Rap1 Regulates E-cadherin-mediated Cell-Cell Adhesion*

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The small GTPase Rap is best characterized as a critical regulator of integrin-mediated cell adhesion, although its mechanism of action is not understood. Rap also influences the properties of other cell-surface receptors and biological processes, although whether these are a consequence of effects on integrins is not clear. We show here that Rap also plays an important role in the regulation of cadherin-mediated cell-cell adhesion in epithelial cells. Expression of constitutively active Rap1A restored cadherin-mediated cell-cell contacts in mesenchymal Ras-transformed Madin-Darby canine kidney cells, resulting in reversion to an epithelial phenotype. Activation of endogenous Rap via the Rap exchange factor Epac also antagonized hepatocyte growth factor-induced disruption of adherens junctions. Inhibition of Rap signaling resulted in disruption of epithelial cell-cell contacts. Rap activity was required for adhesion of cells to recombinant E-cadherin extracellular domains, i.e. in the absence of integrin-mediated adhesion. These findings suggest that Rap signaling positively contributes to cadherin-mediated adhesion and that this occurs independently of effects on integrin-mediated adhesion. Our results imply that Rap may function in a broader manner to regulate the function of cell-surface adhesion receptors.

Rap is a small GTPase that is closely related to the small GTPase archetype, Ras. Like Ras, Rap cycles between an active GTP-bound form and an inactive GDP-bound form. Activation of Rap is mediated by guanine nucleotide exchange factors, such as Epac, which catalyze exchange of GDP for GTP. Rap is inactivated by GTPase-activating proteins (GAPs),1 such as RapGAP, which catalyze the hydrolysis of bound GTP to GDP. Rap is activated by a wide variety of extracellular signals. Second messengers including calcium, diacylglycerol, and cAMP directly activate Rap exchange factors and mediate activation of Rap (1). Rap was first identified in a genome-wide functional screen for proteins that could revert the transformed phenotype of K-Ras (2) and by searches for proteins homologous to Ras (1). This finding was followed by a number of reports that suggested that Rap antagonized Ras signaling; these reports were based principally on experiments using constitutively active mutants of Rap. However, our subsequent studies did not find evidence for antagonism of Ras signaling by endogenous Rap (3, 4). Rap is implicated in the modulation of a growing variety of cellular responses, including calcium signaling (5), secretion (5), and synaptic transmission (6), although the first and best characterized cellular function of Rap is the modulation of integrin-mediated adhesion (for review, see Ref. 7). In general, the capacity of integrins to mediate productive adhesion to the extracellular matrix is dependent on both the affinity of integrins for their extracellular matrix ligand and integrin avidity, which relates to the degree of clustering. Rap has been implicated in the modulation of both of these processes (8). A number of proteins have been shown to interact with Rap and are proposed to function as effectors for Rap. These proteins contain a Rap binding domain or structurally related Rap association RA domain (7). Recently, regulator of adhesion and polarization enriched in lymphoid tissues (RAPL) was identified as a protein that interacts with Rap and promotes adhesion in B-cells (9). Another potential candidate is AF-6, which binds both Rap and the actin-binding protein profilin (10). To what extent these or other putative Rap effectors mediate the effects of Rap has yet to be firmly established.

Cadherins are a large family of transmembrane adhesion molecules that mediate calcium-dependent homotypic adhesion between cells and thus the formation and maintenance of solid tissues (11, 12). E-cadherin is important for the formation and maintenance of contacts between epithelial cells. The modulation of cadherin-cadherin interactions is critical in normal processes such as morphogenesis and wound healing but can also influence the invasiveness of tumor cells. Indeed, loss or mutation of E-cadherin or associated molecules is a common feature in invasive carcinomas. Cadherins are regulated at both the transcriptional and posttranscriptional level. Strong cell-cell contacts are dependent on the linkage of cadherins to the actin cytoskeleton via catenins and the clustering of cadherins and associated proteins into adherens junctions (13). Activation of certain cell-surface receptors, including many growth factor receptors and integrins, results in intracellular signals that either disrupt or strengthen cell-cell contacts (14). GTPases of the Rho family (15, 16) and tyrosine phosphorylation of cadherin and the catenins (17) have been implicated in...
mediating these responses, although the mechanisms involved are poorly understood. Studies in Drosophila showed that loss of the Rap gene resulted in asymmetric positioning of cadherins at cell-cell contact sites, suggesting that Rap may directly modulate cadherin function (18). Furthermore, Dock4, an activator of Rap, which is disrupted in a number of human cancers, was found to enhance the formation of adherens junctions, suggesting a role for Rap in cadherin-mediated cell-cell adhesion in mammalian tissues (19). We have examined the role of Rap in E-cadherin-mediated adhesion. We find that Rap is required for the formation and maintenance of cadherin-mediated adhesion and that this appears to be independent of the effects of Rap on integrins.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Plasmids—**Madin-Darby canine kidney (MDCK) cells stably expressing the ectopic receptor (MDCK-Eco) were provided by Dr. Saskia van Es, The Netherlands Cancer Institute. MDCK cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum in a humidified incubator at 37°C and with 5% CO2. To generate MDCK empty vector and Epac1 stable cell lines, MDCK-Eco cells were infected with Epac1 or empty vector ectopic virus. 48 h after infection, cells were placed under selection with Zeocin (0.2 mg/ml). The Epac1 gene was linked via an IRES sequence to a Zeocin resistance gene and therefore transcribed onto a single mRNA. Thus, cells resistant to Zeocin can be presumed to express Epac1. Epac expression was stable for a period of at least 8 weeks, the maximum period in which cells were used for experiments. Virus for infections was produced as described previously (20, 21) in Phoenix packaging cells that stably expressed Epac1 or vector control. MDCK-GFP-E-cadherin cells were generated similarly using MDCK cells infected with GFP-E-cadherin amphotrophic virus, followed by selection on Zeocin. Ras-transformed MDCK cells (f3 cells) (22) were infected with V12Rap amphotropic virus coexpressing GFP, and a positive polyclonal cell pool was obtained by fluorescence-activated cell sorting. OvCar3 cells were obtained from ATCC and were cultured in RPMI supplemented with 10% fetal calf serum. Canine E-cadherin-human Fc chimeric secretory cells (293-ET7) were provided by Dr. W. J. Nelson (Stanford University) and were cultured in Dulbecco’s modified Eagle’s medium high glucose supplemented with 10% fetal calf serum. Chimeric protein was purified from conditioned medium using a 2-mL protein G-Sepharose column (HI-Trap, Amersham Biosciences), eluted, dialyzed, and concentrated. The chimeras are secreted as dimers, considered to be the minimal functional unit for homotypic E-cadherin interactions (23). V12RapA in LZRS-GFP was from Dr. Rob Roovers, University Medical Centre, Utrecht. Epac1 was subcloned from a PML2 vector (24) using SalINotI into the LZRS-Eco vector (21). GFP-E-cadherin was subcloned into the LZRS-Eco vector. For transient transfections, plasmids RapGAP1, N17Rap1, V12Rap1, and the Ras binding constructs, cells were detached with EDTA (1 mM) at 37°C and recovered for 1 h in serum-free medium with 0.5% BSA. 5 × 105 cells were added to wells of 96-well Nunc Maxisorp plates that had been precoated with the indicated proteins and left to adhere at 37°C for 3 h. Wells were washed three times with wash buffer (PBS, 1.8 mM CaCl2, 2 mM MgCl2) and lysed, and luciferase was measured as described previously (28). Wells were coated with fibronectin (5 μg/ml) overnight at 4°C and blocked with 1% heat-denatured BSA. For E-cadherin binding, wells were coated overnight with 10 μg/ml goat anti-Fc (Jackson Laboratories) and blocked with 1% heat-denatured BSA, followed by incubation with 50 ng/ml recombinant Fc-E-cadherin.

**RESULTS**

**Activated Rap Promotes Cadherin-mediated Cell-Cell Contacts in Mesenchymal Ras-transformed MDCK Cells—**To examine the role of Rap in homotypic cadherin interactions, we examined the effects of expression of constitutively active Rap1A on the morphology of Ras-transformed MDCK-f3 cells (f3 cells) (17). Despite expression of E-cadherin, f3 cells failed to form significant cadherin-mediated cell-cell contacts and displayed a mesenchymal phenotype. Stable expression of V12RapLA by retroviral transduction resulted in a phenotypic reversion of the Ras-transformed phenotype toward that of wild type MDCK cells. Thus, cells lost their fibroblastoid phenotype and instead grew in tightly packed colonies (Fig. 1A). Immunofluorescence microscopy showed that consistent with this phenotypic change was an accumulation of cadherin and β-catenin at sites of cell-cell contact (Fig. 1B). Western blot analysis with antiphosphospecific MAP kinase antibodies showed that there was no effect of stable expression of the constitutively active Rap1 on MAP kinase activation and therefore that V12Rap1 did not exert its effects on cell morphology by antagonizing oncogenic Ras signaling (Fig. 1C). Furthermore, we detected no difference in the expression levels of a variety of different cadherin isoforms between wild type and V12Rap1-expressing f3 cells (not shown). We conclude that constitutive Rap1 activation induces the restoration of cadherin-mediated cell-cell adhesions and reversion of the Ras-transformed phenotype of MDCK-f3 cells.

**Endogenous Rap Signaling Antagonizes HGF-induced Cell Scattering—**To examine the effect of activation of endogenous Rap on cells, we generated MDCK cells in which the level of Rap activation could be directly regulated. To do this, we used retroviral induction to stably express Epac1, an exchange factor for Rap that is regulated by eAmp. Epac1 was found to be stably expressed as determined by Western blotting of cell extracts and by immunofluorescence using a monoclonal anti-Epac1 antibody (Fig. 2). We recently described the use of a CAMP analog, 8CPT-2Me-eAmp, which specifically activates Epac without activating protein kinase A (4). Thus, Rap1 activity could be stimulated with 8CPT-2Me-eAMP in Epac1-expressing MDCK cells. Cells were stimulated with HGF (Sigma) in the presence or absence of 100 μM 8CPT-2Me-eAMP (4). 18 h after treatment, cells were fixed with 3.7% formaldehyde. Phase-contrast images were obtained using a Leica inverted microscope and were photographed.

**Adhesion Assays—**Adhesion assays were performed essentially as described previously (29). Briefly, cells were transfected as above, 48 h after transfection with a luciferase reporter and the indicated constructs, cells were detached with EDTA (1 mM) at 37°C and recovered for 1 h in serum-free medium with 0.5% BSA. 5 × 105 cells were added to wells of 96-well Nunc Maxisorp plates that had been precoated with the indicated proteins and left to adhere at 37°C for 3 h. Wells were washed three times with wash buffer (PBS, 1.8 mM CaCl2, 2 mM MgCl2) and lysed, and luciferase was measured as described previously (28). Wells were coated with fibronectin (5 μg/ml) overnight at 4°C and blocked with 1% heat-denatured BSA. For E-cadherin binding, wells were coated overnight with 10 μg/ml goat anti-Fc (Jackson Laboratories) and blocked with 1% heat-denatured BSA, followed by incubation with 50 ng/ml recombinant Fc-E-cadherin.

**Rap1 Activation Assays and Immunoblotting—**Rap1 was assayed as described previously (30). 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 RaGDS. Precipitates were washed three times with lysis buffer and solubilized 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, which recognize both Rap1A and Rap1B isoforms, but not Rap2 (Santa Cruz Biotechnology). Epac1 in total cell lysates was detected with monoclonal anti-body 5D3. MAP kinase activity was determined with a phosp hospecific MAP kinase antibody (Cell Signaling Technology).
expressing MDCK cells, but not in empty vector-expressing cells, which do not express detectable levels of Epac1 (Fig. 2A). Immunofluorescence studies showed that Epac1 was expressed to varying levels in the polyclonal cell population and was localized predominantly at the plasma membrane, being particularly apparent at cell-cell junctions. The expression of Epac did not result in a discernable effect on E-cadherin distribution, nor did Rap activation produce such an effect when induced by overnight treatment with 8CPT-2Me-cAMP (Fig. 2B).

To further examine the role of endogenous Rap in cadherin-mediated cell-cell adhesion, we examined the effect of increased Rap activity on HGF-induced responses in MDCK cells. HGF treatment of empty vector control cells resulted in the reduction of cell-cell contacts, cell flattening, and acquisition of a migratory phenotype (Fig. 3A, middle panels). These morphological changes were less pronounced in cells expressing Epac, which have a higher basal level of Rap activity. Treatment with the Epac activator 8CPT-2Me-cAMP strongly suppressed HGF-induced morphological changes in Epac1-expressing cells, but had no effect on the HGF-induced response in control cells (Fig. 3A, lower panels). Consistent with these results, immunofluorescence studies showed that HGF treatment led to the reduction of E-cadherin staining at cell-cell contacts and its internalization into intracellular vesicles. Activation of Epac with 8CPT-2Me-cAMP suppressed the HGF-induced loss of E-cadherin from cell-cell junctions, most visibly in those cells expressing higher levels of Epac1 (Fig. 3B). These results suggest that increased activity of endogenous Rap suppresses HGF-induced disruption of cadherin-mediated cell-cell contacts and acquisition of a migratory phenotype.

Activation of c-Met, the cell-surface receptor for HGF, results in activation of a number of intracellular cell signaling pathways that ultimately promote the disruption of cadherin-cadherin adhesions. To determine whether Epac-Rap activation resulted in reduced signaling by c-Met, e.g. because of receptor internalization or reduced expression, we examined the effects on HGF-induced MAP kinase activation. Stimulation of cells with HGF resulted in an equal induction of MAP kinase phosphorylation in both empty vector and Epac1-expressing MDCK cells (Fig. 4). Furthermore, MAP kinase phosphorylation was not affected by activation of Epac-Rap signaling by 8CPT-2Me-cAMP. These results suggest that Rap activation does not prevent HGF-induced disruption of adherens junctions by suppressing signaling by c-Met or activation of the Ras-MAP kinase pathway. HGF stimulation did not stimulate Rap1 activation in MDCK cells. Indeed, in some experiments, such as that shown in Fig. 4, Rap1 activation was reduced following HGF treatment, although this was not a consistent observation.

**Rap1 Activity Is Required for the Maintenance of Adherens Junctions**—The above results suggest that increased Rap acti-
vation promotes cadherin-mediated cell-cell adhesion. To examine whether Rap activation is required for cadherin-mediated adhesion, we first studied the effects of Rap inhibition on the distribution of E-cadherin in OvCar3 ovarian carcinoma cells. Expression of the Rap GTPase activating protein Rap-GAP1, an inhibitor of Rap activity, induced a marked reduction in E-cadherin levels at the sites of cell-cell interaction. Indeed, cell-cell contacts were often broken between RapGAP-expressing cells and often resulted in the acquisition of a migratory phenotype (Fig. 5A). Expression of a dominant negative mutant of Rap1, N17Rap1, led to a similar loss of E-cadherin staining at cell-cell junctions (not shown). This suggests that Rap signaling is required for the maintenance of cadherin-mediated cell-cell contacts. Expression of a constitutively active Rap1 mutant, V12Rap1A, did not result in obvious changes in the distribution of E-cadherin (not shown).

**Homotypic E-cadherin Interactions Are Dependent on Rap1 Activity**—The observations that Rap activity levels influence cell-cell contacts suggest that Rap may directly modulate cadherin-cadherin interactions. However, Rap is also a critical regulator of integrin-mediated adhesion, raising the possibility that the effects of Rap on cell-cell adhesion are an indirect consequence of effects on integrin-mediated adhesion. We therefore examined E-cadherin homotypic interactions in the absence of integrin-extracellular matrix engagement. To do this, we adhered MDCK epithelial cells directly to surfaces coated with canine E-cadherin extracellular domains, expressed as a chimeric protein with Fc (Fc-E-cadherin). Adhesion to Fc-E-cadherin was inhibited by low concentrations of extracellular calcium (50 μM), which is consistent with the high calcium dependence of cadherin homotypic adhesion (Fig. 5B). In contrast, integrin-mediated adhesion to fibronectin was not inhibited at this calcium concentration. To further characterize adhesion to Fc-E-cadherin, MDCK cells stably expressing GFP-E-cadherin were adhered to Fc-E-cadherin-coated coverslips and examined by confocal microscopy. GFP-E-cadherin was expressed over the entire surface of the cells but was particularly enriched at the interface with the Fc-E-cadherin-coated surface and at sites of cell-cell contact (Fig. 5C). Cells also flattened, although they did not tend to spread extensively as observed on fibronectin (not shown). These observations confirm that MDCK cells adhere to Fc-E-cadherin via cellular E-cadherin molecules. They also define an experimental model whereby E-cadherin-mediated cell adhesion can be examined in the absence of integrin-extracellular matrix interactions.

To test the requirement for Rap signaling for cadherin-mediated adhesion, we transiently transfected various modulators of Rap activity and measured the effect on adhesion to Fc-E-cadherin. Expression of a dominant negative inhibitor of Rap, N17Rap1, or the Rap binding domain (RBD) of the Rap/Ras effector RalGDS strongly inhibited adhesion of MDCK cells to Fc-E-cadherin (Fig. 5D). These Rap inhibitors also strongly inhibit adhesion to fibronectin (Fig. 5D) (29), suggesting that integrin-mediated adhesion was also suppressed. Expression of constitutively active Rap1A did not significantly increase adhesion either to Fc-E-cadherin or to fibronectin, suggesting
transfected cells, fixed, and stained with anti-E-cadherin antibodies. Cells were co-transfected with Rap1GAP1 and GFP to identify adhesion. A Fc-E-cadherin was used to induce adhesion to Fc-E-cadherin. MDCK cells were added to wells coated with Fc-E-cadherin (E) or fibronectin (Fn) in the presence of 1.8 mM CaCl₂ (+) or 50 μM CaCl₂ (−), a calcium concentration that does not support E-cadherin-mediated adhesion. The number of adherent cells/microscopic field is presented (n = 4; ± S.D.). B, MDCK cells stably expressing GFP-E-cadherin were seeded on Fc-E-cadherin-coated coverslips for 2 h, fixed, mounted, and visualized by confocal microscopy. The same cells are shown imaged either at the plane of contact with the coverslip (basal) or at the approximate midpoint of the cells (medial). C, MDCK cells were transfected with either Rap1GAP1 (E) or fibronectin (Fn). The percentage of cells adhering to the wells compared with the total number of cells added is shown. RBD, Rap binding domain of RalGDS.

that GTP-Rap levels are not rate-limiting for the formation of cadherin-cadherin interactions under these experimental conditions. These results suggest that Rap signaling is a requirement for the formation of E-cadherin homotypic associations and that this is independent of the effects of Rap on integrin-mediated adhesion.

DISCUSSION

Rap signaling promoted cadherin-mediated cell-cell adhesion in two different experimental models. Ras-transformed MDCK cells (MDCK-f3) display a fibroblastoid phenotype, do not grow in colonies, and are highly invasive as a result of reduced E-cadherin-mediated cell-cell contacts (31, 32). Expression of constitutively active Rap1A induced the formation of cadherin-mediated cell-cell contacts in f3 cells and restored their epithelial phenotype. The mechanism by which Rap1 induces this epithelial reversion is not clear. Oncogenic Ras induces a fibroblastoid phenotype at least in part by the disruption of adherens junctions (AJs), a process that is mediated by mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) (33, 34). Rap1 was first identified as a suppressor of the Ras-transformed phenotype of fibroblasts (2). However, the effect of constitutively active Rap1 on the morphology of f3 cells was apparently not due to antagonism of signaling by oncogenic Ras because activation of a downstream effector of Ras, MAP kinase, was unaffected. Furthermore, results from our laboratory clearly show that Rap does not directly antagonize Ras signaling (3). It is more likely that Ras and Rap signals converge independently at the level of cadherin function. A similar explanation has been proposed for the rescue of the Ras-transformed phenotype, whereby enhanced integrin-mediated adhesion by Rap1 counters the reduced integrin adhesion induced by Ras (7).

Expression of activated GTPase mutants can lead to nonspecific effects that differ from those of transient activation of the endogenous GTPase (35). Therefore, to determine whether activation of endogenous Rap also promoted cell-cell adhesion, we generated an MDCK cell line expressing Epac1 in which Rap activation could be induced with the cAMP analog 8CPT-2Me-cAMP. This analog specifically activates Epac without activating protein kinase A (4). Although expression of Epac1 and subsequent activation with 8CPT-2Me-cAMP did not alone have a discernable effect on epithelial cell morphology, induction of Rap strongly inhibited HGF-induced disruption of cell-cell contacts. Immunofluorescence staining showed that E-cadherin was preserved at cell-cell contacts, particularly in those cells expressing higher levels of Epac. HGF-induced disruption of AJs also occurred in cells that were in the middle of colonies and thus unable to migrate. This suggests that disruption of AJs is an active response to HGF treatment and is not driven solely by migrating cells pulling away from each other. Our findings suggest therefore that Epac-Rap signaling prevented the breakdown of cell-cell contacts by preserving E-cadherin-mediated adhesion rather than inhibiting cell migration. HGF also induces the activation of Ras (36), suggesting a similar mechanism of action to that of oncogenic Ras in the antagonism of adherens junctions.

The integrity of AJs in OvCar3 cells was disrupted by inhibition of Rap signaling via various means. Thus overexpression of Rap1GAP1, which catalyzes the hydrolysis of GTP bound to Rap, results in inactivation of Rap and diminished cell-cell contacts. Similarly, expression of either N17Rap1 or the Rap binding domain of RalGDS, which are predicted to titrate out endogenous Rap strongly inhibited HGF-induced disruption of cell-cell contacts. Immunofluorescence staining showed that E-cadherin was preserved at cell-cell contacts, particularly in those cells expressing higher levels of Epac. HGF-induced disruption of AJs also occurred in cells that were in the middle of colonies and thus unable to migrate. This suggests that disruption of AJs is an active response to HGF treatment and is not driven solely by migrating cells pulling away from each other. Our findings suggest therefore that Epac-Rap signaling prevented the breakdown of cell-cell contacts by preserving E-cadherin-mediated adhesion rather than inhibiting cell migration. HGF also induces the activation of Ras (36), suggesting a similar mechanism of action to that of oncogenic Ras in the antagonism of adherens junctions.

Integrins are also signaling molecules that regulate numerous intracellular signaling pathways including those of the Rho family of small GTPases (37, 38), MAP kinase (39), and Src (40). These signaling pathways are also implicated in the regulation of adherens junctions and may be responsible for “cross-talk” between integrin and cadherin adhesion systems. Indeed, adhesion to different extracellular matrix proteins can either positively or negatively regulate cadherin-cadherin interactions (41–43).

To exclude the possibility that the modulation of cadherin-mediated adhesion by Rap was a result of effects on integrins, we performed adhesion assays using recombinant E-cadherin extracellular domains. Inhibition of Rap signaling potently inhibited adhesion of cells to both Fibroblast and fibronectin, suggesting that Rap activity is required for both integrin- and cadherin-mediated adhesion. The fact that adhesion to Fibroblast
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Like other integrins whose cytoplasmic tails couple to the actin cytoskeleton, association with the actin cytoskeleton appears to be a common theme of Rap activity. However, one exception to this is cadherin. Although association with the actin cytoskeleton is the underlying mechanism of Rap action, the mechanism by which Rap mediates these effects is not clear, and it remains to be seen whether Rap utilizes different effector molecules to control these different adhesion processes or whether one common event such as release of cytoskeletal restraints is the underlying mechanism of Rap action.

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