibility is that it releases IQGAP from β-catenin, allowing β-catenin to bind to E-cadherin (Kuroda et al., 1998). This would then result in stabilisation of E-cadherin at the cell-cell contacts. Transfection of dominant negative Rac into endothelial cells increases endothelial permeability and affects cell-cell junctions (Wojciak-Stothard et al., 2001). Besides regulation of junctions a cross-talk between RhoA and Rac, as observed in other cell-types, can contribute to the RhoA-induced endothelial permeability as well as to Rac induced barrier increase (Wojciak-Stothard et al., 2001). For instance, during thrombin stimulation, Rac activity is down-regulated and possibly works in concert with RhoA activation (Vouret-Craviari et al., 1998; Wojciak-Stothard et al., 2001), although another report found no change in Rac activation during thrombin treatment (van Nieuw Amerongen et al., 2000). Although dominant-negative cdc42 attenuates thrombin-induced actomyosin contractility, it is not activated upon thrombin stimulation (Wojciak-Stothard et al., 2001). As cdc42 and Rac share some guanine-nucleotide exchange factors (GEFs), it is possible that the use of a dominant negative form of cdc42 results in recruitment of these GEFs, thereby inhibiting also Rac-dependent signalling pathways.

S1P

The lysophospholipid sphingosine-1-phosphate

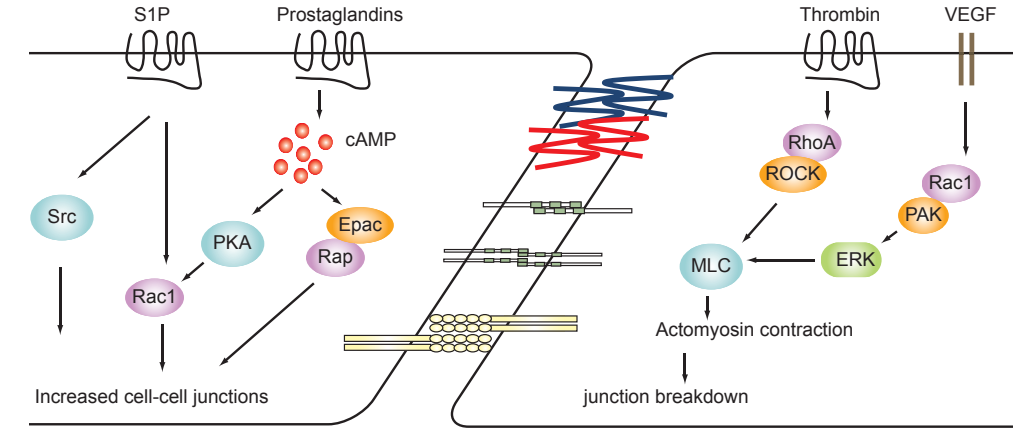

Fig. 2. regulation of endothelial barrier function.

(S1P) represents another stimulus that enhances the endothelial barrier function. S1P is released by activated blood platelets and is proposed to act through the activation of both Rac and Src-kinase. It induces cortical actin formation, which is usually associated with the stabilisation of cell-cell junctions (reviewed in (McVerry and Garcia, 2005)). Interestingly, both Rac and RhoA are activated by S1P stimulation, although RhoA activation is delayed compared to Rac activation (Vouret-Craviari et al., 2002). (Intriguingly,) it was recently shown that enhancement of trans-endothelial electrical resistance (TER) by S1P does not require VE-cadherin or extra-cellular calcium (Xu et al., 2007). This might suggest that S1P regulates the tight junctions directly. However, it is also possible that spreading of cells independent of cell-cell contacts is sufficient to enhance TER.

cAMP

Besides S1P, also prostaglandins increase the endothelial barrier function. Prostaglandins are hormones that via G-protein-coupled receptors, activate adenylate cyclase and produce of cAMP. Treatment of endothelial cells with the adenylate cyclase-agonist Forskolin is sufficient to decrease endothelial permeability and results in cortical actin formation and enhancement of VE-cadherin at the cell-cell contacts.

Elevation of cAMP levels in endothelial cells results in activation of Protein kinase A (PKA) and the exchange protein activated by cAMP (Epac). Protein kinase A consists of two catalytic and two regulatory subunits. Upon cAMP binding to the regulatory subunits, the catalytic subunits are released and can phosphorylate a wide variety of substrates. Epac1 and Epac2 are GEFs for the Ras-like small GTPase Rap1 and Rap2. Although activation of either PKA or Epac is sufficient to enhance the endothelial barrier, their respective downstream mechanisms are distinct. One striking difference is revealed when endothelial resistance is analysed in the absence of integrin-dependent cell-matrix binding. In cells adhering to poly-L-lysine coated electrodes activation of PKA no longer enhances the trans-endothelial electrical resistance, whereas the effect of Epac1-activation is unaffected. This suggests that in contrast with Epac1-mediated barrier function, PKA enhances the endothelial barrier in an integrin-dependent manner (Lorenowicz et al., 2007).

Effectors of cAMP-dependent barrier enhancement

PKA

As the role of Epac1 in endothelial barrier function was only established recently, earlier publications attributed all cAMP-effects to PKA. For instance, vasodilator-stimulated phosphoprotein (VASP) is phosphorylated by PKA in endothelial cells and translocates . from the focal contacts to the cell-cell contacts upon elevation of intracellular cAMP levels. VASP is a member of the Enabled/VASP protein family, involved in cortical actin dynamics. Although their exact function in the regulation of actin is unclear, it is suggested that Ena/VASP is uncaps the actin cytoskeleton, thereby decreasing the barbed actin and enhancing more elongated actin fibres. Indeed, Ena/VASP proteins are involved in the regulation of the actin cytoskeleton in lamellipodia, filopodia and cell-cell junctions. Furthermore, a recent publication proposes that Rac1 is required for the effects of

both PKA and Epac on the endothelial barrier. Upon stimulation with prostaglandins, Rac1 is activated and this activation is attenuated upon PKA-inhibition or knock-down of Epac1. Additionally, siRNA for Rac1 inhibits prostaglandin mediated endothelial barrier function (Birukova et al., 2007b). However, specific activation of either Epac1 or PKA with selective cAMP analogues was not investigated, thereby missing an opportunity to distinguish between the action of these cAMP targets. Also, Forskolin induces phosphorylation of RhoGDI, resulting in an increased interaction with RhoA and thereby keeping RhoA inactive (Qiao et al., 2003). Altogether this suggests that PKA enhances the endothelial barrier through regulation of Rho-like GTPases in an integrin-dependent manner.

Epac1

As the role of Epac in endothelial permeability becomes more and more established, also the underlying mechanisms are being unravelled. Direct activation of Rap1 by Epac1 is required for the effects of the Epac1-specific cAMP analog 8-pCPT-2'O-Me-cAMP (007), since RapGAP transfected cells no longer increase their barrier function upon stimulation (Cullere et al., 2005; Fukuhara et al., 2005; Wittchen et al., 2005). As mentioned above, it is proposed that Rac1-signalling is required for Epac1-induced barrier function (Birukova et al., 2007a; Birukova et al., 2007b). However, recent data also support a role for the Rap1-effector KRIT1 in Epac1-induced endothelial barrier regulation. KRIT1, or CCM1, is a multi-domain protein, consisting of a FERM-domain and several ankyrin repeats and was previously identified as a binding partner for Rap1 in yeast-2-hybrid (Wohlgemuth et al., 2005). Glading and colleagues now show that KRIT1 is localised to the cell-cell contacts and is internalised upon thrombin stimulation. However, when Rap1 is active, either by activation of Epac1 or transfection with dominant active form of Rap1, KRIT1 is retained at the cell-cell contracts. Interestingly, this probably holds true for most junction proteins, as Rap1 activation prevents thrombin-induced contraction. Intriguingly, knock-down of KRIT in endothelial cells results in increase permeability and abrogates the effect of 007 on thrombin-induced permeability. Furthermore, over-expression of KRIT1 also prevented thrombin-induced permeability and rescues RapGAP-induced permeability. The underlying mechanisms for KRIT1 remain elusive, although it is suggested that binding of Rap1 to KRIT1 results in binding to β-catenin and thereby stabilising the junctions (Glading et al., 2007). Interestingly, mutations in KRIT1 can underlie cerebral cavernous malformations (CCM), a disease characterised by lesions in the brain vasculature (Revencu and Vikkula, 2006). Two other proteins involved in these malformations, CCM2 and CCM3 are potentially involved in the endothelial barrier function regulated by Rap1, especially since we found CCM2 and the KRIT1-interacting protein ICAP1 in our siRNA approach (Chapter 5).

Although most reports suggest a role of the actin cytoskeleton in Epac1-mediated barrier function, recent evidence suggests that microtubules (MTs) may play a role as well. Transfected GFP-Epac1 partially co-localises with MTs in endothelial cells and stimulation of endothelial cells with 007 increases the growth of the microtubules. However, the length of the MTs was also claimed to be increased, although the level of detail in these experiments is rather low. Interestingly, the increased length was independent of Rap1 activation, as over-expression of RapGAP had no effect on MT outgrowth (Sehrawat et al., 2008). Previous reports already indicated a role for Epac1 in microtubule regulation (Yarwood, 2005); however, the functional relevance was never established. Also in the data presented by Sehrawat and colleagues the relation between microtubule outgrowth and endothelial barrier function is rather weak. The only evidence for the involvement of microtubules in Epac1-mediated endothelial barrier function is that complete disruption of the microtubule network with the MT-destabilising drug nocodazole perturbed 007-induced TER, suggesting that intact microtubules are required. Interestingly, the MT-stabilizing drug Taxol has no effect on 007-induced TER (Sehrawat et al., 2008), suggesting that the microtubule network is required, but does not need a normal turnover for the effects of Epac1 activation on the endothelial barrier function. The requirement for microtubules is not unexpected since the microtubule and actin networks are linked and perturbation of one affects the other. Altogether, enhancement of the endothelial barrier function by cAMP is mediated both by PKA and Epac1 through distinct signalling pathways. Whether cross-talk between these pathways exists is still elusive.

Scope of this thesis

At the start of the research described in this thesis, two observations suggested a role for Rap in junctions. In *D. melanogaster* the organization of the cell junctions in the wing discs was shown to be dependent on the small GTPase Rap1(Knox and Brown, 2002).

Furthermore, in mammalian cells inhibition of Rap1 prevented the formation of Cadherin-based cell-cell junctions (Hogan et al., 2004; Price et al., 2004). In this thesis we describe our studies on the role of Rap1 in the regulation of cell-cell junctions and its role in endothelial barrier function. In Chapter 1 we summarise our current understanding of the regulation of cell-cell junctions by Rap1. In Chapter 3 and 4 we describe the role of two Rap1 activators in the formation of cell-cell junctions. Our results in Chapter 3 show that the activation of the Rap1 GEF Epac1 results in increased barrier function and that activation of Epac1/Rap1 results in a dramatic re-organization of the actin cytoskeleton in a VE-cadherin independent manner.

In Chapter 4 we investigate the role of another Rap1 activator, PDZGEF2 in epithelial and endothelial cells. Depletion of PDZGEF2 results in zipper-like junctions or retraction fibers, structures that are normally observed as an intermediate during junction maturation. We also show that PDZGEF2 is required for the activation of Rap1 during junction breakdown by chelating calcium. Finally, we could show that re-activation of Rap1 rescues the phenotype of PDZGEF2.

Apart from Rap1 regulators, we also investigated the down-stream signaling pathways. AF-6 is a cell junction protein that has been genetically linked to Rap1 in dorsal closure in *D. melanogaster* and binds directly to Rap1 (Boettner et al., 2003; Yamamoto et al., 1999). In Chapter 5 we investigated the role of AF-6 in Epac1/Rap1-induced barrier function. However, depletion of AF-6 in endothelial cells has no effect on Epac1-induced endothelial resistance. Interestingly, knock-down of AF-6 results in decreased basal endothelial resistance and reorganizes the actin cytoskeleton, whereas the cell junctions are normal.

To identify modifiers of the Rap1-mediated barrier regulation in endothelial cells, we used the siRNA approach described in Chapter 6. We present a small siRNA screen using a customized library on Epac1-induced endothelial resistance. We identify a few potential regulators of Epac1-induced barrier function. In Chapter 7 we investigate one of these regulators, Lamellipodin in more detail. Lamellipodin is required for Epac1-induced endothelial resistance. Translocation of VASP upon 007-treatment is independent of VE-cadherin. Furthermore VE-cadherin is not required for Epac1-induced endothelial resistance, whereas an intact actin cytoskeleton is required.

Finally, in Chapter 8 the mechanisms employed by Rap1 to regulate the endothelial barrier function are discussed.

References

Bazzoni, G., Tonetti, P., Manzi, L., Cera, M. R., Balconi, G. and Dejana, E. (2005). Expression of junctional adhesion molecule-A prevents spontaneous and random motility. J Cell Sci 118, 623-32.

Birukova, A. A., Zagranichnaya, T., Alekseeva, E., Bokoch, G. M. and Birukov, K. G. (2007a). Epac/Rap and PKA are novel mechanisms of ANP-induced Rac-mediated pulmonary endothelial barrier protection. J Cell Physiol.

Birukova, A. A., Zagranichnaya, T., Fu, P., Alekseeva, E., Chen, W., Jacobson, J. R. and Birukov, K. G. (2007b). Prostaglandins PGE(2) and PGI(2) promote endothelial barrier enhancement via PKA- and Epac1/Rap1-dependent Rac activation. Exp Cell Res 313, 2504-20.

Boettner, B., Harjes, P., Ishimaru, S., Heke, M., Fan, H. Q., Qin, Y., Van Aelst, L. and Gaul, U. (2003). The AF-6 homolog canoe acts as a Rap1 effector during dorsal closure of the Drosophila embryo. Genetics 165, 159-69.

Carbajal, J. M. and Schaeffer, R. C., Jr. (1999). RhoA inactivation enhances endothelial barrier function. Am J Physiol 277, C955-64.

Carmeliet, P., Lampugnani, M. G., Moons, L., Breviario, F., Compernolle, V., Bono, F., Balconi, G., Spagnuolo, R., Oostuyse, B., Dewerchin, M. et al. (1999). Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. Cell 98, 147-157.

Corada, M., Liao, F., Lindgren, M., Lampugnani, M. G., Breviario, F., Frank, R., Muller, W. A., Hicklin, D. J., Bohlen, P. and Dejana, E. (2001). Monoclonal antibodies directed to different regions of vascular endothelial cadherin extracellular domain affect adhesion and clustering of the protein and modulate endothelial permeability. Blood 97, 1679-1684.

Cullere, X., Shaw, S. K., Andersson, L., Hirahashi, J., Luscinskas, F. W. and Mayadas, T. N. (2005). Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. Blood 105, 1950-1955.

Dejana, E. (2004). Endothelial cell-cell junctions: happy together. Nat. Rev. Mol. Cell. Biol. 5, 261-270.

Ebnet, K., Aurrand-Lions, M., Kuhn, A., Kiefer, F., Butz, S., Zander, K., Meyer zu Brickwedde, M. K., Suzuki, A., Imhof, B. A. and Vestweber, D. (2003). The junctional adhesion molecule (JAM) family members JAM-2 and JAM-3 associate with the cell polarity protein PAR-3: a possible role for JAMs in endothelial cell polarity. J Cell Sci 116, 3879-91.

Ebnet, K., Suzuki, A., Horikoshi, Y., Hirose, T., Meyer Zu Brickwedde, M. K., Ohno, S. and Vestweber, D. (2001). The cell polarity protein ASIP/PAR-3 directly associates with junctional adhesion molecule (JAM). Embo J 20, 3738-48.

Fukuhara, S., Sakurai, A., Sano, H., Yamagishi, A., Somekawa, S., Takakura, N., Saito, Y., Kangawa, K. and Mochizuki, N. (2005). Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling. pathway. Mol. Cell. Biol. 25, 136-146.

Glading, A., Han, J., Stockton, R. A. and Ginsberg, M. H. (2007). KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell cell junctions. J Cell Biol 179, 247-54.

Hogan, C., Serpente, N., Cogram, P., Hosking, C. R., Bialucha, C. U., Feller, S. M., Braga, V. M., Birchmeier, W. and Fujita, Y. (2004). Rap1 regulates the formation of E-cadherin-based cell-cell contacts. Mol. Cell. Biol. 24, 6690-6700.

Iden, S., Rehder, D., August, B., Suzuki, A., Wolburg-Buchholz, K., Wolburg, H., Ohno, S., Behrens, J., Vestweber, D. and Ebnet, K. (2006). A distinct PAR complex associates physically with VE-cadherin

in vertebrate endothelial cells. EMBO Rep 7, 1239-46.

Knox, A. L. and Brown, N. H. (2002). Rap1 GTPase regulation of adherens junction positioning and cell adhesion. Science 295, 1285-1288.

Kuroda, S., Fukata, M., Nakagawa, M., Fujii, K., Nakamura, T., Ookubo, T., Izawa, I., Nagase, T., Nomura, N., Tani, H. et al. (1998). Role of IQGAP1, a target of the small GTPases Cdc42 and Rac1, in regulation of E-cadherin- mediated cell-cell adhesion. Science 281, 832-5.

Lampugnani, M. G., Resnati, M., Raiteri, M., Pigott, R., Pisacane, A., Houen, G., Ruco, L. P. and Dejana, E. (1992). A novel endothelial-specific membrane protein is a marker of cell-cell contacts. J. Cell. Biol. 118, 1511-1522.

Lampugnani, M. G., Zanetti, A., Breviario, F., Balconi, G., Orsenigo, F., Corada, M., Spagnuolo, R., Betson, M., Braga, V. and Dejana, E. (2002). VE-cadherin regulates endothelial actin activating Rac and increasing membrane association of Tiam. Mol. Biol. Cell 13, 1175-1189.

Lorenowicz, M. J., Fernandez-Borja, M., Kooistra, M. R. H., Bos, J. L. and Hordijk, P. L. (2007). PKA and Epac1 regulate endothelial integrity and cell migration through independent pathways. Submitted, 71-99.

Mandell, K. J., Babbin, B. A., Nusrat, A. and Parkos, C. A. (2005). Junctional adhesion molecule 1 regulates epithelial cell morphology through effects on beta1 integrins and Rap1 activity. J Biol Chem 280, 11665-74.

McVerry, B. J. and Garcia, J. G. (2005). In vitro and in vivo modulation of vascular barrier integrity by sphingosine 1-phosphate: mechanistic insights. Cell Signal 17, 131-9.

Mertens, A. E., Rygiel, T. P., Olivo, C., van der Kammen, R. and Collard, J. G. (2005). The Rac activator Tiam1 controls tight junction biogenesis in keratinocytes through binding to and activation of the Par polarity complex. J Cell Biol 170, 1029-37.

Moy, A. B., Van Engelenhoven, J., Bodmer, J., Kamath, J., Keese, C., Giaever, I., Shasby, S. and Shasby, D. M. (1996). Histamine and thrombin modulate endothelial focal adhesion through centripetal and centrifugal forces. J Clin Invest 97, 1020-7.

Nitta, T., Hata, M., Gotoh, S., Seo, Y., Sasaki, H., Hashimoto, N., Furuse, M. and Tsukita, S. (2003). Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol 161, 653-60.

Orlova, V. V., Economopoulou, M., Lupu, F., Santoso, S. and Chavakis, T. (2006). Junctional adhesion molecule-C regulates vascular endothelial permeability by modulating VE-cadherin-mediated cell-cell contacts. J Exp Med 203, 2703-14.

Price, L. S., Hajdo-Milasinovic, A., Zhao, J., Zwartkruis, F. J., Collard, J. G. and Bos, J. L. (2004). Rap1 regulates E-cadherin-mediated cell-cell adhesion. J. Biol. Chem. 279, 35127-35132.

Qiao, J., Huang, F. and Lum, H. (2003). PKA inhibits RhoA activation: a protection mechanism against endothelial barrier dysfunction. Am J Physiol Lung Cell Mol Physiol 284, L972-80.

Revencu, N. and Vikkula, M. (2006). Cerebral cavernous malformation: new molecular and clinical insights. J Med Genet 43, 716-21.

Saitou, M., Fujimoto, K., Doi, Y., Itoh, M., Fujimoto, T., Furuse, M., Takano, H., Noda, T. and Tsukita, S. (1998). Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. J Cell Biol 141, 397-408.

Sato, T., Fujita, N., Yamada, A., Ooshio, T., Okamoto, R., Irie, K. and Takai, Y. (2006). Regulation of the assembly and adhesion activity of E-cadherin by nectin and afadin for the formation of adherens junctions in Madin-Darby canine kidney cells. J Biol Chem 281, 5288-99.

Sehrawat, S., Cullere, X., Patel, S., Italiano, J., Jr. and Mayadas, T. N. (2008). Role of Epac1, an Exchange Factor for Rap GTPases, in Endothelial Microtubule Dynamics and Barrier Function. Mol Biol Cell.

Soga, N., Namba, N., McAllister, S., Cornelius, L., Teitelbaum, S. L., Dowdy, S. F., Kawamura, J. and Hruska, K. A. (2001). Rho family GTPases regulate VEGF-stimulated endothelial cell motility. Exp Cell Res 269, 73-87.

Stockton, R., Reutershan, J., Scott, D., Sanders, J., Ley, K. and Schwartz, M. A. (2007). Induction of vascular permeability: beta PIX and GIT1 scaffold the activation of extracellular signal-regulated kinase by PAK. Mol Biol Cell 18, 2346-55.

Stockton, R. A., Schaefer, E. and Schwartz, M. A. (2004). p21-activated kinase regulates endothelial permeability through modulation of contractility. J Biol Chem 279, 46621-30.

Takai, Y. and Nakanishi, H. (2003). Nectin and afadin: novel organizers of intercellular junctions. J Cell Sci 116, 17-27.

van Nieuw Amerongen, G. P., Beckers, C. M., Achekar, I. D., Zeeman, S., Musters, R. J. and van Hinsbergh, V. W. (2007). Involvement of Rho kinase in endothelial barrier maintenance. Arterioscler Thromb Vasc Biol 27, 2332-9.

van Nieuw Amerongen, G. P., Draijer, R., Vermeer, M. A. and van Hinsbergh, V. W. (1998). Transient and prolonged increase in endothelial permeability induced by histamine and thrombin: role of protein kinases, calcium, and RhoA. Circ Res 83, 1115-23.

van Nieuw Amerongen, G. P., van Delft, S., Vermeer, M. A., Collard, J. G. and van Hinsbergh, V. W. (2000). Activation of RhoA by thrombin in endothelial hyperpermeability: role of Rho kinase and protein tyrosine kinases. Circ. Res. 87, 335-340.

Vouret-Craviari, V., Boquet, P., Pouyssegur, J. and Van Obberghen-Schilling, E. (1998). Regulation of the actin cytoskeleton by thrombin in human endothelial cells: role of Rho proteins in endothelial barrier function. Mol Biol Cell 9, 2639-53.

Vouret-Craviari, V., Bourcier, C., Boulter, E. and van Obberghen-Schilling, E. (2002). Distinct signals via Rho GTPases and Src drive shape changes by thrombin and sphingosine-1-phosphate in endothelial cells. J Cell Sci 115, 2475-84.

Vouret-Craviari, V., Grall, D., Flatau, G., Pouyssegur, J., Boquet, P. and Van Obberghen-Schilling, E. (1999). Effects of cytotoxic necrotizing factor 1 and lethal toxin on actin cytoskeleton and VE-cadherin localization in human endothelial cell monolayers. Infect Immun 67, 3002-8.

Weber, C., Fraemohs, L. and Dejana, E. (2007). The role of junctional adhesion molecules in vascular inflammation. Nat Rev Immunol 7, 467-77.

Wittchen, E. S., Worthylake, R. A., Kelly, P., Casey, P. J., Quilliam, L. A. and Burridge, K. (2005). Rap1 GTPase inhibits leukocyte transmigration by promoting endothelial barrier function. J. Biol. Chem. 280, 11675-11682.

Wohlgemuth, S., Kiel, C., Kramer, A., Serrano, L., Wittinghofer, F. and Herrmann, C. (2005). Recognizing and defining true Ras binding domains I: biochemical analysis. J Mol Biol 348, 741-58.

Wojciak-Stothard, B., Potempa, S., Eichholtz, T. and Ridley, A. J. (2001). Rho and Rac but not Cdc42 regulate endothelial cell permeability. J Cell Sci 114, 1343-55.

Wojciak-Stothard, B. and Ridley, A. J. (2002). Rho GTPases and the regulation of endothelial permeability. Vascul Pharmacol 39, 187-99.

Xu, M., Waters, C. L., Hu, C., Wysolmerski, R. B., Vincent, P. A. and Minnear, F. L. (2007). Sphingosine 1-phosphate rapidly increases endothelial barrier function independently of VE-cadherin but requires cell spreading and Rho kinase. Am J Physiol Cell Physiol 293, C1309-18.

Yamamoto, T., Harada, N., Kawano, Y., Taya, S. and Kaibuchi, K. (1999). In vivo interaction of AF-6 with activated Ras and ZO-1. Biochem Biophys Res Commun 259, 103-7.

Yarwood, S. J. (2005). Microtubule-associated proteins (MAPs) regulate cAMP signalling through exchange protein directly activated by cAMP (EPAC). Biochem Soc Trans 33, 1327-9.

Zhadanov, A. B., Provance, D. W., Jr., Speer, C. A., Coffin, J. D., Goss, D., Blixt, J. A., Reichert, C. M. and Mercer, J. A. (1999). Absence of the tight junctional protein AF-6 disrupts epithelial cell-cell junctions and cell polarity during mouse development. Curr Biol 9, 880-8.

Chapter 3

Epac1 regulates integrity of endothelial cell junctions through VE-cadherin

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Epac1 regulates integrity of endothelial cell junctions through VE-cadherin

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Abstract

We have previously shown that Rap1 as well as its guanine nucleotide exchange factor Epac1 increase cell-cell junction formation, most likely through the regulation of E-cadherin. Here we show that human umbilical vein endothelial cells express endogenous Epac1 and that the Epac-specific analog 8CPT-2'OMe-cAMP (007) resulted in a tightening of the junctions and a decrease in the permeability of the endothelial cell monolayer. In addition, 007 treatment resulted in the breakdown of actin stress fibers and the formation of cortical actin. These effects were completely inhibited by siRNA against Epac1. To investigate whether VE-cadherin mediates Epac1-induced cell junction formation, we used VE-cadherin knock-out cell lines. In these cells Epac1 did not affect cell permeability, whereas in cells re-expressing VE-cadherin this effect was restored. Finally, the effect of Epac activation on the actin-cytoskeleton was independently of junction formation. From these results we conclude that in HUVEC cells Epac1 controls VE-cadherin-mediated cell junction formation by a process that includes the reorganization of the actin cytoskeleton

Introduction

Epac represents a small family of two guanine nucleotide exchange factors for the small GTPases Rap1 and Rap2. Epac1 and Epac2 are activated by direct binding of cAMP and elevated levels of cAMP results in the activation of both Rap1 and Rap2 in cells that do express Epac1 and/or Epac2. The analysis of the function of these proteins was greatly facilitated by the development of a cAMP analog, 8CPT-2'OMe-cAMP (007) specific for Epac1 and Epac2 (Enserink et al., 2002). This analog does not activate other cAMP targets, i.e. PKA and ion channels. Using this analog it was found that Epac functions, among others, in integrin-mediated adhesion, calcium-induced calcium release and subsequent insulin secretion, activation of phosphoinositol-3-kinase, protein kinase B (PKB) and activation of phospholipase C (PLC) (reviewed in (Bos, 2003)). More recently, Rap proteins were found to regulate cell junction formation as well. The first evidence for involvement of Rap in the formation of adherens junctions came from genetic studies in *Drosophila melanogaster*. Here, loss of the Rap gene resulted in asymmetric positioning of cadherins at cell-cell contact sites, suggesting that Rap may directly modulate cadherin function (Knox and Brown, 2002). Also, DOCK4, an activator of Rap1, enhances the formation of adherens junctions, again hinting towards a role for Rap1 in the regulation of cadherin-mediated cell-cell adhesion (Yajnik et al., 2003). In addition, another GEF for Rap, C3G associates with E-cadherin and through Rap regulates the formation of nascent junctions (Hogan et al., 2004). Finally, inhibition of Rap inhibits E-cadherin function. Interestingly, Epac1 expression in epithelial MDCK cells makes these cells sensitive to cAMP-induced junction formation. This suggests that also Epac could be involved in the control of junction formation (Price et al., 2004).

For endothelial cells it is well established that cAMP stimulates the formation of cell junctions, thereby increasing the barrier function and the protection against various inflammatory mediators has been well. Previously, it was suggested that PKA mediates this cAMP effect. However, inhibition of PKA did not abolish all cAMP effects, suggesting that Epac maybe involved in this process as well (Ishizaki et al., 2003; Moy et al., 1998). In keeping with our previous results that Epac is able to regulated E-cadherin-mediated cell junction formation (Price et al., 2004), we have investigated whether Epac plays a role in endothelial junction formation by siRNA and if so whether VE-cadherin is involved in this process. We observe that in HUVEC Epac1 is the main mediator of cAMP-induced junction formation, a process that requires VE-cadherin, and may involve the formation of cortical actin.

Materials and methods

Cell culture and transfections

HUVEC were isolated according to Jaffe et al. with some modifications

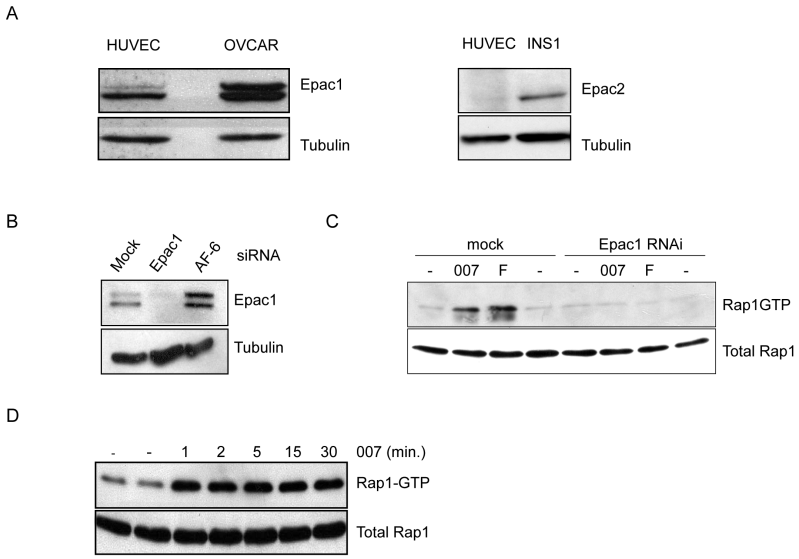


Fig. 1. Epac1 mediates cAMP-induced Rap1 activation in HUVEC. (A) Expression of Epac1 and Epac2 in HUVEC whole cell lysates was determined by Western blotting using the Epac1 monoclonal antibody (5D3) and compared with cell lysates from OVCAR3 and INS1 cells, respectively. (B) Epac1-RNAi was established by transfection of sequence-specific siRNA oligos. HUVEC lysates were analysed 48 hours after transfection by Western blotting against Epac1 and tubulin. (C) Rap1 activity assay was essentially performed as described in the methods section. Cells were stimulated with 007 or Forskolin for 15 minutes. (D) Cells were treated with 007 for the indicated timepoints and Rap1GTP levels were determined as in (C).

as described and cultured in EBM-2 Bulletkit culture medium (EBM-2 supplemented with EGM-2 SingleQuots (hEGF, hydrocortisone, fetal bovine serum, VEGF, hFGF-B, R3-IGF-1, Ascorbic Acid, GA-1000, heparin) (Clonetics)(Jaffe et al., 1973; Willems et al., 1982). Second to fourth passage cells were used. Cells were cultured on 1% gelatin (Sigma) and experiments were performed on 7µg/ml fibronectin (Sigma). 18 hours after plating HUVEC were transfected with or without siRNA oligos (Dharmacon) against Epac1 (GCACCTACGTCTGCAACAA) or AF6 (GAAUAUAGUGAACCAAAGA) as indicated using Oligofectamine (Invitrogen) according to manufacturers recommendation. Transfection was repeated after 24 hours. Samples were analyzed 48 hours after the second round of transfections. Endothelial cells with a homozygous null mutation of the VE-Cadherin-gene and VEC null cells expressing wild-type VE-cadherin (VE-cadherin positive) were described previously (Lampugnani et al., 2002). 8CPT-2'O-Me-cAMP/007 (Biolog) was used at a final concentration of 100µM; Forskolin (Sigma) was used at 10µM.

Antibodies

The mouse mAb 5D3 against Epac1 has been described previously (Price et al., 2004). Mouse mAb against the extracellular domain of human VE-cadherin used was clone TEA 1.31, clone BV9 (Breviario et al., 1995; Lampugnani et al., 1992) and Cadherin-5 (Transduction labs). Rap1 polyclonal antibodies (Santa Cruz), Cortactin monoclonal antibodies (Cell Signaling) and Tubulin monoclonal antibodies (Oncogene) were used.

Rap1 Activation Assay

Rap1 activity was assayed as described previously (van Triest and Bos, 2004). Briefly, cells were washed with cold PBS and lysed with buffer containing 1% Nonidet P-40. Lysates were cleared by centrifugation, and active Rap was precipitated with glutathione-Sepharose beads pre-coupled to a GST fusion protein of the Ras binding domain of RalGDS. Precipitates were washed three times with lysis buffer and solubilised in SDS sample buffer. A portion of the cell lysate was reserved for analysis of total Rap content. Rap1 was detected following Western blotting with anti-Rap1 antibodies.

Permeability assay

Transwell units (6.5-mm diameter, 0.4-µm pore size polycarbonate filters, Corning Costar Corporation) coated with fibronectin (7 µg/mL) were used. Culture medium in the upper and lower compartments was 100 and 600 µL, respectively, as suggested by the manufacturer. Cells were cultured for 72 hours prior to analysis. Cells were incubated with 007 before addition of fluorescein (FITC)-conjugated dextran (38 900 Daltons, final concentration 1 mg/mL, (Sigma)) to measure permeability, immediately followed by thrombin (0,1U/ml). After 30 minutes, a 50-µL sample was removed from the lower compartment and measured using a fluorospectrometer at 492nm.

Immunofluorescence

Cells were cultured on fibronectin-coated (7 µg/mL) glass coverslips (13mm). After stimulation, cells were fixed with 3.8% formaldehyde, permeabilised using 0,2% Triton X-100 and blocked with 2% BSA in PBS. Cells were incubated with indicated antibodies for 1 hour and subsequently incubated with FITC-phalloidin and/or Cy3-labeled anti-mouse antibody (Jackson labs). After mounting the coverslips onto slides, cells were examined using a Zeiss Axiokop2. Quantifications were performed using Metamorph software (Universal Imaging).

Results

To investigate whether and if so, which Epac is functional in HUVE cells, we first tested which of the

two Epac proteins, Epac1 or Epac2, is present. Using monoclonal antibodies to either Epac1 or Epac2 we only observe a positive signal with a mAb against Epac1 (Figure 1A). Epac1 is represented by a double band as previously observed in Ovcar3 cells. To investigate whether both bands represent proteins from the Epac1 gene we used siRNA to knock-down Epac1. Indeed, both bands disappear indicating that they represent two forms of Epac1 (Figure 1B). The difference between the two forms is currently unclear.

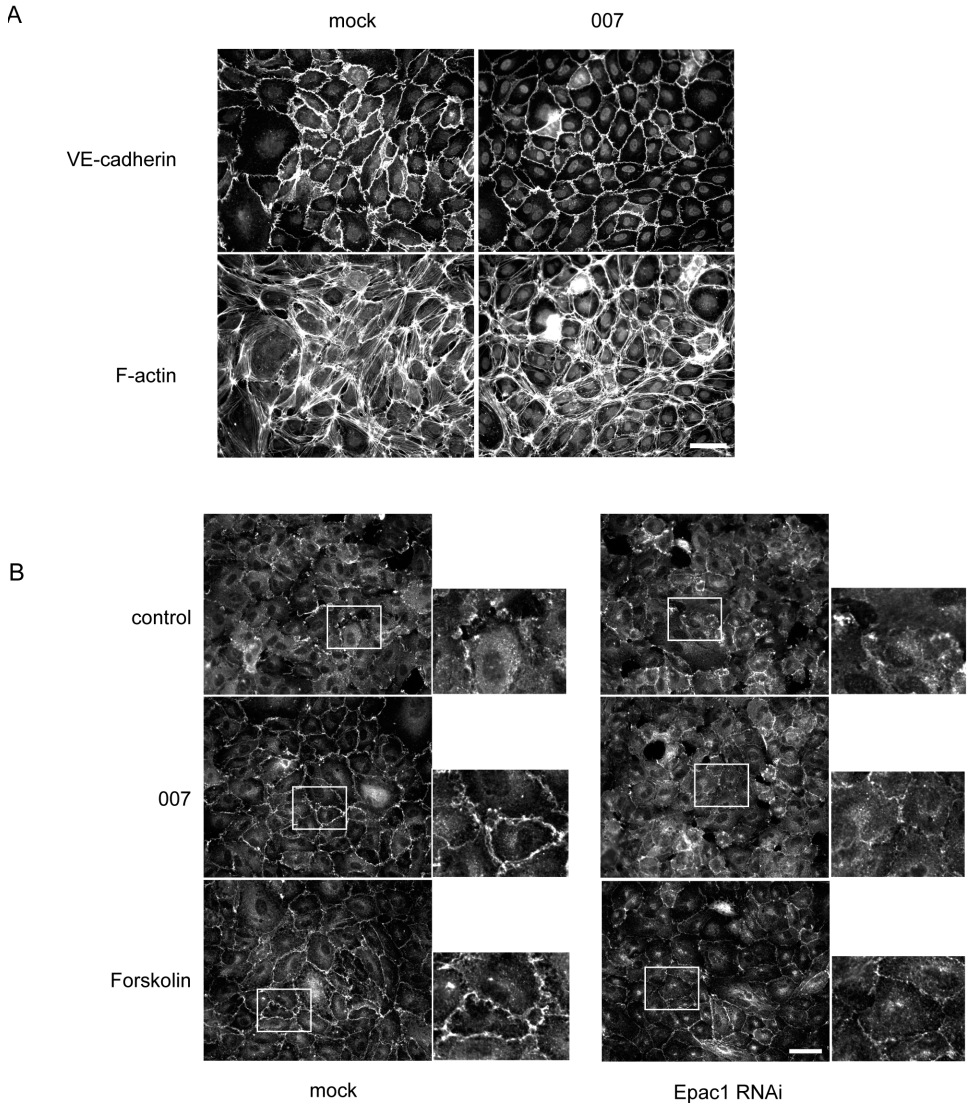
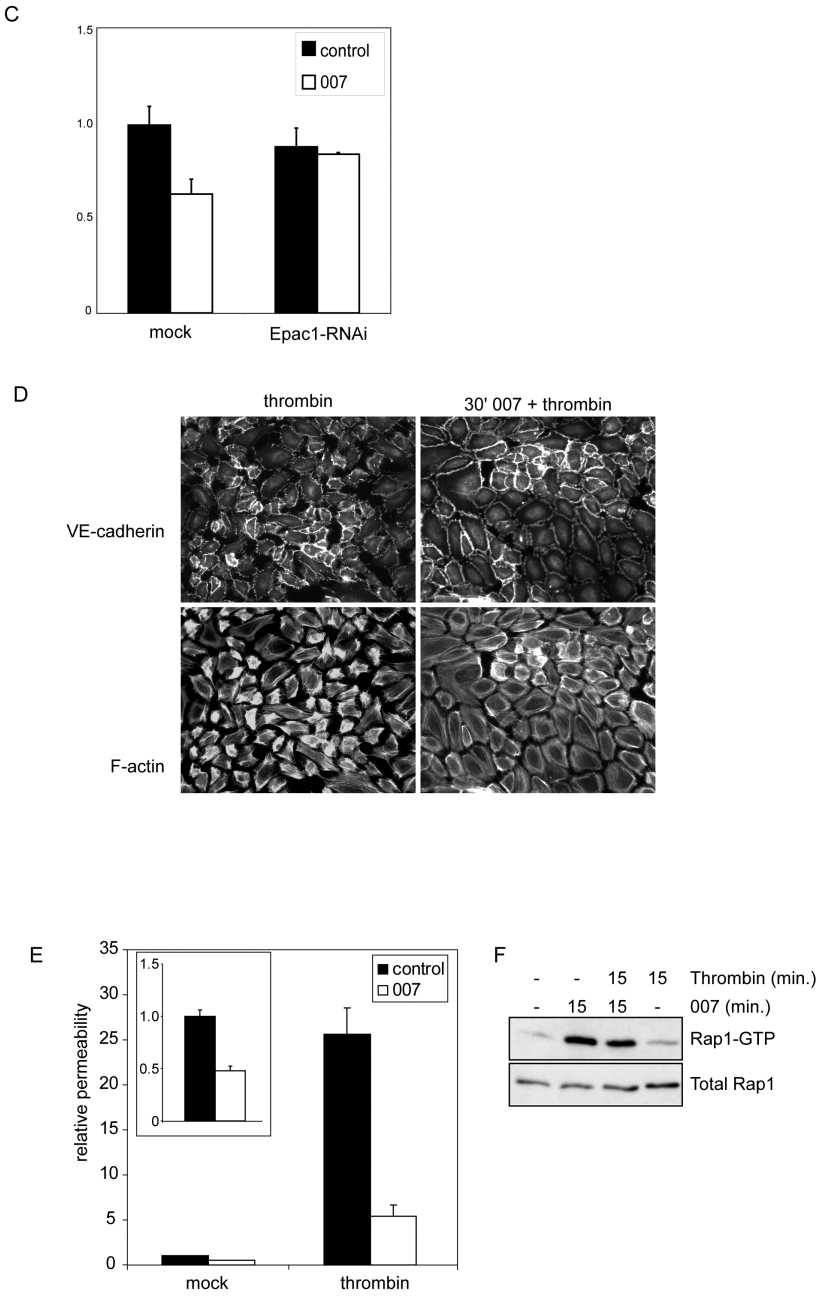


Fig. 2. Epac1 activation induces junction formation and actin remodeling and reduces permeability.
(A) HUVE cells were treated with or without 007 for 30 minutes and stained for VE-cadherin and phalloidin. Bar = 50 µm. (B) Cells wer treated as indicated for 30 minutes and stained for cortactin. Bar = 50 µm. (C) siRNA transfected cells were subjected to a permeability assay in the absence or presence of 007 for 30 minutes. Data shown are representative of three experiments. (D) Cells were treated with or without 007 for 30 minutes prior to treatment with thrombin for 30 minutes. Slides were stained as in (A). (E) HUVEC were treated with or without 007 for 30 minutes previous to the addition of fluorescent dextran in the presence or absence of thrombin and permeability was measured. Average permeability was shown as a relative increase to that observed in unstimulated cells. Differences were significant as determined by student's t-test ($p<0.05$). Data shown are representative of at least three experiments. Inset represents an enlargement of the mock treated samples. (F) Rap1GTP levels were determined after treating HUVE cells as indicated.

Epac is a GEF for the small GTPases Rap1 and Rap2 and is directly activated by cAMP. To investigate whether Epac1 is functional in HUVEC we stimulated the cells with forskolin, a compound that activates adenylate-cyclase, resulting in an increase in cAMP. In addition we used a cAMP analog, 8-CPT-2'0Me-cAMP (007) that specifically activates Epac and not protein kinase A. Both compounds indeed activate Rap1. This activation is completely inhibited by siRNA against Epac1, showing that both cAMP- and 007-



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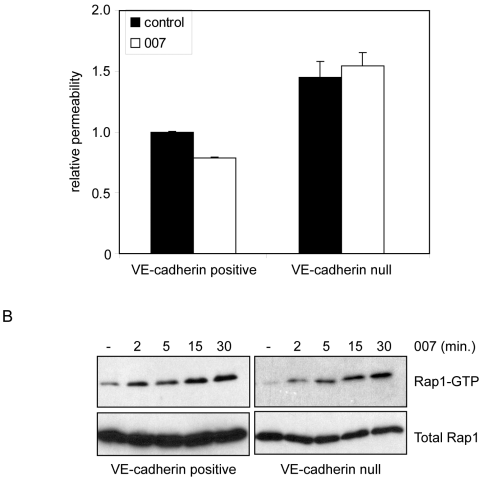

Fig. 3. 007-induced reduction of endothelial permeability is dependent on VE-cadherin.
(A) Endothelial permeability was measured in VE-cadherin null- and VE-cadherin positive cells in the absence or presence of 007 for 5 minutes. (B) Indicated cell-lines were stimulated with 007 for the indicated timepoints and used in a Rap1 activity assay.

induced activation of Rap1 is mediated by Epac1 (Figure 1C). Time course analysis revealed that 007 activates Rap1 rapidly. This activation persists for at least 30 min (Figure 1D). From these results we conclude that HUVECs have a functional cAMP-Epac1-Rap1 signaling pathway.

cAMP is a well established mediator of stimuli that can affect the integrity of the endothelial cell layer by tightening the junctions between endothelial cells. To investigate whether activation of Epac results in more robust cell junctions we stimulated endothelial cells with 007 and stained the cells for both VE-cadherin and actin. VE-cadherin is one of the critical and first components in the formation of cell junctions. Whereas in unstimulated cells the junctions are still rather immature, zipper-like, with small gaps between the cells, 30 min of stimulation with 007 results in much more tightened junctions. In addition, a clear change of the actin cytoskeleton is observed from stress fibers spanning the cell body in the unstimulated cells to filamentous actin at the cell periphery in 007-stimulated cells (Figure 2A).

To further stress the appearance of peripheral filamentous actin in 007-stimulated cells we stained the cells for cortactin, a marker for cortical actin. Clearly, stimulation of HUVECs with 007 results in a remarkable increase in cortactin staining at the cell junctions. This increase is largely abolished when the cells were first treated with siRNA against Epac1. Importantly, also forkolin-induced peripheral cortactin staining is largely inhibited by siRNA against Epac1, indicating that Epac1 is the main mediator of these changes (Figure 2B).

Permeability of endothelial cell layers is regulated in part by the opening and closure of the cell junctions. To investigate whether 007 regulates endothelial permeability in vitro HUVEC were seeded in transwell plates and permeability for FITC-dextran was measured. As shown in Figure 2C, 007 reduced permeability by 30 to 50% dependent on the experiment. Again, Epac1 siRNA completely abolished the 007 effect, supporting the notion that Epac1 mediates the 007 effect. Permeability of endothelial cells is strongly induced by various stimuli, including α-thrombin. Also this effect is strongly reduced by 007 treatment, as shown both by staining for VE-cadherin (Figure 2D) and by measuring cell permeability (Figure 2E). Cell permeability of α-thrombin-treated cells even in the presence of 007 is much higher than untreated cells. To investigate whether this difference may correlate with Rap1 activity, we measure α-thrombin-induced Rap1 activation in the presence and absence of 007. However, we did not observe thrombin-induced Rap1 activation, whereas 007-induced Rap1 activation is similar in α-thrombin-treated compared to untreated cells (Figure 2F). From these results we conclude that in HUVEC activation of Epac1 stimulates the formation of cell junction and reduces cell permeability. In addition, we show that Epac1-Rap1 signaling antagonizes α-thrombin-induced cell junction disruption at a level downstream from Rap1.

One of the first steps in junction formation is a calcium dependent, homotypic interaction between VE-cadherin between two neighboring cells. To investigate whether VE-cadherin is required for 007 induced effects on cell junction formation we used VE-cadherin null mouse cells immortalized with polyoma mT. These cells still form junctions although at a slower rate and with an increased permeability. Importantly, treatment with 007 did not affect the permeability of these cells, whereas cells rescued with a mammalian VE-cadherin did show a consistent 20% decrease in permeability after treatment with 007 (Figure 3A). This difference is not due to the differences in activation of Rap1 by 007, since in both cell types 007 induce similar Rap1 activation (Figure 3B). We therefore conclude that VE cadherin is an essential mediator of Epac-Rap1-induced cell junction formation.

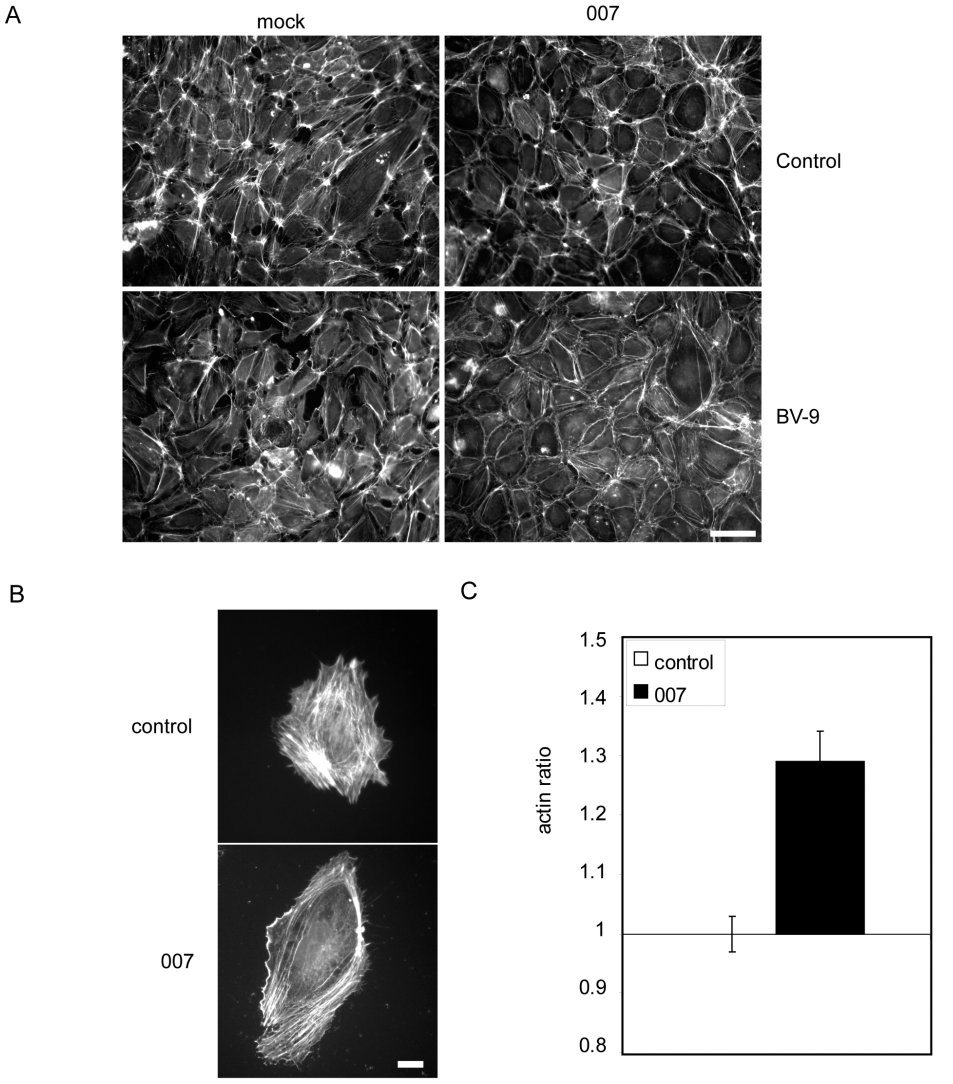

Fig. 4. Actin remodeling by 007 is not dependent on junctions.
(A) HUVEC were grown to confluency in 3-4 days. Indicated antibodies were added to the cells for 7 hours and were subsequently incubated with 007 for 30 minutes before cells were fixed and stained for F-actin. Bar = 50 µm. (B) HUVEC were plated at $1x10^4$ cells/12well. After 24h cells were treated with or without 007 for 30 minutes. Cells were examined using immunofluorescence for F-actin. Bar = 10 µm. (C) Cells were treated as in (B). Intensity of phalloidin staining was measured over an imaginary line through the cell. The average intensity of the outer quarters of the line was divided over the inner two quarters. Average values and SE of 40 cells are shown. ($p< 0.0001$).

Since the actin cytoskeleton and adherens junctions are intimately linked we investigated whether the effects we observed on the actin cytoskeleton are dependent on junction formation. To investigate whether VE-cadherin is required for the actin remodeling, we investigated whether the remodeling also occurs in VE-cadherin null cells. Unfortunately, both in the VE-cadherin positive and VE-cadherin null cells we could not observe 007 induced actin remodeling (data not shown). As an alternative approached we used the VE-cadherin-specific blocking antibody BV9. Binding of BV9 to VE-cadherin causes the disappearance of VE-cadherin from cell-cell contacts (Corada et al., 2001). However, incubation of HUVEC with BV9 did

not affect 007-induced actin rearrangements that resembled the changes observed within an intact monolayer (figure 4A), suggesting that 007-induced actin rearrangements is not due to VE-cadherin-mediated cell junction formation. To exclude the possibility that cell-cell adhesion is still somehow involved in 007-induced actin rearangements we treated a sparse culture of HUVEC with 007. Importantly, the single cells did show rearrangement of the actin cytoskeleton similar to that observed within a monolayer of HUVEC, i.e. a decrease in medial stress fibers and an increase in peripheral stress fibers or cortical actin (figure 4B). Quantification of these actin rearrangements in multiple cells showed a 30% increase in cortical/medial-actin ratio upon 007 treatment (figure 4C). From these results we conclude that the observed 007-induced actin rearrangements are independent of cell junction formation.

Discussion

In this paper we show firstly, that HUVECs express the cAMP-responsive Rap1 GEF, Epac1 predominantly over Epac2 and by siRNA that cAMP-induced Rap1 activation is mediated by Epac1. Secondly, treatment of HUVEC with 007 results in morphologically more tight cell junctions, a rearrangement of the actin cytoskeleton from stress fibers spanning the cell body in unstimulated cells to more cortically located filamentous actin after 007 stimulation. Again this effect was abolished by siRNA against Epac1. Thirdly, we found that VE-cadherin is required for Epac-induced cell junction formation and finally we found that Epac activation results in a rearrangement of the actin cytoskeleton independently of junction formation. While these studies were in progress three papers were published which collectively come to a similar conclusion (Cullere et al., 2004; Fukuhara et al., 2005; Wittchen et al., 2005). In these studies, a role for Epac in endothelial cell junction formation was demonstrated using the Epac specific analog 8CPT-2'OMe-cAMP (007). In all these studies 007 was found to tighten cell junctions and to increase the barrier function of an endothelial monolayer in vitro. Our studies extended these findings by showing that Epac1, and not Epac2 is mediating this effect. This conclusion is based on the observations that we only observe clear Epac1 expression in HUVEC and more importantly, siRNA against Epac1 completely abolished 007-induced Rap1 activation and 007-induced effects on cell junctions.

The mechanism by which Epac regulates endothelial cell junctions is however still largely elusive. However it is clear that VE-cadherin plays a critical role in this process, because, firstly, 007 treatment results in more active VE-cadherin at the cell surface (Fukuhara et al., 2005) and secondly, 007 is unable to increase endothelial barrier function in cells lacking VE-cadherin (this paper). This result is compatible with previous results showing that the Rap1 signalling pathway controls cell junction formation in epithelial cells through E-cadherin (Hogan et al., 2004; Knox and Brown, 2002; Price et al., 2004). In addition, it is clear that Epac induces a rearrangement of the actin cytoskeleton, from stress fibers that span the cell body to cortical actin and the recruitment of the cortical actin-binding protein cortactin to the cell junctions. Importantly, cell junction formation is not required for this cytoskeletal rearrangement because, firstly, disruption of junctions by calcium-depletion or VE-cadherin-specific blocking antibodies is not interfering with actin remodeling and secondly, because actin remodeling is observed in sparse cultures (Cullere et al., 2004), this paper). This opens the attractive hypothesis that the effect of Epac on cell junction formation is mediated by Epac-induced cytoskeletal rearrangements. Indeed a number of proteins that are involved in the organization of the actin cytoskeleton have been implicated as downstream effectors of Rap and thus Epac. These proteins include Vav and Tiam, two GEFs for Rac, ARAP3, a GTPase activating protein for Rho and Arf, and Riam, a profiling binding protein (Arthur et al., 2004; Krugmann et al., 2004; Lafuente et al., 2004). More recently AF-6 was implicated in the Rap1 dependent regulation of E-cadherin endocytosis (Hoshino et al., 2005). Also several studies have implicated Rap signaling upstream from cdc42 (Fukuyama et al., 2005; Schwamborn and Puschel, 2004). Whether these proteins are involved in the observed Epac effects in HUVEC is currently unclear. Intriguingly, 007 was found to inhibit thrombin-induced Rho activation, but not basal Rho activity(Cullere et al., 2004). Indeed, thrombin reduces barrier function of endothelial cells by activation of Rho and thus inhibition of Rho by Epac signaling counteracts the thrombin effect (van Nieuw Amerongen et al., 2000). Since Epac has apparently no effect on basal Rho activity, a different mechanism may be used by Epac to control junction formation.

Vascular leakage as a consequence of a failure in endothelial barrier function often results in serious pathological conditions, including edema and chronic inflammation. The findings that activation of Epac1

can restore endothelial barrier function makes Epac1 a promising target for drugs to restore endothelial integrity. We are currently testing whether indeed the cAMP analog 007 has this protecting effect.

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References

Arthur, W.T., Quilliam, L.A. and Cooper, J.A. (2004) Rap1 promotes cell spreading by localizing Rac guanine nucleotide exchange factors. J Cell Biol, 167, 111-122.

Bos, J.L. (2003) Epac: a new cAMP target and new avenues in cAMP research. Nat Rev Mol Cell Biol, 4, 733-738.

Breviario, F., Caveda, L., Corada, M., Martin-Padura, I., Navarro, P., Golay, J., Introna, M., Gulino, D., Lampugnani, M.G. and Dejana, E. (1995) Functional properties of human vascular endothelial cadherin (7B4/cadherin-5), an endothelium-specific cadherin. Arterioscler Thromb Vasc Biol, 15, 1229-1239.

Corada, M., Liao, F., Lindgren, M., Lampugnani, M.G., Breviario, F., Frank, R., Muller, W.A., Hicklin, D.J., Bohlen, P. and Dejana, E. (2001) Monoclonal antibodies directed to different regions of vascular endothelial cadherin extracellular domain affect adhesion and clustering of the protein and modulate endothelial permeability. Blood, 97, 1679-1684.

Cullere, X., Shaw, S.K., Andersson, L., Hirahashi, J., Luscinskas, F.W. and Mayadas, T.N. (2004) Regulation of vascular endothelial barrier function by Epac, a cAMP activated exchange factor for Rap GTPase. Blood.

Enserink, J.M., Christensen, A.E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H.G., Doskeland, S.O., Blank, J.L. and Bos, J.L. (2002) A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat Cell Biol, 4, 901-906.

Fukuhara, S., Sakurai, A., Sano, H., Yamagishi, A., Somekawa, S., Takakura, N., Saito, Y., Kangawa, K. and Mochizuki, N. (2005) Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling pathway. Mol Cell Biol, 25, 136-146.

Fukuyama, T., Ogita, H., Kawakatsu, T., Fukuhara, T., Yamada, T., Sato, T., Shimizu, K., Nakamura, T., Matsuda, M. and Takai, Y. (2005) Involvement of the c-Src-Crk-C3G-Rap1 signaling in the nectin-induced activation of Cdc42 and formation of adherens junctions. J Biol Chem, 280, 815-825.

Hogan, C., Serpente, N., Cogram, P., Hosking, C.R., Bialucha, C.U., Feller, S.M., Braga, V.M., Birchmeier, W., Fujita, Y., Knox, A.L. and Brown, N.H. (2004) Rap1 regulates the formation of E-cadherin-based cell-cell contacts. Mol Cell Biol, 24, 6690-6700.

Hoshino, T., Sakisaka, T., Baba, T., Yamada, T., Kimura, T. and Takai, Y. (2005) Regulation of E-cadherin endocytosis by nectin through afadin, Rap1, and p120ctn. J Biol Chem.

Ishizaki, T., Chiba, H., Kojima, T., Fujibe, M., Soma, T., Miyajima, H., Nagasawa, K., Wada, I. and Sawada, N. (2003) Cyclic AMP induces phosphorylation of claudin-5 immunoprecipitates and expression of claudin-5 gene in blood-brain-barrier endothelial cells via protein kinase A-dependent and -independent pathways. Exp Cell Res, 290, 275-288.

Jaffe, E.A., Nachman, R.L., Becker, C.G. and Minick, C.R. (1973) Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest, 52, 2745-2756.

Knox, A.L. and Brown, N.H. (2002) Rap1 GTPase regulation of adherens junction positioning and cell adhesion. Science, 295, 1285-1288.

Krugmann, S., Williams, R., Stephens, L. and Hawkins, P.T. (2004) ARAP3 is a PI3K- and rap-regulated GAP for RhoA. Curr Biol, 14, 1380-1384.

Lafuente, E.M., van Puijenbroek, A.A., Krause, M., Carman, C.V., Freeman, G.J., Berezovskaya, A., Constantine, E., Springer, T.A., Gertler, F.B. and Boussiotis, V.A. (2004) RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion. Dev Cell, 7, 585-595.

Lampugnani, M.G., Resnati, M., Raiteri, M., Pigott, R., Pisacane, A., Houen, G., Ruco, L.P. and Dejana, E. (1992) A novel endothelial-specific membrane protein is a marker of cell-cell contacts. J Cell Biol, 118, 1511-1522.

Lampugnani, M.G., Zanetti, A., Breviario, F., Balconi, G., Orsenigo, F., Corada, M., Spagnuolo, R., Betson, M., Braga, V. and Dejana, E. (2002) VE-cadherin regulates endothelial actin activating Rac and increasing membrane association of Tiam. Mol Biol Cell, 13, 1175-1189.

Moy, A.B., Bodmer, J.E., Blackwell, K., Shasby, S. and Shasby, D.M. (1998) cAMP protects endothelial barrier function independent of inhibiting MLC20-dependent tension development. Am J Physiol, 274, L1024-1029.

Price, L.S., Hajdo-Milasinovic, A., Zhao, J., Zwartkruis, F.J., Collard, J.G. and Bos, J.L. (2004) Rap1 regulates E-cadherin-mediated cell-cell adhesion. J Biol Chem, 279, 35127-35132.

Schwamborn, J.C. and Puschel, A.W. (2004) The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity. Nat Neurosci, 7, 923-929.

van Nieuw Amerongen, G.P., van Delft, S., Vermeer, M.A., Collard, J.G. and van Hinsbergh, V.W. (2000) Activation of RhoA by thrombin in endothelial hyperpermeability: role of Rho kinase and protein tyrosine kinases. Circ Res, 87, 335-340.

van Triest, M. and Bos, J.L. (2004) Pull-down assays for guanoside 5'-triphosphate-bound Ras-like guanosine 5'-triphosphatases. Methods Mol Biol, 250, 97-102.

Willems, C., Astaldi, G.C., De Groot, P.G., Janssen, M.C., Gonsalvez, M.D., Zeijlemaker, W.P., Van Mourik, J.A. and Van Aken, W.G. (1982) Media conditioned by cultured human vascular endothelial cells inhibit the growth of vascular smooth muscle cells. Exp Cell Res, 139, 191-197.

Wittchen, E.S., Worthylake, R.A., Kelly, P., Casey, P.J., Quilliam, L.A. and Burridge, K. (2005) Rap1 GTPase inhibits leukocyte transmigration by promoting endothelial barrier function. J Biol Chem, 280, 11675-11682.

Yajnik, V., Paulding, C., Sordella, R., McClatchey, A.I., Saito, M., Wahrer, D.C., Reynolds, P., Bell, D.W., Lake, R., van den Heuvel, S., Settleman, J. and Haber, D.A. (2003) DOCK4, a GTPase activator, is disrupted during tumorigenesis. Cell, 112, 673-684.

Chapter 4

The PDZ-GEF2/Rap1a pathway is required for maturation of cell-cell junctions

Submitted

The PDZ-GEF2/Rap1a pathway is required for maturation of cell-cell junctions

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Abstract

The small G-protein Rap1 is a critical regulator of cell-cell contacts and is activated by the remodeling of adherens junctions. Here we identify the Rap1 guanine nucleotide exchange factor PDZ-GEF2 as an upstream activator of Rap1 required for the maturation of adherens junctions in the lung carcinoma cells A549. Knockdown of PDZ-GEF2 results in the persistence of adhesion zippers at cell-cell contacts and prevents Rap1 activation upon epithelial cadherin internalization. Activation of Rap1A rescues junction maturation in absence of PDZ-GEF2, demonstrating that Rap1A is downstream of PDZ-GEF2 in this process. Moreover, depletion of Rap1A, but not Rap1B, impairs adherens junction maturation. siRNA for PDZ-GEF2 also lowers the levels of E-cadherin, an effect that can be mimicked by Rap1B, but not Rap1A siRNA. Since junctions in Rap1B depleted cells have a mature appearance, these data suggest that PDZ-GEF2 activates Rap1A and Rap1B to perform different functions. Our results present the first direct evidence that the PDZ-GEF2/Rap1A pathway plays a critical role in the maturation of adherens junctions.

Introduction

Rap1 is a small G-protein that acts as a molecular switch in many different processes, including integrin-mediated cell adhesion and epithelial cadherin (E-cadherin)-based adherens junction (AJ) formation (Bos, 2005). AJ are mainly composed of cadherins that interact homophilically with cadherins of neighboring cells in a calcium (Ca^{2+})-dependent manner to mediate cell-cell adhesion. Depending on the cell type, the formation of AJ requires two neighboring cells to extend their filopodia into the membrane of the opposing cell to enhance cell-cell contacts. Cadherin-catenin complexes are recruited at the tip of these filopodial projections, also know as puncta or transmembrane clusters of AJ proteins. Dimers of cadherin-catenin complexes then reorganize to generate a zipper-like structure of assembled puncta. Further junction maturation is ensured by the lateral clustering of cadherins as well as the recruitment of a variety of junctional scaffolding proteins that promote the association of actin-binding and actin polymerization regulators, such as vinculin, zyxin, VASP and Mena (Vasioukhin et al., 2000). Finally, actin polymerization drives the sealing of the cell-cell contacts into a linear shape by generating the mechanical forces to stabilize the junctions and by providing a connection between the E-cadherin complexes and the actin cytoskeleton (Mege et al., 2006; Vaezi et al., 2002; Vasioukhin et al., 2000). Alternatively, in epithelial cells, interactions between opposing lamellipodia initiate E-cadherin clustering while a circumferential actin cable surrounds the cells. As cell-cell adhesion proceeds, E-cadherin molecules cluster into puncta within the cell-cell contact interface and associate with actin filaments. E-cadherin puncta stabilize actin filaments, leading to the dissolution of the circumferential actin cables and their insertion into the cell-cell contact. At the same time, clustering of E-cadherin puncta into E-cadherin plaques at the edges of the cell-cell contacts strengthens cell-cell adhesion (Adams et al., 1998; Perez et al., 2007; Yamada and Nelson, 2007). Moreover, myosin II, myosin VI, as well as microtubule dynamics are thought to be important for the establishment of E-cadherin-based junctions (Maddugoda et al., 2007; Shewan et al., 2005; Stehbens et al., 2006). In addition to the steps and proteins mentioned above, the coordinated actions of several other processes such as trafficking and cell surface organization of E-cadherin are also involved in the formation and maturation of E-cadherin-based cell-cell contacts (Yap et al., 2007). However, the exact molecular mechanisms involved in AJ formation and maturation remain elusive.

Increasing evidence suggests that Rap1 plays a critical role in the formation of AJ. Indeed, Rap1 localizes at the junctions in epithelial and endothelial cells (Hogan et al., 2004; Knox and Brown, 2002; Mandell et al., 2005; Wittchen et al., 2005) and is activated at cell-

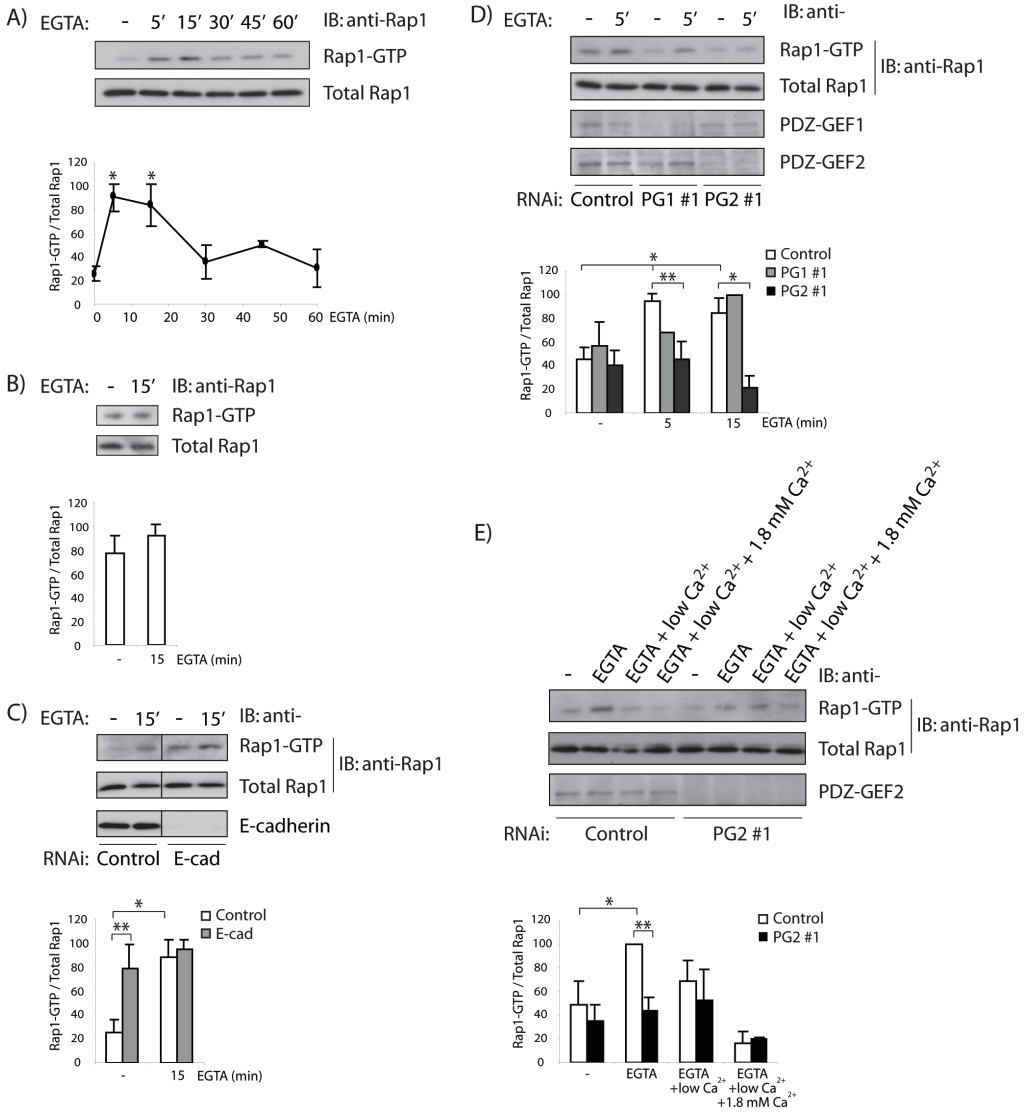

Fig. 1. Activation of Rap1 by Ca2+ depletion is dependent on PDZ-GEF2 expression.

A, B, C and D. Confluent (A), sparse cultures (B), E-cadherin siRNA (C), or control, PDZ-GEF1, PDZ-GEF2 siRNA (D) treated A549 cells were incubated in the presence or absence of EGTA (4 mM) for indicated times and lysates were incubated with GST-RalGDS-RBD to precipitate active (GTP-bound) Rap1. Cell lysates were analyzed by Western blotting with anti-Rap1, anti-E-cadherin, anti-PDZ-GEF1 or anti-PDZ-GEF2 antibodies. E. A549 cells were transfected with control or PDZ-GEF2 (PG2 #1) siRNA and replated 24 hours before the Rap1-GTP pull-down. For the Ca^{2+} switch experiment, cells were incubated in Ca^{2+}-free media supplemented with EGTA (4mM) for 30 minutes (EGTA), followed by two washes in PBS and incubated in 20 μM Ca^{2+} (EGTA + low Ca^{2+}) for 30 minutes, a condition that does not allow the complete reformation of cell-cell junctions. Cells were further incubated in 1.8mM Ca^{2+} (EGTA + low Ca^{2+} + 1.8 mM Ca^{2+}) for 30 minutes. The lysates were incubated with immobilized GST-RalGDS-RBD to precipitate active Rap1. Immunofluorescence studies using anti-E-cadherin antibodies demonstrated that EGTA treatment leads to the loss of E-cadherin staining at cell-cell contacts and that addition of 1.8 mM Ca^{2+} restored E-cadherin-based cell-cell junctions (data not shown). Cell lysates were analyzed by immunoblotting with anti-Rap1 and anti-PDZ-GEF2 antibodies. For the quantification of Rap1-GTP levels relative to total Rap1 protein, Rap1 signals were determined by densiometry and reported as mean ± standard deviation of the mean (SEM). A: 0′ (n=5), 5′ (n=3), 15′ (n=4), 30′, 45′ and 60′ (n=2); B: 0′ and 15′ (n=3); C: Control 0′ and 15′ (n=3), E-cad 0′ and 15′ (n=2); D: Control 0′ (n=5), Control 5′ (n=3), Control 15′ (n=2), PG1 #1 0′ (n=3), PG1 #1 5′ (n=1), PG1 #1 15′ (n=2), PG2 #1 0′ (n=4), PG2 #1 5′ (n=2), PG2 #1 15, (n=2); E: all time points (n=3) except Control and PG2 #1 EGTA + low Ca^{2+} + 1.8mM Ca^{2+} (n=2). T-test: * p<0.03; ** p<0.007.

cell contacts in endothelial cells (Sakurai et al., 2006). In epithelial cells, disruption of AJ activates Rap1 and, inversely, restoration of cell-cell adhesion decreases Rap1 activity in an E-cadherin dependent manner (Balzac et al., 2005).

Several Rap-specific guanine nucleotide exchange factors (GEFs), e.g. C3G, Epac, CalDAG-GEF, and PDZ-GEF, respond to different stimuli to activate Rap1 and are thought to be critical for the spatio-temporal regulation of Rap1 (Hisata et al., 2007; Kooistra et al., 2007). Interestingly, several of these GEFs were found to play a role in the formation of AJ as well (Kooistra et al., 2007; Price et al., 2004; Yajnik et al., 2003). For instance, C3G (RapGEF1) interacts with E-cadherin (Hogan et al., 2004) and is required for E-cadherin recruitment at cell-cell contacts (Fukuyama et al., 2005). Epac1 (RapGEF3), in contrast, is not directly involved in the formation of junctions, but controls endothelial integrity and permeability (Cullere et al., 2005; Fukuhara et al., 2005; Kooistra et al., 2005; Wittchen et al., 2005). PDZ-GEF1 (RapGEF2, RA-GEF-1) was shown to associate with the junctional scaffolding proteins membrane-associated guanylate kinase with inverted orientation-1 and -2 (MAGI-1 and -2), and to bind and colocalize with β-catenin (Dobrosotskaya and James, 2000; Kawajiri et al., 2000; Mino et al., 2000; Ohtsuka et al., 1999; Sakurai et al., 2006). Genetic studies suggest that *C. elegans* PDZ-GEF (pxf-1) is required for maintenance of epithelial integrity (Pellis-van Berkel et al., 2005). Additionally, loss of function of the *Drosophila* PDZ-GEF (dPDZ-GEF, Gef26) impairs AJ formation at the non-dividing somatic cell-germline stem cell interface, causing loss of stem cell identity (Wang et al., 2006). Recently dPDZ-GEF was also shown to modulate cell shape and cell plasticity in epithelial cells (Boettner and Van Aelst, 2007), further stressing a possible role for this GEF in junction formation. However, whether the mammalian PDZ-GEF1 or its family member PDZ-GEF2 (RapGEF6, RA-GEF-2) plays a role in cell-cell junction formation is currently unknown. Here we report two novel and distinct roles of PDZ-GEF2, first in AJ maturation in epithelial and endothelial cells, and second in the regulation of E-cadherin levels. We also find that these functions are differentially regulated by Rap1 isoforms.

Experimental Procedures

Cell culture

A549 cells were maintained in RPMI media (BioWhittaker) supplemented with 10% FBS (Cambrex), 1.2 mM L-Glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin (BioWhittaker). Human vascular endothelial cells (HUVEC) were isolated and cultured as previously described (Kooistra et al., 2005). Second to fifth passage cells were used.

Antibodies

Recombinant protein corresponding to the first atypical cyclic nucleotide binding domain of human PDZ-GEF1 (gene ID 9693; amino acids 1-260) and PDZ-GEF2 (gene ID 51735; amino acids 1-123) were used to produce polyclonal antibodies. Other antibodies used are anti-α-catenin, anti-β-catenin and anti-p120-catenin (BD Transduction Laboratories); anti-ZO-1 and anti-Occludin (Zymed); anti-E-Cadherin (HECD-1) (AbCam); anti-Tubulin (Oncogene); anti-Rap1 (Santa Cruz); anti-HA (12CA5); Alexa Fluor 488 anti-human CD324 (anti-E-cadherin; clone 67A4) (BioLegend); Alexa Fluor 488 Mouse IgG1, κ isotype control (clone MOPC-21) (BioLegend); anti-Epac1 (clone 5D3) (Price et al., 2004); anti-VE-cadherin (clones TEA 1.31 and BV9) (Breviario et al., 1995; Lampugnani et al., 1992); anti-mouse and anti-rabbit Alexa 488 and 568 (Molecular Probes).

Expression vectors

HA-Epac1 (de Rooij et al., 1998) and HA-V12Rap1A (Zwartkruis et al., 1998) vectors were transfected using FuGENE6 (Roche Diagnostics Corporation).

siRNA transfection

Small interfering RNA (siRNA) oligo duplexes were obtained from Dharmacon and transfected using oligofectamine according to the manufacturer's instructions (Invitrogen). For HUVEC, the transfection was repeated after 24 hours and samples were analyzed 48 hours after the second round of transfection. Oligo sequences: PG1 #1 (CTGCCGTATTCTCAATCAA); PG2 #1 (GGATCCAACTTATATAGAA);Rap1A#2(GTGGTGTAACTGTGCCTTT+ CCTGGAAGATGAGCGAGTA);Rap1B#2(CCTAGTGCGGCAAATTAAC + GTGCGGCAAATTAACAGAA); on-target plus SMARTpool: PG2 #2 (L-008593); Rap1A #1 (L-003623); Rap1B #1 (L-010364); E-cadherin (L-003877); α-catenin (L-010505); β-catenin (L-003482); p120-catenin (L-012572).

Immunofluorescence

Cells were fixed in 4% formaldehyde, permeabilized in 0.1% Triton X-100 and blocked with 2% BSA in PBS. Coverslips were incubated with the indicated primary antibodies, followed by incubation with Alexa antibodies and mounted on glass slides. Actin filaments were stained with rhodamine-phalloidin (Molecular Probes). Images were acquired on a DeltaVision Restoration Imaging System (Applied Precision, Seattle) with a 40X/1.42NA Plan-ApoN objective (Olympus) using SoftWorx software. Acquisitions in Z were at 0.2 micron intervals, and stacks were deconvoluted using SoftWorx. Alternatively, images were acquired using a Zeiss LSM 510 Meta confocal microscope. 8-pCPT-2'O-Me-cAMP (007) (Biolog, Bremen, Germany) was used at a final concentration of 50 µM.

Quantification of adhesion zippers

The percentage of adhesion zippers was determined by counting the number of cells that displayed a straight, linear organization of E-cadherin at cell-cell contacts (mature AJ) compared to junctions where the E-cadherin staining presented a non-linear, zipper-like organization (adhesion zippers).

Electron microscopy

siRNA transfected A549 cells were replated and fixed for electron microscopy 24 hours later with a mixture of 2% glutaraldehyde, 5 mM $CaCl_2$, 10 mM $MgCl_2$ in 0.1 M Na-cacodylatebuffer pH 7.4 for four

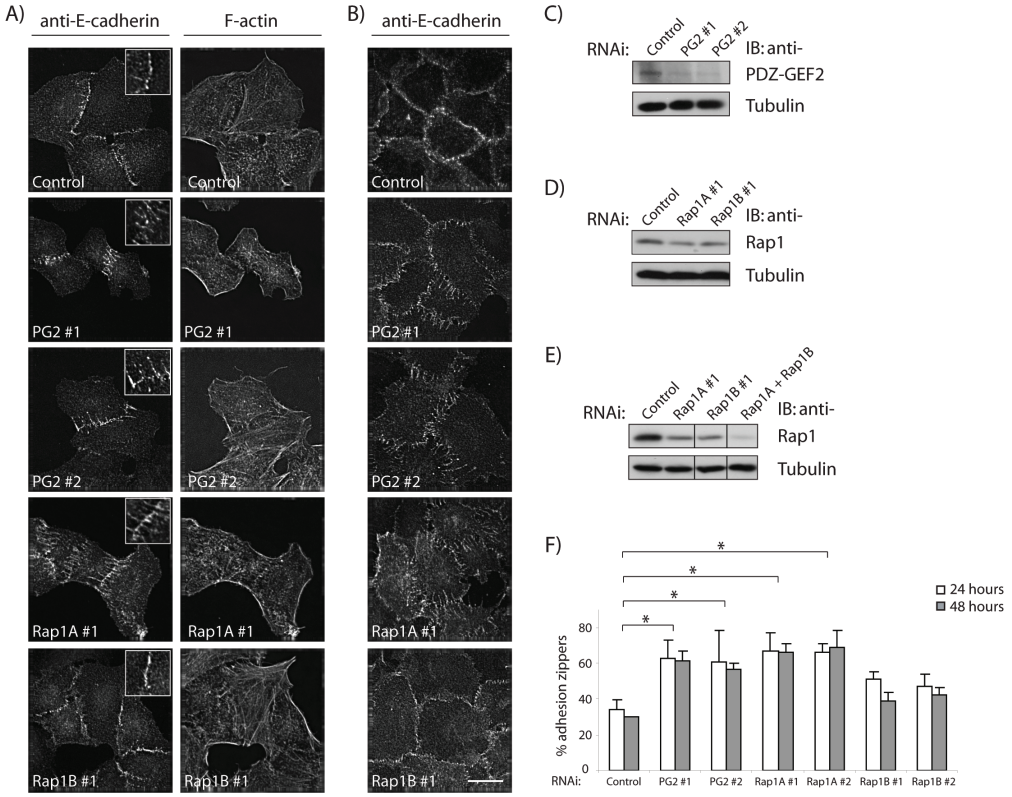

Fig. 2. PDZ-GEF2 or Rap1A depletion in epithelial cells impairs AJ maturation.
A and B. A549 cells transfected with control, PDZ-GEF2 (PG2 #1, PG2 #2), Rap1A (Rap1A #1) or Rap1B (Rap1B #1) siRNA were replated on glass coverslips, fixed and immunostained with anti-E-cadherin antibodies or rhodamine-phalloidin (F-actin) after 24 (A) and 48 hours (B). Similar results were obtained with two different sets of siRNA oligos. A representative picture from at least three independent experiments is shown in each panel. Bar, 20 µm. C, D and E. Cell lysates were analyzed by Western blotting with anti-PDZ-GEF2 or Rap1 antibodies, and anti-Tubulin antibodies were used as a loading control. F. Quantification of adhesion zippers at cell-cell contacts. The ratios were determined blindly by counting the number of junctions that displayed a straight, linear organization of E-cadherin at cell-cell contacts (mature AJ) compared to junctions where the E-cadherin staining presented a non-linear, zipper-like organization (adhesion zippers). The data is expressed as percentage of the number of junctions scoring as "adhesion zippers" over the total E-cadherin junction staining (total of mature AJ + adhesion zippers) of at least 200 junctions per experiment. Averages represent the mean ± SEM of two to five independent experiments. T-test: * $p<0.05$.

hours at room temperature. Post fixation was performed in 1% OsO_4, 1.5% $K_4Fe(Cn)_6$-$3H_2O$ in distilled water for two hours at 4°C. After ethanol dehydration, cells were flat embedded in Epon and sectioned in parallel to the substrate of growth. Ultrathin sections were stained with uranylacetate and lead citrate. To establish the type of contacts between neighboring cells, grids were scanned along a random track, by which all encountered cell-cell interactions were ascribed to one of the following categories: "sealed", where the plasma membranes of the neighboring cells very close together, often running in parallel over extended distances; "intermediate", tight regions of close contact alternating with regions in which the two plasma membranes were at considerable distance; "loose", where the plasma membranes of neighboring cells were mostly at a considerable distance, with very few points of contact, but no regions of membranes running in parallel. Only cell-cell interactions by which both nuclei were visible in the plane of the section were taken into account for the quantification.

E-cadherin cell surface levels

siRNA transfected A549 cells were replated and collected 24 hours later in cold PBS containing 2% FBS on ice. Cell suspensions were incubated with Alexa Fluor 488 anti-human CD324 (anti-E-cadherin; clone 67A4) or Alexa Fluor 488 Mouse IgG1, κ isotype control (clone MOPC-21). Data acquisition was done on a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software, and analysis was performed using WinMDI 2.9 software.

Ca^{2+} switch experiments and Rap-GTP pull-down assay

Cells transfected with siRNA were replated in growth media for 24 hours, rinsed with PBS and incubated in serum-free and Ca^{2+}-free media containing 4 mM EGTA for 30 minutes at 37°C to disrupt E-cadherin-mediated cell-cell contacts. Cells were lysed (200 mM NaCl, 50 mM Tris-HCl pH 7.6, 1% NP-40, 10% glycerol, 2mM $MgCl_2$, containing protease inhibitors, sodium vanadate and sodium fluoride) or incubated in media supplemented with low Ca^{2+} (20 µM) for 30 minutes, then

lysed or further incubated in media containing 1.8 mM Ca^{2+} for 30 minutes. Levels of Rap1-GTP were determined by precipitation with a GST fusion protein of the Ras binding domain of RalGDS, which recognizes only active, GTP-bound Rap. Pull-downs were resolved by SDS-PAGE and immunobloted with anti-Rap1 antibodies to detect precipitated Rap1-GTP (van Triest et al., 2001).

*Western blotting*Cells were washed in ice-cold PBS and lysed in 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1% NP-40 or 200 mM NaCl, 50 mM Tris-HCl pH 7.6, 1% NP-40, 10% glycerol, 2mM $MgCl_2$ containing protease inhibitors, sodium vanadate and sodium fluoride, cleared by centrifugation at 14,000 x g for 10 minutes at 4°C, and subjected to Western Blotting. Signals were quantified using ImageJ software.

Results

PDZ-GEF2 regulates Rap1 activation during Ca^{2+} switch. In our search for a function for PDZ-GEF/Rap1 signaling in E-cadherin-mediated cell adhesion, we used human alveolar cells derived from a basal cell lung carcinoma (A549) as they express both PDZ-GEFs (Fig. 1D) and form E-cadherin-based junctions. We first addressed the question whether disruption of junctions in these cells would lead to Rap1 activation as reported for other epithelial cell lines (Balzac et al., 2005). Indeed, EGTA treatment of A549 cells, which triggered E-cadherin internalization, disrupted AJ and resulted in the activation of Rap1 (Fig. 1A), whereas Rap1-GTP levels remained unchanged in sparse cells lacking cell junctions (Fig. 1B). The basal Rap1 activity in sparse cells was higher compared to confluent cells, compatible with the observation that Rap1 activation is reduced in cells reaching confluence (Posern et al., 1998). Similarly, E-cadherin knockdown by siRNA resulted in higher Rap1 activity (Fig. 1C), indicating that the presence of E-cadherin-based cell-cell junctions is required to maintain Rap1 activity low. These results show that Rap1 is activated when E-cadherin-based cell-cell contacts are disrupted and confirm previously published data (Balzac et al., 2005).

To analyze the putative role of PDZ-GEFs in AJ disruption-induced Rap1 activation, PDZ-GEF1 or PDZ-GEF2 siRNA was introduced in A549 cells. siRNA to PDZ-GEF2, but not to PDZ-GEF1, abolished EGTA-induced Rap1 activation (Fig. 1D). As expected, reformation of AJ by addition of Ca^{2+} resulted in the downregulation of Rap1 in control cells, but the Rap1-GTP levels remained unchanged in PDZ-GEF2 depleted cells (Fig. 1E). This suggests that PDZ-GEF2 responds to E-cadherin-based cell-cell junction disruption by activating Rap1.

PDZ-GEF2/Rap1A pathway is essential for AJ

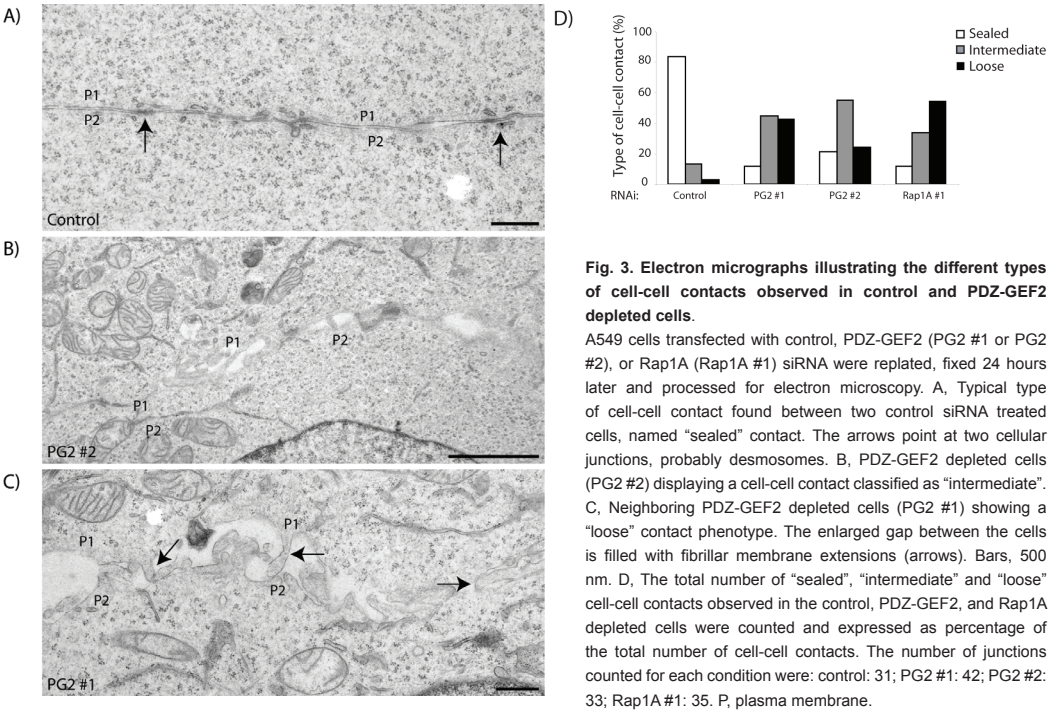

Fig. 3. Electron micrographs illustrating the different types of cell-cell contacts observed in control and PDZ-GEF2 depleted cells.

A549 cells transfected with control, PDZ-GEF2 (PG2 #1 or PG2 #2), or Rap1A (Rap1A #1) siRNA were replated, fixed 24 hours later and processed for electron microscopy. A, Typical type of cell-cell contact found between two control siRNA treated cells, named "sealed" contact. The arrows point at two cellular junctions, probably desmosomes. B, PDZ-GEF2 depleted cells (PG2 #2) displaying a cell-cell contact classified as "intermediate". C, Neighboring PDZ-GEF2 depleted cells (PG2 #1) showing a "loose" contact phenotype. The enlarged gap between the cells is filled with fibrillar membrane extensions (arrows). Bars, 500 nm. D, The total number of "sealed", "intermediate" and "loose" cell-cell contacts observed in the control, PDZ-GEF2, and Rap1A depleted cells were counted and expressed as percentage of the total number of cell-cell contacts. The number of junctions counted for each condition were: control: 31; PG2 #1: 42; PG2 #2: 33; Rap1A #1: 35. P, plasma membrane.

maturation in epithelial cells.

The sustained activation of Rap1 after junction disruption and the downregulation of Rap1 following junction formation seems to be counterintuitive with previous observations that Rap1 activity is required for junction formation (Hogan et al., 2004; Price et al., 2004; Sakurai et al., 2006). However, Rap1 might be activated upon cell-cell contact disruption to maintain junctional integrity. If so, one would predict that PDZ-GEF2 plays a role in junction formation. Therefore we measured the effect of PDZ-GEF2 knockdown on the formation of junctions. E-cadherin staining was analyzed in cells treated with two distinct siRNA for PDZ-GEF2, 24 and 48 hours after replating of the cells (Fig. 2A-C). The junctions observed in PDZ-GEF2 depleted cells were zipper-like structures (Fig. 2A and B), as previously observed in cells establishing cell-cell contacts (Vaezi et al., 2002; Vasioukhin et al., 2000). Both siRNA resulted in a 2-fold increase in the number of adhesion zippers (Fig. 2F), demonstrating that PDZ-GEF2 is involved in junction maturation. The kinetics of E-cadherin recovery at cell-cell contacts following a Ca^{2+} switch was similar in control and PDZ-GEF2 depleted cells (data not shown), implying that E-cadherin is recruited at cell-cell contacts but the junctions cannot proceed to complete maturation and are "trapped" in the zipper state. PDZ-GEF1 depletion had no measurable effect on junction formation (data not shown), indicating that this effect is specific to PDZ-GEF2.

Since PDZ-GEF2 is a GEF for Rap1A as well as for Rap1B, we tested isoform specific effects of Rap1A and Rap1B knockdown by siRNA on AJ maturation. Rap1A or Rap1B depletion gave an approximately 40% reduction of the total Rap1 levels (Fig. 2D) and together a further decrease (Fig. 2E). This indicates that both proteins are expressed in A549 cells and that each of the isoforms is significantly depleted by its respective siRNA. Interestingly, Rap1A siRNA impaired AJ maturation and gave a similar increase in the number of adhesion zippers as PDZ-GEF2 depletion, whereas Rap1B depleted cells did not present significant alterations in AJ maturation (Fig. 2A, B and F). Hence, Rap1A, and not Rap1B, mediates junction maturation in A549 cells. Importantly, if the A549 cells were not replated following siRNA transfection, adhesion zippers were not observed in PDZ-GEF2 depleted (data not shown), suggesting that the Rap1 pathway is required for the establishment rather than the maintenance of cell-cell contacts.

The structure of the adhesion zippers was further investigated by electron microscopy. Control siRNA treated cells presented in general a typical "sealed" cell-cell contact phenotype, with the closely opposed adjacent plasma membranes (P1 and P2) running in parallel (Fig. 3A). In contrast, PDZ-GEF2 depleted cells presented strongly meandering cell-cell attachment sites, with frequent openings between the two membranes and extensions crossing the gaps, supporting the presence of adhesion zippers (Fig. 3B and C). The cell-cell contacts were phenotyped as "sealed" (Fig. 3A), "intermediate" (Fig. 3B), and "loose" (Fig. 3C). Quantification of the type of junctions revealed a dramatic increase in intermediate and loose junctions following PDZ-GEF2 knockdown, from 16% in control cells compared to 79-88% in PDZ-GEF2 depleted cells (Fig. 3D). Similarly, a strong increase in intermediate and loose cell-cell contact types was observed in Rap1A depleted cells (Fig. 3D). These results indicate that the PDZ-GEF2/Rap1A pathway is a predominant mediator of AJ maturation.

Expression of activated Rap1A or Epac1 rescues AJ maturation in A549 PDZ-GEF2 depleted cells.

To further corroborate that the PDZ-GEF2/Rap1A pathway is involved in AJ maturation, we introduced active Rap1A (V12Rap1A) in PDZ-GEF2 depleted cells to rescue the defective AJ maturation. The linear E-cadherin organization at cell-cell contacts indicated that V12Rap1A restored junction maturation to levels observed in control cells (Fig. 4A and C). Similar results were obtained when we activated endogenous Rap1 by overexpressing a Rap1 GEF (Epac1), whose endogenous levels are low in A549 cells (Fig. 4A and C).

PDZ-GEF2 knockdown in human vascular endothelial cells leads to adhesion zippers.

PDZ-GEF2 depletion also impaired AJ maturation in primary HUVEC. VE-cadherin staining of PDZ-GEF2 depleted HUVEC showed adhesion zippers (Fig. 4D and E). Activation of Rap1 using the Epac1 selective agonist 8-pCPT-2'-O-Me-cAMP restored junction maturation in PDZ-GEF2 depleted cells (Fig. 4D and E).

The PDZ-GEF2/Rap1B pathway regulates cell surface E-cadherin levels.

To gain insight into the maturation defect observed in PDZ-GEF2 and Rap1A knockdown A549 cells, we investigated whether the levels of junctional proteins were modified by the introduction of the siRNA. E-

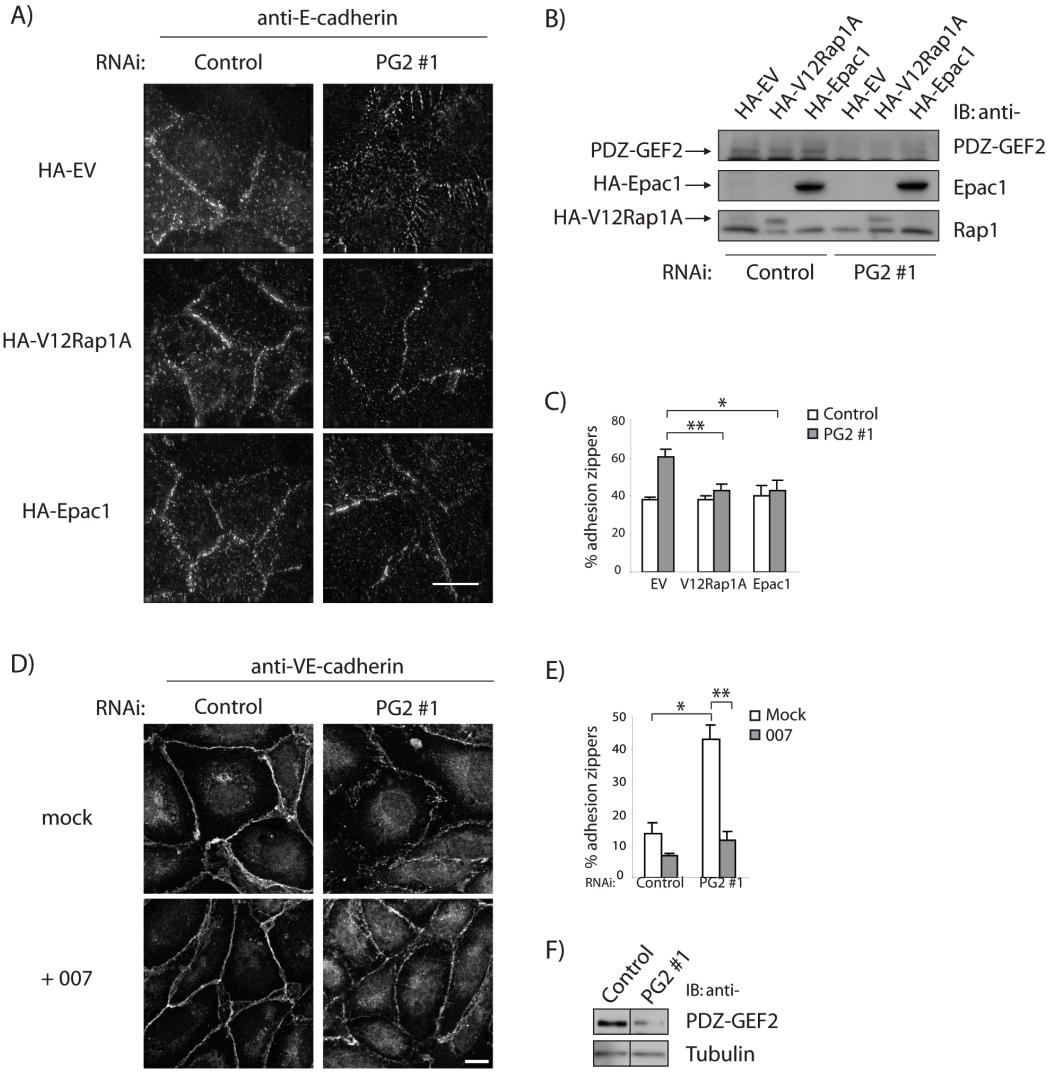

Fig. 4. Expression of constitutively activated Rap1A or Epac1 restores AJ maturation in PDZ-GEF2 depleted cells.
A. A549 cells were treated with control or PDZ-GEF2 (PG2 #1) siRNA and transfected 24 hours later with HA-tagged constructs (EV, V12Rap1A or Epac1). Cells were replated on glass coverslips the next day, grown for 24 hours, fixed and immunostained with anti-E-cadherin antibodies. Bar, 15 µm. B. Cell lysates were analyzed by Western blotting with anti-PDZ-GEF2 or anti-HA antibodies to assess PDZ-GEF2, Epac1 and V12Rap1A expression. C. E-cadherin junction staining was quantified as described in Fig. 2F. Averages represent the mean ± SEM of two to six independent experiments. T-test: * $p<0.04$; ** $p<0.006$. D. PDZ-GEF2 knockdown impairs adherens junction maturation in endothelial cells. HUVEC were transfected twice with control or PDZ-GEF2 (PG2 #1) siRNA and replated on glass coverslips. When the cells formed a monolayer, they were treated with vehicule (mock) or 8-pCPT-2'-O-Me-cAMP (007) for 30 minutes, then fixed and immunostained with anti-VE-cadherin antibodies. Bar, 10µm. E. Quantification of zipper-like cell-cell contacts. The ratios were determined has described in Fig. 2F, by counting blindly at least 100 cells per condition. Averages represent the mean ± SEM. T-test: * $p<0.00003$; ** $p<0.00001$. F. HUVEC lysates were analyzed by Western blotting with anti-PDZ-GEF2 antibodies, and anti-Tubulin antibodies were used as a loading control.

cadherin levels were slightly reduced in PDZ-GEF2 depleted cells, whereas the other AJ or tight junction (TJ) proteins were unaffected (Fig. 5A). The TJ proteins ZO-1 and Occludin were also normally present at junctional membranes in PDZ-GEF2 depleted cells (data not shown). Depletion of α-catenin, β-catenin and p120-catenin was previously reported to reduce E-cadherin levels in whole cell lysates (Davis et al., 2003; Haegel et al., 1995; Torres et al., 1997), but the levels of these proteins were unchanged in PDZ-GEF2 deficient cells (Fig. 5A). We next analyzed E-cadherin surface levels by flow cytometry using Alexa Fluor 488 labeled anti-E-cadherin antibodies and observed a reduction of cell surface E-cadherin in PDZ-GEF2 depleted cells (Fig. 5B and summarized in F). To determine if the Rap1 pathway is downstream of PDZ-GEF2 in this process, we measured E-cadherin levels in Rap1A and Rap1B deficient cells. Interestingly, Rap1B depletion, but not Rap1A, gave a slight reduction in E-cadherin levels (Fig. 5E). The cell surface E-cadherin levels were also significantly lower in Rap1B deficient cells as demonstrated by the shift in mean fluorescence measured by flow cytometry (Fig. 5D and summarized in F). Since Rap1B knockdown had no effect on AJ maturation but decreased E-cadherin levels, our results suggest that AJ maturation can occur in part independently of E-cadherin levels. Taken together, our findings indicate that the PDZ-GEF2/Rap1A pathway is required for AJ maturation, whereas PDZ-GEF2/Rap1B signaling regulates E-cadherin levels.

Discussion

Our results illustrate that Rap1 activation upon AJ disruption is mediated by PDZ-GEF2 in epithelial cells. Furthermore, we show that PDZ-GEF2 depleted epithelial and endothelial cells present adhesion zippers, demonstrated by E-cadherin organization at cell-cell contacts and further confirmed by electron microscopy studies, where the adjacent plasma membranes were rarely seen in parallel and cell-cell contacts presented irregular membrane invaginations. Since E-cadherin is still at the junctions, it is likely that they form homotypic interactions with neighboring cells but the junctions fail to adopt a linear shape (Vasioukhin et al., 2000). Adhesion zippers were also observed after depletion of Rap1A, and overexpression of Rap1A or activation of endogenous Rap1 could rescue the defect caused by PDZ-GEF2 depletion. Interestingly, Rap1B knockdown did not impair junction maturation. From these results we conclude that PDZ-GEF2 is required for AJ maturation through the activation of Rap1A, while Rap1B signaling may play a different role in this process.

Mechanism of PDZ-GEF2 activation.

Although it is currently unknown how PDZ-GEF2 is activated, it could occur through protein translocation, conformational change, or by a small molecule. Our data indicate that EGTA treatment or knockdown of E-cadherin in epithelial cells results in the activation of Rap1, suggesting that the dissociation of the E-cadherin-catenin complex may trigger Rap1 activation. It was previously shown that EGTA or Src-induced endocytosis of E-cadherin might be an essential step in disruption-induced Rap1 activation (Balzac et al., 2005). One possibility is that the E-cadherin complex keeps PDZ-GEF2 in an inactive conformation and dissociation of the complex, either by E-cadherin internalization or depletion, results in the activation and/or relocalization of PDZ-GEF2. In that respect, PDZ-GEF2 binds to MAGI-3 (N.D. and J.L.B., unpublished data), a protein that localizes at cell-cell contacts (Adamsky et al., 2003). Interestingly, MAGI-1 is required for Rap1 activation upon cell-cell contact formation (Sakurai et al., 2006), further suggesting that the E-cadherin complex as well as the scaffolding proteins regulate Rap1 activation. However, thus far we were unable to detect PDZ-GEF2 at cell-cell contacts and overexpressed PDZ-GEF2 is located in the cytosol (N.D. and J.L.B., unpublished data). Nonetheless, our results suggest that the absence of trans-linked E-cadherin may play a role in the activation of PDZ-GEF2. Alternatively, PDZ-GEF2 may be regulated similarly as Epac, where its auto-inhibited state is released by binding of cAMP (Rehmann et al., 2003). Like Epac, PDZ-GEF2 has a cyclic nucleotide-like binding domain, but currently no molecules that can activate the protein are known. As well, PDZ-GEF2 binds to the small G-protein M-Ras, resulting in the translocation of PDZ-GEF2 to the plasma membrane (Gao et al., 2001; Yoshikawa et al., 2007), but the stimulus that activates M-Ras is currently unclear.

Distinct functions of Rap1A and Rap1B.

One intriguing observation is that the knockdown of Rap1A or Rap1B led to distinct cellular phenotypes: Rap1A depletion impaired AJ maturation and cells presented normal E-cadherin levels, whereas Rap1B deficiency did not affect junction maturation but reduced E-cadherin levels. Although Rap1A

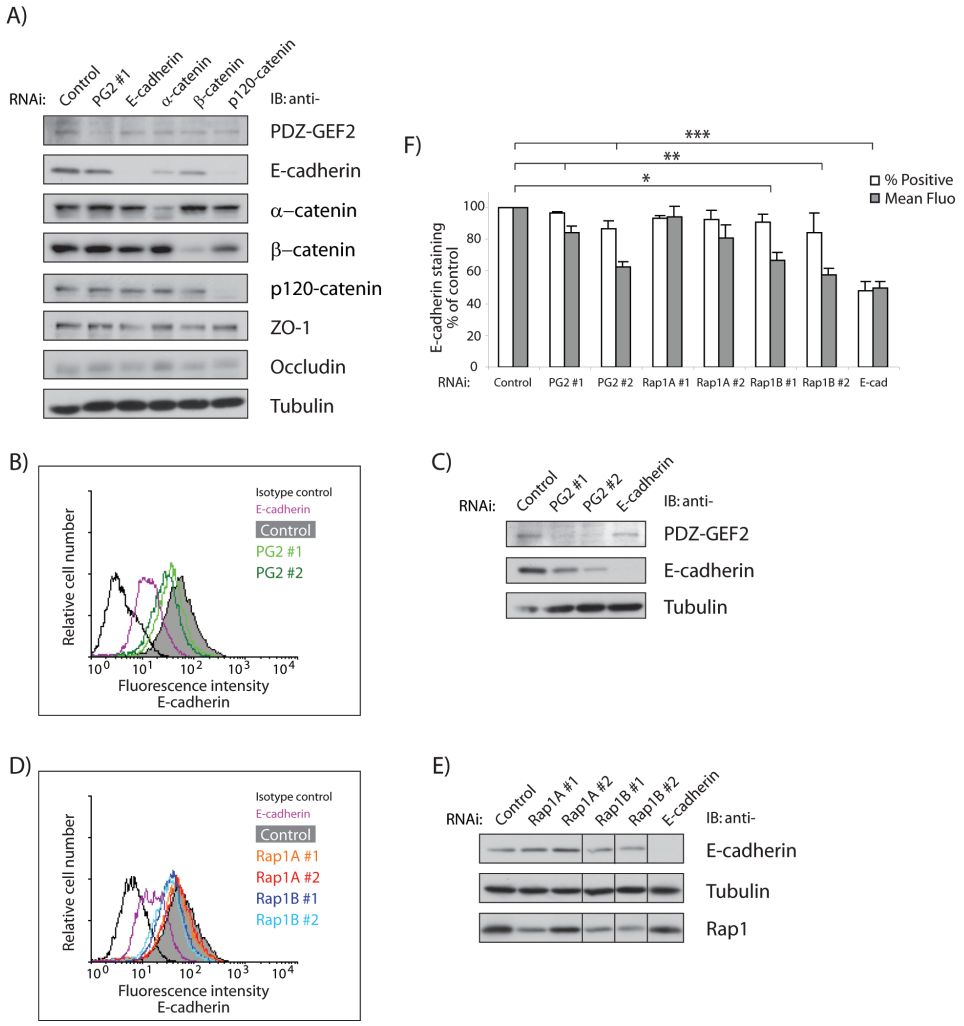

Fig. 5. The PDZ-GEF2/Rap1B pathway regulates E-cadherin levels. A549 cells were transfected with the indicated siRNA, grown for 24 hours and replated 24 hours before the experiments. A. Lysates from A549 cells treated with control, PDZ-GEF2 (PG2 #1), E-cadherin, α-catenin, β-catenin or p120-catenin siRNA were immublotted for the indicated junctional proteins. siRNA against E-cadherin, α-catenin, β-catenin and p120-catenin were used as controls to ensure the proper identity of the bands observed by immunoblotting. B and D. E-cadherin cell surface staining was determined by flow cytometry analysis of Alexa Fluor 488 anti-human CD324-stained A549 cells treated with control, PDZ-GEF2 (PG2 #1, PG2 #2), Rap1A (Rap1A #1, Rap1A #2), Rap1B (Rap1B #1, Rap1B #2) or E-cadherin siRNA. Cells were grown for 24 hours following siRNA transfection and replated 24 hours before flow cytometry analysis. Alexa Fluor 488 Mouse IgG1, κ isotype control was used as a control. A representative result from at least three independent experiments is shown in each panel. C and E. Lysates of the samples used for the flow cytometry experiment were analyzed by Western blotting with anti-PDZ-GEF2, anti-Rap1 and anti-E-cadherin antibodies to ensure protein knockdown, and anti-Tubulin antibodies were used as a loading control. F. Summary of E-cadherin surface levels by flow cytometry analysis. Relative values of E-cadherin levels at the cell surface from PDZ-GEF2, Rap1A, Rap1B and E-cadherin samples were calculated by fixing the control values to 100%. "% Positive" represents the percentage of cells stained with anti-E-cadherin antibodies and "Mean Fluo" indicates the average fluorescence of E-cadherin on the cells. Data are mean ± SEM from at least three independent experiments. T-test: * $p<0.01$; ** $p<0.0004$; *** $p<0.00004$.

and Rap1B are 95% identical and only differ by 9 amino acids mostly located in the hypervariable C-terminal region, their respective null mice phenotypes are different. Rap1A null mice are viable and their hematopoietic cells present decreased adhesion on ICAM and fibronectin, suggesting that Rap1A regulates integrins (Duchniewicz et al., 2006). In contrast, Rap1B inactivation by gene-targeting is

embryonic lethal and results in a bleeding defect due to a reduction in platelet aggregation (Chrzanowska-Wodnicka et al., 2005). Whether these phenotypic differences reflect a distinct function is currently unclear. A different function for very homologous small G-proteins is not without a precedent: Ral and Ras have highly similar isoforms but with apparent different roles (Camonis and White, 2005; Ehrhardt et al., 2002).

PDZ-GEF2 is a GEF for both Rap1A and Rap1B and the phenotype obtained by depleting PDZ-GEF2 is a combination of simultaneous Rap1A and Rap1B knockdown, where cells presented adhesion zippers and reduced surface E-cadherin levels. Although we were unable to measure activation of Rap1A and Rap1B separately in the Rap1-GTP pull-down, we conclude that PDZ-GEF2 activates Rap1A to mediate junction maturation, and Rap1B to regulate E-cadherin levels. Indeed, introduction of Rap1GAP, which blocks all Rap proteins, prevents junction formation (Hogan et al., 2004; Price et al., 2004). Since the knockdown of both Rap1A and Rap1B did not result in a phenotype as severe as Rap1GAP on AJ formation, it is possible that more Rap proteins such as Rap2 are involved in this process. However, other explanations are possible and we are currently investigating the role of other Rap GEFs in further detail.

Which processes are regulated by the two Rap1 proteins in AJ formation? In Rap1A depleted cells, E-cadherin levels are normal but the junctions fail to stretch and to form tight cell-cell contacts. The extensions observed are decorated with E-cadherin, representing immature adhesions. This suggests either a defect in the linkage of E-cadherin to the actin cytoskeleton or a failure to generate tension at cell-cell contacts, as actomyosin contractility and adequate cytoskeletal tension are required for proper AJ assembly (Miyake et al., 2006; Shewan et al., 2005). In that respect, it is important to note that activation of Rap1 can result in accumulation of cortical actin, independent of junction formation (Kooistra et al., 2005). In PDZ-GEF2 depleted cells, F-actin is not properly organized at cell-cell contacts. This phenocopies the inhibition of actin assembly at cadherin adhesive contacts blocked by inhibiting Arp2/3 activity, which is known to be necessary for the formation of cadherin-based cell junctions (Verma et al., 2004). Furthermore, replating A549 cells in media containing the ROCK inhibitor Y27632, which inhibits actin contractility (Yamada and Nelson, 2007), resulted in impaired actin organization at cell-cell contacts, mimicking the PDZ-GEF2 knockdown phenotype (data not shown). An alternative explanation for the presence of adhesion zippers is that PDZ-GEF2 depletion decreases the strength of the homotypic interaction between cadherins. We assessed this possibility by plating A549 cells on a Fc-E-cadherin-coated surface, but we were unable to measure clear adhesiveness, both in the control and PDZ-GEF2 depleted cells. Perhaps the relatively low levels of p120-catenin expressed in these cells are responsible for the weak adhesive properties (Fox and Peifer, 2007; Thoreson et al., 2000). In contrast, Rap1B may play a role either in E-cadherin translocation or E-cadherin stabilization, but this possibility remains to be determined. In this respect, it is interesting to note that Rap null Drosophila wing cells show a defect in DE-cadherin localization and junction integrity, but DE-cadherin is still present at cell-cell contacts (Knox and Brown, 2002). Similarly, in C. elegans, Rap1 nulls do not show defect in junction formation, whereas interfering with both the Rap1 pathway and the basolateral protein transport via the exocyst complex results in the loss of the cadherin-catenin complex from the membrane (Frische et al., 2007).

Mechanistic details of how Rap1 regulates junction formation are currently lacking. However, a number of proteins have been assigned as Rap1 effectors, including Tiam, Vav2, AF6 and Riam, all with connections to the actin cytoskeleton. Recently the Rap1 effector KRIT-1 (Serebriiskii et al., 1997) was shown to colocalize with junctional proteins and is involved in endothelial junction integrity in a Rap1-dependent fashion (Glading et al., 2007), further supporting a role for Rap1 in junction formation. Clearly, the interconnectivity between the Rap1 GEFs and the Rap1 effectors involved in this process needs further investigation.

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References

Adams, C. L., Chen, Y. T., Smith, S. J. and Nelson, W. J. (1998). Mechanisms of epithelial cell-cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin-green fluorescent protein. J Cell Biol 142, 1105-19.

Adamsky, K., Arnold, K., Sabanay, H. and Peles, E. (2003). Junctional protein MAGI-3 interacts with receptor tyrosine phosphatase beta (RPTP beta) and tyrosine-phosphorylated proteins. J Cell Sci 116, 1279-89.

Balzac, F., Avolio, M., Degani, S., Kaverina, I., Torti, M., Silengo, L., Small, J. V. and Retta, S. F. (2005). E-cadherin endocytosis regulates the activity of Rap1: a traffic light GTPase at the crossroads between cadherin and integrin function. J Cell Sci 118, 4765-83.

Boettner, B. and Van Aelst, L. (2007). The Rap GTPase Activator dPDZ-GEF Regulates Cell Shape in Epithelial Migration and Morphogenesis. Mol Cell Biol.

Bos, J. L. (2005). Linking Rap to cell adhesion. Curr Opin Cell Biol 17, 123-8.

Breviario, F., Caveda, L., Corada, M., Martin-Padura, I., Navarro, P., Golay, J., Introna, M., Gulino, D., Lampugnani, M. G. and Dejana, E. (1995). Functional properties of human vascular endothelial cadherin (7B4/cadherin-5), an endothelium-specific cadherin. Arterioscler Thromb Vasc Biol 15, 1229-39.

Camonis, J. H. and White, M. A. (2005). Ral GTPases: corrupting the exocyst in cancer cells. Trends Cell Biol 15, 327-32.

Chrzanowska-Wodnicka, M., Smyth, S. S., Schoenwaelder, S. M., Fischer, T. H. and White, G. C., 2nd. (2005). Rap1b is required for normal platelet function and hemostasis in mice. J Clin Invest 115, 680-7.

Cullere, X., Shaw, S. K., Andersson, L., Hirahashi, J., Luscinskas, F. W. and Mayadas, T. N. (2005). Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. Blood 105, 1950-5.

Davis, M. A., Ireton, R. C. and Reynolds, A. B. (2003). A core function for p120-catenin in cadherin turnover. J Cell Biol 163, 525-34.

de Rooij, J., Zwartkruis, F. J., Verheijen, M. H., Cool, R. H., Nijman, S. M., Wittinghofer, A. and Bos, J. L. (1998). Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature 396, 474-7.

Dobrosotskaya, I. Y. and James, G. L. (2000). MAGI-1 interacts with beta-catenin and is associated with cell-cell adhesion structures. Biochem Biophys Res Commun 270, 903-9.

Duchniewicz, M., Zemojtel, T., Kolanczyk, M., Grossmann, S., Scheele, J. S. and Zwartkruis, F. J. (2006). Rap1A-deficient T and B cells show impaired integrin-mediated cell adhesion. Mol Cell Biol 26, 643-53.

Ehrhardt, A., Ehrhardt, G. R., Guo, X. and Schrader, J. W. (2002). Ras and relatives--job sharing and networking keep an old family together. Exp Hematol 30, 1089-106.

Fox, D. T. and Peifer, M. (2007). Cell adhesion: separation of p120's powers? Curr Biol 17, R24-7.

Frische, E. W., Pellis-van Berkel, W., van Haaften, G., Cuppen, E., Plasterk, R. H., Tijsterman, M., Bos, J. L. and Zwartkruis, F. J. (2007). RAP-1 and the RAL-1/exocyst pathway coordinate hypodermal cell organization in Caenorhabditis elegans. Embo J 26, 5083-92.

Fukuhara, S., Sakurai, A., Sano, H., Yamagishi, A., Somekawa, S., Takakura, N., Saito, Y., Kangawa, K. and Mochizuki, N. (2005). Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling pathway. Mol Cell Biol 25, 136-46.

Fukuyama, T., Ogita, H., Kawakatsu, T., Fukuhara, T., Yamada, T., Sato, T., Shimizu, K., Nakamura, T., Matsuda, M. and Takai, Y. (2005). Involvement of the c-Src-Crk-C3G-Rap1 signaling in the nectin-induced activation of Cdc42 and formation of adherens junctions. J Biol Chem 280, 815-25.

Gao, X., Satoh, T., Liao, Y., Song, C., Hu, C. D., Kariya Ki, K. and Kataoka, T. (2001). Identification and characterization of RA-GEF-2, a Rap guanine nucleotide exchange factor that serves as a downstream target of M-Ras. J Biol Chem 276, 42219-25.

Glading, A., Han, J., Stockton, R. A. and Ginsberg, M. H. (2007). KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell cell junctions. J Cell Biol 179, 247-54.

Haegel, H., Larue, L., Ohsugi, M., Fedorov, L., Herrenknecht, K. and Kemler, R. (1995). Lack of beta-catenin affects mouse development at gastrulation. Development 121, 3529-37.

Hisata, S., Sakisaka, T., Baba, T., Yamada, T., Aoki, K., Matsuda, M. and Takai, Y. (2007). Rap1-PDZ-GEF1 interacts with a neurotrophin receptor at late endosomes, leading to sustained activation of Rap1 and ERK and neurite outgrowth. J Cell Biol 178, 843-60.

Hogan, C., Serpente, N., Cogram, P., Hosking, C. R., Bialucha, C. U., Feller, S. M., Braga, V. M., Birchmeier, W. and Fujita, Y. (2004). Rap1 regulates the formation of E-cadherin-based cell-cell contacts. Mol Cell Biol 24, 6690-700.

Kawajiri, A., Itoh, N., Fukata, M., Nakagawa, M., Yamaga, M., Iwamatsu, A. and Kaibuchi, K. (2000). Identification of a novel beta-catenin-interacting protein. Biochem Biophys Res Commun 273, 712-7.

Knox, A. L. and Brown, N. H. (2002). Rap1 GTPase regulation of adherens junction positioning and cell adhesion. Science 295, 1285-8.

Kooistra, M. R., Corada, M., Dejana, E. and Bos, J. L. (2005). Epac1 regulates integrity of endothelial cell junctions through VE-cadherin. FEBS Lett 579, 4966-72.

Kooistra, M. R., Dube, N. and Bos, J. L. (2007). Rap1: a key regulator in cell-cell junction formation. J Cell Sci 120, 17-22.

Lampugnani, M. G., Resnati, M., Raiteri, M., Pigott, R., Pisacane, A., Houen, G., Ruco, L. P. and Dejana, E. (1992). A novel endothelial-specific membrane protein is a marker of cell-cell contacts. J Cell Biol 118, 1511-22.

Maddugoda, M. P., Crampton, M. S., Shewan, A. M. and Yap, A. S. (2007). Myosin VI and vinculin cooperate during the morphogenesis of cadherin cell cell contacts in mammalian epithelial cells. J Cell Biol 178, 529-40.

Mandell, K. J., Babbin, B. A., Nusrat, A. and Parkos, C. A. (2005). Junctional adhesion molecule 1 regulates epithelial cell morphology through effects on beta1 integrins and Rap1 activity. J Biol Chem 280, 11665-74.

Mege, R. M., Gavard, J. and Lambert, M. (2006). Regulation of cell-cell junctions by the cytoskeleton. Curr Opin Cell Biol 18, 541-8.

Mino, A., Ohtsuka, T., Inoue, E. and Takai, Y. (2000). Membrane-associated guanylate kinase with inverted orientation (MAGI)-1/brain angiogenesis inhibitor 1-associated protein (BAP1) as a scaffolding molecule for Rap small G protein GDP/GTP exchange protein at tight junctions. Genes Cells 5, 1009-16.

Miyake, Y., Inoue, N., Nishimura, K., Kinoshita, N., Hosoya, H. and Yonemura, S. (2006). Actomyosin tension is required for correct recruitment of adherens junction components and zonula occludens formation. Exp Cell Res 312, 1637-50.

Ohtsuka, T., Hata, Y., Ide, N., Yasuda, T., Inoue, E., Inoue, T., Mizoguchi, A. and Takai, Y. (1999). nRap GEP: a novel neural GDP/GTP exchange protein for rap1 small G protein that interacts with synaptic scaffolding molecule (S-SCAM). Biochem Biophys Res Commun 265, 38-44.

Pellis-van Berkel, W., Verheijen, M. H., Cuppen, E., Asahina, M., de Rooij, J., Jansen, G., Plasterk, R. H., Bos, J. L. and Zwartkruis, F. J. (2005). Requirement of the Caenorhabditis elegans RapGEF pxf-1 and

rap-1 for epithelial integrity. Mol Biol Cell 16, 106-16.

Perez, T. D., Tamada, M., Sheetz, M. P. and Nelson, W. J. (2007). Immediate-early signaling induced by E-cadherin engagement and adhesion. J Biol Chem.

Posern, G., Weber, C. K., Rapp, U. R. and Feller, S. M. (1998). Activity of Rap1 is regulated by bombesin, cell adhesion, and cell density in NIH3T3 fibroblasts. J Biol Chem 273, 24297-300.

Price, L. S., Hajdo-Milasinovic, A., Zhao, J., Zwartkruis, F. J., Collard, J. G. and Bos, J. L. (2004). Rap1 regulates E-cadherin-mediated cell-cell adhesion. J Biol Chem 279, 35127-32.

Rehmann, H., Prakash, B., Wolf, E., Rueppel, A., de Rooij, J., Bos, J. L. and Wittinghofer, A. (2003). Structure and regulation of the cAMP-binding domains of Epac2. Nat Struct Biol 10, 26-32.

Sakurai, A., Fukuhara, S., Yamagishi, A., Sako, K., Kamioka, Y., Masuda, M., Nakaoka, Y. and Mochizuki, N. (2006). MAGI-1 is required for Rap1 activation upon cell-cell contact and for enhancement of vascular endothelial cadherin-mediated cell adhesion. Mol Biol Cell 17, 966-76.

Serebriiskii, I., Estojak, J., Sonoda, G., Testa, J. R. and Golemis, E. A. (1997). Association of Krev-1/rap1a with Krit1, a novel ankyrin repeat-containing protein encoded by a gene mapping to 7q21-22. Oncogene 15, 1043-9.

Shewan, A. M., Maddugoda, M., Kraemer, A., Stehbens, S. J., Verma, S., Kovacs, E. M. and Yap, A. S. (2005). Myosin 2 is a key Rho kinase target necessary for the local concentration of E-cadherin at cell-cell contacts. Mol Biol Cell 16, 4531-42.

Stehbens, S. J., Paterson, A. D., Crampton, M. S., Shewan, A. M., Ferguson, C., Akhmanova, A., Parton, R. G. and Yap, A. S. (2006). Dynamic microtubules regulate the local concentration of E-cadherin at cell-cell contacts. J Cell Sci 119, 1801-11.

Thoreson, M. A., Anastasiadis, P. Z., Daniel, J. M., Ireton, R. C., Wheelock, M. J., Johnson, K. R., Hummingbird, D. K. and Reynolds, A. B. (2000). Selective uncoupling of p120(ctn) from E-cadherin disrupts strong adhesion. J Cell Biol 148, 189-202.

Torres, M., Stoykova, A., Huber, O., Chowdhury, K., Bonaldo, P., Mansouri, A., Butz, S., Kemler, R. and Gruss, P. (1997). An alpha-E-catenin gene trap mutation defines its function in preimplantation development. Proc Natl Acad Sci U S A 94, 901-6.

Vaezi, A., Bauer, C., Vasioukhin, V. and Fuchs, E. (2002). Actin cable dynamics and Rho/Rock orchestrate a polarized cytoskeletal architecture in the early steps of assembling a stratified epithelium. Dev Cell 3, 367-81.

van Triest, M., de Rooij, J. and Bos, J. L. (2001). Measurement of GTP-bound Ras-like GTPases by activation-specific probes. Methods Enzymol 333, 343-8.

Vasioukhin, V., Bauer, C., Yin, M. and Fuchs, E. (2000). Directed actin polymerization is the driving force for epithelial cell-cell adhesion. Cell 100, 209-19.

Verma, S., Shewan, A. M., Scott, J. A., Helwani, F. M., den Elzen, N. R., Miki, H., Takenawa, T. and Yap, A. S. (2004). Arp2/3 activity is necessary for efficient formation of E-cadherin adhesive contacts. J Biol Chem 279, 34062-70.

Wang, H., Singh, S. R., Zheng, Z., Oh, S. W., Chen, X., Edwards, K. and Hou, S. X. (2006). Rap-GEF signaling controls stem cell anchoring to their niche through regulating DE-cadherin-mediated cell adhesion in the Drosophila testis. Dev Cell 10, 117-26.

Wittchen, E. S., Worthylake, R. A., Kelly, P., Casey, P. J., Quilliam, L. A. and Burridge, K. (2005). Rap1 GTPase inhibits leukocyte transmigration by promoting endothelial barrier function. J Biol Chem 280, 11675-82.

Yajnik, V., Paulding, C., Sordella, R., McClatchey, A. I., Saito, M., Wahrer, D. C., Reynolds, P., Bell, D. W., Lake, R., van den Heuvel, S. et al. (2003). DOCK4, a GTPase activator, is disrupted during tumorigenesis. Cell 112, 673-84.

Yamada, S. and Nelson, W. J. (2007). Localized zones of Rho and Rac activities drive initiation and expansion of epithelial cell-cell adhesion. J Cell Biol 178, 517-27.

Yap, A. S., Crampton, M. S. and Hardin, J. (2007). Making and breaking contacts: the cellular biology of cadherin regulation. Curr Opin Cell Biol 19, 508-14.

Yoshikawa, Y., Satoh, T., Tamura, T., Wei, P., Bilasy, S. E., Edamatsu, H., Aiba, A., Katagiri, K., Kinashi, T., Nakao, K. et al. (2007). The M-Ras-RA-GEF-2-Rap1 pathway mediates tumor necrosis factor-alpha dependent regulation of integrin activation in splenocytes. Mol Biol Cell 18, 2949-59.

Zwartkruis, F. J., Wolthuis, R. M., Nabben, N. M., Franke, B. and Bos, J. L. (1998). Extracellular signal-regulated activation of Rap1 fails to interfere in Ras effector signalling. Embo J 17, 5905-12.

Chapter 5

Activation of Epac1 rescues AF6 knock-down-induced electrical resistance defect in endothelial cell-cell junctions

Activation of Epac1 rescues AF6 knock-down-induced electrical resistance defect in endothelial cell-cell junctions

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Abstract

Regulation of endothelial cell-cell contacts is essential for the integrity of the endothelium. We showed previously that Rap1 and its guanine-nucleotide exchange factor Epac1 regulate endothelial cell-cell junctions, resulting in increased endothelial integrity. Here, we studied the role of the Rap1 binding protein AF6 in this process. AF6 is a junction protein that can bind to active Rap1 and Ras and is required for proper cell-cell junctions in epithelial cells. We found that knock-down of AF6 in HUVE cells had no effect on the recruitment of VE-cadherin, JAM-A and ZO-1 to the junctions, but resulted in a reorganization of the cytoskeleton. This reorganization was also observed in single cells indicating that the effect of AF6 on actin is independent of the formation of cell-cell junctions. AF6 KD did also result in a decreased trans-endothelial resistance, showing that also in endothelial cells AF6 is involved in the formation of cell-cell junctions. However, AF6 knock-down did not affect Epac1-mediated increase in endothelial resistance, suggesting that activation of Rap1 can overcome a defect in AF6. We conclude that AF6 is involved in the regulation of endothelial cell-cell junction formation and that the AF6 knock-down-induced electrical resistance defect of endothelial cell-cell junctions can be rescued by activation of Epac1. This suggests that AF6 operates in a pathway parallel to Rap1 in the control of endothelial integrity.

Introduction

The small GTPase Rap1 is a member of the Ras family of GTPases, that plays a role in, among others, the control of integrin-mediated cell adhesion and cadherin-mediated cell junction formation. Rap1 cycles between an active GTP-bound and an inactive GDP-bound form. Activation of Rap1 is induced by guanine-nucleotide exchange factors (GEF), and GTPase activating proteins (GAP) enhance the hydrolysis of GTP into GDP. Recently one of the GEFs for Rap1, Epac1, was found to be an effector for cAMP-mediated control of endothelial cell-cell junction formation. Using the Epac1-specific cAMP-analog 8-pCPT-2O-Me-cAMP (007), activation of Rap1 resulted in a decrease in permeability for larger molecules and an increase in electrical resistance of the endothelial monolayer. Also the trans-endothelial migration of HL-60 cells was inhibited by the increased junction formation upon activation of Rap1 (Wittchen et al., 2005). This increased junction formation coincides with increased adherens junction maturation as well as increased cortical actin formation (Cullere et al., 2005; Fukuhara et al., 2005; Wittchen et al., 2005). Although the increased endothelial integrity appears to depend on VE-cadherin, the reorganization of the actin cytoskeleton in HUVE cells by 007 was independent of cell-cell contacts (Cullere et al., 2005; Kooistra et al., 2005). The molecular mechanism of how Rap1 regulation controls junction formation is currently unclear, but recently KRIT/CCM1 has been implicated as a possible intermediate between Rap1 and junction proteins, including beta-catenin (Glading et al., 2007).

An additional interesting candidate involved in Rap1-mediated junction formation is AF6/Afadin. The AF6 gene, also called MLLT-4, was identified as the fusion partner of the myeloid/lymphoid or mixed-lineage leukemia (MLL) gene (Prasad et al., 1993). The MLL/AF6 chimeric protein is the critical product of the t(6;11) abnormality associated with some forms of leukemia. Furthermore, AF6/Afadin was found to be localized at the cell-cell contacts and binds various junctional proteins, such as JAM-A, ZO-1 and nectins (Ebnet et al., 2000; Takahashi et al., 1999; Yamamoto et al., 1997) as well as the regulator of the actin cytoskeleton profilin (Boettner et al., 2000). Indeed, evidence is accumulating that AF6 plays an important role in the formation of cell-cell junctions. For instance, although E-cadherin and ZO-1 localize normally to the junctions in the intestine of AF6 knock-

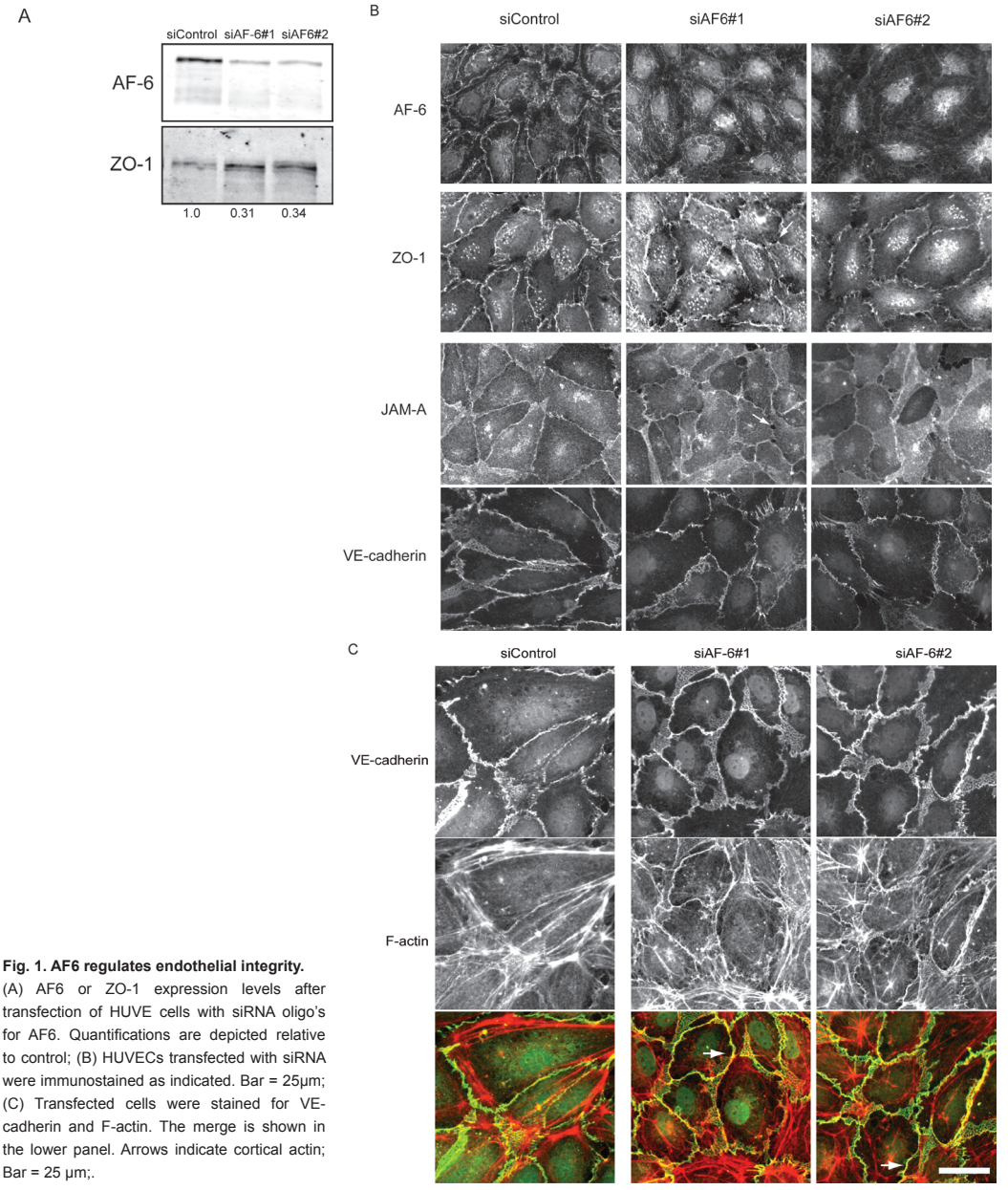

Fig. 1. AF6 regulates endothelial integrity.
(A) AF6 or ZO-1 expression levels after transfection of HUVE cells with siRNA oligo's for AF6. Quantifications are depicted relative to control; (B) HUVECs transfected with siRNA were immunostained as indicated. Bar = 25µm; (C) Transfected cells were stained for VE-cadherin and F-actin. The merge is shown in the lower panel. Arrows indicate cortical actin; Bar = 25 µm;.

out mice, the integrity of the intestinal epithelium is affected (Zhadanov et al., 1999) and knock-down of AF6 in epithelial cells results in loss of tight junctions (Sato et al., 2006). The first RA-domain of AF6 has been shown to bind to the effector region of both active Ras and Rap, marking AF6 as a putative target protein(Boettner et al., 2000; Yamamoto et al., 1999). However, most evidence indicates that AF6 is most likely an effector of Rap1. For instance, in Drosophila melanogaster, both Rap1 and the AF6 homolog, canoe, are required for dorsal closure of the embryo. Furthermore, active Rap1 could not rescue the phenotype of a canoe loss-of-function, suggesting that canoe is located downstream of Rap1 (Boettner

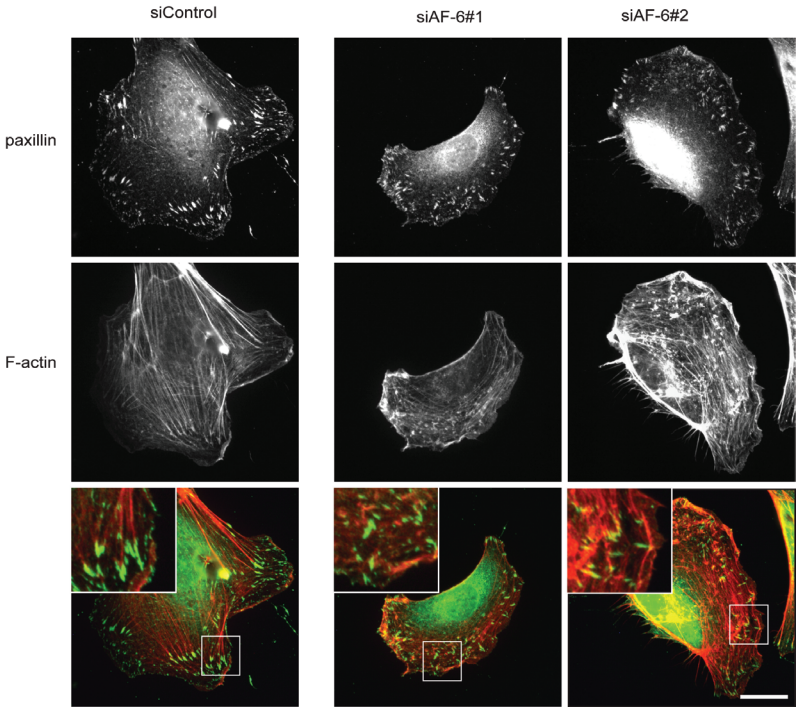

Fig. 2. Organization of the actin cytoskeleton requires AF6. HUVEC were seeded sparsely for 16 hours, fixed and stained for paxillin and F-actin. Bar = 10 µm.

et al., 2003). More recently, the Rap1-GEF dPDZ-GEF was analysed in *D. melanogaster.* dPDZ-GEF mutants show failure of dorsal closure, as reported for Rap1 and canoe. Interestingly, over-expression of Rap1 or Canoe rescues the phenotype partially, suggesting a PDZ-GEF, Rap1, canoe axis in flies (Boettner and Van Aelst, 2007). Furthermore, it was found that inhibition of endocytosis of E-cadherin in vitro by dominant negative Rap1 can be rescued by mutant of AF6 lacking the Rap1 binding domains but not by full length AF6, suggesting that AF6 also functions as effector of Rap1 in mammalian epithelial cells (Hoshino et al., 2005). AF6 also binds to actin and to the actin binding protein profilin (Boettner et al., 2000). For instance, it was shown that the actin binding domain of AF6 is required for cell-cell junction formation and migration of epithelial cells (Lorger and Moelling, 2006). Finally, AF6 also plays a role in the regulation of Rap1 activity. Indeed, AF6 binds Rap1GAP1 and the RapGAP Spa-1 to negatively control Rap1 activity, but in addition binding of AF6 protects Rap1 from GAP-induced GTP- hydrolysis (Su et al., 2003; Zhang et al., 2005). However, whether AF6 plays a role in endothelial cell-cell junction formation is currently unclear.

Here we investigated the role of AF6/Afadin in Epac1-induced junction enhancement in endothelial cells using real-time analysis of endothelial resistance. We find that reduced levels of AF6 in endothelial cells result in decreased endothelial resistance. Although the constitution of the endothelial junctions is normal, we observed changes in the actin cytoskeleton. Surprisingly we observe that activation of endogenous Rap1 in endothelial cells rescued the reduction in endothelial resistance to basal values. Together these data suggest that AF6 is required for the integrity of the endothelial monolayer and that activation of Rap1 through Epac1 can overcome this defect.

Materials and Methods

Cell culture

HUVEC were isolated and cultured in EBM-2 Bulletkit culture medium (EBM-2 supplemented with EGM-2 SingleQuots (hEGF, hydrocortisone, fetal bovine serum, VEGF, hFGF-B, R3-IGF-1, Ascorbic Acid, GA-1000, heparin) (Clonetics) as described (Jaffe et al., 1973; Willems et al., 1982). Second to fourth passage cells were used. Cells were cultured on 1% gelatin (Sigma) and experiments were performed on 7 µg/ml fibronectin (Sigma). 18 hours after plating HUVEC were transfected with siRNA oligos (Dharmacon) against AF6 (#1: GAAUAUAGUGAACCAAAGA; #2: UGAGAAACCUCUAGUUGUA) or Epac1 (GCACCUACGUCUGCAACAA) as indicated using Oligofectamine (Invitrogen) according to manufacturers recommendations. Transfection was repeated after 24 hours. Cells were replated 24 hours later and analyzed 48 hours after the second round of transfections, unless stated differently.

Antibodies

Mouse mAb against the extracellular domain of human VE-cadherin used was clone TEA 1.31 (Breviario et al., 1995; Lampugnani et al., 1992). Rap1 polyclonal antibodies (Santa Cruz), JAM-A (Abcam), AF6 monoclonal antibody (Transduction labs), ZO-1 polyclonal antibodies (Transduction labs) were used.

Immunofluorescence

Cells were replated 24 hrs after transfection on fibronectin-coated (7µg/ml) glass coverslips (12mm) for 24 hrs. After stimulation, cells were fixed with 3.8% formaldehyde, permeabilised using 0,2% Triton X-100 and blocked with 2% BSA in PBS. Cells were incubated with indicated antibodies for 1 hour and subsequently incubated with Alexa-568 labeled phalloidin to stain F-actin and/or Alexa-488/568-labeled secondary antibodies (Molecular Probes). After mounting the coverslips onto slides, cells were examined using a Axiokop2 microscope (Zeiss).

Transendothelial electrical resistance measurement

Unless otherwise stated, HUVEC were seeded at $1.5x10^5$ cells per well (0.8 cm^2) on fibronectin coated electrode arrays and grown to confluency. Measurements of transendothelial electrical resistance were performed in real time at 4000Hz, 37ºC, 5% CO_2, using an electrical cell-substrate impedance sensing system (ECIS; Applied BioPhisics Inc.Troy NY, USA)(Tiruppathi et al., 1992).

Results

Knock down of AF6 affects the actin cytoskeleton independent of cell-cell junction formation

To investigate the role of AF6 in Epac1-mediated junction formation in endothelial cells, we used siRNA to knock down AF6 in human umbilical vein endothelial cells (HUVECs). Using 2 different siRNAs targeting different regions of the protein, AF6 expression was reduced to approximately 30% as monitored by Western blotting. (figure 1a). Also in immunofluorescence staining of AF6, particularly at the cell-cell junctions, was strongly reduced by both siRNAs. Importantly, knock-down of AF6 did not affect cell-cell junction formation and the junctional proteins VE-cadherin, ZO-1, and JAM-A are normally present (Figure 1b). However, a clear difference in the organization of the actin-cytoskeleton was observed (figure 1c). Whereas in HUVE cells expressing control siRNA the actin cytoskeleton comprises stress fibers through out the cell, cells with reduced AF6 levels show a remarkable reorganization of the actin cytoskeleton. Mainly, the thickness of the stress fibers through the cells was reduced and there was an increase of F-actin in the junctions (figure 1c, arrows). Altogether these observations suggest that AF6 is not required for the proper localization of junctional adhesion molecules, but rather appears to be involved in the proper organization of the actin-cytoskeleton.

To exclude that this effect of AF6 KD on the actin cytoskeleton was mediated by the cell-cell junction we analyzed the effect on single cells. We observed that similar to endothelial cells in a confluent layer knock-down of AF6 reduced the amount of stress and induced an accumulation of actin at the edges of the cells. When co-stained with paxillin to mark focal contacts, we found that in AF6 KD cells the focal contacts are smaller, but also show accumulation of actin (figure 2). From these results we concluded that the effect of AF6 KD on the actin cytoskeleton is independent of cell-cell junction formation.

Knock down of AF6 reduces electrical resistance of junctions

Although knock-down of AF6 had no apparent effect on the formation of cell-cell junctions, it may affect its functionality as a barrier for trans-endothelial migration. This barrier function can be measured using the Electrical Cell Impedence System (ECIS) (Tiruppathi et al., 1992). This system measures the electrical resistance of the endothelial layer at low frequency, which is a measurement for the integrity of the junctions. We observed that knock-down of AF6 in a confluent monolayer of endothelial cells using two different siRNAs reduces the electrical resistance by 40% (figure 3). This indicates that AF6 plays a role in the integrity of cell junctions.

Epac1 activation restores endothelial integrity in AF6 knock-down cells

cAMP is a well-known inducer of the integrity of endothelial cell-cell junctions and recently a number

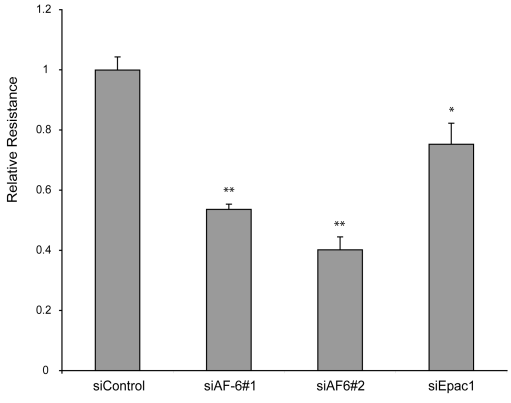

Fig 3. Knock-down of AF6 reduces endothelial resistance.
HUVEC were transfected with siRNA oligo as indicated. Basal transendothelial electrical resistance was measured under confluent conditions 14 hours after plating at 4000Hz.Significant differences from the control determined by Student's t test are indicated by a single asterisk ($p<0.01$) or double asterisks ($p<0.0001$).

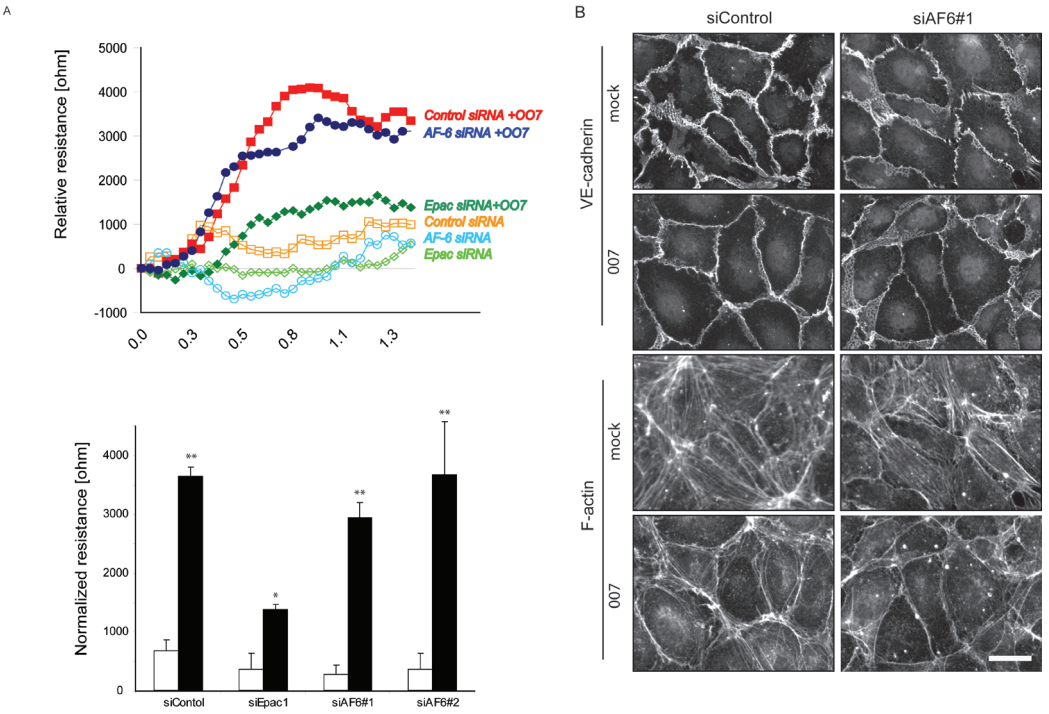

Fig. 4. Activation of Rap1 increases endothelial integrity independent of AF6.
(A) Transendothelial resistance of HUVEC monolayers on fibronectin coated gold electrodes was measured. Cells were stimulated with vehicle (open) of 100 µM 8pCPT-2'OMe-cAMP (closed) at the timepoint indicated by the arrow; ■- siControl; ▲- siEpac1; ●- siAF6#1; Bar-graph (lower panel) represents the average of three independent experiments at 1,5 hours after treatment. *p<0.05, **p< 0.01; (B) HUVEC were transfected with AF6 or control siRNA and 48 hours later treated with or without 007 for 30 minutes, fixed and stained for VE-cadherin and F-actin Bar = 25 µm.

of groups including ours have shown that this cAMP effect is in part mediated by Epac1 and can be mimicked by the Epac1 selective analog of cAMP, 8pCPT-2'OMe-cAMP (007). This effect is mediated by Rap and we reasoned that if AF6 is indeed an effector of Rap in the induction of endothelial integrity, knock down of AF6 should inhibit or at least reduce 007-induced increase in electrical resistance. Indeed, as shown previously (M.J. Lorenowicz et al., unpublished observations) 007 rapidly and strongly increases electrical resistance of the endothelial monolayer, which is sensitive to siRNA of Epac1 (figure 4a). Importantly, although the electrical resistance of AF6-KD cells is reduced, the increase in electrical resistance by 007 is not affected (figure 4a, lower panel). Similarly, 007 reduces irregularities in cell junctions as observed after staining for VE-cadherin both in control and AF6-KD cells (figure 4b). This suggest that AF6 is not essential for 007-induced endothelial integrity, or at least that AF6 is not a rate-limiting step in this process, in contrast to the effect of AF6 KD on basal endothelial integrity.

Previously we have also shown that 007 induces the formation of cortical actin both in a monolayer of endothelial cells as in single cells. We therefore analyzed the effect of 007 on the actin cytoskeleton. Although knock-down of AF6 already induced some cortical actin formation, this was further enhanced by 007 treatment. (figure 4b). From these results we conclude that 007 still can induce the formation of cortical actin formation in AF6-KD cells.

Discussion

We have shown that knock-down of AF6 to about 30% of its original level in endothelial cells has no effect on the formation of cell-cell junctions, as demonstrated by the normal staining of VE-cadherin, ZO-1 and

JAM-A, but clearly affects the integrity of the junctions as demonstrated by the reduced electrical resistance of the endothelial monolayer. AF6 knock down also clearly affects the actin cytoskeleton, by reducing the formation of thick stress fibers and the induction of cortical actin formation. However, AF6 knock-down did not affect the ability of the Epac1 agonist 007 to increase endothelial resistance and to increase cortical actin formation.

These results clearly establish a role for AF6 in the control of endothelial barrier function. A role for AF-6 in cell-cell junctions was previously reported for epithelial cells, both in the intestine of mice and in polarized MDCK cells. In MDCK cells knock down of AF6 affects the localization of tight junction proteins like JAM-A. We do not observe this effect in endothelial cells, but rather observe an effect on the actin cytoskeleton and endothelial resistance. Since endothelial integrity is regulated by the actin cytoskeleton (Dudek and Garcia, 2001), it is plausible to assume that the effect of AF6-KD on the actin cytoskeleton is causative to the reduction of electrical resistance. However, we cannot exclude that AF6 knock-down affects both processes independently.

Previously it has been shown that cAMP reduces the permeability of an endothelial cell layer in part through Epac1 and this effect can be mimicked by the Epac1 selective analog of cAMP, 007. This effect is mediated by Rap1, but the effectors of Rap1 for this effect are currently elusive. However, several observations suggest that AF6 may be involved in this process. First, AF6 has an RA domain, which can bind Rap1 in vitro and in vivo. Secondly, in Drosophila melanogaster, both Rap1 and the AF6 homolog, canoe, are required for dorsal closure of the embryo. Furthermore active Rap1 could not rescue the phenotype of a canoe loss-of-function, suggesting that canoe is located downstream of Rap1 (Boettner et al., 2003). Thirdly, inhibition of endocytosis of E-cadherin in vitro by dominant negative Rap1 can be rescued by mutant of AF6 lacking the Rap1 binding domains, but not by full length AF6 (Hoshino et al., 2005). Our results now show that knock-down of AF6 does not abolish the induction of electrical resistance by 007 nor does it inhibit the effect of 007 on the formation of cortical actin. However, the level of electrical resistance of 007-treated cells is reduced in AF6 KD cells compared to control cells, suggesting that AF6 and Rap1 regulate independent additive effects on cell-cell junction integrity. In keeping with the notion that AF6 is a likely effector of Rap1, a plausible explanation could be that Rap1 critically regulates the level of active AF6 in junction formation and that a reduced level of AF6 is still sensitive to Rap1 to increase junctional activity. Alternatively, AF6 does not function as an effector of Rap1 in endothelial junction formation, but operates in parallel and both affect the integrity of the cell junction. Finally, AF6 may mediate Rap1-dependent effects in a process independent of Rap1-Epac signaling. Indeed, Rap1 acts in a sequential process during junction formation. First, C3G is found to transiently bind to E-cadherin in MCF-7 cells during junction formation (Hogan et al., 2004). Furthermore, in endothelial cells Sakurai and colleagues have shown that Rap1 is activated during junction formation in a MAGI-1 dependent-manner (Sakurai et al., 2006). MAGI-1 is a scaffold protein that binds to PDZ-GEF directly. Finally, Epac1 can increase the junctional integrity of confluent endothelial monolayers (Cullere et al., 2005; Kooistra et al., 2005). It is well possible that AF6 is involved in Epac1-independent Rap1-induced processes of junction formation.

Thus AF6 is clearly involved in the regulation of junctional integrity, most likely in a pathway parallel to Rap1.

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References

Boettner, B., Govek, E. E., Cross, J. and Van Aelst, L. (2000). The junctional multidomain protein AF-6 is a binding partner of the Rap1A GTPase and associates with the actin cytoskeletal regulator profilin. Proc Natl Acad Sci U S A 97, 9064-9.

Boettner, B., Harjes, P., Ishimaru, S., Heke, M., Fan, H. Q., Qin, Y., Van Aelst, L. and Gaul, U. (2003). The AF-6 homolog canoe acts as a Rap1 effector during dorsal closure of the Drosophila embryo. Genetics 165, 159-69.

Boettner, B. and Van Aelst, L. (2007). The Rap GTPase activator Drosophila PDZ-GEF regulates cell shape in epithelial migration and morphogenesis. Mol Cell Biol 27, 7966-80.

Breviario, F., Caveda, L., Corada, M., Martin-Padura, I., Navarro, P., Golay, J., Introna, M., Gulino, D., Lampugnani, M. G. and Dejana, E. (1995). Functional properties of human vascular endothelial cadherin (7B4/cadherin-5), an endothelium-specific cadherin. Arterioscler. Thromb. Vasc. Biol. 15, 1229-1239.

Cullere, X., Shaw, S. K., Andersson, L., Hirahashi, J., Luscinskas, F. W. and Mayadas, T. N. (2005). Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. Blood 105, 1950-1955.

Dudek, S. M. and Garcia, J. G. (2001). Cytoskeletal regulation of pulmonary vascular permeability. J Appl Physiol 91, 1487-500.

Ebnet, K., Schulz, C. U., Meyer Zu Brickwedde, M. K., Pendl, G. G. and Vestweber, D. (2000). Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1. J Biol Chem

275, 27979-88.

Fukuhara, S., Sakurai, A., Sano, H., Yamagishi, A., Somekawa, S., Takakura, N., Saito, Y., Kangawa, K. and Mochizuki, N. (2005). Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling. pathway. Mol. Cell. Biol. 25, 136-146.

Glading, A., Han, J., Stockton, R. A. and Ginsberg, M. H. (2007). KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell cell junctions. J Cell Biol 179, 247-54.

Hogan, C., Serpente, N., Cogram, P., Hosking, C. R., Bialucha, C. U., Feller, S. M., Braga, V. M., Birchmeier, W. and Fujita, Y. (2004). Rap1 regulates the formation of E-cadherin-based cell-cell contacts. Mol. Cell. Biol. 24, 6690-6700.

Hoshino, T., Sakisaka, T., Baba, T., Yamada, T., Kimura, T. and Takai, Y. (2005). Regulation of E-cadherin endocytosis by nectin through afadin, Rap1, and p120ctn. J. Biol. Chem. 280, 24095-24103.

Jaffe, E. A., Nachman, R. L., Becker, C. G. and Minick, C. R. (1973). Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J. Clin. Invest. 52, 2745-2756.

Kooistra, M. R., Corada, M., Dejana, E. and Bos, J. L. (2005). Epac1 regulates integrity of endothelial cell junctions through VE-cadherin. FEBS Lett 579, 4966-72.

Lampugnani, M. G., Resnati, M., Raiteri, M., Pigott, R., Pisacane, A., Houen, G., Ruco, L. P. and Dejana, E. (1992). A novel endothelial-specific membrane protein is a marker of cell-cell contacts. J. Cell. Biol. 118, 1511-1522.

Lorger, M. and Moelling, K. (2006). Regulation of epithelial wound closure and intercellular adhesion by interaction of AF6 with actin cytoskeleton. J Cell Sci 119, 3385-98.

Sakurai, A., Fukuhara, S., Yamagishi, A., Sako, K., Kamioka, Y., Masuda, M., Nakaoka, Y. and Mochizuki, N. (2006). MAGI-1 is required for Rap1 activation upon cell-cell contact and for enhancement of vascular endothelial cadherin-mediated cell adhesion. Mol Biol Cell 17, 966-76.

Sato, T., Fujita, N., Yamada, A., Ooshio, T., Okamoto, R., Irie, K. and Takai, Y. (2006). Regulation of the assembly and adhesion activity of E-cadherin by nectin and afadin for the formation of adherens junctions in Madin-Darby canine kidney cells. J Biol Chem 281, 5288-99.

Su, L., Hattori, M., Moriyama, M., Murata, N., Harazaki, M., Kaibuchi, K. and Minato, N. (2003). AF-6 controls integrin-mediated cell adhesion by regulating Rap1 activation through the specific recruitment of Rap1GTP and SPA-1. J Biol Chem 278, 15232-8.

Takahashi, K., Nakanishi, H., Miyahara, M., Mandai, K., Satoh, K., Satoh, A., Nishioka, H., Aoki, J., Nomoto, A., Mizoguchi, A. et al. (1999). Nectin/PRR: an immunoglobulin-like cell adhesion molecule recruited to cadherin-based adherens junctions through interaction with Afadin, a PDZ domain-containing protein. J Cell Biol 145, 539-49.

Tiruppathi, C., Malik, A. B., Del Vecchio, P. J., Keese, C. R. and Giaever, I. (1992). Electrical method for detection of endothelial cell shape change in real time: assessment of endothelial barrier function. Proc Natl Acad Sci U S A 89, 7919-23.

Willems, C., Astaldi, G. C., De Groot, P. G., Janssen, M. C., Gonsalvez, M. D., Zeijlemaker, W. P., Van Mourik, J. A. and Van Aken, W. G. (1982). Media conditioned by cultured human vascular endothelial cells inhibit the growth of vascular smooth muscle cells. Exp. Cell. Res. 139, 191-197.

Wittchen, E. S., Worthylake, R. A., Kelly, P., Casey, P. J., Quilliam, L. A. and Burridge, K. (2005). Rap1 GTPase inhibits leukocyte transmigration by promoting endothelial barrier function. J. Biol. Chem. 280, 11675-11682.

Yamamoto, T., Harada, N., Kano, K., Taya, S., Canaani, E., Matsuura, Y., Mizoguchi, A., Ide, C. and Kaibuchi, K. (1997). The Ras target AF-6 interacts with ZO-1 and serves as a peripheral component of tight junctions in epithelial cells. J Cell Biol 139, 785-95.

Yamamoto, T., Harada, N., Kawano, Y., Taya, S. and Kaibuchi, K. (1999). In vivo interaction of AF-6 with activated Ras and ZO-1. Biochem Biophys Res Commun 259, 103-7.

Zhadanov, A. B., Provance, D. W., Jr., Speer, C. A., Coffin, J. D., Goss, D., Blixt, J. A., Reichert, C. M. and Mercer, J. A. (1999). Absence of the tight junctional protein AF-6 disrupts epithelial cell-cell junctions and cell polarity during mouse development. Curr Biol 9, 880-8.

Zhang, Z., Rehmann, H., Price, L. S., Riedl, J. and Bos, J. L. (2005). AF6 negatively regulates Rap1-induced cell adhesion. J Biol Chem 280, 33200-5.

Chapter 6

siRNA approach to identify mediators of 007-induced trans-endothelial resistance

siRNA approach to identify mediators of 007-induced trans-endothelial resistance

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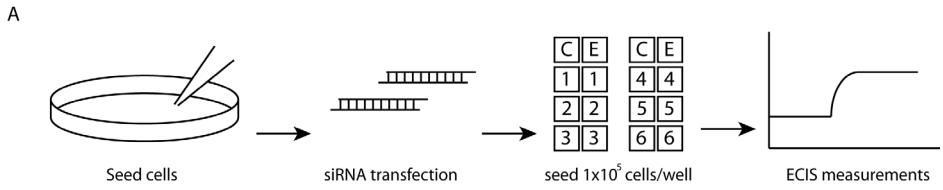
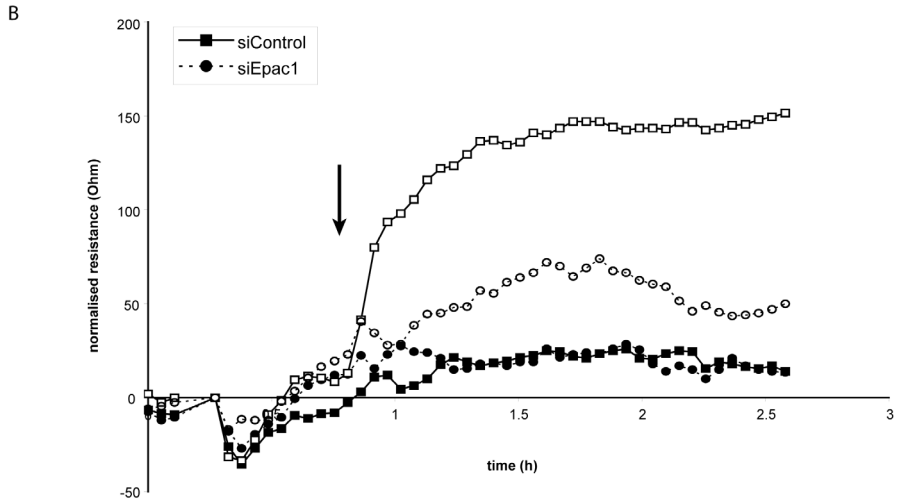
Abstract
The vascular endothelium provides a semi-permeable barrier, which restricts the passage of fluid, macromolecules and cells to the surrounding tissues. Specific activation of the Rap1 guanine-nucleotide exchange factor Epac1 by the cAMP analogue 8-pCPT-2'O-Me-cAMP (007) results in increased barrier function in endothelial cells both in vitro and in vivo. We used real time measurement of trans-endothelial electrical resistance, in combination with siRNA technology to investigate the role of 45 candidate proteins in this process. We identified ten genes, whose knock-down significantly reduced the effects of Epac1 activation on the endothelial barrier. Among these are both known regulators of Epac1 induced resistance, such as Rac1, as well as putative interactors of Rap1 with unknown function. Our findings can be used as basis for further exploration of the Epac1-dependent signalling networks.

Introduction
The Exchange proteins activated by cAMP (Epac) represents a small family of two guanine nucleotide exchange factors (GEFs) for the small GTPases Rap1 and Rap2. Epac1 and Epac2 are activated by direct binding of cAMP and elevated levels of cAMP result in the GTP-loading of both Rap1 and Rap2 in cells that express Epac1 and/or Epac2. The analysis of these proteins in a cellular context was greatly enhanced by an Epac-specific cAMP analog, 8-pCPT-2'O-Me-cAMP (007). This analogue does not activate PKA or other cAMP targets and activates Epac1 better than cAMP (Enserink et al., 2002; Rehmann et al., 2003). Using this analogue, Epac1 was implicated, amongst others, in integrin-mediated adhesion and the inhibition of Na+/H+ exchanger (Honegger et al., 2006; Rangarajan et al., 2003).
More recent reports suggest a role for Epac1 and Rap proteins in the regulation of cell-cell contact formation. Activation of Epac1 was shown to inhibit HGF-induced junction breakdown in MDCK cells. Furthermore, inhibition of Rap1 by over-expression of Rap1GAP inhibits E-cadherin function (Hogan et al., 2004; Price et al., 2004). Although Epac1 is not abundantly expressed in epithelial cells, recent observations show that Epac1 is expressed in endothelial cells and that stimulation of these cells with 007 results in the activation of Rap1 and Rap2. Various groups reported that activation of Epac1 results in increased VE-cadherin localization at the cell-cell contacts, as well as increased adhesion of endothelial cells to Fc-VE-cadherin. Furthermore, we and others have shown that 007 treatment resulted in decreased permeability and VE-cadherin independent reorganization of the actin cytoskeleton (Cullere et al., 2005; Fukuhara et al., 2005; Kooistra et al., 2005). Finally, it was shown that Epac1 activation increases trans-endothelial resistance in a Rap1 dependent manner (Wittchen et al., 2005). Although the role of Epac1-Rap1 in endothelial barrier function is well established, very little is known about the underlying signalling pathways.
The endothelium of the vascular system forms a barrier between the blood and the extra-vascular space that controls the extravasation of solutes, macromolecules, and blood cells. Failure to control the vascular integrity results in various pathological conditions, such as chronic inflammation and oedema. We used Electrical Cell-Substrate Impedance Sensing (ECIS) to measure the trans-endothelial electrical resistance in real-time, as measure for barrier function. In this assay, a small target electrode and a large electrode are lithographed on the surface of a slide and positioned within a chamber that holds tissue culture media. A one volt AC current passes through the culture media electrolyte, and a lock-in amplifier measures the current flow through this circuit, detecting the resistance over time. Cells binding to the target electrode surface cause changes in the resistance of the circuit. Also, when the electrode is covered fully by a monolayer of cells, differences in cell-cell contacts influence the resistance (Tiruppathi et al., 1992).
Here, we employed a small scale siRNA screen to identify proteins required for 007-induced resistance. Although the number of proteins tested was quite





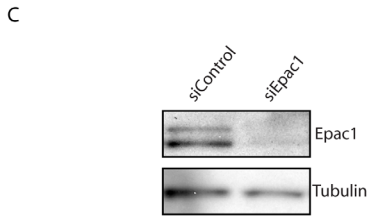


Fig. 1. (A) Schematic representation of the siRNA screen. Cells were seeded and transfected the next day at approx. 40% confluence. Hereafter, cells were replated and electrical resistance was measured as described in Material & Methods. (B) HUVECs were transfected with siRNA oligo's as indicated and trans-endothelial electrical resistance was measured. Cells were grown to confluence and either stimulated (arrow) with PBS (closed) or 100μM 007 (open). (C) Western blot for Epac1 indicating the knock-down.

limited we found various regulators of Epac1 induced barrier function.

Materials and Methods

Trans-endothelial electrical resistance measurements

Endothelial cells were seeded at $1.0x10^5$ cells per well ($0.8cm^2$) on fibronectin-coated electrode arrays and grown to confluency. Measurements of trans-endothelial electrical resistance were performed in real time at 37°C, 5% CO_2, using an electrical cell-substrate impedance sensing system at 400Hz (ECIS; Applied Biophysics Inc, Troy, NY, USA) as previously described (Tiruppathi et al., 1992). Endothelial resistance was normalized by subtracting the basal resistance of the monolayer.

Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured in EBM-2 Bulletkit culture medium (EBM-2 supplemented with EGM-2 SingleQuots (hEGF, hydrocortisone, fetal bovine serum, VEGF, hFGF-B, R3-IGF-1, Ascorbic Acid, GA-1000, heparin)) (Clonetics) as described (Jaffe et al., 1973; Willems et al., 1982). Second to fourth passage cells were used. Cells were cultured on 1% gelatin (Sigma) and experiments were performed on 7μg/ml fibronectin (Sigma). 18 hours after plating HUVEC were transfected with siRNA smartpool oligos (Dharmacon) using Interferin (Polyplus-transfection Inc., New York, NY, USA) according to manufacturer's recommendation. 16 hours after transfection, medium was replaced and cells were replated 8 hours later and grown to confluency. Basal resistance was subtracted to be able to compare individual electrodes.

Results and Discussion

Previously, we and others have shown that activation of Epac1 in endothelial cells leads to decreased

permeability (Cullere et al., 2005; Kooistra et al., 2005). It was also shown that the endothelial electrical resistance was increased upon 007 treatment in HUVEC (Wittchen et al., 2005). Despite the differences between these assays, both of them are commonly used to measure the endothelial barrier function. Given that trans-endothelial electrical resistance (TER) measurements are more amenable to high throughput approaches, we used this system to investigate the downstream signalling mechanisms in the 007-induced barrier function. To quantify the changes of TER over time, we seeded HUVEC on fibronectin-coated gold-covered electrodes and measured the resistance using the Electrical Cell Impedance System (ECIS) in real time (figure 1a). Indeed, when 007 was added to a confluent monolayer of endothelial cells, we observed a vast increase in endothelial resistance, reaching a maximum after one hour (figure 1b). To find proteins involved in the 007-mediated endothelial barrier function we set up a screening method using siRNA. As a positive control we investigated whether the increase by 007 could be abolished by siRNA for Epac1. A pronounced knock-down of Epac1 reduced the effect of 007 on TER significantly, although a residual effect was observed (figure 1b and c).

First we designed a custom library of 45 genes consisting of (putative) Rap-effectors and regulators of Rap1 activity. After transfection, cells were re-seeded onto electrodes and we measured 6 different siRNAs per experiment. To decrease the variability between electrodes, we measured two electrodes per siRNA (figure 1a). The data shown in figure 2a is the normalized resistance, represented as percentage of their respective controls for comparison between different experiments. Although 10 siRNAs gave a clear reduction of the 007-induced endothelial resistance, there were also 10 smartpool oligo's that gave more ambiguous results. This was either due to a small window between siControl and siEpac1 (e.g. PDZK10) or due to severe differences between the duplicate measurements (e.g. RasGRP3). Nevertheless, as an extra internal control, Epac1 (RAPGEF3) was clearly picked up in the screen.

To solidify the effects of the positive smartpools on 007 induced TER, we re-screened both the siRNAs that clearly reduced the effect of 007, as well as the more ambiguous siRNAs. To further decrease the variability between electrodes, we measured all siRNAs in quadruplicate. We found 11 siRNA smartpools that significantly decreased the effect of 007 on TER (Figure 2b and Table 1). To verify the siRNA targets, we are currently validating these smartpools with single siRNAs. This approach has excluded R-Ras and RasGRP3 as targets, since we could not repeat the results obtained from the screen with any single oligo or smartpool, whereas the knock-down was complete (data not shown). Also Lamellipodin was tested with single oligo's and these gave comparable knock-down and comparable results on the 007-induced TER (Chapter 6 of this thesis). Although the verification of the smartpools is still ongoing there is functional overlap between various smartpool-targets initially pick up in this screen.

The small GTPase Rac1 is an important regulator of the cytoskeleton and is involved in the regulation of endothelial barrier function (Wojciak-Stothard and Ridley, 2002). Here we found that Rac1 GTPase and one of its guanine-nucleotide exchange factors, Vav1, are involved in the regulation of the endothelial barrier function. Fukuyama and colleagues have shown that E-cadherin mediated activation of Rac1 is dependent on the activity of Rap1. Interestingly, they suggest that Vav2 is involved in this process (Fukuyama et al., 2006). Also, Vav2 interacts with Rap1-GTP and Rap1 is required for Vav2-induced cell spreading (Arthur et al., 2004). Recent publications also indicate that Vav2 and Rac1 are required for the cAMP mediated regulation of the endothelial barrier function. Endothelial cells treated with cAMP elevating agents, such as PGE2 and ANP, increased the TER, coinciding with Rac1 activation. Chemical inhibition of PKA or knock-down of Epac1 decreases both the TER-induction and the Rac1 activation by these agents (Birukova et al., 2007a; Birukova et al., 2007b). Typically, in our siRNA screen Vav1 was the only RacGEF identified in 007-mediated electrical resistance, whereas also Vav2, vav3 and Tiam were tested. Interestingly, previous reports indicate Vav2 and Tiam as Rap1 effectors (Arthur et al., 2004).

Smartpool siRNA targeting CCM2 decreases the 007-induced TER up to 61% in our screen. CCM2 is mutated in familial cerebral cavernous malformation (CCM). Patients bearing this mutation suffer from neurovascular lesions such as enlarged vascular channels. There are two other genes found to be mutated in familial CCM: KRIT1/CCM1 and CCM3. CCM1 has been shown to bind to Rap1 in yeast-2-hybrd assays and in vitro (Beraud-Dufour et al., 2007; Serebriiskii et al., 1997). Furthermore CCM2 interacts with CCM1 directly (Zhang et al., 2007). Another CCM1-binding protein, ITGB1BP1 or ICAP1

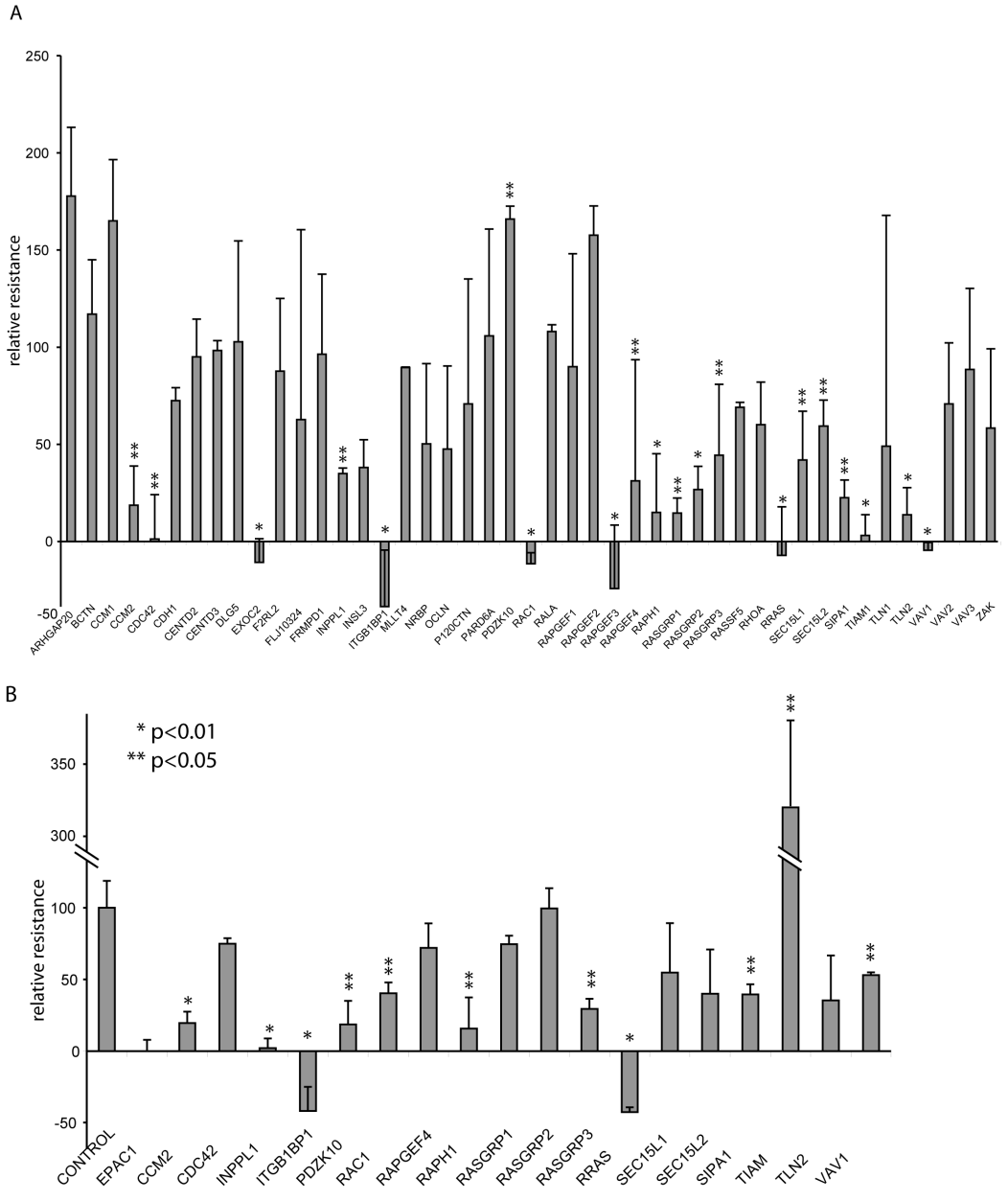


Fig. 2. (A) Results of first screening round. The experiments were performed as in Figure 1C. The increase in normalized resistance is represented as a percentage, where siControl is set to 100% and siEpac1 to 0%. Averages of duplicate measurements are shown; * clear reduction in 007-response; ** ambiguous results (see text). (B) Results of second screening round. The experiments were performed as in Figure 1C. Data are mean (+/- SEM) of four measurements. Statistical differences from siControl were determined by Student's T-test (* = p<0.01; ** p=<0.05).

gene	geneID	007 induction in 2nd round (%)	p-value T-test with siControl)	Link to Rap1 signalling	Refs
Control		100			
CCM2	83605	40	0.008	Interacts with Rap-effector KRIT/CCM1	(Glading et al., 2007; Zhang et al., 2007)
CDC42	998	85	0.243	Downstream of Rap1	(Schwamborn and Puschel, 2004)
INPPL1	3636	27	0.002	Interacts with Rap-effector ARAP3	(Raaijmakers et al.,(2007)
ITGB1BP1	9270	8	0.001	Interacts with Rap-effector KRIT1/CCM1	(Beraud -Dufour et al., 2007; Glading et al., 2007)
PDZK10	9758	8	0.018	Interacts with Rap1	(Riedl et al., 2005)
RAC1	5879	39	0.027	Downstream Rap1	(Arthur et al., 2004)
RAPGEF4	11069	82	0.315	Rap-GEF	(Bos, 2003)
RAPH1	65059	21	0.027	Homolog interacts with Rap1	(Krause et al., 2004; Lafuente et al., 2004)
RASGRP1	10125	97	0.249	Rap/Ras-GEF	(Bos et al., 2001)
RASGRP2	10235	117	0.988	Rap/Ras-GEF	(Bos et al., 2001)
RASGRP3	25780	47	0.013	Rap/Ras-GEF	(Bos et al., 2001)
RRAS	6237	22	0.001	Activated by Epac1?	(Lopez De Jesus et al., 2006)
SEC15L1	54536	115	0.295	In complex with Rap1 in S.cerivisiae	(France et al., 2006)
SEC15L2	23233	104	0.149	In complex with Rap1 in S.cerevisiae	(France et al., 2006)
SIPA1	6494	53	0.024	RapGAP	(Bos et al., 2001)
TIAM	7074	139	0.011	Interacts with Rap1	(Arthur et al., 2004)
TLN2	83660	45	0.129	Homolog interacts with Rap1 effector	(Han et al., 2006)
VAV1	7409	98	0.049	Homolog interacts with Rap1	(Arthur et al., 2004)

Table 1: Effects of specific smartpool siRNAs on 007 induced TER.

(Zawistowski et al., 2002), gave over 90% reduction of 007 induced TER. ITGB1BP1 binds to beta1-integrins and was shown to be involved in migration of C2C12 cells (Alvarez et al., 2008). Remarkably, itgb1bp1 knock-out mice do not display any obvious endothelial defects but suffer from bone malformations, probably due to increased integrin-mediated adhesion of osteoblasts (Bouvard et al., 2007). The observation that ITGB1BP1 is required for 007-induced TER, suggests a role for integrin in this process. However, when HUVEC were plated on poly-L-Lysine, 007 still increased the TER to same extend as cells plated on fibronectin (M.Lorenowicz, P. Hordijk, unpublished results). Recently, Glading et al. showed that CCM1 is required for 007-mediated barrier function. They observed that CCM1 is localized in the cell-cell junctions and knock-down of CCM1 increased the endothelial permeability. Furthermore, CCM1-depleted cells were no longer capable to decrease the permeability upon stimulation with 007. Finally, transfection of Rap1GAP into endothelial cells resulted in increased permeability, which was rescued by over-expression of CCM1 (Glading et al., 2007). Although CCM1 was not picked up in the screen initially, later testing revealed that knockdown of CCM1 results in a decreased effect of 007 on TER (data not shown).

In our screen we also picked up two putative Rap1-effectors. PDZK10, or KIAA0316, contains a FERM-, WW- and PDZ-domain. The C .elegans homologue of PDZK10, frm-8, was initially found to interact with the Ras-homolog let-60 (Li et al., 2004). Independently our lab has previously found that both C. elegans frm-8 and its human isoform PDZK10 interact with active Rap1 in a yeast-2-hybrid screen. Although its function remains elusive, previous observations in human ovarian carcinoma cells indicate that PDZK10 synergizes with Rap1 to form actin based dorsal ruffles, suggesting a role in actin remodelling (Riedl

et al., 2005).

We also identified RAPH1, or Lamellipodin (LPD), as a regulator for 007-induced TER. LPD has similarity to RIAM, a Rap1 effector involved in Rap1-mediated integrin-dependent adhesion (Han et al., 2006; Lafuente et al., 2004). Both RIAM and LPD bind to Ena/VASP family of proteins through their EVL-binding motifs and have a well conserved lipid-binding PH-domain, as well as a Ras-association domain (RA). LPD contains seven EVL-binding sites in its C-terminus, whereas RIAM has four. RIAM was shown to bind to Rap1 directly, but although tested extensively, this was not found for LPD thus far (Rodriguez-Viciana et al., 2004). LPD is localized in lamellipodia and knock-down of LPD blocks the formation of these lamellipodia. The mechanism through which LPD induces formation of lamellipodia is by recruitment of Ena/VASP-proteins. Localization of LPD to the lamellipodia is in part dependent on the PH-domain, that binds specifically to PI(3,4)P2 (Krause et al., 2004). Surprisingly, siRNA for SHIP2 was found to inhibit 007-induced TER. SHIP2 is a lipid-phosphatase that dephosphorylates PI(3,4,5)P3 to PI(3,4)P2, suggesting that localization of LPD to lamellipodia is required for 007-induced TER. SHIP2 and the Rap1-effector ARAP3 bind directly through their SAM domains (Raaijmakers et al., 2007). However, knock-down of ARAP3 was extensively tested, but had no effect on 007-induced TER (Figure 2a and data not shown).

We and others have previously shown that the induction of TER by 007 requires an intact actin-cytoskeleton. Furthermore, we have shown that independent of the effects on cell-cell junctions, 007 re-organizes the actin cytoskeleton. Since both LPD and PDZK10 have been reported to localize to actin-based structures, an obvious experiment would be to investigate the requirement of both proteins for the observed cytoskeletal rearrangements.

SIPA1, or SPA1, is a GTPase activating protein specific for Rap1 and Rap2. Knock-down of SIPA1 reduced the effects of 007-induced TER. Although we did not investigate this, it is likely that siRNA for a GTPase activating protein increases the basal GTP-loading for Rap1, thereby decreasing the effect of a GEF on it. Furthermore, a polymorphism in sipa1 leads to metastatic enhancement in a mouse mammary tumour model. Also they showed that siRNA of SIPA1 reduces the metastatic potential of Mvt1 cells in vivo, thereby increasing the tumour size. In addition, over-expression of SIPA1 enhances the metastatic potential of this cell-line in vivo (Park et al., 2005). In addition, the Sipa-1 null mice show a variety of hematopoietic stem cell disorders, but no obvious phenotype involving the endothelium has been described (Ishida et al., 2006; Ishida et al., 2003; Kometani et al., 2006). However, a role for SIPA1 in the regulation of endothelial barrier function is not addressed thus far.

Overall, the targets we found involved in 007 mediated TER using siRNA based screening will provide new insights in the mechanisms employed by Epac1 to increase the barrier function. They also further support a model where the reorganization of the actin cytoskeleton precedes the actual increase in VE-cadherin localization, especially since both beta-catenin and p120-catenin had no effect on the 007 induced TER. Although all the targets have to be properly validated, the consistency with previously validated mediators of endothelial barrier function, such as Rac, founds a basis for further investigation of the Epac1/Rap1 dependent signaling pathways in endothelial barrier function.

References

Alvarez, B., Stroeken, P. J., Edel, M. J. and Roos, E. (2008). Integrin Cytoplasmic domain-Associated Protein-1 (ICAP-1) promotes migration of myoblasts and affects focal adhesions. J Cell Physiol 214, 474-82.

Arthur, W. T., Quilliam, L. A. and Cooper, J. A. (2004). Rap1 promotes cell spreading by localizing Rac guanine nucleotide exchange factors. J. Cell. Biol. 167, 111-122.

Beraud-Dufour, S., Gautier, R., Albiges-Rizo, C., Chardin, P. and Faurobert, E. (2007). Krit 1 interactions with microtubules and membranes are regulated by Rap1 and integrin cytoplasmic domain associated protein-1. Febs J 274, 5518-32.

Birukova, A. A., Zagranichnaya, T., Alekseeva, E., Bokoch, G. M. and Birukov, K. G. (2007a). Epac/Rap and PKA are novel mechanisms of ANP-induced Rac-mediated pulmonary endothelial barrier protection. J Cell Physiol.

Birukova, A. A., Zagranichnaya, T., Fu, P., Alekseeva, E., Chen, W., Jacobson, J. R. and Birukov, K. G. (2007b). Prostaglandins PGE(2) and PGI(2) promote endothelial barrier enhancement via PKA- and Epac1/Rap1-dependent Rac activation. Exp Cell Res 313, 2504-20.

Bos, J. L. (2003). Epac: a new cAMP target and new avenues in cAMP research. Nat. Rev. Mol. Cell. Biol. 4, 733-738.

Bos, J. L., de Rooij, J. and Reedquist, K. A. (2001). Rap1 signalling: adhering to new models. Nat. Rev. Mol. Cell. Biol. 2, 369-377.

Bouvard, D., Aszodi, A., Kostka, G., Block, M. R., Albiges-Rizo, C. and Fassler, R. (2007). Defective osteoblast function in ICAP-1-deficient mice. Development 134, 2615-25.

Cullere, X., Shaw, S. K., Andersson, L., Hirahashi, J., Luscinskas, F. W. and Mayadas, T. N. (2005). Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. Blood 105, 1950-1955.

Enserink, J. M., Christensen, A. E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H. G., Doskeland, S. O., Blank, J. L. and Bos, J. L. (2002). A novel Epac-specific cAMP analogue demonstrates

independent regulation of Rap1 and ERK. Nat. Cell. Biol. 4, 901-906.

France, Y. E., Boyd, C., Coleman, J. and Novick, P. J. (2006). The polarity-establishment component Bem1p interacts with the exocyst complex through the Sec15p subunit. J Cell Sci 119, 876-88.

Fukuhara, S., Sakurai, A., Sano, H., Yamagishi, A., Somekawa, S., Takakura, N., Saito, Y., Kangawa, K. and Mochizuki, N. (2005). Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling. pathway. Mol. Cell. Biol. 25, 136-146.

Fukuyama, T., Ogita, H., Kawakatsu, T., Inagaki, M. and Takai, Y. (2006). Activation of Rac by cadherin through the c-Src-Rap1-phosphatidylinositol 3-kinase-Vav2 pathway. Oncogene 25, 8-19.

Glading, A., Han, J., Stockton, R. A. and Ginsberg, M. H. (2007). KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell cell junctions. J Cell Biol 179, 247-54.

Han, J., Lim, C. J., Watanabe, N., Soriani, A., Ratnikov, B., Calderwood, D. A., Puzon-McLaughlin, W., Lafuente, E. M., Boussiotis, V. A., Shattil, S. J. et al. (2006). Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. Curr Biol 16, 1796-806.

Hogan, C., Serpente, N., Cogram, P., Hosking, C. R., Bialucha, C. U., Feller, S. M., Braga, V. M., Birchmeier, W. and Fujita, Y. (2004). Rap1 regulates the formation of E-cadherin-based cell-cell contacts. Mol. Cell. Biol. 24, 6690-6700.

Honegger, K. J., Capuano, P., Winter, C., Bacic, D., Stange, G., Wagner, C. A., Biber, J., Murer, H. and Hernando, N. (2006). Regulation of sodium-proton exchanger isoform 3 (NHE3) by PKA and exchange protein directly activated by cAMP (EPAC). Proc Natl Acad Sci U S A 103, 803-8.

Ishida, D., Su, L., Tamura, A., Katayama, Y., Kawai, Y., Wang, S. F., Taniwaki, M., Hamazaki, Y., Hattori, M. and Minato, N. (2006). Rap1 signal controls B cell receptor repertoire and generation of self-reactive B1a cells. Immunity 24, 417-27.

Ishida, D., Yang, H., Masuda, K., Uesugi, K., Kawamoto, H., Hattori, M. and Minato, N. (2003). Antigen-driven T cell anergy and defective memory T cell response via deregulated Rap1 activation in SPA-1-deficient mice. Proc Natl Acad Sci U S A 100, 10919-24.

Jaffe, E. A., Nachman, R. L., Becker, C. G. and Minick, C. R. (1973). Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J. Clin. Invest. 52, 2745-2756.

Kometani, K., Aoki, M., Kawamata, S., Shinozuka, Y., Era, T., Taniwaki, M., Hattori, M. and Minato, N. (2006). Role of SPA-1 in phenotypes of chronic myelogenous leukemia induced by BCR-ABL-expressing hematopoietic progenitors in a mouse model. Cancer Res 66, 9967-76.

Kooistra, M. R., Corada, M., Dejana, E. and Bos, J. L. (2005). Epac1 regulates integrity of endothelial cell junctions through VE-cadherin. FEBS Lett 579, 4966-72.

Krause, M., Leslie, J. D., Stewart, M., Lafuente, E. M., Valderrama, F., Jagannathan, R., Strasser, G. A., Rubinson, D. A., Liu, H., Way, M. et al. (2004). Lamellipodin, an Ena/VASP ligand, is implicated in the regulation of lamellipodial dynamics. Dev Cell 7, 571-83.

Lafuente, E. M., van Puijenbroek, A. A., Krause, M., Carman, C. V., Freeman, G. J., Berezovskaya, A., Constantine, E., Springer, T. A., Gertler, F. B. and Boussiotis, V. A. (2004). RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion. Dev. Cell 7, 585-595.

Li, S., Armstrong, C. M., Bertin, N., Ge, H., Milstein, S., Boxem, M., Vidalain, P. O., Han, J. D., Chesneau, A., Hao, T. et al. (2004). A map of the interactome network of the metazoan C. elegans. Science 303, 540-3.

Lopez De Jesus, M., Stope, M. B., Oude Weernink, P. A., Mahlke, Y., Borgermann, C., Ananaba, V. N., Rimmbach, C., Rosskopf, D., Michel, M. C., Jakobs, K. H. et al. (2006). Cyclic AMP-dependent and Epac-mediated activation of R-Ras by G protein-coupled receptors leads to phospholipase D stimulation. J Biol Chem 281, 21837-47.

Park, Y. G., Zhao, X., Lesueur, F., Lowy, D. R., Lancaster, M., Pharoah, P., Qian, X. and Hunter, K. W. (2005). Sipa1 is a candidate for underlying the metastasis efficiency modifier locus Mtes1. Nat Genet 37, 1055-62.

Price, L. S., Hajdo-Milasinovic, A., Zhao, J., Zwartkruis, F. J., Collard, J. G. and Bos, J. L. (2004). Rap1 regulates E-cadherin-mediated cell-cell adhesion. J. Biol. Chem. 279, 35127-35132.

Raaijmakers, J. H., Deneubourg, L., Rehmann, H., de Koning, J., Zhang, Z., Krugmann, S., Erneux, C. and Bos, J. L. (2007). The PI3K effector Arap3 interacts with the PI(3,4,5)P3 phosphatase SHIP2 in a SAM domain-dependent manner. Cell Signal 19, 1249-57.

Rangarajan, S., Enserink, J. M., Kuiperij, H. B., de Rooij, J., Price, L. S., Schwede, F. and Bos, J. L. (2003). Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the beta 2-adrenergic receptor. J Cell Biol 160, 487-93.

Rehmann, H., Schwede, F., Doskeland, S. O., Wittinghofer, A. and Bos, J. L. (2003). Ligand-mediated activation of the cAMP-responsive guanine nucleotide exchange factor Epac. J Biol Chem 278, 38548-56.

Riedl, J. A., Pellis-van Berkel, W., Barbanti, F., Bos, J. L. and Zwartkruis, G. J. T. (2005). Thesis: Analysis of the Rap1 signalling network: Utrecht University.

Rodriguez-Viciana, P., Sabatier, C. and McCormick, F. (2004). Signaling specificity by Ras family GTPases is determined by the full spectrum of effectors they regulate. Mol Cell Biol 24, 4943-54.

Schwamborn, J. C. and Puschel, A. W. (2004). The sequential activity of the GTPases Rap1B and Cdc42 determines neuronal polarity. Nat. Neurosci. 7, 923-929.

Serebriiskii, I., Estojak, J., Sonoda, G., Testa, J. R. and Golemis, E. A. (1997). Association of Krev-1/rap1a with Krit1, a novel ankyrin repeat-containing protein encoded by a gene mapping to 7q21-22. Oncogene 15, 1043-9.

Tiruppathi, C., Malik, A. B., Del Vecchio, P. J., Keese, C. R. and Giaever, I. (1992). Electrical method for detection of endothelial cell shape change in real time: assessment of endothelial barrier function. Proc Natl Acad Sci U S A 89, 7919-23.

Willems, C., Astaldi, G. C., De Groot, P. G., Janssen, M. C., Gonsalvez, M. D., Zeijlemaker, W. P., Van Mourik, J. A. and Van Aken, W. G. (1982). Media conditioned by cultured human vascular endothelial cells inhibit the growth of vascular smooth muscle cells. Exp. Cell. Res. 139, 191-197.

Wittchen, E. S., Worthylake, R. A., Kelly, P., Casey, P. J., Quilliam, L. A. and Burridge, K. (2005). Rap1 GTPase inhibits leukocyte transmigration by promoting endothelial barrier function. J. Biol. Chem. 280, 11675-11682.

Wojciak-Stothard, B. and Ridley, A. J. (2002). Rho GTPases and the regulation of endothelial permeability. Vascul Pharmacol 39, 187-99.

Zawistowski, J. S., Serebriiskii, I. G., Lee, M. F., Golemis, E. A. and Marchuk, D. A. (2002). KRIT1 association with the integrin-binding protein ICAP-1: a new direction in the elucidation of cerebral cavernous malformations (CCM1) pathogenesis. Hum Mol Genet 11, 389-96.

Zhang, J., Rigamonti, D., Dietz, H. C. and Clatterbuck, R. E. (2007). Interaction between krit1 and malcavernin: implications for the pathogenesis of cerebral cavernous malformations. Neurosurgery 60, 353-9; discussion 359.

Chapter 7

Lamellipodin is required for 007-induced endothelial barrier function

Lamellipodin is required for 007-induced endothelial barrier function

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Abstract

The endothelial barrier regulates the passage of fluids, leukocytes and solutes from blood to the surrounding tissues. We have previously shown that Epac1-activation results in increased barrier function, presumably through regulation of the actin cytoskeleton. In a screen for possible mediators of this effect we observed that downregulation of the RA-domain containing protein Lamellipodin (LPD) inhibits Epac1-induced trans-endothelial electrical resistance of a monolayer of human umbilical vein endothelial cells. LPD is an Ena/VASP binding protein, involved in actin-mediated lamellipodia formation. Indeed, activation of Epac induces the localisation of VASP cell-cell contacts. However, depletion of LPD did not affect the translocation of actin to junctions, suggesting that LPD in not required for Epac-induced recruitment of actin to the junctions. Importantly, knock-down of VE-cadherin did not affect Epac-induced VASP translocations and trans-endothelial electrical resistance. We conclude that in addition to actin-recruitment to junctions, Epac1 induced trans-endothelial electrical resistance requires the Ena/VASP binding protein Lamellipodin, presumably to regulate protrusive activity.

Introduction

The vascular system is lined with endothelial cells that function as a barrier to separate the blood from the underlying tissues. Regulation of the endothelial barrier function is necessary for the passage of immune cells, solutes and fluids. Barrier function requires cell-cell contacts to form a physical barrier between the blood and the underlying tissue.

These cell-cell contacts are formed by adherens and tight junction proteins that are linked to the actin cytoskeleton. Perturbation of the barrier function plays a role in various pathological conditions such as oedema, chronic inflammation, atherosclerosis and sepsis (Wysolmerski and Lagunoff, 1985).

Epac1 and Epac2 are two nucleotide exchange factors for the small GTPases Rap1 and Rap2 that are activated by direct binding of cAMP. The use of an Epac-selective cAMP analog, 8-pCPT-2'O-Me-cAMP (007) (Enserink et al., 2002), implicated Epac in various processes, such as cell adhesion, inhibition of Na+/H+ exchanger and insulin secretion (Honegger et al., 2006; Rangarajan et al., 2003; Shibasaki et al., 2007). We have previously found that Epac1 can regulate cell-cell junctions in epithelial cells (Price et al., 2004) and subsequently several group described a similar role in endothelial cells (Cullere et al., 2005; Fukuhara et al., 2005; Wittchen et al., 2005).

Indeed, treatment of endothelial cells with 007 results in an Epac1-dependent activation of Rap1, resulting in a decrease in endothelial permeability. Also, activation of Epac1 results in increased VE-cadherin localisation at the cell-cell contacts, as well as increased VE-cadherin-based adhesion (Cullere et al., 2005; Fukuhara et al., 2005). The underlying mechanism for the enhancement of the endothelial barrier is presumably orchestrated by the actin cytoskeleton as de-polymerisation of the actin with Cytochalasin D abolishes the effects of 007 on endothelial permeability (Fukuhara et al., 2005). Furthermore, 007 stimulation results in reorganisation of the actin cytoskeleton, resulting in decreased stress-fibres and increased cortical actin, in a VE-cadherin independent manner (Kooistra et al., 2005).

Members of the Ena/VASP family of actin regulators localise at the leading edge of lamellipodia and filopodia to sites of dynamic actin reorganisation. Ena/VASP promotes actin polymerisation, presumably through de-capping of barbed ends of the actin cytoskeleton, resulting in elongation of the actin fibres (Krause et al., 2003). Recently, Ena/VASP proteins have been implicated in the actin reorganisation during the formation of epithelial adherens junctions. Ena/VASP is recruited to E-cadherin mediated contacts and regulates actin filament density at cell-cell contacts. Perturbation of Ena/VASP activity results in diffuse F-actin at the cell-cell contacts, indicating that Ena/

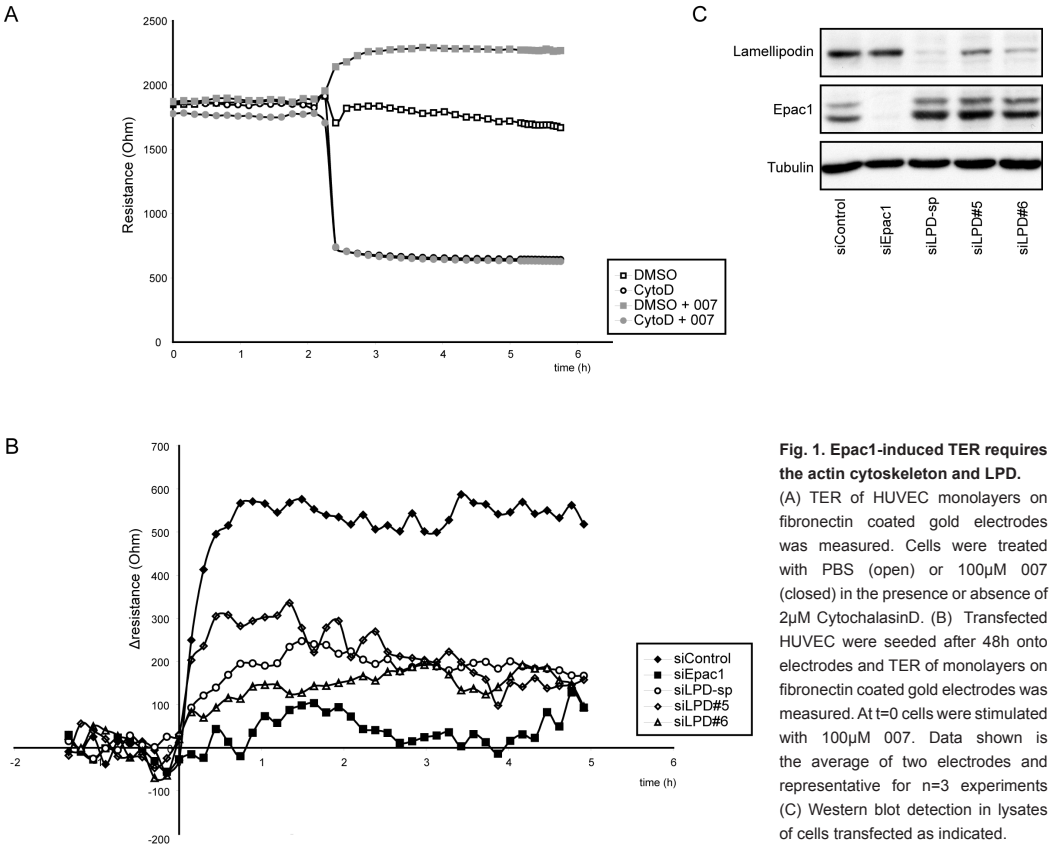

Fig. 1. Epac1-induced TER requires the actin cytoskeleton and LPD. (A) TER of HUVEC monolayers on fibronectin coated gold electrodes was measured. Cells were treated with PBS (open) or 100μM 007 (closed) in the presence or absence of 2μM CytochalasinD. (B) Transfected HUVEC were seeded after 48h onto electrodes and TER of monolayers on fibronectin coated gold electrodes was measured. At t=0 cells were stimulated with 100μM 007. Data shown is the average of two electrodes and representative for n=3 experiments (C) Western blot detection in lysates of cells transfected as indicated.

VASP plays a role in the organisation of the actin cytoskeleton in cell junctions (Scott et al., 2006). In endothelial cells the predominantly expressed family member VASP localises to focal adhesions and cell-cell contacts. VASP is phosphorylated by PKA in endothelial cells on various sites and stimulation of endothelial cells with Forskolin increases the localisation of VASP at the cell-cell contacts (Comerford et al., 2002). Interestingly, VASP null-mice show mainly alterations in platelet functioning (Massberg et al., 2004). However, ablation of all three Ena/VASP genes in mice resulted in embryonic death due to severe oedema, caused by impaired endothelial barrier function (Furman et al., 2007).

Ena/VASP is recruited by FP4-motif containing proteins, such as zyxin and vinculin. Recently, LPD was identified as an interactor for Ena/VASP proteins. LPD consists of seven FP4-motifs for Ena/VASP binding as well as a Ras-association (RA) domain and a PH-domain that is involved in lipid-binding. The RA- and PH-domains are involved in targeting LPD to the leading edge of lamellipodia where it enhances the protrusive activity through the recruitment of Ena/VASP. Furthermore, cells depleted from LPD were void of lamellipodial structures (Krause et al., 2004).

Here we report a role for LPD in Epac1-induced trans-endothelial electrical resistance. We show that the increase in resistance requires an intact actin cytoskeleton, as well as LPD expression. Additionally, we show that VASP is recruited to the cell-cell contacts upon Epac1-activation, but that the interaction between LPD and VASP is unaltered. Intriguingly, we show that the actin reorganisation by Epac1 is unaltered in the absence of LPD. Furthermore, we report that Epac1-induced VASP translocation is not due to increase VE-cadherin interactions. Finally, we show that VE-cadherin-based cell-cell contacts are not required for Epac1-induced barrier function. We propose a model where Epac1 induces local reorganisation of the actin cytoskeleton through LPD, resulting in increased trans-endothelial electrical resistance.

Materials and Methods

Cell culture and transfection

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured in EBM-2 Bulletkit culture medium (EBM-2 supplemented with EGM-2 SingleQuots (hEGF, hydrocortisone, fetal bovine serum, VEGF, hFGF-B, R3-IGF-1, Ascorbic Acid, GA-1000, heparin)) (Clonetics) as described (Jaffe et al., 1973; Willems et al., 1982). Second to fourth passage cells were used. 100 and 100VE-cells were kindly provided by Dr. E. Dejana and were cultured as previously described (Kooistra et al., 2005).

Cells were cultured on 1% gelatin (Sigma) and experiments were performed on 7µg/ml fibronectin (Sigma). 18 hours after plating HUVEC were transfected with OnTarget siRNA oligo's (Dharmacon) using Interferin (Polyplus-transfection Inc., New York, NY, USA) according to manufacturer's recommendation. Where indicated, we used oligofectamin (Invitrogen) for transfection of siRNA oligo's as previously described (Kooistra et al., 2005). 16 hours after transfection, medium was replaced and cells were replated 8 hours later and grown to confluency. Sequences of the oligo's used are: LPD#5: GGAAACAGUAAGCGUCAAAUU; LPD#6: GAACAGGCCUVUUUGAGUAUU. LPD-smartpool is a combinatrion of 4 oligo's Besides LPD#5 and #6 this also includes: GGAAGCAGCUCUUAUGAAUU and CCACUGCGGUUAUAGUUUAUU; Epac1 smartpool consists of: CGUGGGAACUCAUGAGAUGUU, GGACCGAGAUGCCCAAUUCUU; GAGCGUCUCUUUGUUGUCAUU and CGUGGUACAUUAUCUGGAAUU; VE-cadherin, β-catenin and p120-catenin sequences were still disclosed at that time. For over-expression experiments Cos-7 cells were transfected with indicated constructs using Fugene6 (Roche Diagnostics) according to manufacturers protocol. Experiments were performed 48 after transfection.

Antibodies and reagents

Mouse mAb against the extracellular domain of human VE-cadherin used was clone TEA 1.31 (Breviario et al., 1995; Lampugnani et al., 1992). Rabbit pAb against LPD was a kind gift of M. Krause. Anti-HA monoclonal 12CA5, Rap1 polyclonal (Santa Cruz), beta-catenin polyclonal (Abcam), VASP monoclonal (Cell Signaling Technology), GFP monoclonal (Roche Diagnostics), ZO-1 polyclonal antibodies (Transduction labs) were used.. 8-pCPT-2O-Me-cAMP (Biolog) was used at 100µM and CytochalasinD (Sigma) at 2µM. pcDNA3-GFP-VASP was a generous gift from Dr. J. de Rooij, pcDNA3-GFP-N3-RIAM was obtained from Dr. van Puijenbroek and HA-LPD was cloned from the Kazusa KIAA1681 cDNA by Dr. J. Riedl

Immunofluorescence

Cells were replated 24 hrs after transfection on fibronectin-coated (7µg/ml) glass coverslips (12mm) for 24 hrs. After stimulation, cells were fixed with 3.8% formaldehyde, permeabilised using 0,2% Triton X-100 and blocked with 2% BSA in PBS. Cells were incubated with indicated antibodies for 1 hour and subsequently incubated with Alexa-568 labelled phalloidin to stain F-actin and/or Alexa-488/568-labeled secondary antibodies (Molecular Probes). After mounting the coverslips onto slides, cells were examined using a Axioskop2 CLSM microscope (Zeiss). RBG Profiles were generated using ImageJ and the RBG profiles tool.

Immunoprecipitation and Western Blotting

Cells were washed with ice-cold PBS and scraped in lysis buffer (50mM Tris-Cl pH 7.5, 200mM NaCl, 1% NP-40, 2mM $CaCl_2$, 10% glycerol, NaF, protease inhibitors). Lysates were centrifuged for 10 minutes at 12000xg at 4°C. Supernatants were incubated with protein-agarose and indicated antibodies for 2 hours. After washing, immunoprecipitated proteins were analysed on 7.5% SDS-PAGE gels and transferred to PVDF-membrane. Blots were incubated with indicated antibodies and analysed using Odyssey (Li-Cor)

Rap1 activation assay

Rap1 activity was assayed as described previously (van Triest and Bos, 2004). Briefly, cells were washed with cold PBS and lysed with buffer containing 1% NP-40. Lysates were cleared by centrifugation and active Rap was precipitated with glutathione-Sepharose beads pre-coupled to a GST fusion protein of the Ras-binding domain of RalGDS. Precipitates were washed and analysed on Western blot.

Trans-endothelial electrical resistance measurement

HUVEC were seeded at $1.5x10^5$ cells per well (0.8 cm^2) on fibronectin coated electrode arrays and grown to confluency. Measurements of trans-endothelial electrical resistance were performed in real time at 400Hz, 37ºC, 5% CO_2, using an electrical cell-substrate impedance sensing system (ECIS; Applied BioPhisics Inc.Troy NY, USA)(Tiruppathi et al., 1992). Basal resistance was subtracted to be able to compare individual electrodes.

Results

Epac1-induced trans-endothelial electrical resistance is dependent on actin

Epac1 regulates the actin cytoskeleton in endothelial cells, resulting in a reorganisation from stress-fibres to cortical actin (Kooistra et al., 2005). To investigate the underlying mechanisms for Epac1-induced barrier function we tested whether trans-endothelial electrical resistance (TER) induced by 007-treatment requires the actin cytoskeleton. Activation of Epac1 with the 8-pCPT-2O-Me-cAMP (007) results in a rapid increased in trans-endothelial electrical resistance (TER). Treatment of cells with the actin-depolymerising agent CytochalasinD resulted in a vast decrease of TER. When cells were treated with CytochalasinD and 007 together, there was no difference with CytochalasinD alone. This indicates that activation of Epac1 by 007 results in actin-dependent increase in TER (figure 1A).

LPD is required for Epac1-induced TER

To identify the proteins involved in Epac1-mediated TER, we screened for various RA-domain containing proteins using siRNA. We observed that the response of cells depleted from LPD using Dharmacon OnTarget smartpool siRNA to 007 was reduced significantly compared to control cells. Also when we used single siRNAs from this smartpool the effects of 007 on TER was decreased (figure 1B).

The interaction between LPD and VASP is not regulated by Rap1

Since the Epac1-mediated effect on TER is dependent on LPD and requires the actin cytoskeleton, we

investigated the role of the LPD binding protein and actin regulator Ena/VASP. Since VASP is the most predominantly expressed member of this family in endothelial cells, we investigated whether the previously reported interaction between LPD and VASP was regulated by the activation of Epac1. Although we could precipitate VASP and LPD together, treatment with 007 had no effect on the interaction between HA-LPD and GFP-VASP (figure 2A). Also co-expression of RapV12 had no effect (data not shown). Furthermore, although we could identify the interaction between VASP and LPD in endothelial cells, treatment with 007 had no effect on the interaction, indicating that the VASP-LPD interaction is not regulated by the activation of Epac1 and Rap1 (figure 2B).

LPD interacts with the Rap1 effector RIAM

Although LPD is a close relative of the Rap1 effector protein RIAM (Lafuente et al., 2004), previous results suggest that LPD does not bind Rap1 (Krause et al., 2004; Rodriguez-Viciana et al., 2004). Indeed, HA-LPD and GST-Rap1aV12 did not co-precipiate (data not shown). This implies that LPD is not a direct effector of Rap1. Since both LPD and RIAM bind to Ena/VASP and Ena/VASP tetramerises, we investigated whether LPD and RIAM may form a complex. Indeed, when both RIAM and LPD are overexpressed we identified RIAM in a LPD immunoprecipitation (figure 2c). This may imply hat Rap1 through RIAM regulates LPD, and thus that RIAM also plays a role in Epac-mediated regulation of TER. However thus far we were unable to demonstrate a role for RIAM in this process.

Epac1 induces translocation of VASP to the cell-cell contacts

Previous reports showed that treatment of endothelial cells with cAMP results in the translocation of VASP to cell-cell contacts (Comerford et al., 2002). This was attributed to Protein kinase A (PKA), as VASP is directly phosphorylated by PKA. We investigated whether activation of Epac1 is sufficient for the cAMP-induced translocation of VASP. In unstimulated HUVEC, VASP

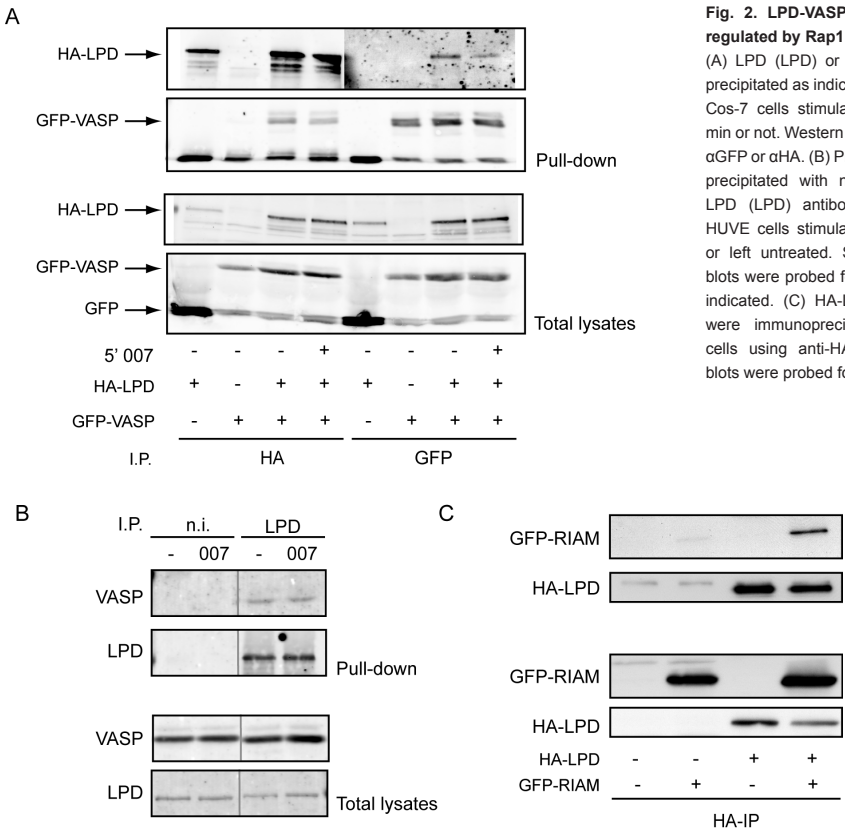

Fig. 2. LPD-VASP interaction is not regulated by Rap1 activation.
(A) LPD (LPD) or VASP was immuno-precipitated as indicated from transfected Cos-7 cells stimulated with 007 for 30 min or not. Western blots were probed for αGFP or αHA. (B) Proteins were immuno-precipitated with non-immune (n.i.) or LPD (LPD) antibodies from confluent HUVE cells stimulated with 100μM 007 or left untreated. Subsequent Western blots were probed for LPD and VASP as indicated. (C) HA-LPD and GFP-RIAM were immunoprecipitated from Cos-7 cells using anti-HA antibody. Western blots were probed for αGFP or αHA.

is mainly localised at the focal adhesions and at actin fibres. Upon stimulation with 007 however, this localisation is lost and VASP accumulates at the cell-cell contacts (figure 3A). This shows that activation of Epac1 is sufficient to translocate VASP to the cell-cell contacts. Cells stimulated with the adenylate cyclase activator Forskolin also show translocation of VASP to the cell-cell contacts as reported previously. When Epac1 was depleted from these cells, 007-induced translocation of VASP was completely inhibited, but forskolin-induced translocation partially, indicating a role for PKA-dependent phosphorylation in VASP translocation (figure 3A). Importantly whereas forskolin induced a clear shift of VASP to the phosphorylated form, 007 had no effect (Figure 3B). Therefore we conclude that activation of Epac1 is

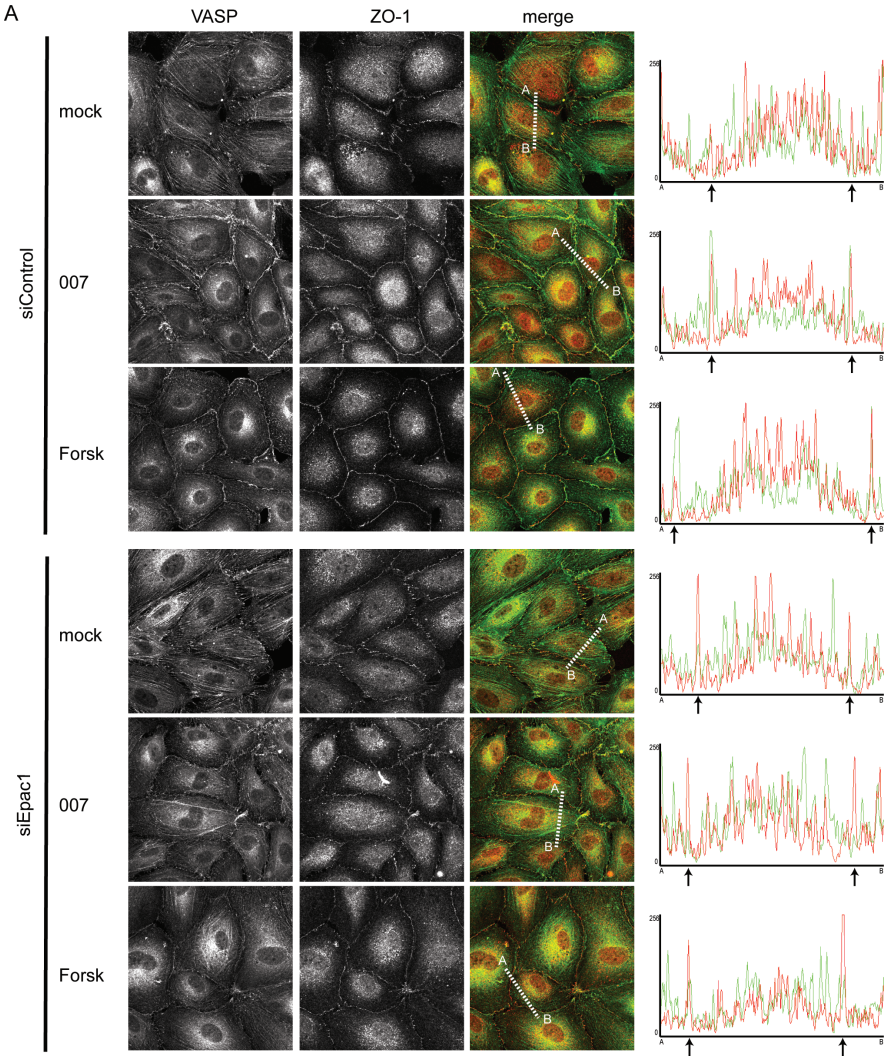

Fig. 3. VASP translocates to cell-cell contacts upon Epac1 activation. (A) HUVECs transfected with siRNA were immunostained as indicated after 30' treatment with 007 or Forskolin. Intensity profiles along dashed line between points A and B are shown. Arrows indicate cell-cell contacts. (B) HUVECs were stimulated for 5' as indicated and Rap1GTP was determined by the pull-down assay. Lysated were also probed for VASP to determine a phosphorylation-dependent shift. (C) HUVEC transfected with siRNA were treated with or without 007 for 30 minutes. Cells were stained with phalloidin to show F-actin. (D) HUVECs transfected with siRNA were stimulated with 007 or left untreated. Cells were immunostained as indicated. Bar= 10 μM.

sufficient to translocate VASP to the cell-cell contacts, independent of VASP phosphorylation.

Epac1-induced cortical actin formation is independent of LPD

We next investigated whether LPD is mediating Epac1-induced actin re-arrangements. To that end we depleted cells from LPD and determined actin rearrangements induced by 007. Surprisingly, 007 normally induced the translocation of actin to the cell-cell contacts (figure 3C). From these results we conclude that although LPD is required for Epac-induced TER, it is not required for Epac-induced formation of junctional actin.

VASP translocation is independent of VE-cadherin

Previously, we have shown that the formation of cortical actin by 007 is independent of VE-cadherin. To investigate whether VASP translocation requires VE-cadherin based cell-cell contacts, we depleted cells for VE-cadherin and examined the localisation of VASP. Cells depleted for VE-cadherin using siRNA lost beta-catenin localisation from the junctions, but the VASP localisation remained unchanged (figure 3D). Importantly, 007 induces the translocation of VASP to the cell-cell contacts in the absence of VE-cadherin.

VE-cadherin is dispensable in Epac1-induced TER

We have previously shown that VE-cadherin is required for 007-mediated permeability, measured by the flux of dextran over a monolayer of endothelial cells. Here we found that the TER is dependent on the actin cytoskeleton. Since VASP translocation is independent of VE-cadherin, we re-investigated the role of VE-cadherin based cell-cell contacts in

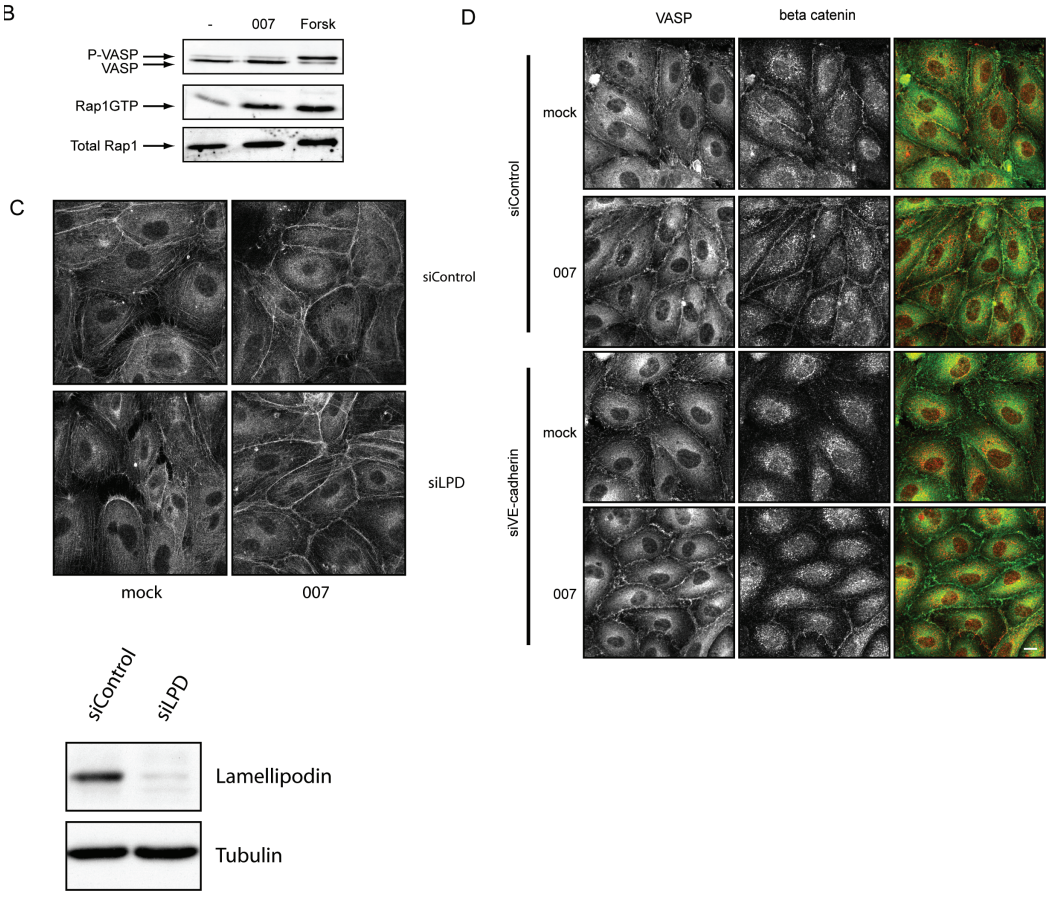

Figure 3 (cont.)

007-induced TER. Strikingly, cells transfected with siRNA targeting either VE-cadherin, β-catenin or p120-catenin responded normally to 007-treatment, whereas Epac1-siRNA inhibited the effect (figure 4A). Since it is likely that siRNA does not achieve complete knock-down, we also tested VE-cadherin null cells (100) and cells reconstituted with human VE-cadherin (100VE). Indeed also in these cells, the loss of VE-cadherin has no effect on 007-induced TER (figure 4B). Finally, we also investigated the effects of 007 under conditions where all Ca^{2+}-dependent junctions are prohibited by treatment with EGTA. Also in the absence of extra cellular calcium, 007 increases TER (figure 4C). Altogether these results show that the Epac1-mediated increase in TER is independent of VE-cadherin.

Discussion

Here we report that LPD is required for 007-induced TER. Also, we show that the binding between LPD and VASP is not regulated by 007 in endothelial cells. However, we could show that LPD interacts with the Rap1-effector RIAM. Furthermore, we show that translocation of VASP to the cell-cell contacts upon Epac1 activation is independent of VE-cadherin. Finally, we show that Epac1-induced TER is independent of VE-cadherin. Altogether these results point towards a model where Epac1/Rap1 activation employs LPD to enhance endothelial resistance.

Induction of endothelial barrier function by Epac1 was previously shown to be dependent on the actin cytoskeleton (Cullere et al., 2005; Sehrawat et al., 2008). Likewise, the enhancement of VE-cadherin adhesion required F-actin (Fukuhara et al., 2005). Our results extend these previous observations in that Epac1-mediated barrier regulation functions through the actin cytoskeleton. Firstly, we show that disruption of the actin-cytoskeleton abrogates 007-induced TER. Secondly, our results show the involvement of LPD in Epac1-induced TER. In single cells, LPD is required for the localisation of Ena/VASP to the leading edge of lamellipodia, resulting in elongation of actin fibres. In the endothelial monolayer we observed normal 007-induced actin reorganisation, i.e. the formation of cortical actin, in the absence of LPD, suggesting that this Epac-mediated effect is not mediated by LPD, and thus not the only determinant of Epac-induced TER. We propose Epac-induced TER additionally requires protrusive actively generated by the Lamelipodin/VASP complex. This would imply that VASP is also essential for Epac-induced TER. Indeed, it has been reported that siRNA for VASP results in increased permeability, whereas over expression of VASP reduces the endothelial permeability (Rosenberger et al., 2007). Although VASP null mice exhibit no phenotype in the endothelial barrier function (Hauser et al., 1999), Ena/VASP/Mena triple null mice suffer from embryonic oedema, due to increased vascular permeability. Interestingly, when endothelial cells were transfected with a construct to interfere with Ena/VASP signalling, the endothelial permeability is increased (Furman et al., 2007). A role for VASP in cAMP-mediated barrier function also has been suggested, since cAMP treatment of endothelial cells results in localisation of VASP at the cell-cell contacts (Comerford et al., 2002). This was previously attributed to PKA because VASP is directly phosphorylated by PKA on various sites (Butt et al., 1994). However, we now show that VASP translocates to the cell-cell contacts directly upon Epac1 activation and that this does not coincide with its phosphorylation. Finally, VASP was found in complex with Rap1 in a mass-spectrometry approach (Goldfinger et al., 2007). Given all this evidence, it is likely that Ena/VASP is employed by Epac1 to enhance the TER.

How is LPD regulated by Epac1 and Rap1? Binding of Rap1a to LPD was never observed in various studies (Krause et al., 2004; Rodriguez-Viciana et al., 2004), despite the presence of an RA domain and the observation that the LPD homolog RIAM does bind to Rap1. However, we observed that LPD can interact with RIAM, suggesting that LPD forms a complex with Rap1 through RIAM. This needs further exploration. Additionally the involvement of phosphatidylinositol (3,4)P2, with is required for LPD binding to the plasma membrane (Krause et al., 2004), in TER needs further investigations. However it is interesting to note that our siRNA screen also identified SHIP2 to be required for Epac1-induced TER. SHIP2 generates PI(3,4)P2 from PI(3,4,5)P3 lipids. The requirement of SHIP2 in Epac1-induced TER is presumably due to its requirement for the localisation of LPD to the plasma membrane.

Previous data show that activation of either Epac1 or PKA is sufficient to enhance the TER (Lorenowicz et al., 2008). Interestingly, Epac1 and PKA use distinct pathways to enhance the endothelial barrier, since PKA mediated TER is dependent on cell-matrix adhesion, whereas Epac1 mediated TER is not (Lorenowicz et al., 2008). Recently, it was shown that VASP binds to αII-Spectrin. Interestingly, αII-Spectin only binds

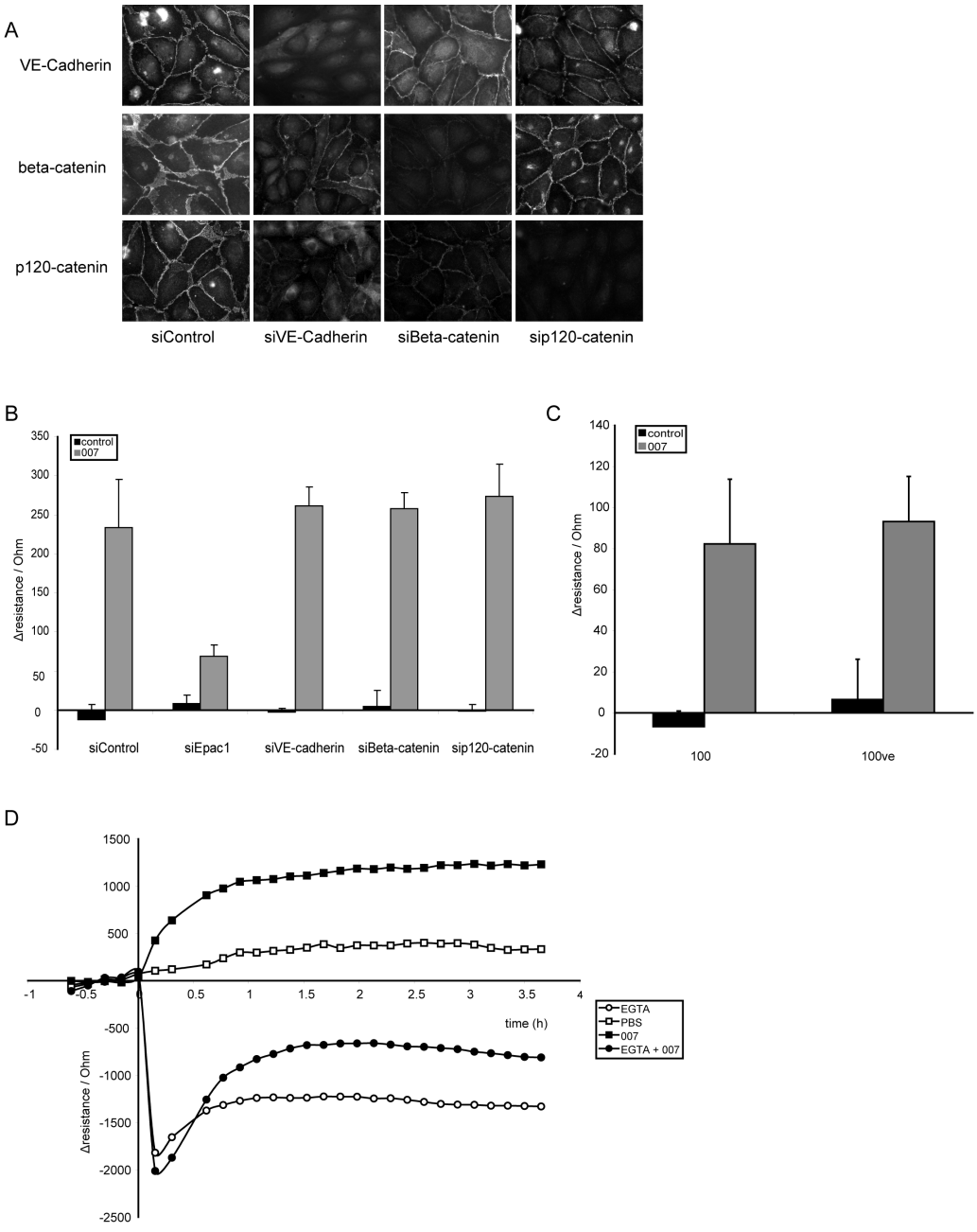

Fig. 4. VE-cadherin is not required for Epac1 induced TER.

(A) Transfected HUVEC were immunostained as indicated. (B) Transfected HUVEC were seeded after 48h onto electrodes and TER of monolayers on fibronectin coated gold electrodes was measured. Cells were treated with PBS (black) or 100μM 007 (grey) for 1 hour and averages +/- SEM are shown. (C) 100 and 100VE cells were seeded onto electrodes and TER of monolayers on fibronectin coated gold electrodes was measured. Cells were treated with PBS (black) or 100μM 007 (grey) for 1 hour and averages +/- SEM are shown. (C) TER of HUVEC monolayers on fibronectin coated gold electrodes was measured. Cells were treated with PBS (open) or 100μM 007 (closed) in the presence or absence of 2mM EGTA.

to the unphosphorylated form of VASP and does so in confluent cells. Under sparse conditions, VASP is localised to focal adhesions and phosphorylated at the predominant PKA site (S157) and that when the culture is denser, VASP is localised at the cell-cell contacts in it unphosphorylated form (Benz et al., 2008). This is in concordance with our observation that 007 induces the translocation to the cell-cell contacts without increasing phosphorylation. It will be interesting to see whether SpectinIIα is required for Epac1-induced translocation of VASP and TER.

Previously, we have shown that VE-cadherin is required for the 007-induced permeability decrease using VE-cadherin knock-out cells (Kooistra et al., 2005). Here we show that trans-endothelial electrical resistance regulation by Epac1 is independent of VE-cadherin. This raises questions regarding the comparability of these assays. In the permeability assays the passage of fluorescent dextran over the endothelial monolayer is measured, a process driven by diffusion. To prevent the passage of such a large molecule (40kD) through the cell-cell contacts of endothelial cells it is plausible that this requires a physical barrier provided by the adhesion molecules present in the cell-cell junctions. In TER, the current of electrons is measured. The resistance and is driven by the voltage over the system and depends on the insulating properties of the cell-cell contacts. Although an increase in cell adhesion molecules will contribute to this resistance, closing the space between endothelial cells by protruding membranes could be sufficient to increase the resistance in the absence of adhesion-molecules. Therefore it would be interesting to measure the effects of 007 on cells void of all adhesion molecules. Interestingly, in epithelial cells resistance is considered to be dependent on the tight junctional compartment (Capaldo and Macara, 2007). However, the presence of tight junctions in endothelial cells is dependent on the localisation in the vascular tree; in the blood-brain barrier, tight junctions are well organised, whereas in capillary veins, the tight junctions are absent. Although HUVEC are widely used, the role of tight junctions in HUVEC is hardly investigated. Nevertheless, expression of occludin at the cell-cell contacts in HUVEC is reported (Burns et al., 2000). However, we could not detect occludin at the cell-cell contacts in HUVEC (data not shown).

Increased leakage of the endothelial barrier is involved in various pathological conditions, such as oedema and inflammation. We propose that enhancement of the endothelial barrier by Epac1 is mediated by the RIAM/LPD/Ena/VASP complex. We are currently investigating whether Epac1 activation induces protrusion dependent on RIAM and LPD and whether this is involved in cAMP-induced TER.

References

Benz, P. M., Blume, C., Moebius, J., Oschatz, C., Schuh, K., Sickmann, A., Walter, U., Feller, S. M. and Renne, T. (2008). Cytoskeleton assembly at endothelial cell-cell contacts is regulated by alphaII-spectrin-VASP complexes. J Cell Biol 180, 205-19.

Breviario, F., Caveda, L., Corada, M., Martin-Padura, I., Navarro, P., Golay, J., Introna, M., Gulino, D., Lampugnani, M. G. and Dejana, E. (1995). Functional properties of human vascular endothelial cadherin (7B4/cadherin-5), an endothelium-specific cadherin. Arterioscler. Thromb. Vasc. Biol. 15, 1229-1239.

Burns, A. R., Bowden, R. A., MacDonell, S. D., Walker, D. C., Odebunmi, T. O., Donnachie, E. M., Simon, S. I., Entman, M. L. and Smith, C. W. (2000). Analysis of tight junctions during neutrophil transendothelial migration. J Cell Sci 113 (Pt 1), 45-57.

Butt, E., Abel, K., Krieger, M., Palm, D., Hoppe, V., Hoppe, J. and Walter, U. (1994). cAMP- and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-stimulated phosphoprotein (VASP) in vitro and in intact human platelets. J Biol Chem 269, 14509-17.

Capaldo, C. T. and Macara, I. G. (2007). Depletion of E-cadherin disrupts establishment but not maintenance of cell junctions in Madin-Darby canine kidney epithelial cells. Mol Biol Cell 18, 189-200.

Comerford, K. M., Lawrence, D. W., Synnestvedt, K., Levi, B. P. and Colgan, S. P. (2002). Role of vasodilator-stimulated phosphoprotein in PKA-induced changes in endothelial junctional permeability. Faseb J 16, 583-5.

Cullere, X., Shaw, S. K., Andersson, L., Hirahashi, J., Luscinskas, F. W. and Mayadas, T. N. (2005). Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. Blood 105, 1950-1955.

Enserink, J. M., Christensen, A. E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H. G., Doskeland, S. O., Blank, J. L. and Bos, J. L. (2002). A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat. Cell. Biol. 4, 901-906.

Fukuhara, S., Sakurai, A., Sano, H., Yamagishi, A., Somekawa, S., Takakura, N., Saito, Y., Kangawa, K. and Mochizuki, N. (2005). Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling. pathway. Mol. Cell. Biol. 25, 136-146.

Furman, C., Sieminski, A. L., Kwiatkowski, A. V., Rubinson, D. A., Vasile, E., Bronson, R. T., Fassler, R. and Gertler, F. B. (2007). Ena/VASP is required for endothelial barrier function in vivo. J Cell Biol 179, 761-75.

Goldfinger, L. E., Ptak, C., Jeffery, E. D., Shabanowitz, J., Han, J., Haling, J. R., Sherman, N. E., Fox, J. W., Hunt, D. F. and Ginsberg, M. H. (2007). An experimentally derived database of candidate Ras-interacting proteins. J Proteome Res 6, 1806-11.

Hauser, W., Knobeloch, K. P., Eigenthaler, M., Gambaryan, S., Krenn, V., Geiger, J., Glazova, M., Rohde, E., Horak, I., Walter, U. et al. (1999). Megakaryocyte hyperplasia and enhanced agonist-induced platelet activation in vasodilator-stimulated phosphoprotein knockout mice. Proc Natl Acad Sci U S A 96, 8120-5.

Honegger, K. J., Capuano, P., Winter, C., Bacic, D., Stange, G., Wagner, C. A., Biber, J., Murer, H. and Hernando, N. (2006). Regulation of sodium-proton exchanger isoform 3 (NHE3) by PKA and exchange protein directly activated by cAMP (EPAC). Proc Natl Acad Sci U S A

103, 803-8.

Jaffe, E. A., Nachman, R. L., Becker, C. G. and Minick, C. R. (1973). Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J. Clin. Invest. 52, 2745-2756.

Kooistra, M. R., Corada, M., Dejana, E. and Bos, J. L. (2005). Epac1 regulates integrity of endothelial cell junctions through VE-cadherin. FEBS Lett 579, 4966-72.

Krause, M., Dent, E. W., Bear, J. E., Loureiro, J. J. and Gertler, F. B. (2003). Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration. Annu Rev Cell Dev Biol 19, 541-64.

Krause, M., Leslie, J. D., Stewart, M., Lafuente, E. M., Valderrama, F., Jagannathan, R., Strasser, G. A., Rubinson, D. A., Liu, H., Way, M. et al. (2004). Lamellipodin, an Ena/VASP ligand, is implicated in the regulation of lamellipodial dynamics. Dev Cell 7, 571-83.

Lafuente, E. M., van Puijenbroek, A. A., Krause, M., Carman, C. V., Freeman, G. J., Berezovskaya, A., Constantine, E., Springer, T. A., Gertler, F. B. and Boussiotis, V. A. (2004). RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion. Dev. Cell 7, 585-595.

Lampugnani, M. G., Resnati, M., Raiteri, M., Pigott, R., Pisacane, A., Houen, G., Ruco, L. P. and Dejana, E. (1992). A novel endothelial-specific membrane protein is a marker of cell-cell contacts. J. Cell. Biol. 118, 1511-1522.

Lorenowicz, M. J., Fernandez-Borja, M., Kooistra, M. R. H., Bos, J. L. and Hordijk, P. L. (2008). PKA and Epac1 regulate endothelial integrity and cell migration through independent pathways. Submitted, 71-99.

Massberg, S., Gruner, S., Konrad, I., Garcia Arguinzonis, M. I., Eigenthaler, M., Hemler, K., Kersting, J., Schulz, C., Muller, I., Besta, F. et al. (2004). Enhanced in vivo platelet adhesion in vasodilator-stimulated phosphoprotein (VASP)-deficient mice. Blood 103, 136-42.

Price, L. S., Hajdo-Milasinovic, A., Zhao, J., Zwartkruis, F. J., Collard, J. G. and Bos, J. L. (2004). Rap1 regulates E-cadherin-mediated cell-cell adhesion. J. Biol. Chem. 279, 35127-35132.

Rangarajan, S., Enserink, J. M., Kuiperij, H. B., de Rooij, J., Price, L. S., Schwede, F. and Bos, J. L. (2003). Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the beta 2-adrenergic receptor. J Cell Biol 160, 487-93.

Rodriguez-Viciana, P., Sabatier, C. and McCormick, F. (2004). Signaling specificity by Ras family GTPases is determined by the full spectrum of effectors they regulate. Mol Cell Biol 24, 4943-54.

Rosenberger, P., Khoury, J., Kong, T., Weissmuller, T., Robinson, A. M. and Colgan, S. P. (2007). Identification of vasodilator-stimulated phosphoprotein (VASP) as an HIF-regulated tissue permeability factor during hypoxia. Faseb J 21, 2613-21.

Scott, J. A., Shewan, A. M., den Elzen, N. R., Loureiro, J. J., Gertler, F. B. and Yap, A. S. (2006). Ena/VASP proteins can regulate distinct modes of actin organization at cadherin-adhesive contacts. Mol Biol Cell 17, 1085-95.

Sehrawat, S., Cullere, X., Patel, S., Italiano, J., Jr. and Mayadas, T. N. (2008). Role of Epac1, an Exchange Factor for Rap GTPases, in Endothelial Microtubule Dynamics and Barrier Function. Mol Biol Cell.

Shibasaki, T., Takahashi, H., Miki, T., Sunaga, Y., Matsumura, K., Yamanaka, M., Zhang, C., Tamamoto, A., Satoh, T., Miyazaki, J. et al. (2007). Essential role of Epac2/Rap1 signaling in regulation of insulin granule dynamics by cAMP. Proc Natl Acad Sci U S A 104, 19333-8.

Tiruppathi, C., Malik, A. B., Del Vecchio, P. J., Keese, C. R. and Giaever, I. (1992). Electrical method for detection of endothelial cell shape change in real time: assessment of endothelial barrier function. Proc Natl Acad Sci U S A 89, 7919-23.

van Triest, M. and Bos, J. L. (2004). Pull-down assays for guanoside 5'-triphosphate-bound Ras-like guanosine 5'-triphosphatases. Methods

Chapter 8

Discussion

In this thesis I describe the role of the Rap1 signaling pathway in the regulation of adherens junctions. More specifically, we have analyzed the role of two distinct guanine-nucleotide exchange factors for Rap1, Epac1 and PDZ-GEF2, in endothelial cells and in the lung carcinoma cell A549. In addition we have tried to identify proteins that act in concert with these exchange factors to regulate endothelial junctions. At the start of the research described here, a role for Rap1 in junction formation had just been found in Drosophila melanogaster, where rap1-mutant cell clones in the wing of Drosophila melanogaster have a different shape and disperse into the surrounding normal tissue. The predominant cell-cell adhesion molecule in these cells, Drosophila E-cadherin (DE-cadherin), is normally distributed along the basolateral axis, but is not uniformly distributed around the cell periphery in rap1-mutant cells (Knox and Brown, 2002). In addition, Price at al, found that Rap1 regulates E-cadherin-mediated cell junction formation in mammalian cells (Price et al., 2004). At this point, both the regulation of Rap1 and the mechanisms employed by Rap1 to regulate junctions were still elusive.

Activation of Rap during junction formation

Activation of Rap GTPases is induced by a variety of stimuli through guanine-nucleotide exchange factors (GEFs) (reviewed in (Bos et al., 2007)). What GEFs are used to regulate Rap1-dependent junction formation? In order to answer this question we started to investigate the role of the exchange-protein directly activated by cAMP (Epac) in endothelial cell-cell junctions for various reasons. First, we can easily activate Epac1 specifically with the cAMP-analog 8-pCPT-2OMe-cAMP (007). Furthermore, Epac1 is expressed in endothelial cells, whereas we could detect no expression in epithelial cells. Finally, endothelial cell-cell junctions are more dependent on cadherins then epithelial cell junctions, where depletion of E-cadherin has no effect once tight junctions are formed (Capaldo and Macara, 2007). We and others found that Epac1 is involved in endothelial cell junction formation (Chapter 3). Treatment of endothelial cells with the Epac-selective cAMP analog 8-pCPT-2O-Me-cAMP (007) results in increased junctional integrity and increased localization of VE-cadherin at cell-cell contacts (Cullere et al., 2005; Fukuhara et al., 2005; Wittchen et al., 2005). Similarly, activation of Epac1 results in increased VE-cadherin dependent adhesion (Fukuhara et al., 2005). Although Epac1 enhances the formation of cell-cell junctions, siRNA for Epac1 has no effect on the localization of VE-cadherin (Chapter 3). This suggests that Epac1 activation enhances the junctions, but is not required for the formation or maintenance of junctions. However, trans-endothelial electrical resistance is reduced upon depletion for Epac1 (Lorenowicz et al., 2008), compatible with a role for Epac1/Rap in the regulation of the actin cytoskeleton as discussed below.

Since complete inactivation of Rap by Rap1GAP prevents cells to form complete junctions both in epithelial and endothelial cells (Hogan et al., 2004; Price et al., 2004; Wittchen et al., 2005) we expected that other GEFs are involved in the regulation of Rap1 for the formation of cell-cell junctions. Since PDZ-GEFs were suggested to localize to cell-cell contacts (Mino et al., 2000), we studied the role of PDZ-GEFs in this process. Where depletion of PDZ-GEF1 had no obvious effect on cell-cell junctions in A549 cells, knock-down of PDZ-GEF2 results in the persistence of adhesion zippers consisting of membrane extensions decorated with (V)E-cadherin both in endothelial and epithelial cells (Chapter 4). This suggests either a defect in the linkage of (V)E-cadherin to the actin cytoskeleton or a failure to generate tension at cell-cell contacts, as actomyosin contractility and adequate cytoskeletal tension are required for proper AJ assembly (Miyake et al., 2006; Shewan et al., 2005). Also PDZ-GEF2 depleted cells show decreased expression of E-cadherin. Intriguingly, in PDZ-GEF2 knock-out mice no junctional aberrations have been reported. Loss of PDZ-GEF2 in mice results in decreased integrin adhesion of B-cell by TNF-α (Yoshikawa et al., 2007).

Disruption of cell-cell junctions by depletion of calcium results in activation of Rap1 (Balzac et al., 2005). We could show that this occurs in a PDZ-GEF2 dependent manner (Chapter 4). Together with the role of PDZ-GEF2 in junction maturation, this suggests that PDZ-GEF2/Rap1 is activated upon junctional disruption to counteract the breakdown of junctions. How PDZ-GEF2 is activated is still unknown. Although PDZ-GEF2 has a cyclic-nucleotide binding domain, thus far no second messenger was found to interact with that domain. During junctional re-arrangements the cell undergoes quite some changes at the cell-cell contacts, such as membrane extension and actomyosin contractions. Therefore, it is possible that PDZ-GEF2 is activated by mechanical stress during junction rearrangements, similar to the

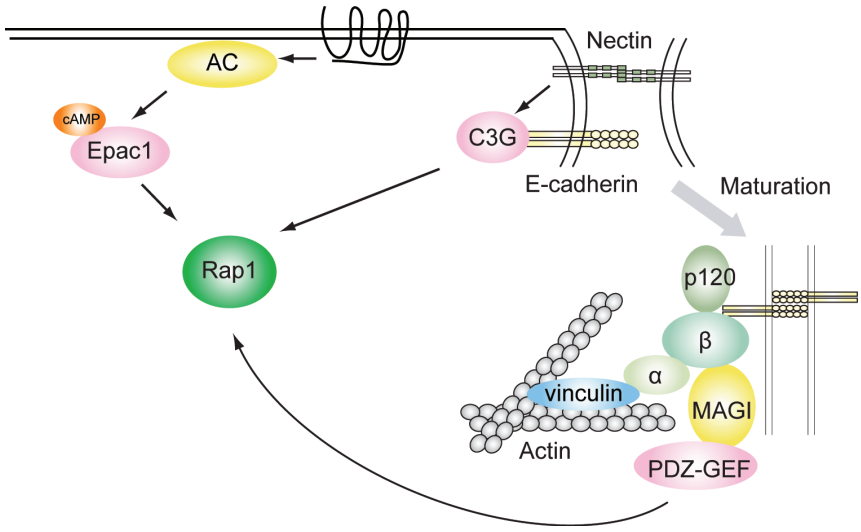

Fig. 1. Activation of Rap1 during junction formation.

mechanisms of Rap1 activation in focal adhesions, where a mechanical extension of p130Cas results in activation of the RapGEF C3G and subsequently of Rap1(Sawada et al., 2006). Furthermore, transient activation of Rap1 during junction formation at the cell-cell contacts is dependent on MAGI-1 in endothelial cells (Sakurai et al., 2006). PDZGEF-1 binds to MAGI-1, whereas PDZ-GEF2 interacts with the homolog MAGI-3. Perhaps binding of MAGI to the C-terminus is involved in the activation of PDZ-GEF2.

Besides a role for Epac1 and PDZ-GEF2, also C3G has been implicated in the regulation of junctions by Rap1. In early experiments implicating Rap1 in junction formation, the RapGEF C3G was shown to interact with E-cadherin directly (Hogan et al., 2004). Furthermore, C3G transiently binds to E-cadherin during formation of cell-cell junctions and it was shown that this interaction was mutually exclusive with beta-catenin but not with p120 catenin binding to E-cadherin. Also, transfection of a fragment of C3G that binds to E-cadherin directly, prevents cell-cell junction formation (Hogan et al., 2004). This suggests that C3G is involved in the initial steps of junction formation. Although studies on c3g null mice focused on the role of C3G in migration and integrin regulation (Ohba et al., 2001), the investigation of a C3G mutant in vivo indicates that C3G is not required for endothelial cell-cell junctions, since PECAM localization is unaltered. However, these mice suffer from angiogenesis defects, probably due to aberrant migration of supporting mesenchymal cells (Voss et al., 2003).

Other Rap-GEFs have not been studied in the regulation of cell-cell junctions thus far. However, like C3G various GEFs have been ablated in mice. For instance, mice lacking PDZ-GEF1 expression are defective in early angiogenesis (Wei et al., 2007) and ablation of another Rap1-GEF, CalDAGEF-I results in defects in integrin regulation in leukocytes and platelets (Bergmeier et al., 2007). Although ablation of these GEFs in mice gives no junctional phenotypes, we cannot exclude a role for these GEFs in junction formation. Especially since the complexity of an organism also provides compensatory mechanisms during embryogenesis. Therefore it will be interesting to further address the role of these GEFs in Rap-mediated cell-cell junctions using siRNA approaches.

Besides different means to activate Rap, divergence also occurs through the various isoforms of the Rap1-family. In mammalian cells, two Rap1 genes and three Rap2 genes have been identified thus far. In Chapter 4 we could show that siRNA for Rap1a in epithelial cells results in a similar junctional structure as PDZ-GEF2 knock-down, whereas Rap1b knock-down results in normal junctions with reduced levels of E-cadherin (Chapter 4). Complete inhibition of Rap1 activity results in reduced presence of cadherin at the cell-cell contacts (Hogan et al., 2004; Wittchen et al., 2005), whereas knock-down of single Rap-isoforms

have a more delicate effect. This suggests that there is either redundancy of Rap-isoforms or that they function in parallel pathways, all contributing to cell-cell junction formation via different mechanisms. The role of the various Rap2 isoforms in junction formation is currently under investigation and will give us more information on the role of the Rap-signaling network in cell-cell junction formation.

Altogether, three structurally different RapGEFs have been implicated in different roles during cell-cell junction formation thus far. First, when cell-cell junctions are formed, initial cell-cell contact results in the activation of Rap by various means, including the engagement of nectins and the interaction of C3G with E-cadherin. This activation of Rap1 is required for the maturation of cell-cell junctions. Further maturation is then mediated by PDZGEF2, which interacts with MAGI-3, or by extracellular stimuli that activate Rap1 through Epac1.

Rap1 regulates junctions through multiple effector molecules

As discussed above, activation of Epac1 in endothelial cells results in increased VE-cadherin at the cell-cell contacts (Cullere et al., 2005; Wittchen et al., 2005). In the second part of this thesis we investigated what mechanisms Rap1 employs to regulate the increased stability of cell-cell junctions by Epac1, since we can activate Epac1 directly through 007.

There are various ways to measure Rap1-dependent junctional regulation, especially in endothelial cells. Is there a common theme for Rap-induced junction regulation in these various assay? For instance, Epac1 activation results in increased VE-cadherin at the cell-cell contacts, whereas cell surface accumulation of cadherins measured by flow cytometry is not increased in cells with activated Epac1 (Lorenowicz et al., 2008). This shows that Epac1/Rap activation is not regulating the vesicular transport of cadherins to the plasma membrane. Furthermore, adhesion of cells to Fc-VE-cadherin coated surfaces is increased upon stimulation with 007 (Fukuhara et al., 2005) suggesting that Epac1 induces increased affinity of VE-cadherin. However, these assays are not performed under limiting conditions, resulting in activation of cell spreading by VE-cadherin dependent signaling pathways. Since Rap activation enhances cell-spreading (Arthur et al., 2004), the increase in adhesion onto Fc-VE-cadherin might be due to increased cell spreading. Furthermore, we show in Chapter 3 that VE-cadherin is required for the reduction in endothelial permeability by 007, but the increase in trans-endothelial electrical resistance by Epac1 is not dependent on VE-cadherin (Chapter 7). This discrepancy is extensively discussed in Chapter 7, but indicates that Epac1/Rap can induce TER in the absence of VE-cadherin. Altogether, these results suggest that the regulation of cell-cell junctions by Epac1/Rap is, at least not exclusively, dependent on direct regulation of VE-cadherin. Interestingly, all assays described above have in common that the disruption of the actin cytoskeleton with CytochalasinD inhibits the effects of 007 (Cullere et al., 2005; Fukuhara et al., 2005)(Chapter 7). Is Epac1/Rap1 regulating the cell-cell contacts then by regulating the actin cytoskeleton? One clear observation is that Epac1 activation results in reorganization of the actin cytoskeleton in endothelial cells independently of VE-cadherin (Chapter 3). Although not as striking as in endothelial cells, also in epithelial cells we have indications that Rap regulates the junctions through the actin cytoskeleton. Knock-down of Rap1a results in adhesion zippers, but gives no decrease in E-cadherin levels (Chapter 4). On the other hand, knock-down of Rap1b in epithelial cells does decrease the amount of E-cadherin at the surface (Chapter 4) and complete inhibition of Rap with RapGAP clearly decrease the amount of cadherin at cell-cell contacts in both epithelial and endothelial cells. Altogether, the regulation of cell-cell junctions by Rap1 includes regulation of the actin cytoskeleton, although direct regulation of junctional proteins by Rap is probably also involved. How does Rap regulate the actin cytosketon? Rap does not bind actin directly. Therefore, we will discuss three Rap-effectors and their involvement in the regulation of the actin cytoskeleton and cell-cell junction formation.

AF-6

AF-6 is genetically linked to Rap1 in dorsal closure in D. melanogaster (Boettner et al., 2003). AF-6 localizes at cell-cell contacts where it interacts with various adhesion molecules. Although we could show that cells depleted from AF-6 have decreased endothelial electrical resistance, we found that AF-6 is dispensable for Epac1-mediated TER (Chapter 5). Also in epithelial cells, AF-6 is localized at cell-cell junctions, but siRNA for AF-6 results in loss of tight junctions (Sato et al., 2006). How AF-6 regulates the junctions is still poorly understood, although it is suggested that AF-6 prevent E-cadherin endocytosis through recruitment of p120catenin. Since we observe clear effects on the actin cytoskeleton in

endothelial cells and AF-6 can bind to actin directly (Lorger and Moelling, 2006), it might be that depletion of AF-6 results in release of actin filaments. Recently, it was shown that AF-6 binds to Src-kinase directly, which results in decreased kinase activity of Src. In contrast, siRNA for AF-6 results in phosphorylation of increased number of substrates (Radziwill et al., 2007). Since Src is required for both the formation and breakdown of cell-cell junctions, dependent on its activity (McLachlan et al., 2007), it might be that AF-6 is required in endothelial cells to control the activity of Src in order to balance between junction formation and breakdown. Interestingly, a role for AF-6 in integrin-mediated adhesion is also proposed. For instance, over-expression of AF-6 in T-cells results in decreased adhesion, whereas siRNA for AF-6 results in an opposite effect (Zhang et al., 2005). Also, knock-down of AF-6 in epithelial cells results in increased directional migration (Lorger and Moelling, 2006) Intriguingly, in endothelial cells, loss of AF-6 reduces the amount of stress fibres arising from focal adhesions in singles cells (Chapter 5). Therefore it is interesting to investigate whether AF-6 is involved in the interplay between cell-matrix and cell-cell adhesion molecules. Cells from C3G-mutant mice also show increased migration (Voss et al., 2003) and previous studies suggest that C3G and AF-6 are involved in the same pathway regulating cell-cell junction formation (Fukuyama et al., 2005; Hoshino et al., 2005). Furthermore, in D. melanogaster the AF-6 homolog canoe is regulated by PDZ-GEF and Rap. Although we did not find a role for AF-6 in Epac1-mediated junction formation, it might be that AF-6 is involved in Rap-dependent processes induced by PDZ-GEF or C3G.

RIAM/Lamellipodin

In Chapter 7 we could show that Lamellipodin is

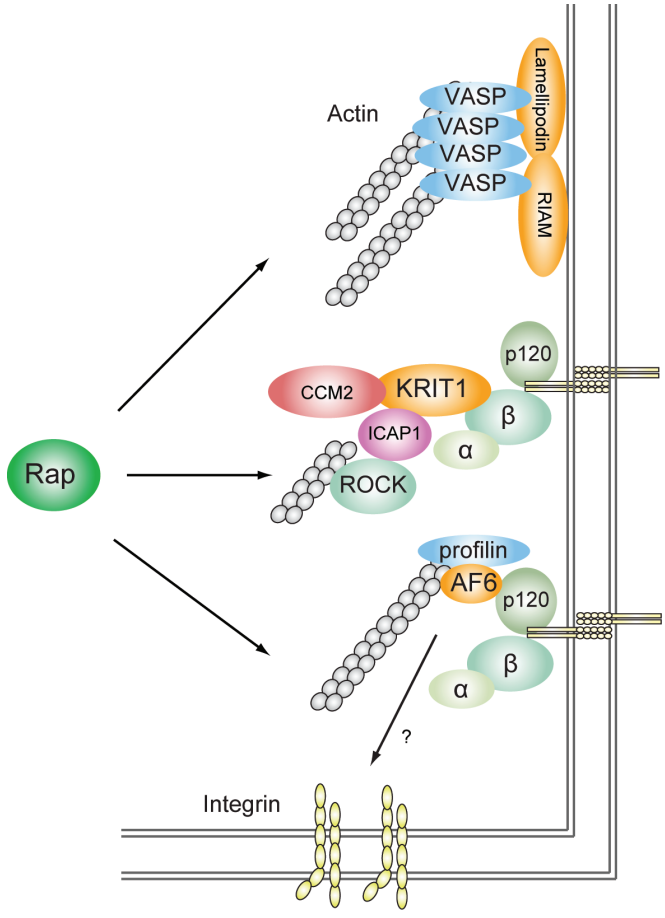

Fig. 2 Rap-effectors in junction formation.

required for Epac1-induced TER. Lamellipodin binds to the Ena/VASP family of proteins, which are involved in actin remodeling through decapping of branched actin filaments. Artificial localization of Ena/VASP at the plasma membrane results in elongated actin, whereas mislocalization of Ena/VASP to the mitochondria results in more branched actin filaments at the plasma membrane (reviewed in (Krause et al., 2003). Lamellipodin localizes Ena/VASP protein to the lamellipodia and knock-down of Lamellipodin reduces the protrusive activity of cells (Krause et al., 2004). The evidence that Lamellipodin interacts directly with Rap1 is lacking. However, we could show that Lamellipodin and RIAM interact. The interaction between RIAM and Lamellipodin could be mediated by Ena/VASP, especially since Ena/VASP forms tetramers and both Lamellipodin and RIAM have multiple Ena/VASP binding sites (Bachmann et al., 1999; Jenzora et al., 2005; Krause et al., 2004). Thus far RIAM is only implicated in Rap-mediated integrin adhesion (Han et al., 2006; Lafuente et al., 2004) and it is suggested that RIAM regulates integrins independent of Ena/VASP, but through the activation of Talin (Han et al., 2006). The involvement of Lamellipodin in Epac1-induced TER, suggests that RIAM uses different mechanisms in cell-cell junction enhancement.

The role of Lamellipodin in lamellipodia formation suggests a role for actin-based protrusions in Rap1-induced barrier function. In epithelial cells, interactions between opposing lamellipodia initiate E-cadherin clustering while a circumferential actin cable surrounds the cells. As cell-cell adhesion proceeds, E-cadherin molecules cluster into so called puncta within the cell-cell contact interface and associate with actin filaments. (Adams and Nelson, 1998; Perez et al., 2008; Yamada and Nelson, 2007). The requirement for Lamellipodin suggests that 007 induces the formation of lamellipodia, even within a monolayer of cells, and that this increased protrusive activity then results in increased cell-cell contacts and cell-cell junction formation. Recently, the small GTPase Rac1 was implicated in cAMP-induced barrier function together with its GEFs Vav2 and Tiam (Birukova et al., 2007). However, these studies do not discriminate between Epac1 and PKA-dependent signaling. Results from our screen suggest that knock-down of Rac1 inhibits 007-induced TER (Chapter 6). Previously, it was shown that the Rac-GEFs Vav2 and Tiam bind to Rap1 directly and that Rap1 is required for the localization of Vav2 and Tiam to the cell periphery to induce cell spreading (Arthur et al., 2004). In line with our finding for the requirement of Lamellipodin in Epac1-induced TER, it should be noted that lamellipodia formation classically involves the activation of Rac1 (Nobes and Hall, 1995). Overall, it will be interesting to investigate whether 007 induces protrusions or increases their growth speed and whether this is dependent on Lamellipodin possibly through RIAM. The observation that the actin cytoskeleton remodeling by Epac1 is still induced even in the absence of Lamellipodin would suggest that beside the increased protrusive activity (Chapter 7), Epac1 also enhances the cell-cell junctions through a different pathway. Although we only investigated the role of Lamellipodin and Ena/VASP in Epac1-dependent junctional enhancement in endothelial cells, it is possible that PDZ-GEF2 uses a similar mechanism. It will be interesting to investigate whether active Ena/VASP can rescue the PDZ-GEF2/Rap1a phenotype in epithelial cells.

KRIT1

Recently, the Rap1 binding protein KRIT1 was shown to be involved in Epac1/Rap-induced permeability. KRIT1 (CCM1) is mutated in familial cerebral cavernous malformation. Patients bearing this mutation suffer from neurovascular lesions such as enlarged vascular channels. Glading and colleagues show that KRIT1 is localized to the cell-cell contacts and is internalized upon thrombin stimulation. Knock-down of KRIT in endothelial cells, results in increased permeability and abrogates the effect of 007 on thrombin-induced permeability. Furthermore, over expression of KRIT1 also prevented thrombin-induced permeability and rescues Rap1GAP-induced permeability (Glading et al., 2007). It is suggested that binding of Rap1 to KRIT1 results in binding to β-catenin and AF-6 and thereby stabilizes the junctions. Interestingly, our data indicate that Epac1-induced TER does not involve β-catenin or AF-6 (Chapters 5 and 7). In our screen we found that ICAP1α and CCM2 are required for Epac1-induced TER (Chapter 6). Both ICAP1α and CCM2 bind to KRIT1 directly (Zawistowski et al., 2002; Zhang et al., 2007). Furthermore, ICAP1α binds to the Rho-effector ROCK (Stroeken et al., 2006). ROCK is, amongst others, involved in the regulation of actomyosin contractility (Vicente-Manzanares et al., 2005). Recently it was shown that although ROCK is required for thrombin-induced permeability, inhibition of ROCK results in decreased localization of VE-cadherin, indicating that ROCK is required for maintenance of cell-cell junctions

(van Nieuw Amerongen et al., 2007). Perhaps Rap1 signaling through the KRIT1/ICAP1/CCM2 complex locally pulls the switch on ROCK to enhance the cell-cell contacts.

It might be that the various effector molecules discussed above comprise one signaling cascade, but it is more likely that Rap enhances junctions through multiple parallel effector pathways. It will be interesting to determine the combinatory effects of these pathways on cell-cell junctions. This will provide more insight into their relative importance in the various steps of junction regulation by Rap.

In conclusion, Rap1 activation results in enrichment of cadherins in the cell-cell junction through a combination of various signals. This variety of signals is not only generated by divergence through the various effector molecules, but also through activation of different GEFs at different stages of junction formation. It will be interesting to investigate whether a certain GEF/Rap combination regulates the various effectors and what determines the specificity.

References

Adams, C. L. and Nelson, W. J. (1998). Cytomechanics of cadherin-mediated cell-cell adhesion. Curr Opin Cell Biol 10, 572-7.

Arthur, W. T., Quilliam, L. A. and Cooper, J. A. (2004). Rap1 promotes cell spreading by localizing Rac guanine nucleotide exchange factors. J. Cell. Biol. 167, 111-122.

Bachmann, C., Fischer, L., Walter, U. and Reinhard, M. (1999). The EVH2 domain of the vasodilator-stimulated phosphoprotein mediates tetramerization, F-actin binding, and actin bundle formation. J Biol Chem 274, 23549-57.

Balzac, F., Avolio, M., Degani, S., Kaverina, I., Torti, M., Silengo, L., Small, J. V. and Retta, S. F. (2005). E-cadherin endocytosis regulates the activity of Rap1: a traffic light GTPase at the crossroads between cadherin and integrin function. J Cell Sci 118, 4765-83.

Bergmeier, W., Goerge, T., Wang, H. W., Crittenden, J. R., Baldwin, A. C., Cifuni, S. M., Housman, D. E., Graybiel, A. M. and Wagner, D. D. (2007). Mice lacking the signaling molecule CalDAG-GEFI represent a model for leukocyte adhesion deficiency type III. J Clin Invest 117, 1699-707.

Birukova, A. A., Zagranichnaya, T., Fu, P., Alekseeva, E., Chen, W., Jacobson, J. R. and Birukov, K. G. (2007). Prostaglandins PGE(2) and PGI(2) promote endothelial barrier enhancement via PKA- and Epac1/Rap1-dependent Rac activation. Exp Cell Res 313, 2504-20.

Boettner, B., Harjes, P., Ishimaru, S., Heke, M., Fan, H. Q., Qin, Y., Van Aelst, L. and Gaul, U. (2003). The AF-6 homolog canoe acts as a Rap1 effector during dorsal closure of the Drosophila embryo. Genetics 165, 159-69.

Bos, J. L., Rehmann, H. and Wittinghofer, A. (2007). GEFs and GAPs: critical elements in the control of small G proteins. Cell 129, 865-77.

Capaldo, C. T. and Macara, I. G. (2007). Depletion of E-cadherin disrupts establishment but not maintenance of cell junctions in Madin-Darby canine kidney epithelial cells. Mol Biol Cell 18, 189-200.

Cullere, X., Shaw, S. K., Andersson, L., Hirahashi, J., Luscinskas, F. W. and Mayadas, T. N. (2005). Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. Blood 105, 1950-1955.

Fukuhara, S., Sakurai, A., Sano, H., Yamagishi, A., Somekawa, S., Takakura, N., Saito, Y., Kangawa, K. and Mochizuki, N. (2005). Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling. pathway. Mol. Cell. Biol. 25, 136-146.

Fukuyama, T., Ogita, H., Kawakatsu, T., Fukuhara, T., Yamada, T., Sato, T., Shimizu, K., Nakamura, T., Matsuda, M. and Takai, Y. (2005). Involvement of the c-Src-Crk-C3G-Rap1 signaling in the nectin-induced activation of Cdc42 and formation of adherens junctions. J. Biol. Chem. 280, 815-825.

Glading, A., Han, J., Stockton, R. A. and Ginsberg, M. H. (2007). KRIT-1/CCM1 is a Rap1 effector that regulates endothelial cell cell junctions. J Cell Biol 179, 247-54.

Han, J., Lim, C. J., Watanabe, N., Soriani, A., Ratnikov, B., Calderwood, D. A., Puzon-McLaughlin, W., Lafuente, E. M., Boussiotis, V. A., Shattil, S. J. et al. (2006). Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. Curr Biol 16, 1796-806.

Hogan, C., Serpente, N., Cogram, P., Hosking, C. R., Bialucha, C. U., Feller, S. M., Braga, V. M., Birchmeier, W. and Fujita, Y. (2004). Rap1 regulates the formation of E-cadherin-based cell-cell contacts. Mol. Cell. Biol. 24, 6690-6700.

Hoshino, T., Sakisaka, T., Baba, T., Yamada, T., Kimura, T. and Takai, Y. (2005). Regulation of E-cadherin endocytosis by nectin through afadin, Rap1, and p120ctn. J. Biol. Chem. 280, 24095-24103.

Jenzora, A., Behrendt, B., Small, J. V., Wehland, J. and Stradal, T. E. (2005). PREL1 provides a link from Ras signalling to the actin cytoskeleton via Ena/VASP proteins. FEBS Lett 579, 455-63.

Knox, A. L. and Brown, N. H. (2002). Rap1 GTPase regulation of adherens junction positioning and cell adhesion. Science 295, 1285-1288.

Krause, M., Dent, E. W., Bear, J. E., Loureiro, J. J. and Gertler, F. B. (2003). Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration. Annu Rev Cell Dev Biol 19, 541-64.

Krause, M., Leslie, J. D., Stewart, M., Lafuente, E. M., Valderrama, F., Jagannathan, R., Strasser, G. A., Rubinson, D. A., Liu, H., Way, M. et al. (2004). Lamellipodin, an Ena/VASP ligand, is implicated in the regulation of lamellipodial dynamics. Dev Cell 7, 571-83.

Lafuente, E. M., van Puijenbroek, A. A., Krause, M., Carman, C. V., Freeman, G. J., Berezovskaya, A., Constantine, E., Springer, T. A., Gertler, F. B. and Boussiotis, V. A. (2004). RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion. Dev. Cell 7, 585-595.

Lorenowicz, M. J., Fernandez-Borja, M., Kooistra, M. R. H., Bos, J. L. and Hordijk, P. L. (2008). PKA and Epac1 regulate endothelial integrity and cell migration through independent pathways. Submitted, 71-99.

Lorger, M. and Moelling, K. (2006). Regulation of epithelial wound closure and intercellular adhesion by interaction of AF6 with actin cytoskeleton. J Cell Sci 119, 3385-98.

McLachlan, R. W., Kraemer, A., Helwani, F. M., Kovacs, E. M. and Yap, A. S. (2007). E-cadherin adhesion activates c-Src signaling at cell-cell contacts. Mol Biol Cell 18, 3214-23.

Mino, A., Ohtsuka, T., Inoue, E. and Takai, Y. (2000). Membrane-associated guanylate kinase with inverted orientation (MAGI)-1/brain angiogenesis inhibitor 1-associated protein (BAP1) as a scaffolding molecule for Rap small G protein GDP/GTP exchange protein at tight junctions. Genes Cells 5, 1009-16.

Miyake, Y., Inoue, N., Nishimura, K., Kinoshita, N., Hosoya, H. and Yonemura, S. (2006). Actomyosin tension is required for correct

recruitment of adherens junction components and zonula occludens formation. Exp Cell Res 312, 1637-50.

Nobes, C. D. and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81, 53-62.

Ohba, Y., Ikuta, K., Ogura, A., Matsuda, J., Mochizuki, N., Nagashima, K., Kurokawa, K., Mayer, B. J., Maki, K., Miyazaki, J. et al. (2001). Requirement for C3G-dependent Rap1 activation for cell adhesion and embryogenesis. Embo J 20, 3333-41.

Perez, T. D., Tamada, M., Sheetz, M. P. and Nelson, W. J. (2008). Immediate-Early Signaling Induced by E-cadherin Engagement and Adhesion. J Biol Chem 283, 5014-22.

Price, L. S., Hajdo-Milasinovic, A., Zhao, J., Zwartkruis, F. J., Collard, J. G. and Bos, J. L. (2004). Rap1 regulates E-cadherin-mediated cell-cell adhesion. J. Biol. Chem. 279, 35127-35132.

Radziwill, G., Weiss, A., Heinrich, J., Baumgartner, M., Boisguerin, P., Owada, K. and Moelling, K. (2007). Regulation of c-Src by binding to the PDZ domain of AF-6. Embo J 26, 2633-44.

Sakurai, A., Fukuhara, S., Yamagishi, A., Sako, K., Kamioka, Y., Masuda, M., Nakaoka, Y. and Mochizuki, N. (2006). MAGI-1 is required for Rap1 activation upon cell-cell contact and for enhancement of vascular endothelial cadherin-mediated cell adhesion. Mol Biol Cell 17, 966-76.

Sato, T., Fujita, N., Yamada, A., Ooshio, T., Okamoto, R., Irie, K. and Takai, Y. (2006). Regulation of the assembly and adhesion activity of E-cadherin by nectin and afadin for the formation of adherens junctions in Madin-Darby canine kidney cells. J Biol Chem 281, 5288-99.

Sawada, Y., Tamada, M., Dubin-Thaler, B. J., Cherniavskaya, O., Sakai, R., Tanaka, S. and Sheetz, M. P. (2006). Force sensing by mechanical extension of the Src family kinase substrate p130Cas. Cell 127, 1015-26.

Shewan, A. M., Maddugoda, M., Kraemer, A., Stehbens, S. J., Verma, S., Kovacs, E. M. and Yap, A. S. (2005). Myosin 2 is a key Rho kinase target necessary for the local concentration of E-cadherin at cell-cell contacts. Mol Biol Cell 16, 4531-42.

Stroeken, P. J., Alvarez, B., Van Rheenen, J., Wijnands, Y. M., Geerts, D., Jalink, K. and Roos, E. (2006). Integrin cytoplasmic domain-associated protein-1 (ICAP-1) interacts with the ROCK-I kinase at the plasma membrane. J Cell Physiol 208, 620-8.

van Nieuw Amerongen, G. P., Beckers, C. M., Achekar, I. D., Zeeman, S., Musters, R. J. and van Hinsbergh, V. W. (2007). Involvement of Rho kinase in endothelial barrier maintenance. Arterioscler Thromb Vasc Biol 27, 2332-9.

Vicente-Manzanares, M., Webb, D. J. and Horwitz, A. R. (2005). Cell migration at a glance. J Cell Sci 118, 4917-9.

Voss, A. K., Gruss, P. and Thomas, T. (2003). The guanine nucleotide exchange factor C3G is necessary for the formation of focal adhesions and vascular maturation. Development 130, 355-67.

Wei, P., Satoh, T., Edamatsu, H., Aiba, A., Setsu, T., Terashima, T., Kitazawa, S., Nakao, K., Yoshikawa, Y., Tamada, M. et al. (2007). Defective vascular morphogenesis and mid-gestation embryonic death in mice lacking RA-GEF-1. Biochem Biophys Res Commun 363, 106-12.

Wittchen, E. S., Worthylake, R. A., Kelly, P., Casey, P. J., Quilliam, L. A. and Burridge, K. (2005). Rap1 GTPase inhibits leukocyte transmigration by promoting endothelial barrier function. J. Biol. Chem. 280, 11675-11682.

Yamada, S. and Nelson, W. J. (2007). Localized zones of Rho and Rac activities drive initiation and expansion of epithelial cell-cell adhesion. J Cell Biol 178, 517-27.

Yoshikawa, Y., Satoh, T., Tamura, T., Wei, P., Bilasy, S. E., Edamatsu, H., Aiba, A., Katagiri, K., Kinashi, T., Nakao, K. et al. (2007). The M-Ras-RA-GEF-2-Rap1 pathway mediates tumor necrosis factor-alpha dependent regulation of integrin activation in splenocytes. Mol Biol Cell 18, 2949-59.

Zawistowski, J. S., Serebriiskii, I. G., Lee, M. F., Golemis, E. A. and Marchuk, D. A. (2002). KRIT1 association with the integrin-binding protein ICAP-1: a new direction in the elucidation of cerebral cavernous malformations (CCM1) pathogenesis. Hum Mol Genet 11, 389-96.

Zhang, J., Rigamonti, D., Dietz, H. C. and Clatterbuck, R. E. (2007). Interaction between krit1 and malcavernin: implications for the pathogenesis of cerebral cavernous malformations. Neurosurgery 60, 353-9; discussion 359.

Zhang, Z., Rehmann, H., Price, L. S., Riedl, J. and Bos, J. L. (2005). AF6 negatively regulates Rap1-induced cell adhesion. J Biol Chem 280, 33200-5.

Samenvatting in het Nederlands

De wand van bloedvaten is bekleed met endotheelcellen. De belangrijkste functie van endotheelcellen is het vormen van een barrière om de passage van macromoleculen, bloedcellen en vloeistoffen te reguleren. Onder verschillende pathologische condities, zoals oedeem en chronische ontsteking, is deze regulatie verstoord. De integriteit van de endotheelcellen is afhankelijk van intercellulaire juncties. Deze juncties worden gevormd door een verscheidenheid aan adhesie eiwitten zoals VE-cadherine. In dit proefschrift beschrijf ik het onderzoek dat we de afgelopen vier jaar hebben verricht naar de rol van het Rap-GTPase in regulatie van deze juncties. In de eerste twee hoofdstukken van dit proefschrift geef ik een uiteenzetting van de huidige stand van zaken betreffende de rol van Rap in regulatie van cel-cel juncties (Hoofdstuk 1) en de regulatie van de endotheliale barrière (Hoofdstuk 2).

In Hoofdstuk 3 beschrijf ik het onderzoek naar de Rap activator Epac1 in endotheelcellen. We laten zien dat we Epac1 kunnen activeren in endotheelcellen met de Epac-selectieve cAMP-analoog 8-pCPT-2OMe-cAMP (007). Tevens laten we zien dat activatie van Epac1 leidt tot versterking van de cel-cel juncties gebaseerd op VE-cadherine evenals een verlaging van de permeabiliteit van de monolaag. Het is interessant om te zien dat hoewel verlaging van Epac1-levels geen zichtbaar effect heeft op de cel-cel juncties, de 007-geinduceerde versterking van de barrière verdwenen is zonder Epac1. Ten slotte laten we zien dat Epac1 het actine skelet reguleert door haar organisatie te veranderen van ‘stress-fibers’ naar corticaal actine, onafhankelijk van het adhesie eiwit VE-cadherine. Hieruit blijkt dat Epac1 activatie leidt to versterking van de endotheliale barriere en dat dit samengaat met veranderingen in het actine skelet.

Vervolgens hebben we een andere activator van Rap onderzocht. In Hoofdstuk 4 beschrijven we de rol van PDZ-GEF2 in de formatie van cel-cel juncties. Rap1 wordt geactiveerd wanneer de contacten tussen cellen artificieel verbroken worden door het verwijderen van extracellulair calcium. We laten zien dat deze activatie van Rap1 afhankelijk is van de activator PDZ-GEF2. Interessant genoeg is PDZ-GEF2, in tegenstelling tot Epac1, ook nodig voor het formeren van complete cel-cel juncties. In cellen waarin PDZ-GEF2-levels gereduceerd zijn zien we dat de juncties niet met de membranen strak tegen elkaar zitten, maar dat er gaten zitten tussen de cellen en dat er membraan uitstulpingen achterblijven. Het lijkt erop dat de cellen als het ware blijven steken halverwege het proces van junctie formatie. Ook hebben deze cellen minder E-cadherine op het membraan. Interessant is het dat wanneer we Rap1 opnieuw activeren in deze cellen door middel van een actieve vorm van Rap1 of door overexpressie van Epac1 we dit fenotype kunnen herstellen en cellen weer normale cel-cel juncties hebben. Verder blijkt dat verlaging van de expressie-levels van Rap1a en Rap1b door middel van siRNA technieken, verschillende effecten heeft op de formatie van cel-cel juncties. Verwijdering van Rap1a leidt tot vergelijkbare structuren als bij PDZ-GEF2 depletie, maar heeft geen effect op de expressie van E-cadherine. Daarentegen resulteert Rap1b siRNA in normale cel-cel juncties, ondanks dat de hoeveelheden E-cadherine op het plasma membraan verlaagd zijn. Samengevat laten we zien dat de PDZ-GEF2/Rap1 signaleringsroute essentieel is een goede vorming van cel-cel juncties. PDZ-GEF2 reguleert via Rap1a de maturatie van de cel-cel juncties en daarnaast de hoeveelheid E-cadherine op het membraan via Rap1b.

In het vervolg van ons onderzoek hebben wij ons gericht op de onderliggende mechanismes van de regulatie van cel-cel juncties door Rap1. Allereerst hebben we de Rap1-effector AF-6 onderzocht (Hoofdstuk 5). In het verleden is aangetoond dat AF-6 direct aan Rap1 kan binden en dat AF-6 gelokaliseerd is in de cel-cel juncties. Gezamenlijk maakt dit dat AF-6 een goede kandidaat om betrokken te zijn bij de Rap-gemedieerde formatie van cel-cel juncties. Daarom hebben we knock-down gedaan van AF-6 in endotheel cellen en de juncties bestudeerd. Tegen de verwachting in had de depletie van AF-6 geen effect op de lokalisatie van verscheidene junctie eiwitten. Wel zagen we een duidelijk effect op de organisatie van het actine skelet waarbij de stress fibers verminderd waren en er corticaal actine ontstaan was. Tevens laten we zien dat AF-6 siRNA leidt tot verminderde stress fibers in losse cellen wat suggereert dat het actine fenotype niet afhankelijk is van de cel-cel juncties. Het is daarom des te verrassender dat cellen zonder AF-6 wel een verminderde trans-endotheliale elektrische weerstand (TER) hebben. Echter, depletie van AF-6 heeft geen effect op Epac1-geinduceerde verhoging van TER. Dit laat dus zien dat AF-6 niet nodig is voor de regulatie van cel-cel juncties door Epac1.

Om de eiwitten die betrokken zijn bij Epac1/Rap1 geïnduceerde regulatie van juncties te identificeren, hebben we een set van siRNAs getest die gericht zijn tegen eiwitten die mogelijk betrokken zijn bij Rap1-signalering. De resultaten van deze exercitie staan beschreven in Hoofdstuk 6. Uiteindelijk identificeren we 11 eiwitten die mogelijk een rol spelen bij de regulatie van TER door Epac1. We kunnen deze eiwitten grofweg in drie groepen delen. Allereerst vinden we CCM2 en ICAP1, welke beide aan KRIT of CCM1 kunnen binden. Deze eiwitten zijn vaak gemuteerd in het DNA van patiënten die lijden aan cerebrale caverneuze malformaties (CCM), een ziekte die zich manifesteert door bloedingen in de hersenen. Ook hebben we eiwitten gevonden, zoals Rac1 en Vav1, die al beschreven zijn in Rap1-signalering en betrokken zijn bij vele algemene celbiologische processen. Tot slot vinden we dat Lamellipodin en PDZK10 siRNA het effect van Epac1 op TER reduceren. Hoewel er over PDZK10 weinig bekend is, lijken beide eiwitten betrokken te zijn bij de regulatie van het actine skelet.

In Hoofdstuk 7 beschrijven we de rol van Lamellipodin en het actine skelet in Epac1-geinduceerde TER. Allereerst laten we zien dat het actine skelet nodig is voor het effect van Epac1 op TER. Lamellipodin bind aan de actine regulator VASP en we laten zien dat deze interactie niet verandert na Rap1 activatie. Ook laten we zien dat Lamellipodin een complex vormt met de Rap1 effector Riam. Ten slotte vinden we dat VASP in de cel-cel contacten lokaliseert na activatie van Epac1. We laten zien dat dit niet gepaard gaat met de fosforylering van VASP en dat het onafhankelijk is van VE-cadherine. Tevens zien we dat VE-cadherine niet nodig is voor de effecten van Epac1 op de TER. Hieruit concluderen we dat Epac1 de endotheliale elektrische weerstand afhankelijk is van het actine cytoskelet en Lamellipodin. Waarschijnlijk leidt de activatie van Epac1 en Rap1 tot een activatie of lokalisatie van Lamellipodin, mogelijk via RIAM. Dit leidt vervolgens locaal tot veranderingen in het actine cytoskelet.

De resultaten in dit proefschrift ondersteunen eerder observaties dat de Rap-GTPases een belangrijke rol spelen in de regulatie van cel-cel juncties. We laten zien dat verschillende activatoren van Rap op verschillende manieren bijdragen aan de vorming van een complete junctie. Tevens laten we zien dat verschillende effector complexen deze activiteit omzetten in sterkere juncties. Vooral het onderzoek naar de rol van Rap in endotheelcel juncties kan bijdrage aan de reductie van lekkage, tijdens bijvoorbeeld ontstekingen wanneer de barrière functie van de endotheelcellen verminderd is.

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In dit laatste deel van mijn boekje wil ik nog een aantal mensen noemen die een rol hebben gespeeld bij het tot stand komen van dit boekje. Allereerst wil ik natuurlijk mijn promotor bedanken. Hans, het waren jou colleges over signaal-transductie in het tweede jaar van mijn studie, die mij richting jouw lab dirigeerden. Ik heb de afgelopen vier jaar veel geleerd op wetenschappelijk gebied en ook veel daarbuiten. Ik wil je bedanken voor je begeleiding en dat je me de kans en de ruimte hebt gegeven om mijn sterke kanten te benadrukken en mijn zwaktes te leren kennen. Vooral de mogelijkheid om ondanks je drukke agenda altijd bij je binnen te kunnen lopen was voor mij erg prettig. Hoewel dat we het niet altijd eens waren, kwamen we meestal wel op hetzelfde model uit. Bedankt voor het vertrouwen met de vele samenwerkingen die we zijn begonnen en veel succes met het combineren van goed onderzoek en de bestuurlijke functies.
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Nadia, I think we were a really good team. I have never met anyone who could do more experiments in a week than you did. I really enjoyed working with you on the PG2 project and the review. I also haven't met anyone who could drink the wine at Spetses faster than you! Good luck in Heidelberg and in science. I also want to thank the weird American (ALL Americans are weird), Shannon. What can I say, maybe you have kept me from my work more than it benefited from it, thanks for that! I really enjoyed having you in our office and when you're in England, please renew you permits in time. Have a good time over there with Guy and I wish you all the best!
Verder wil ik de analisten bedanken die mij hebben geholpen bij het vullen van dit boekje: Marjolein, niemand kan zulke mooie blotjes maken als jij. Erg bedankt dat je het laatste jaar zoveel proeven hebt kunnen doen. Sander, bedankt voor het installeren van de ECIS en bij het uitvoeren van de screen. Succes met je promotie bij oncologie; de aanhouder wint!
Tijdens mijn promotie heb ik ook twee studenten mogen begeleiden. Luc, succes met afstuderen. Jenny, thanks for you enthousiasm and good luck in New York.
Fried, hoewel je kritiek soms erg streng is, kon ik het meestal wel waarderen. Het is erg fijn om zo'n ervaren persoon op het lab te hebben. Erg bedankt voor al het lezen en corrigeren van mijn manuscript. Holger, jij weet een discussie soms erg abstract te maken, desalniettemin is kritische massa altijd prettig. Je bent de koning van de kristallen. Er zijn nog veel GEFs te gaan…
Boudewijn, je eigengereidheid wordt geroemd en ik heb veel van je opmerkingen geleerd. Het is goed om kritiek en suggesties te krijgen uit een andere hoek. Tobias, succes met de groep Dansen en misschien kom ik je nog wel eens tegen op de heuvelrug.
Daarnaast zijn er natuurlijk de andere AIOs op het Bos/Burgering lab. Ik zal jullie niet snel vergeten: de vele pizza-meetings, biertjes and natuurlijk de trip naar Barcelona. Diana, onze lab-banketbakker. Het gaat je gewoon lukken! Ik ben benieuwd of je weer in Italië gaat wonen. Mijn bench-makkers: Martijn en Willem-Jan. Ik heb met niemand zoveel goede en slechte ideeën besproken als met jullie. Het enige nadeel was dat mijn bench af en toe zoek was en anders mijn buffers wel. Misschien kunnen we binnenkort weer eens "goedkoop" uit eten bij de Reunie? Willem-Jan, maak nog wat moois van Lamellipodin; succes! Martijn, Houten is echt ver! Anna, good luck with your mice! It takes a while, but hopefully you can finish it nicely. Lars, succes met het onderhouden van de relaties met het heck-lab, de mass-spec en Epac. Maaike, Krit is echt een Rap1-effector die toevallig aan foxo bindt. Veel succes daarmee! Jantine, je enthousiasme is erg aanstekelijk, hou dat vast, dan kom je er wel. Succes met de HUVEC enne, die ECIS, wat meet je nu eigenlijk? On Ying, het was erg gezellig op onze kamer. Helaas is de plant wel overleden. Veel succes met CCM1/2/3 en ICAP. Anouk, het wordt inderdaad hoog tijd dat iemand uitzoekt hoe PDZGEF werkt. Jij gaat hier vast achterkomen. Ten slotte David, de man die aan de kleinste dingetjes werkt van het hele lab. Succes met je RNA moleculen en misschien kun er één Deak noemen.
Wendy, veel geluk met de kids. Ik wil natuurlijk ook Lydia, Miranda, Hester, Iris, Harm-Jan, Paulien, Marije, Arjan, Niels, Mark, Annelies en Fons bedanken voor alle discussies en gezelligheid. Wim en consorten, bedankt voor de bereidheid om altijd te helpen, vooral toen tijdens het schrijven mijn computer kuren kreeg. Marcel en Kees, bedankt voor de ondersteuning.
Een paar oud-labgenoten wil ik ook graag noemen. Marta, je verjaardag in Cerano was echt fantastisch.

En Hoevec zjin inderdaad een beetje rare cellen. Veel geluk samen met Bart! Leo, de typische Hollandse Englishman. Bedankt voor al je adviezen en begeleiding in het eerste deel van mijn promotie. Ik vond het erg prettig om met je te kunnen sparren over ideeën van mij. Jürgen, je was al aan het afronden toen ik begon, maar de biertjes op het terras waren voor mij wel een goed begin in de wereld die Rap1 heet. Armando, veel succes in Australië en veel geluk met Barbara. Pieter, mijn stage bij jou ging als een speer. Ik vond het erg leuk om met je samen te werken. Mike and Jun, it was fun to know you guys. Good luck. Ook wil ik de Medemaatjes bedanken voor de gezelligheid. Ik zei toch dat het in Utrecht gezellig is. Gerben, Renske, Marvin, René, Barbara en Marcel, bedankt voor de gezelligheid in Amsterdam en Utrecht. Jamila, good luck in Amsterdam and thanks for the guidance during my stage. Timmertjes, Kopsjes, Holstegetjes en Voestjes, bedankt voor de goede sfeer.
Behalve al de mensen in Utrecht heb ik ook veel samenwerkingen buiten Utrecht gehad. Vooral de samenwerking met Magdalena Lorenowicz en Peter Hordijk op het Sanquin was erg leuk en vruchtbaar. Magdalena, good luck at the Hubrecht. Verder wil ik ook Marcel Schouten, Kees van 't Veer en Tom van der Poll van het AMC bedanken voor de samenwerking op het muizen project. Hopelijk krijgt het nog een mooi staartje. Dear Monica and Elisabetta, thank you for giving me the opportunity to give my PhD a kick start by visiting your lab. Your lab showed me what endothelial research is like.
Een AIO ben je natuurlijk 24 uur per dag, dus daarom wil ik ook mijn vrienden buiten het lab noemen. Eelke, het is erg prettig om met jou over wetenschap, literatuur en de politiek te praten. Succes met geneeskunde en veel geluk met Anke. Als jullie nog een keer 'per ongeluk' off-piste willen skiën dan hoor ik het wel. Jelger, ik heb veel respect voor de manier waarop je je er doorheen slaat. Binnenkort wordt jij ook dr. en je hebt het dik verdient. We moeten weer eens een goede film gaan bekijken. Heren van VV, bedankt voor de vele gezellige uren en activiteiten. Het is goed om een netwerk te hebben met goede vrienden. Dat het tot in het einde der dagen zo mag blijven! Pro Viro! Emile, Lidwien, Martine, Michiel, Margreet, Bart-Jan, Lonneke, Sander, Sandra, Roel, Annette en Mark. Bedankt voor jullie interesse en steun. Binnenkort barbecue in de Bilt.
Lida en Henk, bedankt voor jullie onaflatende interesse in mijn promotie. Eveline en René, het is leuk om een zusje te hebben. We stick together! René, als PSV weer europa in gaat of je weer ergens kaartjes voor zwemmen hebt kunnen ritselen hou ik me aanbevolen. Binnenkort weer eens een avondje met z'n vieren afspreken? Papa en mama, ik ga nu echt weg bij de universiteit. Jullie hebben me goed voorbereid op de grote mensen wereld en het gaat best goed tot nu toe. Ik prijs me erg gelukkig met jullie.
En tot slot natuurlijk mijn lievie. Eigenlijk schieten woorden hier tekort, maar ik ga het toch proberen. Met jou kan ik lachen en huilen, rennen en stilstaan, zien en gezien worden. Samen kunnen we de hele wereld aan! We hebben 8 jaar gehad en mogen er hopelijk nog 80. Ik hou van je.

Matthijs

Curriculum vitae and Publication list

Curriculum Vitae

Matthijs Kooistra werd geboren op 20 juni 1979 te Eindhoven. Het VWO diploma werd in 1997 aan het Christiaan Huygens College te Eindhoven behaald, waarna in hetzelfde jaar werd aangevangen met de studie Medische Biologie aan de Universteit Utrecht. Gedurende deze studie werd onderzoekservaring opgedaan met stages bij de afdeling Fysiologische Chemie van het Universitair Medisch Centrum te Utrecht onder begeleiding van Dr. Pieter van de Heuvel en Prof. Dr. Ir. Boudewijn Burgering en op het Nederlands Kanker Instituut te Amsterdam onder begeleiding van Dr. Jamila Laoukili en Prof. Dr. René Medema. Na het afronden van deze studie in 2004 werd aangevangen met het in dit proefschrift beschreven onderzoek onder begeleiding van Prof. Dr. Hans Bos bij de afdeling Fysiologische Chemie van het Universitair Medisch Centrum te Utrecht.

List of Publications

Laoukili, J., Kooistra, M.R.H., Brás, A., Kauw, J., Kerkhoven, R.M., Morrison, A., Clevers, H. and Medema, R.H.
FoxM1 is required for execution of the mitotic programme and chromosomal stability.
Nature Cell Biology (2005); 7(2): 126-36

Kooistra, M.R.H., Corada, M., Dejana, E. and Bos, J.L.
Epac1 regulates integrity of endothelial cell junctions through VE-cadherin.
FEBS Letters (2005); 579(22): 4966-72

Kooistra, M.R.H., Dubé, N. and Bos, J.L.
Rap1: a key regulator in cell-cell junction formation.
Journal of Cell Science (2007); 120(1): 17-22

Dubé, N., Kooistra, M.R.H., Pannekoek, W-J., Vliem, M.J., Oorschot, V., Klumperman, J., Rehmann, H. and Bos, J.L.,
The PDZ-GEF2/Rap1a pathway is required for maturation of cell-cell junctions.
Submitted for publication

Bieringss, G., Sellink, E., Kooistra, M.R.H., Gijzen, K., Fernandez-Borja, M., van Mourik, J.A., and Voorberg, J.
Epac is involved in cAMP-mediated exocytosis of Weibel-Palade bodies in endothelial cells through the activation of the small GTPase Rap1.
Submitted for publication

Lorenowicz, M.J., Fernandez-Borja, M., Kooistra, M.R.H., Bos, J.L. and Hordijk, P.L.
PKA and Epac1 regulate endothelial integrity and migration through parallel and independent pathways.
Submitted for publication

Vliem, M.J., Ponsioen, B., Schwede, F., Pannekoek, W-J., Riedl, J., Kooistra, M.R.H., Jalink, K., Genieser, H-G., Bos, J.L., and Rehmann, H.
8-pCPT-2'-O-Me-cAMP-AM: An improved Epac-selective cAMP analogue.
Submitted for publication

Color figures

Chapter 1

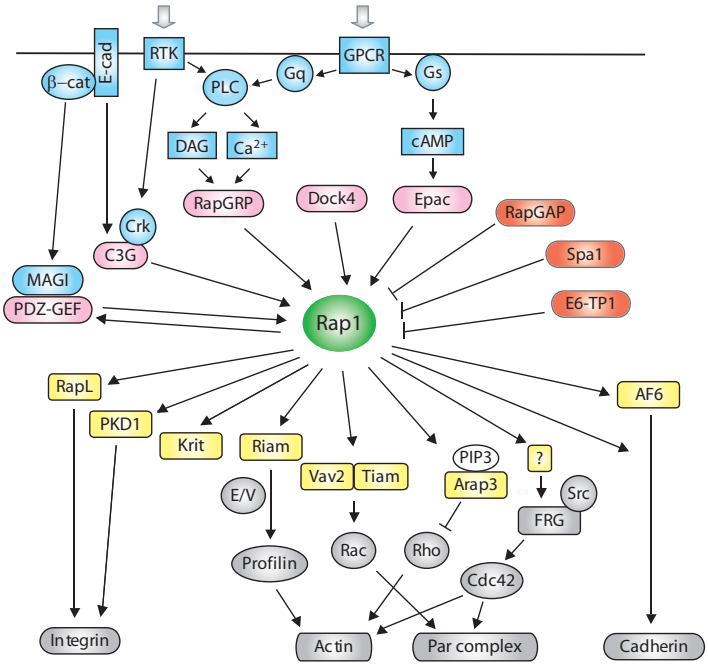


Fig. 1. Overview of the Rap1 signalling network
Adapted from (Bos, 2005). DAG, Diacylglycerol; E/N, Ena/Vasp; GPCR, G-protein coupled receptor; PIP3, Phosphoinositol-tri-phosphate; PLC, phospholipase C; RTK, Receptor Tyrosine Kinase.

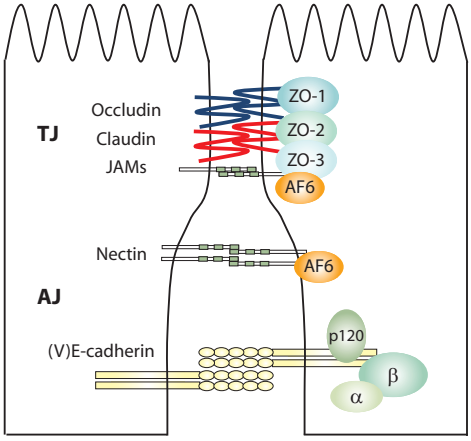
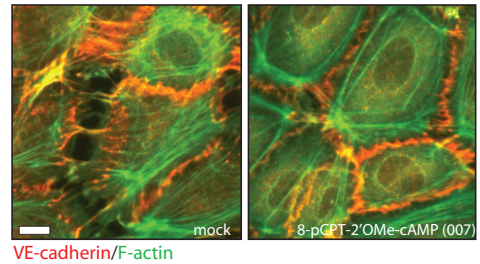


Fig. 2. Schematic overview of adherens and tight junctions.
AJ, adherens junctions; TJ, tight junctions; JAMs, ZO, Zona occludens; junctional adhesion molecules; AF-6, Afadin/AF-6; p120, p120catenin; β, β-catenin; α, α-catenin.



VE-cadherin/F-actin

Fig. 3. Activation of Rap1 in endothelial cells induces cell-cell junction maturation. Anti-VE-cadherin and F-actin staining of HUVE cells treated for 30 minutes with vehicle or the Epac-specific cAMP analog 007. Stimulation with 007 clearly increased cell-cell junction maturation as the adherens junctions form a smoother line between the cells. Endothelial cell permeability is reduced as a biological consequence of the junction tightening (bar = 10μm).

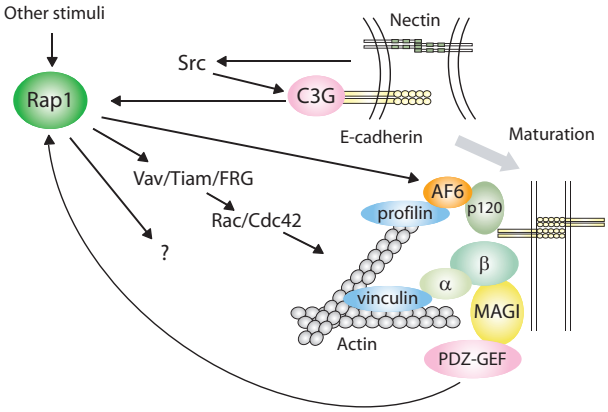


Fig. 4. Model for the involvement of Rap1 in cell-cell junction formation.
At initial cell-cell contacts, C3G bound to E-cadherin is activated, resulting in Rap1 activation. Upon maturation, C3G is displaced by β-catenin, which interacts with PDZ-GEF to further activate Rap1. Also, extracellular stimuli, including stimuli that stimulate production of cAMP which activates Epac, induce Rap1 activation.

Chapter 2

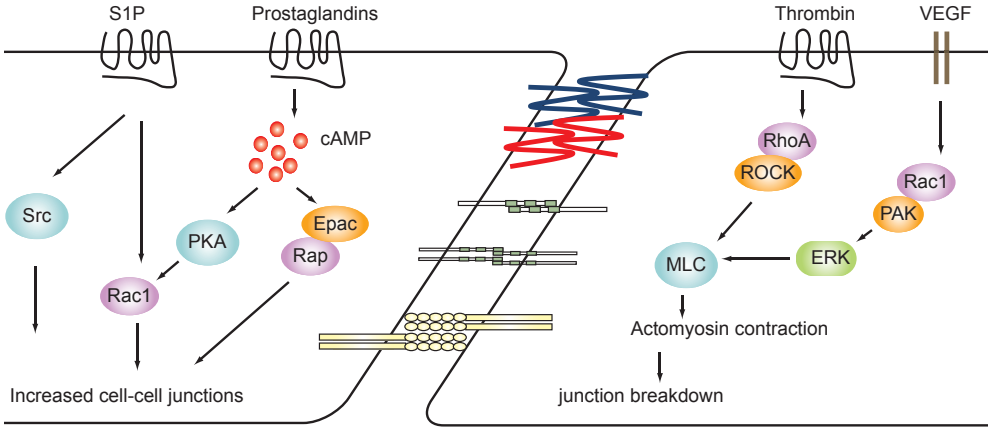


Fig. 2. regulation of endothelial barrier function.

Chapter3

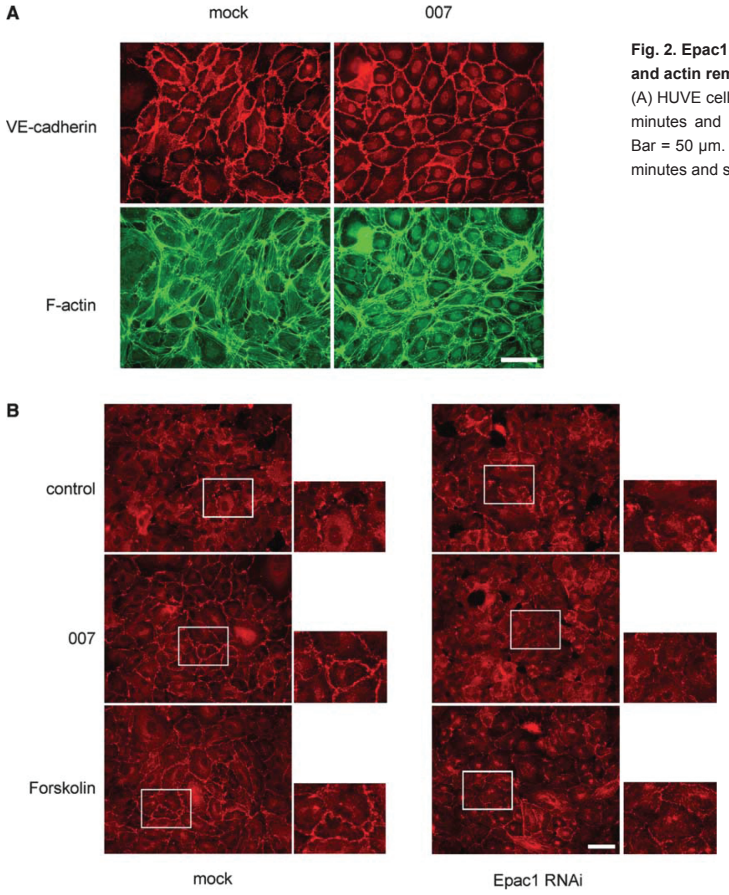


Fig. 2. Epac1 activation induces junction formation and actin remodeling and reduces permeability. (A) HUVE cells were treated with or without 007 for 30 minutes and stained for VE-cadherin and phalloidin. Bar = 50 μm. (B) Cells wer treated as indicated for 30 minutes and stained for cortactin. Bar = 50 μm.

Chapter 4

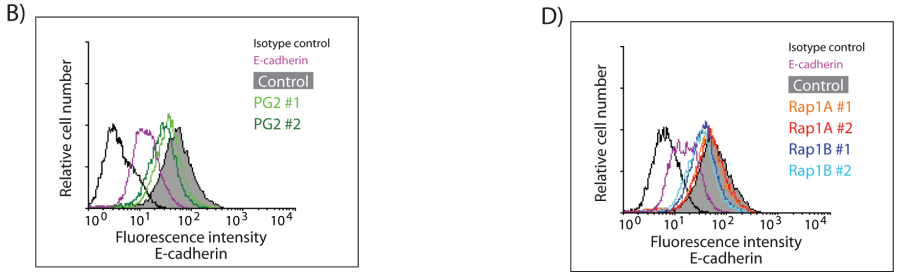


Fig. 5. The PDZ-GEF2/Rap1B pathway regulates E-cadherin levels. B and D. E-cadherin cell surface staining was determined by flow cytometry analysis of Alexa Fluor 488 anti-human CD324-stained A549 cells treated with control, PDZ-GEF2 (PG2 #1, PG2 #2), Rap1A (Rap1A #1, Rap1A #2), Rap1B (Rap1B #1, Rap1B #2) or E-cadherin siRNA. Cells were grown for 24 hours following siRNA transfection and replated 24 hours before flow cytometry analysis. Alexa Fluor 488 Mouse IgG1, κ isotype control was used as a control. A representative result from at least three independent experiments is shown in each panel.

Chapter 5

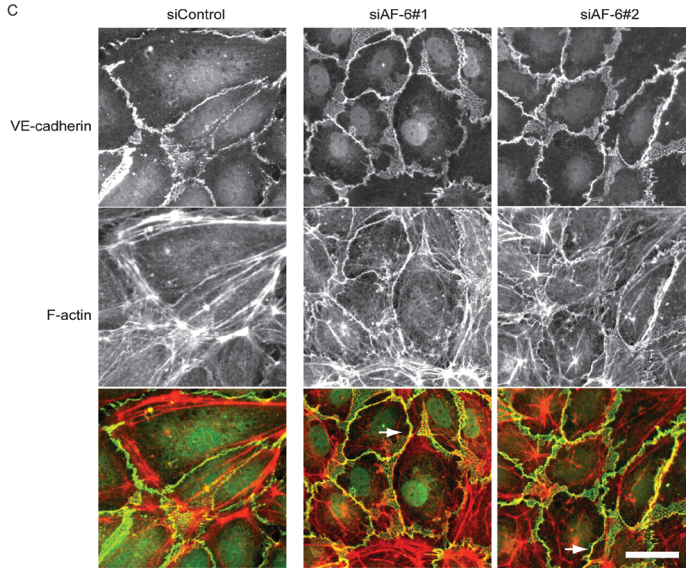


Fig. 1. AF6 regulates endothelial integrity. (C) Transfected cells were stained for VE-cadherin and F-actin. The merge is shown in the lower panel. Arrows indicate cortical actin; Bar = 25 µm.

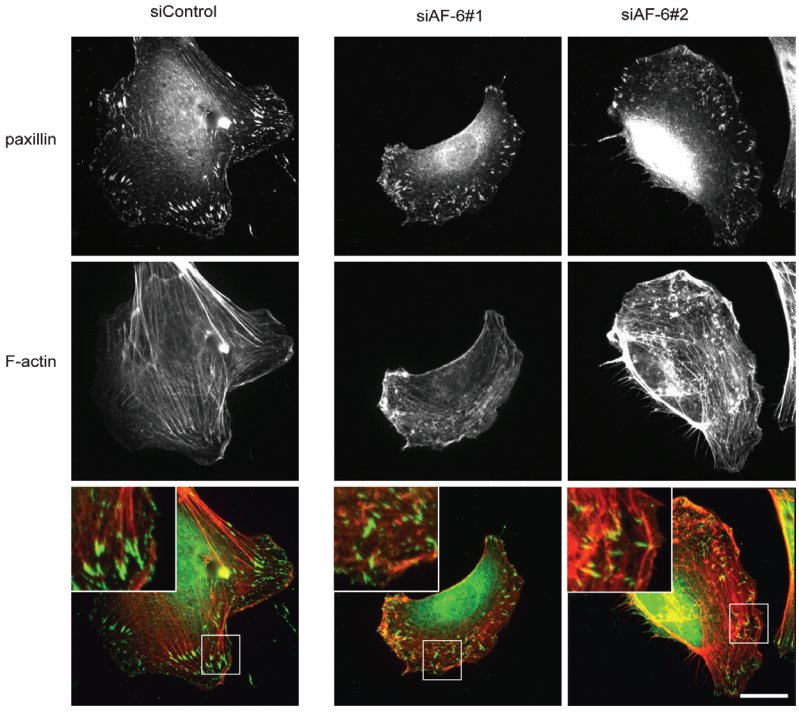


Fig. 2. Organization of the actin cytoskeleton requires AF6. HUVEC were seeded sparsely for 16 hours, fixed and stained for paxillin and F-actin. Bar = 10 µm.

Chapter 5

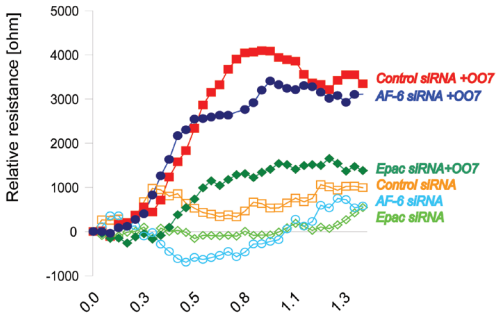


Fig. 4. Activation of Rap1 increases endothelial integrity independent of AF6.
(A) Transendothelial resistance of HUVEC monolayers on fibronectin coated gold electrodes was measured. Cells were stimulated with vehicle (open) of 100 μM 8pCPT-2'OMe-cAMP (closed) at the timepoint indicated by the arrow; ■- siControl; ▲- siEpac1; ●- siAF6#1; Bar-graph (lower panel) represents the average of three independent experiments at 1,5 hours after treatment. *p<0.05, **p< 0.01.

Chapter 7

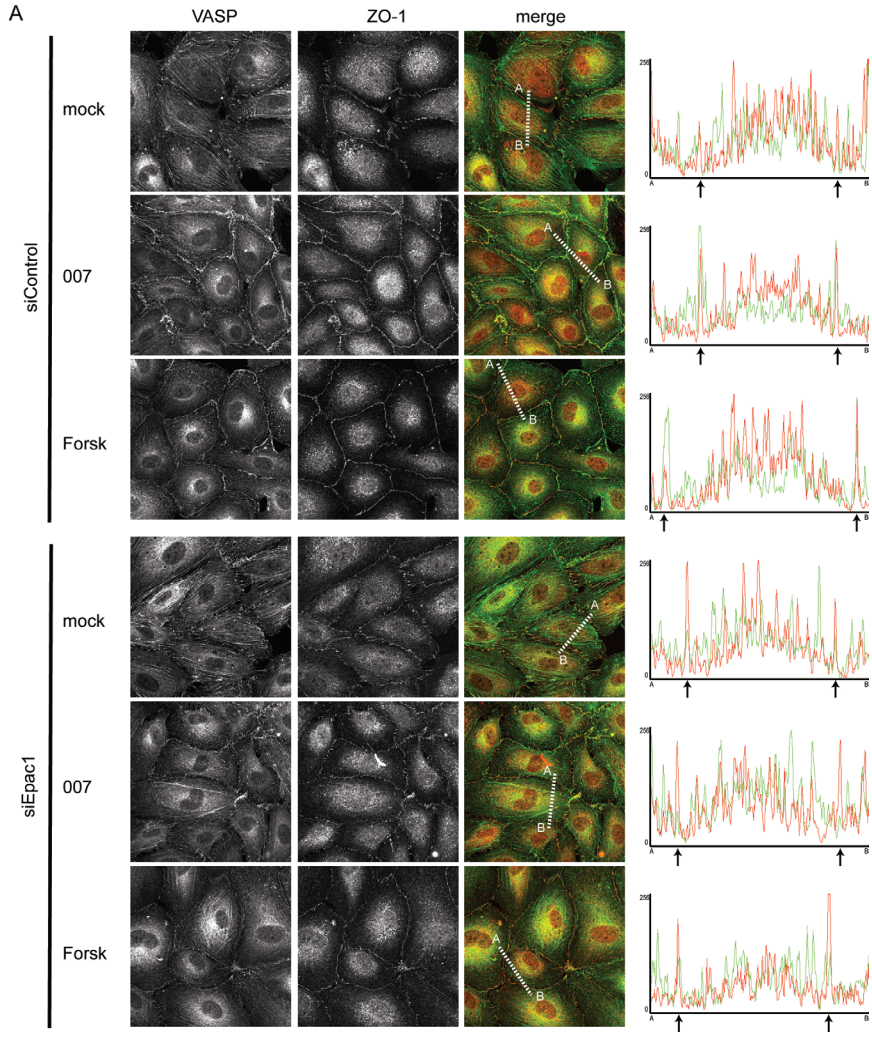


Figure 3

Chapter 7

Figure 3 (cont.)

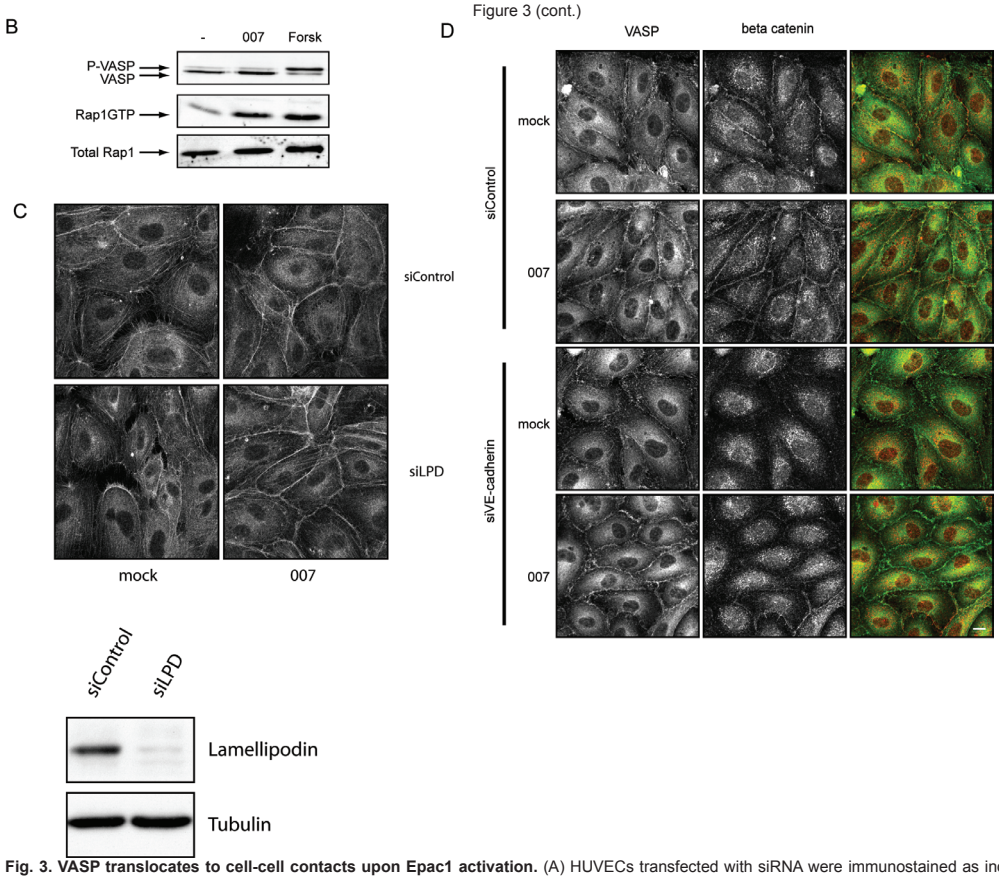


Fig. 3. VASP translocates to cell-cell contacts upon Epac1 activation. (A) HUVECs transfected with siRNA were immunostained as indicated after 30' treatment with 007 or Forskolin. Intensity profiles along dashed line between points A and B are shown. Arrows indicate cell-cell contacts. (B) HUVECs were stimulated for 5' as indicated and Rap1GTP was determined by the pull-down assay. Lysated were also probed for VASP to determine a phosphorylation-dependent shift. (C) HUVEC transfected with siRNA were treated with or without 007 for 30 minutes. Cells were stained with phalloidin to show F-actin. (D) HUVECs transfected with siRNA were stimulated with 007 or left untreated. Cells were immunostained as indicated. Bar= 10 μM.

Chapter 8

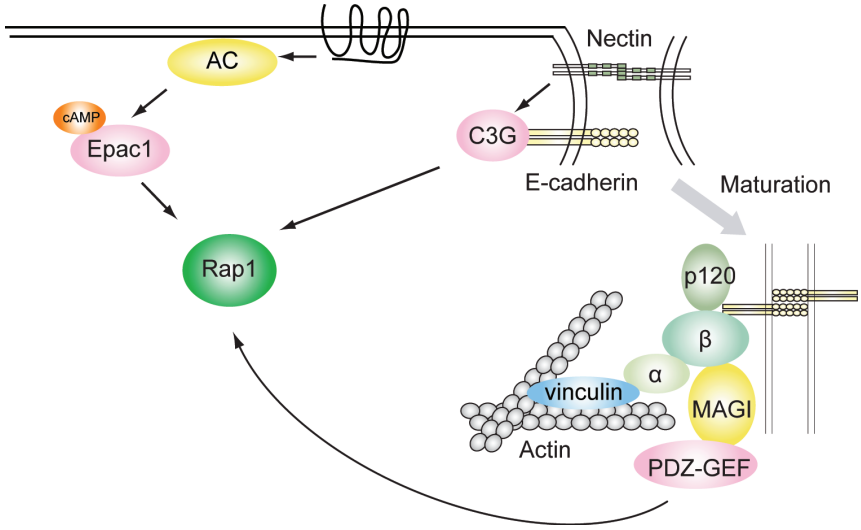


Fig. 1. Activation of Rap1 during junction formation.

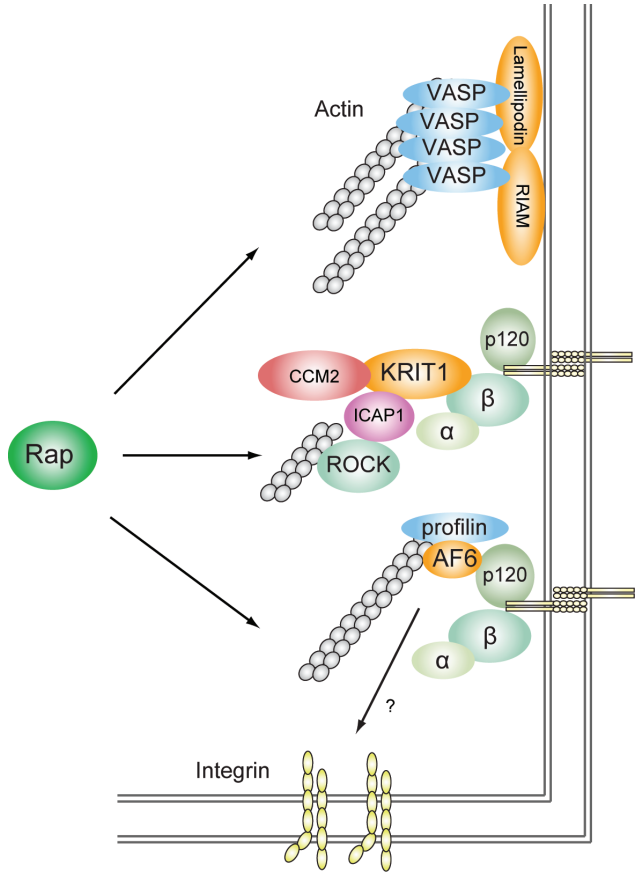


Fig. 2 Rap-effectors in junction formation.