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.

Key words: Rap1, Cadherin, Adherens junctions, GTPase, Actin

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 extracellular 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 Ca^{2+}, diacylglycerol and cyclic AMP (cAMP) (Fig. 1). Inactivation of Rap1 is mediated by several 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 by 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-cadherin and 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). Their cytoplasmic tails comprise 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-cadherin and 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 endocytic or exocytic compartments (Davis et al., 2003; Xiao et al., 2005) are among the processes that regulate these junctions. Notably, disruption of the integrity of cell-cell junctions has serious pathological consequences, including unwanted vessel leakage and, in cancer, 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*

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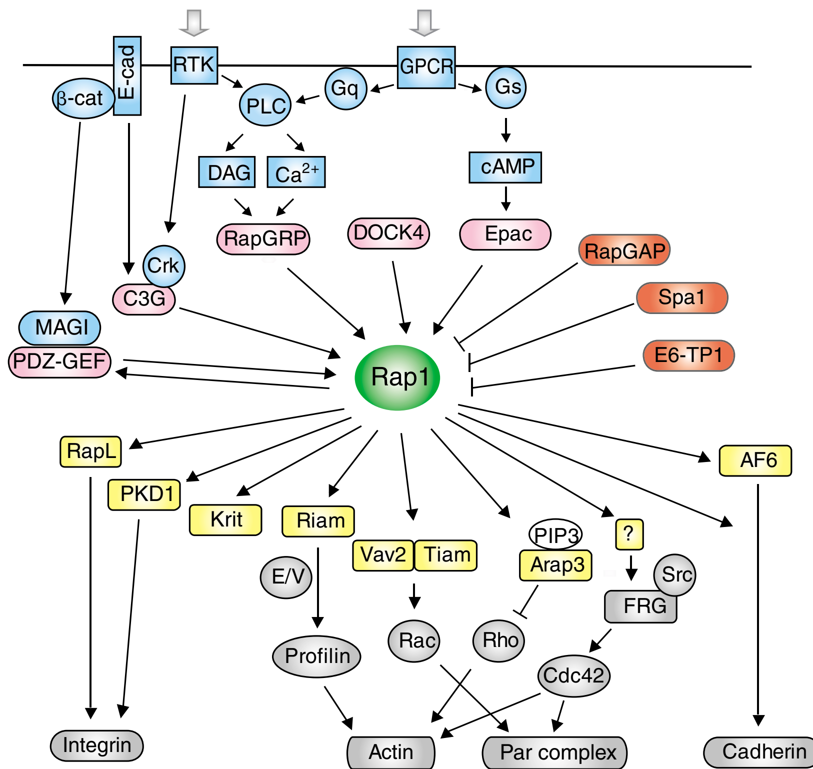

Fig. 1. Overview of the Rap1 signalling network. Adapted from Bos, 2005 with permission (Bos, 2005). DAG, diacylglycerol; E/V, Ena/Vasp; GPCR, G-protein coupled receptor; PIP3, phosphoinositol-tri-phosphate; PLC, phospholipase C; RTK, receptor tyrosine kinase.

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/AF6 ortholog Canoe. Importantly, apical-basolateral polarity and the localization 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 maintain the circumferential distribution of adherens junctions. This is compatible with the observation that green fluorescent protein (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 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 the 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 the idea that Rap1 has a role 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 junction 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).

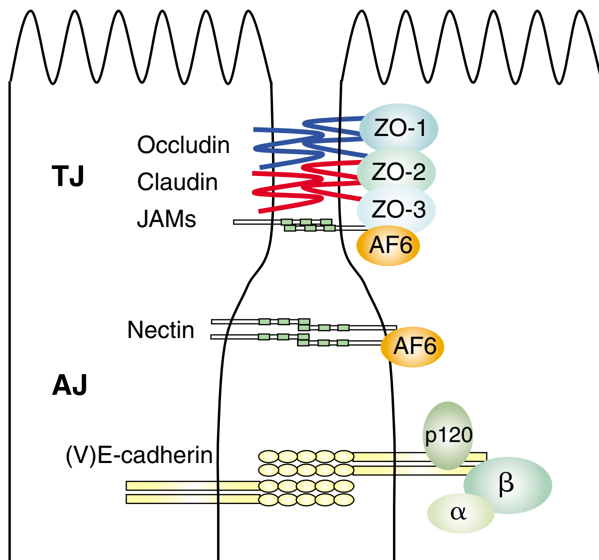

Fig. 2. Schematic overview of adherens junctions and tight junctions. α, α-catenin; AF6, afadin/AF6; AJ, adherens junctions; β, β-catenin; JAMs, junctional adhesion molecules; p120, p120catenin; TJ, tight junctions; ZO, zona occludens.

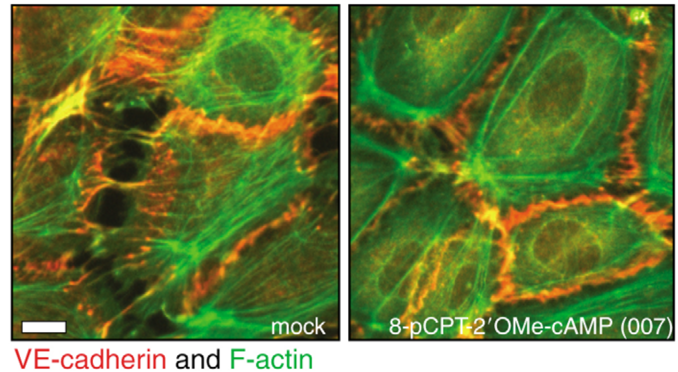

Fig. 3. Activation of Rap1 in endothelial cells induces cell-cell junction maturation. Anti-VE-cadherin and F-actin staining of HUVE cells treated for 30 minutes with vehicle or the Epac-specific cAMP analog 007. Stimulation with 007 clearly increased cell-cell junction maturation: the adherens junctions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

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 the 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 a 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 to the observation in MCF7 cells that junction disassembly is not initiated by RapGAP expression, this 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-cadherin 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

Once it is established that Rap1 is involved in the control of cell-cell adhesion, probably through E-cadherin and VE-cadherin, two questions arise. First, how is Rap1 activated and, second, how does Rap1 regulate cell-cell junctions? The accumulation of Rap1 at cell-cell contacts suggests that it 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 Ca^{2+} 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 immunoglobulin (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, which results 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). Both the presence of MAGI-1 at 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.B., 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 downregulated 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 internalization 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 the 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 E-cadherin 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

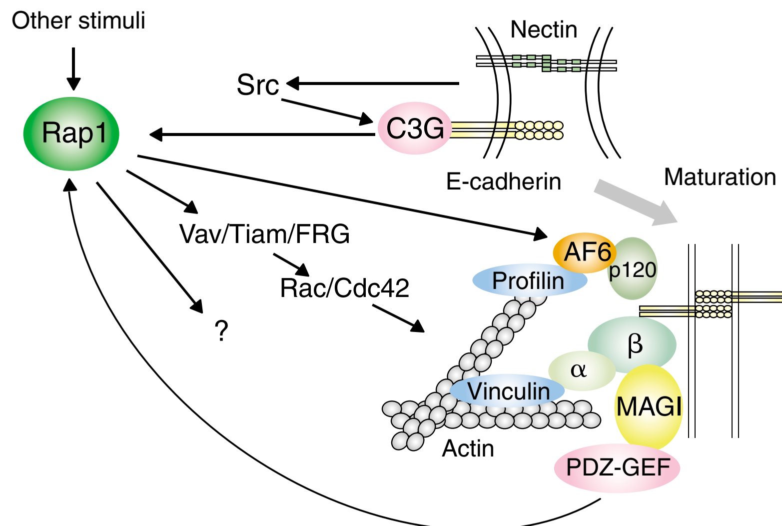

Fig. 4. Model for the involvement of Rap1 in cell-cell junction formation. At initial cell-cell contacts, C3G bound to E-cadherin is activated, resulting in Rap1 activation. Upon maturation, C3G is displaced by β-catenin, which interacts with PDZ-GEF to further activate Rap1. Also, extracellular stimuli,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

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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. Such a 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 for mediating Rap1-dependent effects on E-cadherin (Boettner et al., 2000; Davis et al., 2003; Sato et al., 2006). In addition, 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 process is not clear. For instance, inhibition of Rap1 in MCF7 cells does not affect localization 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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