Rac Activation upon Cell-Cell Contact Formation Is Dependent on Signaling from the Epidermal Growth Factor Receptor*

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Cadherins are transmembrane receptors that mediate cell-cell adhesion. They play an essential role in embryonic development and maintenance of tissue architecture. The Rho family small GTPases regulate actin cytoskeletal dynamics in different cell types. The function of two family members, Rho and Rac, is required for the stability of cadherins at cell-cell contacts. Consistent with the published data we have found that Rac is activated upon induction of intercellular adhesion in epithelial cells. This activation is dependent on functional cadherins (Nakagawa, M., Fukata, M., Yamaga, M., Itoh, N., and Kaibuchi, K. (2001) J. Cell Sci. 114, 1829–1838; Noren, N. K., Niessen, C. M., Gumbiner, B. M., and Burridge, K. (2001) J. Biol. Chem. 276, 3305–3308). Here we show for the first time that clustering of cadherins using antibody-coated beads is sufficient to promote Rac activation. In the presence of Latrunculin B, Rac can be partially activated by antibody-clustered cadherins. These results suggest that actin polymerization is not required for initial Rac activation. Contrary to what has been described before, phosphatidylinositol 3-kinases are not involved in Rac activation following cell-cell adhesion in keratinocytes. Interestingly, inhibition of epidermal growth factor receptor signaling efficiently blocks the increased Rac-GTP levels observed after contact formation. We conclude that cadherin-dependent adhesion can activate Rac via epidermal growth factor receptor signaling.

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Cadherin-dependent adhesion is subject to regulation by cytoplasmic proteins, and recent work has focused on modulation by the Rho family of small GTPases. Members of this family of GTP-binding proteins regulate actin cytoskeletal dynamics and focal complex formation in different cell types (4). The function of two family members, Rho and Rac, is required for the stability of cadherins at cell-cell contacts (5). The exact mechanisms by which Rho and Rac operate are unclear, but we and others have shown that Rac plays a role in actin recruitment to junctions (6) and to clustered cadherin receptors (7, 8).

Rho and Rac function in signal transduction cascades downstream of a variety of cell surface receptors. For example, Rho mediates stress fiber formation in response to lysophosphatidic acid (LPA) stimulation, whereas Rac is required for growth factor-induced membrane ruffling (9). More recently it has been directly demonstrated that ligand binding to cell surface receptors can activate Rho and Rac (10, 11). In addition, the regulation of the activity of Rho, Rac, and Cdc42 by integrin engagement has been determined and shown to be dependent on time, matrix composition, and matrix concentration (12–15).

Lately, it has become apparent that cadherin-mediated adhesion can trigger intracellular signaling events including activation of phosphatidylinositol 3-kinases (PI3-kinases) and the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase pathway (16–18). In addition, the epidermal growth factor (EGF) receptor has been coimmunoprecipitated with the cadherin adhesion complex from epithelial cells and is activated upon cell-cell contact formation (18, 19).

Furthermore, recent work has demonstrated that cell-cell contact formation can activate Rac in Madine-Darby canine kidney and Chinese hamster ovary cells transfected with C-cadherin (20, 21). Spreading of E-cadherin-expressing Chinese hamster ovary cells on immobilized E-cadherin ectodomain also induces Rac activation (22). In addition, levels of GTP-bound Rac are higher in VE-cadherin-expressing endothelial cells than in VE-cadherin-null cells (23). Although these studies showed that Rac activation is dependent on cadherin function, they did not establish the functional significance of Rac activation upon cell-cell contact formation, nor did they address the question of whether cadherin clustering is sufficient to enhance GTP loading on Rac. Although two studies found that PI3-kinase activity was required for full Rac activation, inhibition of PI3-kinase function did not reduce Rac activity to basal levels (20, 22), suggesting that other signaling pathways

The abbreviations used are: LPA, lysophosphatidic acid; ERK, extracellular signal-regulated kinase; EGF, epidermal growth factor; PI3-kinase, phosphatidylinositol 3-kinase; GEF, guanine nucleotide exchange factor; CRIB, Cdc42/Rac interactive binding; PAK, p21-activated kinase; GAP, GTPase activating protein; MEK, mitogen-activated protein kinase/ERK kinase; BSA, bovine serum albumin; VE, vascular endothelial.
may be involved in Rac activation downstream of cadherin-mediated adhesion.

In light of these results, we were interested to determine whether cadherin-mediated adhesion could also activate Rac in keratinocytes. Normal keratinocytes provide the ideal system to look at Rac activation on induction of cell-cell adhesion. They may be involved in Rac activation downstream of cadherin-mediated adhesion.

To determine whether Rac does indeed play a role in regulation of cadherin localization at intercellular junctions, we investigated whether an activated version of Rac (L61Rac) can induce intercellular adhesion calcium chloride was added to 2 mM.

EXPERIMENTAL PROCEDURES

Cell Culture and Microinjection—Normal human keratinocytes (strains Kb and Sa, passages 3–7) were cultured as described previously (25). Cells were grown to confluence in low calcium medium (25). To induce intercellular adhesion calcium chloride was added to 2 mM.

Antibodies and Immunofluorescence—Immunofluorescence was performed as described previously (7). Primary monoclonal antibodies used were: anti-E-cadherin (HECD-1, mouse and ECCD-2, rat) (26); anti-P-cadherin (NCC-CAD-299, mouse) (27); anti-α,β,ε-integrin (VM-2, mouse) (28); anti-Rac (23A8, mouse); anti-activated EGF receptor from Transduction Laboratories (clone 74, mouse); and anti-diphosphorylated ERK1/2 from Sigma (mouse). Rabbit polyclonal antibodies were used: anti-α-catenin (VB1) (29); anti-phosphoSer473-Akt (PW66); anti-Akt (PW56) (30); anti-phosphoFyr1173-EGF receptor (R42pY1173) (31); anti-EGF receptor (Cell Signaling Technology); and anti-ERK2 (Santa-Cruz). Secondary antibodies and Dextran-Texas Red were from Jackson Immuno Research Laboratories (Stratech Scientific). Rabbit anti-mouse IgG and fluorescein isothiocyanate-phalloidin were bought from Sigma.

Pull-down Assays—Pull-down assays, using a GST-PAK-CRIB fusion protein, were performed essentially as described (32). One 9-cm dish of confluent keratinocytes was used per data point. Cells were lysed in lysis buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris-HCl pH 7.5, 150 mM sodium chloride, 10 mM magnesium chloride and 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM pefabloc). Lysates were incubated with PAK-CRIB beads (20 μg/dish) for 45 min, and beads were washed three times with wash buffer (1% Triton X-100, 50 mM Tris-HCl pH 7.5, 150 mM sodium chloride, and 10 mM magnesium chloride). Precipitated proteins and 2% of each lysate were separated by SDS-PAGE, blotted, and probed for Rac.

For antibody blocking experiments, cells were incubated with 5 μg/ml HECD-1 and 2 μg/ml NCC-CAD-299 for 30 min at 37 °C prior to addition of calcium. As a control, cells were preincubated with 7 μg/ml rabbit anti-mouse IgG. For clustering experiments, keratinocytes grown in low calcium medium were incubated for 15 min on ice with 5 μg/ml HECD-1 and 2 μg/ml NCC-CAD-299. Anti-mouse IgG was added to 35 μg/ml, and cells were incubated for 30 min at 37 °C.

For bead experiments, 15 μm latex beads (Polysciences) were coated with HECD-1 described as previously (7). Latrunculin B (Calbiochem) was titrated to 0.3 μM, which prevented actin cytoskeleton remodeling in keratinocytes without causing cellular retraction. Cells were treated with Latrunculin B or Me2SO control for 10 min and incubated with HECD-1-coated beads (2.4 × 10^5 beads per dish) for 10 min before lysis. In inhibition experiments, cells were incubated with 30 μg/ml LY294002 (Sigma), 316 nM tyrphostin AG1478 (Calbiochem) (18), 35 μM PD98059 (Calbiochem), or MeSO vehicle for 30 min prior to stimulation with 2 μM EGF for 5 min. In EGF stimulation experiments, cells were incubated with LY294002 or MeSO vehicle as described above before stimulation with 10 ng/ml EGF for 10 min.

Akt Phosphorylation—To probe for Akt phosphorylation, cells were lysed in lysis buffer (1% Triton X-100, 20 mM β-glycerophosphate, 20 mM sodium fluoride, 2 mM EDTA, 0.2 mM sodium vanadate, 10 mM benzamidine, 2.5 μg/ml microcystin, 1 μg/ml leupeptin, 1 μg/ml pepsta-
As reported previously (7), N17Rac inhibited localization of cadherin receptors at boundaries between injected cells (Fig. 1, A and B). Microinjection of L61Rac alone had no effect on cadherin localization at junctions after incubation for 1 h (Fig. 1, C and D). Interestingly, co-injection of L61Rac and N17Rac completely rescued the inhibitory effect of N17Rac on cadherin-mediated adhesion (Fig. 1, E and F). This effect is specific for L61Rac because L61Cdc42 cannot restore junctional cadherin staining in N17Rac-injected cells.2 Together these results provide strong evidence that N17Rac blocks localization of cadherin receptors at junctions by inhibiting Rac function. Thus, Rac plays an important role in the regulation of cadherin-mediated adhesion.

2 J. K. Zhang, unpublished observations.

**FIG. 2.** Rac is activated upon induction of cadherin-mediated adhesion in keratinocytes. *a*, GST-PAK-CRIB specifically pulls down GTP-loaded Rac. Lysates from confluent dishes of keratinocytes grown in the absence of cell-cell adhesion were loaded with GTPγS or GDP. GTP-Rac was pulled down with GST-PAK-CRIB and detected by SDS-PAGE, Western blotting, and probing for Rac. Samples of lysates were also fractionated to determine total Rac levels. *b* and *c*, confluent dishes of keratinocytes grown in the absence of intercellular contacts were stimulated with 2 mM calcium for various time periods (*b*, 0–20 min; *c*, 0–120 min), and the levels of GTP-Rac and total Rac were determined as described above. *d* and *e*, Rac activation is dependent on cadherin receptors. *d*, keratinocytes were incubated with a mixture of anti-E- and anti-P-cadherin antibodies or anti-mouse IgG for 30 min at 37°C. Cells were treated with calcium for 30 min before fixing and staining for E-cadherin. Bar, 50 μm. *e*, keratinocytes were incubated as described in *d* and treated with (+) or without (−) calcium for 30 min before lysing to assay for Rac activation. Results are representative of three experiments.

**Rac Is Activated upon Cell-Cell Contact Formation in Keratinocytes**—Because Rac activity is required for the stability of cadherin receptors at junctions, is Rac activated upon cell-cell contact formation in keratinocytes? To evaluate Rac activation, we performed pull-down assays utilizing the Cdc42/Rac interactive binding (CRIB) domain of the Rac effector p21-activated kinase (PAK) (32). We confirmed the specificity of the assay under our conditions by loading keratinocyte lysates with GTPγS or GDP and assessing Rac activity (Fig. 2a).

To investigate whether cell-cell adhesion can activate the small GTPase Rac, we grew keratinocytes in low calcium medium and induced cell-cell contacts for various time periods before measuring Rac activation. We found that endogenous Rac was activated 2–4-fold within 5 min of addition of calcium, and this activation was sustained up to 120 min (Fig. 2, *b* and *c*).
Cadherins and Rac Activation

Rac Activation Is Dependent on Functional Cadherin Receptors—It is possible that the up-regulation of Rac described above was caused by stimulation of calcium-dependent intracellular signaling pathways rather than induction of cadherin-dependent adhesion. To distinguish between these two possibilities, we inhibited cadherin function during the calcium switch. Keratinocytes express at least two members of the cadherin family (E- and P-cadherin) (26). Cells were preincubated with anti-E- and anti-P-cadherin antibodies at concentrations known to block cadherin function in keratinocytes (24). To confirm that the antibodies blocked cadherin function, we demonstrated that, in the presence of the inhibitory antibodies, no cadherin clustering was found at intercellular boundaries (Fig. 2d). Incubation with anti-cadherin antibodies prevented Rac up-regulation upon addition of calcium ions, whereas control IgG did not (Fig. 2e). Thus, the Rac activation is dependent on functional cadherin receptors, and addition of calcium ions per se is not sufficient to trigger Rac activation.

Clustering of Cadherins Activates Rac—These results demonstrated that cell-cell adhesion mediated by cadherins could activate Rac in keratinocytes. Because junction formation is accompanied by the clustering of cadherin receptors (3), we investigated whether clustering of cadherins with antibodies was sufficient to activate Rac. Keratinocytes grown in low calcium medium were incubated with anti-E- and anti-P-cadherin antibodies (mouse monoclonals). Clustering of the receptors was then induced by addition of anti-mouse IgG, before assaying for Rac activity. Under these conditions, we could observe clusters of cadherins on the cell surface by immunofluorescence (Fig. 3A). We found that clustering induced a modest increase in Rac activity relative to samples where the anti-cadherin antibodies were omitted (Fig. 3B). Quantification of the relative Rac activation in several experiments revealed that treatment of cells with anti-cadherin antibodies alone only induced a 1.2-fold increase in Rac activation relative to cells treated with anti-mouse IgG alone. Clustering of surface cadherins with two layers of antibodies induced a larger increase in Rac activation (1.7-fold; Fig. 3, B and F).

However, the degree of Rac activation after antibody clustering is lower than the activation observed following cell-cell contact formation (2–4-fold). We hypothesized that this weak Rac activation may be due to the small number of cadherin molecules per cluster formed. To overcome this problem, we incubated cells for 10 min in low calcium medium with beads coated with anti-E-cadherin antibodies. Immunofluorescence of cells treated with these beads revealed binding of anti-E-cadherin beads to the cell surface and recruitment of α-catenin and F-actin to the beads (Fig. 3C, arrowheads). In contrast, few beads coated with BSA alone bound to the cell surface, and those that did recruited little α-catenin or F-actin (Fig. 3C, arrowheads).
E-cadherin beads showed increased Rac activation in comparison with cells treated with BSA beads (Fig. 3D). Quantification of the results of three experiments revealed that anti-E-cadherin beads enhanced Rac activation by a factor of 2.1 compared with BSA beads (Fig. 3G), suggesting that clustering of cadherins is sufficient to activate Rac.

To determine whether new actin polymerization is required for Rac activation by clustered cadherin molecules, we treated cells with Latrunculin B prior to receptor clustering. Latrunculin B inhibits actin polymerization by sequestering actin monomers (36, 37). Treatment of cells with Latrunculin B for 10 min before adding anti-E-cadherin beads still allowed binding of beads to the cell surface and recruitment of α-catenin but blocked actin recruitment (Fig. 3C, arrows, bottom panels). Upon assaying for Rac activation, we found that Latrunculin B treatment per se caused an increase in Rac activation. Incubation with E-cadherin beads in the presence of Latrunculin B induced a further 1.4-fold increase in Rac activation (Fig. 3, D and G). Thus, Rac activation can occur in the absence of de novo actin polymerization, but full induction of Rac activity does require formation of new actin filaments.

However, the use of beads to artificially cluster cadherin receptors may resemble the phagocytic process. Rac activity is necessary for Fcγ-mediated phagocytosis (38). Therefore, the possibility exists that anti-E-cadherin beads may induce Rac activation by triggering early events in phagocytosis. As a control to confirm that this was not the case, we treated cells with beads coated with antibodies against another cell surface receptor, αβ3-integrin. As expected, these beads showed little staining for α-catenin but did recruit F-actin (Fig. 3C, asterisks). However, anti-αβ3-integrin beads did not induce increased Rac activity relative to BSA control beads (Fig. 3E). Thus, the clustering of cadherin receptors can specifically activate Rac.

Rac Activation Is Dependent on EGF Receptor Signaling but Not PI3-kinases—We next explored which signaling pathways operate downstream of cadherin-dependent contact formation to induce Rac activity. We investigated three different classes of signaling molecule: PI3-kinases, the EGF receptor, and ERK1/2.

We made use of the compounds LY294002, tyrphostin AG1478, and PD98059, specific inhibitors of PI3-kinases, the EGF receptor, and mitogen-activated kinase or ERK kinase-1 (MEK1), respectively (39–41). We confirmed that the inhibitors were functional under our experimental conditions by probing lysates for Akt phosphorylated on serine 473 (for LY294002; Fig. 4A) or activated ERK1/2 (AG1478 and PD98059; Fig. 4, B and C). As expected, phosphorylation and activation of these molecules were blocked.

Inhibition of keratinocytes with LY294002 increased Rac activity (compare with low calcium sample; Fig. 4D) but did not inhibit Rac activation induced by cell-cell adhesion. We therefore concluded that PI3-kinases do not play a major role in Rac activation downstream of cadherin adhesion in keratinocytes.

Similarly to LY294002, treatment of keratinocytes with tyrphostin AG1478 increased Rac activity (Fig. 4D). However, the drug dramatically decreased Rac activation upon induction of cell-cell adhesion. Therefore we concluded that EGF receptor function participates in cadherin-dependent Rac stimulation (Fig. 4D). Consistent with this conclusion, calcium-induced cell-cell adhesion induces EGF receptor activation in normal keratinocytes3 as has been observed in HaCat cells (18).

Rac activation in these experiments was quantified relative to the control low calcium cultures treated with MeSO4 (Fig. 4G; −calcium, DMSO). However, there was some variability in the degree of Rac activation induced by drug treatment per se. For this reason Rac activity was also calculated relative to the control low calcium cultures treated with each inhibitor and was arbitrarily set at 1 (Fig. 4F; −calcium).

The inhibitors Latrunculin B, AG1478, or LY294002 can all activate Rac in keratinocytes. To investigate whether this Rac activation is a nonspecific consequence of treatment with different inhibitors, we incubated cells with the MEK1 inhibitor PD98059. Treatment with PD98059 compound did not increase the basal level of Rac activity in low calcium medium, nor did it inhibit activation of Rac induced by cell-cell contact formation (Fig. 4E). Quantification of these experiments is shown in Fig. 4, H and I. Thus, blocking signaling pathways in general does not lead to Rac activation. In addition, these results suggested that the ERK/mitogen-activated kinase cascade is not involved in induction of GTP loading on Rac downstream of cadherin-dependent adhesion.

EGF-stimulated activation of Rac is PI3-kinase-dependent in fibroblasts (42). Given our results, we were interested to determine whether a similar pathway exists in keratinocytes. Cells grown in the absence of cell-cell contacts were incubated with the PI3-kinase inhibitor LY294002 and stimulated with EGF for 10 min. Whereas in untreated cells EGF stimulation induces a strong Rac activation, this is blocked by inhibition of PI3-kinase (Fig. 4F). Thus, as in fibroblasts, EGF-induced Rac activation is PI3-kinase-dependent in keratinocytes.

DISCUSSION

Our results demonstrate for the first time that activated Rac can rescue the inhibitory effect of a dominant negative Rac mutant on the localization of cadherin receptors at cell-cell contact sites. This effect is specific for activated Rac and cannot be mimicked by activated Cdc42,2 thus providing strong evidence that Rac plays a role in the regulation of intercellular junctions in keratinocytes. Similarly, our previous work has shown that active Rac cannot compensate for the loss of Rho function in cadherin-mediated adhesion and epithelial morphology (7, 35). Taken together, our results indicate that Rho, Rac, and Cdc42 have distinct functions during junction formation in keratinocytes.

We have shown that induction of cell-cell adhesion in keratinocytes specifically causes an up-regulation of Rac activity. This increased Rac activation is blocked by inhibition of cadherin function, consistent with published data (20, 21). Most importantly, here we have demonstrated that clustering of cadherins using antibody-coated beads per se is indeed sufficient to induce GTP loading on Rac. This response is specific to clustering of cadherins and is not achieved upon clustering of αβ3-integrins in keratinocytes.

We have shown previously that Rac plays a role in actin recruitment to clustered E-cadherin receptors (7). Now we demonstrate for the first time that Rac activation can occur independently of de novo actin polymerization. However, full induction of GTP loading on Rac requires formation of new actin filaments. Approximately 50% of cadherin receptors are associated with actin filaments in the absence of cell-cell adhesion (43). Thus, clustering of this pool of receptors would provide a framework for new actin polymerization. Subsequent adhesion-dependent Rac activation further enhances actin recruitment and polymerization, thereby stabilizing cadherin receptors at junctions.

In the course of these experiments, we observed that treatment of keratinocytes with two drugs that affect the actin cytoskeleton, Latrunculin B and cytochalasin D, induces Rac

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3 M. Betson, E. Lozano, and V. M. M. Braga, unpublished observations.
activation (Fig. 3D and data not shown). Both drugs inhibit actin polymerization, but they do so by different mechanisms (36, 37, 44). Interestingly, cytochalasin D treatment has also been shown to activate Rho (10). In addition, drugs that affect the microtubule network have been shown to influence Rho and Rac activity (10, 45). Thus, the activity of Rho family GTPases appears to be acutely sensitive to the polymerization state of microfilaments and microtubules. It is conceivable that regulatory proteins such as GEFs and GTPase activating proteins (GAPs) localize at cytoskeletal filaments and that upon disruption of the network they may be released/modified to up-regulate Rho and Rac activity.4

Similarly to what is observed with disruption of actin filaments, inhibition of PI3-kinases (LY294002) and the EGF receptor (AG1478) increased the basal levels of Rac activation. This was not a general consequence of treating cells with inhibitors of signaling molecules because the PD98059 inhibitor, which blocks MEK1 activation, did not enhance Rac activity. Although these results are surprising, the mechanisms via which these drugs may interfere with activation of Rho proteins are beyond the scope of this manuscript.

How can clustering of cadherin receptors promote Rac activation? To address this question we investigated which signaling pathways might be involved. In contrast to the work of Nakagawa et al. (20), we have found that Rac activation following cell-cell contact formation is not dependent on PI3-kinase activity. Our results are consistent with another report in which initial Rac activation following adhesion of E-cadherin-expressing Chinese hamster ovary cells to E-cadherin ectodomain is PI3-kinase-independent (22). Another situation in which Rac activation occurs independently of PI3-kinase activity is in chemoattractant-stimulated neutrophils (46).

In contrast, we have demonstrated that EGF receptor signaling participates in the stimulation of Rac activity in kera-

4 Braga, V. M. M. (2002) Curr. Opin. Cell Biol., in press.
tocytes upon junction formation. Inhibition of EGF receptor signaling blocks Rac activation both initially (Fig. 4D) and at later time points after induction of cell-cell adhesion.\textsuperscript{3} Consistent with this, in normal keratinocytes cell-cell adhesion activates the EGF receptor, and phosphorylated EGF receptor is recruited to cadherin clustered by beads.\textsuperscript{3} Preliminary work suggests that EGF receptor signaling is also required for increased GTP loading on Rac induced by clustering of cadherin molecules on beads.\textsuperscript{3} Our data are in agreement with the proposal that cadherin clustering leads to EGF receptor recruitment and activation at cell-cell contact sites (18, 19).

Similar to other cell systems (e.g. fibroblasts), EGF-stimulated Rac activation is PI3-kinase-dependent in keratinocytes. This suggests that EGF receptor-induced Rac activation proceeds by two different mechanisms, depending on how the EGF receptor is activated (i.e. by clustering with cadherins or by ligand binding). In support of this theory, there is some evidence in the literature suggesting that the consequences of EGF receptor activation may depend on both the ligand and the localization of the receptor in the cell (apical or basolateral domains) (47–49).

Based on the fact that Rac function stabilizes cadherin receptors at junctions (7) and our present results, we expected that the EGF receptor inhibitor (AG1478) would interfere with establishment of cell-cell contacts. To our surprise, the inhibition of EGF receptor signaling does not block localization of cadherins at junctions upon addition of calcium ions.\textsuperscript{3} Similar observations were made in Madine-Darby canine kidney cells; although PI3-kinase inhibition diminished Rac activation, it was not observed in Madine-Darby canine kidney cells; although PI3-kinase inhibition diminished Rac activation, it was not observed in endogenous keratinocytes (20).

Preliminary work has recruited to cadherin clustered by beads.\textsuperscript{3} Based on the fact that Rac function stabilizes cadherin receptors at junctions (7) and our present results, we expected that the EGF receptor inhibitor (AG1478) would interfere with establishment of cell-cell contacts. To our surprise, the inhibition of EGF receptor signaling does not block localization of cadherins at junctions upon addition of calcium ions.\textsuperscript{3} Similar observations were made in Madine-Darby canine kidney cells; although PI3-kinase inhibition diminished Rac activation, it did not block junction formation or recruitment of GEF-Rac to junctions (20).

One explanation for these observations is that the pull-down assay measures global Rac activation, it is possible that the results do not reflect localized changes in Rac activity at cell-cell contacts. Alternatively, EGF receptor inhibition may cause an increased rate of GTP cycling on Rac, so no net increase in GTP-bound Rac is observed (50).

Two further considerations should be taken into account here: (a) Rac activity is required for the stabilization of cell-cell contacts rather than cadherin clustering per se\textsuperscript{5} and (b) blocking Rac function only inhibits 50% of actin recruitment to clustered cadherin receptors (7), suggesting that alternative pathways also contribute to actin polymerization at junctions. One possibility is Cdc42, which can promote actin accumulation at cell-cell contact sites and is activated upon intercellular junction formation in MCF-7 cells (16, 51). Other GTPase-independent pathways may also be involved, including calcium signaling and Ena/VASP (52, 53). These pathways may compensate for the reduction in Rac activity caused by EGF receptor inhibition. Nevertheless, basal levels of activated Rac are still necessary for stable cell-cell contacts because complete inhibition of Rac activity destabilizes cadherin-mediated adhesion (Fig. 1) (7).

If EGF receptor-dependent Rac activation does not play a role in cadherin stabilization at junctions, which other signaling events would Rac activation trigger? Our recent results suggest that when EGF receptor signaling is inhibited, there is no delay in actin recruitment to junctions or formation of other adhesive structures such as desmosomes.\textsuperscript{5} We speculate that the EGF receptor-dependent Rac activation may play a role in other cadherin-dependent cellular processes, e.g. regulation of growth and/or differentiation of epithelia. Clearly more experiments are required to resolve this issue.

The link between EGF receptor activation and the increase in GTP loading on Rac is unclear presently. It is likely to involve activation of a Rac-GEF (promoting GTP loading on Rac) or inhibition of a Rac-GAP (thereby preventing GTP hydrolysis). Both mechanisms would result in an increase in the levels of Rac-GTP. Work is in progress to identify the GEFs or GAPs responsible for Rac activation during junction formation in keratinocytes.

Evidence is accumulating for the existence of a cross-talk between cadherin-mediated adhesion and growth factor signaling. For example, non-adhesive cadherin receptors associate with insulin receptor substrate 1 (54), whereas VE-cadherin interacts with VEGFR-2 in endothelial cells (55). The communication between cadherins and growth factor receptors may be analogous to the cross-talk observed between integrins and growth factor receptors, which regulates cell survival and proliferation (56). This cross-talk may also contribute to the differences in cadherin signaling in different cell types, depending on the growth factor receptor that is able to associate with the cadherin complexes.

Here we demonstrate in epithelia the first example of cross-talk between cadherins, growth factors, and small GTPases. In keratinocytes, cadherin receptor clustering is sufficient to activate Rac, and cadherin-dependent Rac activation may play a role in cadherin stabilization at junctions, which other signal-transduction pathways also contribute to actin polymerization at junctions. For example, non-adhesive cadherin receptors associate with VEGFR-2 (55). The crosstalk between cadherins and growth factor receptors may be analogous to the cross-talk observed between integrins and growth factor receptors, which regulates cell survival and proliferation (56). This cross-talk may also contribute to the differences in cadherin signaling in different cell types, depending on the growth factor receptor that is able to associate with the cadherin complexes.

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