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Activation of the Small GTPase Rac Is Sufficient to Disrupt Cadherin-dependent Cell-Cell Adhesion in Normal Human Keratinocytes

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To achieve strong adhesion to their neighbors and sustain stress and tension, epithelial cells develop many different specialized adhesive structures. Breakdown of these structures occurs during tumor progression, with the development of a fibroblastic morphology characteristic of metastatic cells. During Ras transformation, Rac-signaling pathways participate in the disruption of cadherin-dependent adhesion. We show that sustained Rac activation per se is sufficient to disassemble cadherin-mediated contacts in keratinocytes, in a concentration- and time-dependent manner. Cadherin receptors are removed from junctions before integrin receptors, suggesting that pathways activated by Rac can specifically interfere with cadherin function. We mapped an important region for disruption of junctions to the putative second effector domain of the Rac protein. Interestingly, although this region overlaps the domain necessary to induce lamellipodia, we demonstrate that the disassembly of cadherin complexes is a new Rac activity, distinct from Rac-dependent lamellipodia formation. Because Rac activity is also necessary for migration, Rac is a good candidate to coordinately regulate cell-cell and cell-substratum adhesion during tumorigenesis.

INTRODUCTION

Cell-cell adhesion is an essential feature of epithelia that ensures their polarized status and therefore their differentiation and physiological function. During tumorigenesis, the breakdown of intercellular adhesion has two main consequences: loss of epithelial characteristics and, as dedifferentiation proceeds, increased migration and metastasis of the dissociated cells. Cell-cell adhesion receptors of the cadherin family have been implicated in these cellular processes. First, it is well established that cadherin receptors play an important role in the development and maintenance of the differentiated epithelial phenotype during organogenesis and adult life (reviewed by Gumbiner, 1996). Second, cadherins participate in the contact inhibition of growth shown by nonimmortalized cells (St Croix *et al.*, 1998) and alterations in cadherin function are frequently found, and have a causal role, during tumor progression (Perl *et al.*, 1998).

Cadherins are transmembrane proteins that promote calcium-dependent intercellular adhesion between cells containing the same type of receptor (homophilic binding; Gumbiner, 1996). At the intracellular side, cadherin molecules associate with cytoplasmic proteins known collectively as catenins. At the surface of individual cells, it is believed that cadherin complexes are found as dimers in a lateral association mediated by their extracellular domains (Shapiro *et al.*, 1995; Brieher *et al.*, 1996; Nagar *et al.*, 1996; Yap *et al.*, 1997). Dimers from two opposing cells interact in an antiparallel manner (adhesive association) to form the structural unit of cadherin-mediated cell-cell adhesion (Chitaev and Troyanovsky, 1998). This adhesive interaction requires and is stabilized by extracellular calcium ions and, at the cytoplasmic side, by the association of cadherin receptors with the catenins and actin cytoskeleton (reviewed by Kemler, 1993; Yap *et al.*, 1997; Chitaev and Troyanovsky, 1998). Although nonadhesive cadherin complexes can weakly interact with the cytoskeleton (Sako *et al.* 1998), the cytoskeletal interaction is greatly enhanced during the formation of cell-cell adhesion, by the clustering of the adhesive complexes at contact sites (reviewed by Kemler, 1993; Brieher *et al.*, 1996; Yap *et al.*, 1997; Chitaev and Troyanovsky, 1998).

Over the past few years, much effort has been put into understanding how cadherin function adhesion is regulated from the cytoplasm. Recently, we and others have demonstrated that cadherin-mediated adhesion requires the activity of the cytosolic proteins of the Rho family of small GTPases (Braga *et al.*, 1997, 1999; Hordijk *et al.*, 1997; Takaishi *et al.*, 1997; Zhong *et al.*, 1997). They belong to the Ras superfamily of small GTPases, proteins whose function is

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regulated depending on the type of guanine nucleotide bound (reviewed by Van Aelst and D'Souza-Schorey, 1997). When GTP is associated, the small GTPases are in an activated form and competent for signaling. Upon GTP hydrolysis and liberation of phosphate, the small GTPases are inactivated, in a cycle that is tightly modulated by regulatory proteins (Van Aelst and D'Souza-Schorey, 1997). The output signal is dependent on the amount of time that GTP remains associated as well as the localization of the GTP-bound protein within the cell (brought about by GDP-GTP exchange factors or GEF) (Bokoch *et al.*, 1994; Michiels *et al.*, 1997).

The RHO subfamily members, Rho, Rac, and Cdc42, participate in a variety of cellular processes primarily involving actin cytoskeleton reorganization, such as cell-cell adhesion, cell-extracellular matrix adhesion, cytokinesis, and cell motility (Van Aelst and D'Souza-Schorey, 1997). Rho is involved in cell contractility and stress fiber formation, whereas Rac drives actin polymerization and formation of lamellipodia (Ridley and Hall; 1992; Ridley *et al.*, 1992). In epithelial cells, the activity of small GTPases is required both for the formation of new cadherin-mediated contacts and for the maintenance of stable junctions (Braga *et al.*, 1997, 1999; Hordijk *et al.*, 1997; Takaishi *et al.*, 1997; Zhong *et al.*, 1997). Rho and Rac effects on cadherin receptors are modulated by the maturation of the junctions and the cellular context in which the cadherin molecule is expressed (Braga *et al.*, 1999).

In simple epithelial cells such as Madin-Darby canine kidney (MDCK), exogenously expressed myc-tagged Rho and Rac localize at sites of cell-cell adhesion (Adamson *et al.*, 1992; Takaishi *et al.*, 1995, 1997; Jou and Nelson, 1998). Moreover, proteins that can interact with the small GTPase Rac can also localize at intercellular junctions, but the functional significance of their localization is not known (reviewed by Van Aelst and D'Souza Schorey, 1997; Hordijk *et al.*, 1997; Kuroda *et al.*, 1998). In MDCK cells, Rac activation correlates with an increased staining of cadherin receptors and actin at cell-cell borders, suggesting that Rac may strengthen cadherin-dependent adhesion (Hordijk *et al.*, 1997; Takaishi *et al.*, 1997).

However, although Rac function is necessary for cadherin-dependent adhesion, there is evidence in the literature that Rac can play a role during tumor progression. Rac activation is required for the full transformed phenotype induced by oncogenes such as Tiam-1, Ras, and Mas (Habets *et al.*, 1994; Khosravi-Far *et al.*, 1995; Qiu *et al.*, 1995; van Leeuwen *et al.*, 1995; Roux *et al.*, 1997; Zohn *et al.*, 1998). In addition, it has been shown that Rac activation can promote invasion of carcinoma and lymphoma cell lines (Habets *et al.*, 1994; Keely *et al.*, 1997; Shaw *et al.*, 1997). Although Rac clearly participates in cell migration, the question remains of how to reconcile its role in migration with the "strengthening" effect on cell-cell contacts.

In this article, we investigated in more detail the effects of Rac activation on the stability of cadherin receptors in human keratinocytes. Rac activation does not lead to increased levels of cadherin staining at the keratinocyte junctions, contrary to what has been shown for MDCK cells. Moreover, our results suggest that sustained Rac activation can specifically remove cadherin receptors from newly formed and stable cell-cell contacts in a concentration- and time-dependent manner. Interestingly, although the Rac-dependent loss of cadherin function was accompanied by changes in cell shape and protusion formation, we demonstrate that this is a new Rac activity, distinct from its reported cytoskeletal role in lamellipodia formation.

MATERIALS AND METHODS

Cells

Normal human keratinocytes (strain Kb, passages 3 to 7) were cultured on a mitomycin C-treated monolayer of 3T3 fibroblasts at 37°C and 5% CO_2 as reported previously (Rheinwald, 1989). Cells were routinely cultured in standard medium (DMEM:F-12 medium, 1:3 mixture; Imperial Laboratories, Hampshire, United Kingdom) containing 1.8 mM calcium ions and supplements as described, but with 5% fetal calf serum. Cultures grown in the absence of calcium-dependent cell-cell contacts used the same medium formulation as described above, but with 0.1 mM calcium ions and serum depleted of divalent ions by treatment with Chelex-100 resin (Bio-Rad, Richmond, CA; Hodivala and Watt, 1994). HaCat cells (immortalized, nontumorigenic human keratinocytes) were a kind gift from N. Fusenig, Deutches Krebsforschungszentrum, Heidelberg, Germany (Ryle *et al.*, 1989). In experiments in which the calcium switch was performed, HaCat cells were transferred to low-calcium medium (1–2 days after plating) and cultured until confluence as described above. Swiss 3T3 cells were routinely cultured as described previously (Ridley and Hall, 1992; Ridley *et al.*, 1992); cells were allowed to reach confluence and become quiescent for 6–10 d before seeding onto coverslips.

Antibodies

E-cadherin staining was performed using either ECCD-2 antibody (rat monoclonal) (Hirai *et al.*, 1989) or HECD-1 (mouse monoclonal; gift from M. Takeichi, Kyoto University, Japan; Shimoyama *et al.*, 1989). Integrin labeling was done using the anti-β1 integrin antibody P5D2 (mouse monoclonal) (Dittel *et al.*, 1993). The other monoclonal antibody used was anti-myc (mouse monoclonal 9E10). Secondary antibodies were bought from Jackson ImmunoResearch Laboratories, West Grove, PA (Stratech Scientific, Luton, United Kingdom): indodicarbocyanine (Cy5)-conjugated donkey anti-mouse IgG; fluorescein isothiocyanate-conjugated goat anti-mouse IgG and FITC-conjugated donkey anti-rat IgG. FITC-phalloidin was purchased from Sigma (Poole, United Kingdom).

Mutagenesis and Subcloning

Point mutations were introduced into constitutively active Rac (L61Rac) by polymerase chain reaction (PCR) with 5′ primers and 3′ primers containing the respective alanine-alanine substitutions in the putative second effector domain as follows: A147 A148 (lys^{147} glu^{148} converted to ala^{147} ala^{148}, respectively; or using single letter code; KE to AA); A162 A163 (glu^{162} arg^{163} to ala^{162} ala^{163}, respectively; QR to AA); A166 A167 (lys^{166} thr^{167} to ala^{166} ala^{167}, respectively; KT to AA), and A170 A171 (asp^{170} glu^{171} to ala^{170} ala^{171}, respectively; DE to AA). The PCR fragments were then subcloned as *Nco*I-*Eco*RI inserts into pGEX-2T-L61Rac. All L61Rac mutants were fully sequenced using the Stratagene kit.

L61Rac second effector domain mutants were PCR amplified and subcloned into the *Eco*RI/*Bam*HI sites of the yeast two hybrid vector pYTH9. The constructs were sequenced to confirm that the Rac sequence was fused in frame with the sequence encoding the GAL4 DNA-binding domain. To create pACTII-NIQGAP2, the sequence corresponding to amino acids 711-1579 of IQGAP2 was amplified using the primers GTG CTA CAT CAT CAT CGG AAG AG and CCT TGA TTG GAG ACT TGA CC and subcloned into the *Nco*I-*Bam*HI site of the GAL4-activation domain vector pACTII. Rac targets (ROK-α, MLK2, MLK3, and PAK) and RhoGAP subcloned

into pACTII vector were a kind gift from Alan Hall (Aspenstrom and Olson, 1995; Nagata *et al.*, 1998).

Recombinant Proteins

Recombinant proteins were purified as glutathione *S*-transferase-fusion proteins from *Escherichia coli* by using glutathione beads, thrombin cleaved (unless otherwise stated), dialysed, and concentrated essentially as described (Ridley *et al.*, 1992). The protein concentration of each batch was determined by bicinchoninic acid assay (Pierce, Rockford, IL), by using bovine serum albumin as standard, and the purity of the preparation was evaluated by separation in SDS-PAGE followed by Coomassie blue staining. Biological activity was determined beforehand in fibroblasts and keratinocytes as reported (Ridley and Hall, 1992; Ridley *et al.*, 1992; Braga *et al.*, 1997).

Recombinant proteins used were as follows: C3 transferase (used at 0.1 mg/ml), constitutively active forms of Rac (L61Rac, 4 mg/ml), Rho (L63Rho, 3.76 mg/ml), or H-Ras (V12Ras, 3.77 mg/ml). RacRho chimeras used were as follows: $Rac^{73}Rho$ (3.35 mg/ml), $Rac^{126}Rho$ (0.53 mg/ml), $Rac^{143}Rho$ (0.43 mg/ml), and $Rac^{175}Rho$ (2.39 mg/ml). L61Rac second effector domain mutant recombinant proteins were also prepared (see above for details): A147 A148 (0.89 mg/ml), A162 A163 (2.26 mg/ml), A166 A167 (2.91 mg/ml), and A170 A171 (3.57 mg/ml). In addition to GST, the following proteins were used uncleaved: RhoGAP (1.84 mg/ml); ROK-α (GBD, GTPase binding domain only, 4.44 mg/ml; gift from David Drechsel, Heidelberg, Germany; Burbelo *et al.*, 1995); PAK (GBD only, 4.42 mg/ml; Sander *et al.*, 1998); MLK2 (leucine-zipper and GBD domain; Nagata *et al.*, 1998); and POSH (GBD only, 2 mg/ml, kind gift from Anne Bishop (MRC-LMCB, London, UK); Tapon *et al.*, 1998).

Microinjection

Microinjection was performed essentially as described (Braga *et al.*, 1997). Confluent patches of keratinocytes grown in the absence of contacts were microinjected with the different recombinant proteins mixed with Dextran Texas-Red (Molecular Probes, Eugene, OR) to visualize the injected patches. Within 5 to 15 min after injection, cells were transferred to standard medium to induce calcium-dependent cell-cell contacts for additional 1 to 5 h. Alternatively, medium-sized colonies of keratinocytes cultured in standard medium (mature junctions) were injected with distinct recombinant proteins and incubated for different amounts of time in the same medium. Swiss 3T3 cells were seeded onto coverslips subconfluent and prepared for microinjection as reported (Puls *et al.*, 1999). After microinjection, cells were incubated for 15 to 30 min in the same medium.

Recombinant proteins were injected either neat or at the stated dilutions to better assess their effects on junction disassembly in keratinocytes or lamellipodia formation in Swiss 3T3 cells. Quantification of the effects of Rac mutants on cadherin-mediated adhesion was performed using the following criteria. Patches containing three or more cell-cell borders with perturbed cadherin staining between at least two different injected cells were scored and expressed as a percentage of the total number of microinjected patches. Between 30 and 50 patches (containing 4 to 10 cells each) were analyzed for any given mutant. Quantification of lamellipodium formation in Swiss 3T3 cells is expressed as the percentage of injected cells with lamellipodia/ruffles. Between 60 and 180 injected cells (Swiss 3T3) were scored for each recombinant protein tested. Statistical analysis was performed using Student's *t* test, assuming unequal variances. Activated Rac DNA (L61Rac-pRK5myc; Lamarche *et al.*, 1996) was microinjected into the nucleus of HaCat cells grown in low-calcium medium. After 2 h of expression, cells were transferred to standard calcium medium to induce junction formation for 4 h. Activated H-Ras (V12 Ras-pRK5myc) and dominant-negative Rac (N17Rac-pRK5myc) were injected in HaCat cells grown in standard medium (mature junctions) and incubated for 5 h. DNA was injected at 0.1 mg/ml.

Immunofluorescence

Cells were fixed in 3% paraformaldehyde for 10 min at room temperature, permeabilized, and stained as described previously (Braga *et al.*, 1997). In some experiments, cells were extracted with CSK buffer containing 0.5% Triton X-100 for 10 min at room temperature before fixation (Braga *et al.*, 1995). Single labeling for E-cadherin was performed by using the mouse monoclonal HECD-1 and FITC-conjugated anti-mouse IgG. Double labeling for cadherins and integrins was performed by sequential incubation with rat anti-E-cadherin monoclonal (ECCD-2), FITC-conjugated anti-rat IgG, followed by mouse anti-1 integrin antibody (P5D2), and Cy5-conjugated anti-mouse IgG. Stainining for myc-tagged proteins was performed using the mouse monoclonal 9E10 and Cy5-conjugated anti-mouse IgG. Filamentous actin in Swiss 3T3 cells was labeled with FITC-phalloidin. Confocal images were obtained (1-μm slices) at the plane in which the majority of cadherin staining in the injected patch was found and processed as reported (Braga *et al.*, 1999). For the Dextran-Texas Red image, the optical section is taken at a different plane (usually a few microns below) to show that the cells are still touching each other and not retracted at the end of the experiment.

Slot Blots

Fusion proteins were immobilized onto PVDF membranes (Millipore, Bedford, MA) by using a slot blot apparatus (Hoeffer, San Francisco, CA). Equal amounts of L61Rac and L61Rac containing additional mutations in the second effector region (A162 A163, and A170 A171) were loaded with radioactive GTP ([γ-^{32}P]GTP, 6000 Ci/mmol; NEN-DuPont, Boston, MA) and allowed to interact with the immobilized proteins as described (Lamarche *et al.*, 1996).

Yeast Two-Hybrid Interactions

Integrated yeast strains were created containing L61Rac and the L61Rac second effector domain mutants fused to the GAL-4 DBD (Aspenstrom and Olson, 1995). Yeast strains were transformed with cDNAs encoding various Rac binding partners in a GAL4-activation domain vector: pACTII-RhoGAP, pACTII-PAK, pACTα-ROK-α (GTPase binding domain only), pACTII-IQGAP2, pACTII-MLK2, and pACTII-MLK3 (Nagata *et al.*, 1998). Interactions were assayed by testing growth of colonies in the presence of 3-amino-1,2,4-triazole and by filter-lift β-galactosidase assay (Aspenstrom and Olson, 1995). IQGAP2 interactions were tested on 10 mM 3AT plates, as the association with activated Rac was barely detectable at the standard concentration used for the other targets (25 mM).

Mammalian Cell Transfections and c-Jun NH_2-Terminal Kinase 1(JNK1) Activation Assay

Cos-7 cells were transfected by DEAE-dextran method as described (Lamarche *et al.*, 1996). Plasmid amounts per 10-cm Petri dish were as follows: 5 μg of pCMV-FLAG-JNK1 with 1 μg each of pRK5myc, pRK5myc-RacL61, or the various RacL61 mutants. Twenty-four hours later, transfected cells were serum-starved for 16 h before lysis in 25 mM HEPES (pH 7.6), 1% (vol/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, 0.3 M NaCl, 50 mM NaF, 0.1 mM vanadate, 5 mM EDTA, 5 mM EGTA, 40 mM sodium pyrophosphate, and protease inhibitors. To quantitate the amount of JNK1 present in each experiment, one-tenth of each lysate was loaded onto 15% SDS-PAGE and transferred to nitrocellulose membrane. Flag-tagged JNK1 was visualized with an anti-FLAG monoclonal antibody (Sigma) and 0.1 Ci/ml protein A-^{125}I and quantitated by phosphorimage analysis. An equal amount of JNK1 protein was loaded onto 7.5% SDS-PAGE and transferred to nitrocellulose membrane. Activated JNK1 was determined with an antiphospho-JNK1 (Thr 183/Thr185) monoclonal antibody (New England Biolabs, Beverly, MA) and protein A-^{125}I and revealed by autoradiography. The relative levels of activated JNK1 were deter-

mined by phosphorimage analysis. To determine the amount of JNK1 in each lane, the membrane was stripped in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7, at 50°C for 30 min and incubated with an anti-FLAG antibody and revealed by chemiluminescence.

RESULTS

A Role for Rac during Tumorigenesis

As suggested by experiments in MDCK cells, Rac activation may strengthen cadherin-dependent contacts because it induces an increased localization of the receptors and actin at junctions (Hordijk *et al.*, 1997; Takaishi *et al.*, 1997). To test this possibility in normal keratinocytes, L61Rac was microinjected into cells grown in standard medium (mature contacts) and incubated for 2 h (0.5 mg/ml, Figure 1, a and b). Under these conditions, the concentration of cadherin receptors at cell-cell contacts (Figure 1, a and b) and their detergent solubility (Figure 1, c and d) were not increased in the injected keratinocytes compared with neighboring cells.

In addition, our results indicate that Rac activation was not sufficient to protect cadherin receptors from different destabilizing effects. One such stimulus is the expression of oncogenic Ras (V12H-Ras), which in keratinocytes interferes with stable cell-cell adhesion (Figure 1, e and f; our unpublished results; Espada *et al.*, 1999). Controls showed that when injected alone, H-Ras disrupted cadherin adhesion within 2 h (Figure 1, e and f). This effect was not changed or delayed by coinjection of activated Rac (Figure 1, g and h). Similar results were observed if junctions were perturbed by inhibition of endogenous Rho (our unpublished results; Braga *et al.*, 1997; Jou and Nelson, 1998). Our data support the conclusion that Rac activation does not increase the localization of cadherin receptors at junctions in normal human keratinocytes as shown in MDCK cell lines.

If Rac is not providing a protective effect for the keratinocyte junctions, what are the consequences of Rac activation? We asked whether Rac-signaling pathways could contribute to the destabilization of cell-cell adhesion seen during oncogenic transformation. Expression of activated H-Ras (V12Ras) in HaCat cells, a keratinocyte cell line, disrupted cadherin adhesion (Figure 2, d–f) similarly to what was observed in normal human keratinocytes (Figure 1, e and f). A dominant-negative form of Rac was expressed in HaCats to levels not high enough to perturb junctions (N17Rac, Figure 2, a–c). When N17Rac was coexpressed with activated Ras (V12Ras), a reduction in the signaling from Rac was sufficient to restore cadherin localization at cell-cell contacts (Figure 2, g–i). In contrast, inhibition of other pathways such as phosphoinositide 3-OH kinase (PI3 kinase) and mitogen-activated protein kinase (MAPK) during Ras activation in normal keratinocytes could only partially rescue the localization of cadherins at junctions (our unpublished results).

Rac Activation Specifically Perturbs Cadherin-dependent Cell-Cell Contacts

The above-mentioned results suggested that Rac can be activated during transformation and its activity may contribute to destabilization of junctions. We next addressed two questions: 1) whether Rac activation per se was sufficient to disrupt cadherin-dependent adhesion in human keratinocytes, and 2) whether Rac could specifically interfere with cadherin-mediated adhesion. Keratinocytes grown in standard medium (mature junctions) were microinjected with different concentrations of the same batch of activated Rac (L61Rac, 0.25–2.0 mg/ml, Figure 3) and incubated for 2 h. Cells were then fixed and double labeled for E-cadherin (Figure 3, b, e, h, and k) and β1-integrins (Figure 3, c, f, i, and l). With increasing concentrations of activated Rac, cadherin receptors were selectively removed from intercellular junctions (arrowheads, Figure 3), whereas localization of integrins remained unchanged over 2 h (arrows, Figure 3). Our data suggest that Rac activation can specifically destabilize cadherin receptors from mature cell-cell junctions in normal keratinocytes, because it had no significant effect on the localization of integrin receptors.

Activation of Rac Is Sufficient to Perturb Cadherin-dependent Adhesion in Human Keratinocytes

We have previously shown that newly formed junctions are more sensitive to the effects of the small GTPases (Braga *et al.*, 1999). The above-mentioned results with Rac activation were also confirmed during induction of intercellular junctions. Keratinocytes grown in low-calcium medium were microinjected with different dilutions of the same batch of activated Rac (L61Rac, 0.25–2 mg/ml, Figure 4). Cell-cell adhesion was induced for 2 h and cells were labeled for E-cadherin (Figure 4, b, d, f, and h). Our results showed that at lower concentration, L61Rac activity was compatible with stable cell-cell contacts (0.25 mg/ml, Figure 4, a and b; Braga *et al.*, 1997). However, increasing amounts of activated Rac clearly perturbed cadherin localization at junctions and cell morphology (0.5–2.0 mg/ml, Figure 4, c–h), with a concomitant formation of protusions and lamellae (Figure 4, e–h). Thus, lower concentrations of active Rac disrupted newly formed junctions (0.5 mg/ml) compared with the amount necessary to perturb mature junctions (1 mg/ml, Figure 3).

A time course was also performed by microinjecting L61Rac at a given concentration (0.5 mg/ml) into keratinocytes without cell-cell contacts, and transferring the cells to standard medium to induce intercellular adhesion for 1 to 5 h (Figure 5). Although a shorter incubation did not affect the cadherin staining or cell morphology (1 h, Figure 5, a and b), prolonged incubation after Rac activation interfered with both (2 and 5 h, Figure 5, c–f). These results are consistent with the data shown in Figure 4, and suggest that they did not result from toxicity of the more concentrated L61Rac protein solutions injected into keratinocytes.

To confirm the disruptive effect of activated Rac on junctions and exclude the contribution of any bacterial protein contaminants, we performed microinjections of L61Rac DNA into the nucleus. We were unable to obtain good expression levels when normal keratinocytes were used, despite testing a variety of different expression vectors. Instead, we used HaCat cells, a human keratinocyte cell line. After induction of cell-cell contacts for 4 h (total expression time 6 h), we observed a qualitative disruption of cadherin adhesion (Figure 5, g and h) as seen in primary keratinocytes (see also Figures 4, c–f, and 5, e and f). This result can be obtained with microinjection of either recombinant proteins or DNA encoding activated Rac in HaCat cells. Taken to-

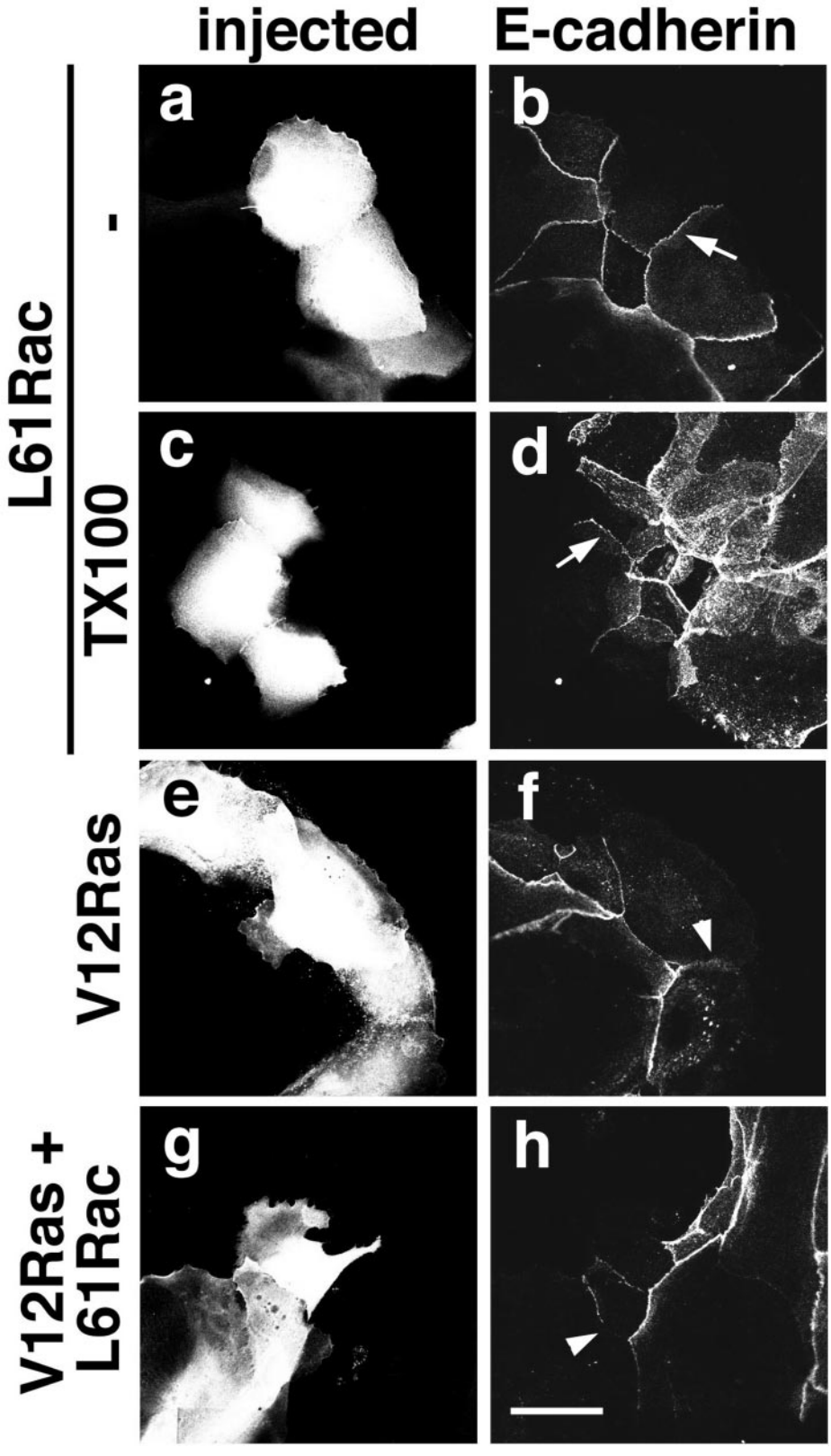


Figure 1. Rac activation does not strengthen cadherin-dependent adhesion in keratinocytes. Cells containing mature intercellular junctions were microinjected with constitutively active Rac (L61Rac, 0.5 mg/ml). Activated Rac was injected either alone (a–d), or in combination with activated H-Ras (V12Ras + L61Rac, g and h). Controls show keratinocytes injected with H-Ras alone (V12Ras, e and f). After 2 h of incubation, cells were fixed and stained for E-cadherin (b, d, f, and h). In c and d, keratinocytes were preextracted with Triton X-100 before fixation (see MATERIALS AND METHODS). Microinjected patches of cells are seen in a, c, e, and g. Arrowheads (f and h) show absence of cadherin staining at junctions. Arrows (b and d) point to similar levels of cadherin staining in microinjected cells. Bar, 50 μm.

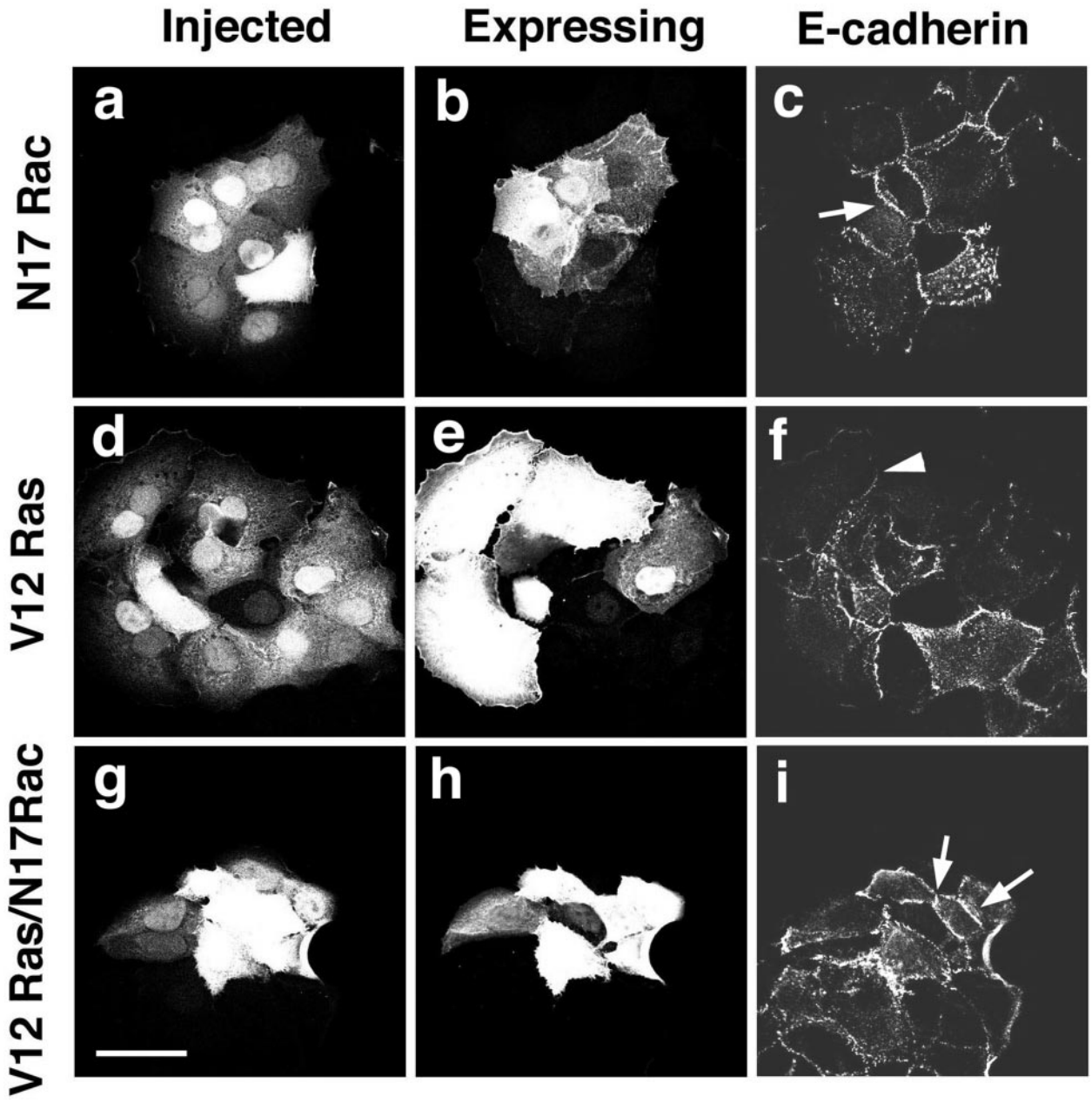


Figure 2. Rac signaling pathways contribute to the destabilization of cadherin receptors after Ras activation. HaCat cells were microinjected with an expression vector containing dominant-negative Rac (N17Rac, a–c), oncogenic Ras (V12Ras, d–f), or both together (g–i). After 5 h, cells were fixed and double labeled for the myc tag (b, e, and h) and E-cadherin (c, f, and i). Injected patches were identified by coinjection of Dextran-Texas Red (a, d, and g). Arrows (c and i) point to the presence of cadherin staining at junctions; arrowhead (f) shows the absence of cadherin staining between two expressing cells. Bar, 50 μm.

gether, our data indicate that sustained Rac activation during junction formation resulted in changes in cell shape and E-cadherin localization in a time- and concentration-dependent manner.

Although the L61Rac effects on cadherin adhesion in mature junctions were similar to those observed when new cell-cell contacts were established, epithelial cell shape was not significantly perturbed when keratinocytes have stable junctions (Figures 3, j–l, and 4, g and h) and higher levels of active Rac were required to disrupt the intercellular contacts (Figures 3, d and f, and 4, c and d). The same differences were observed in HaCat cells: although formation of junctions was readily affected by expression of active Rac, it was more difficult to disrupt mature contacts (our unpublished

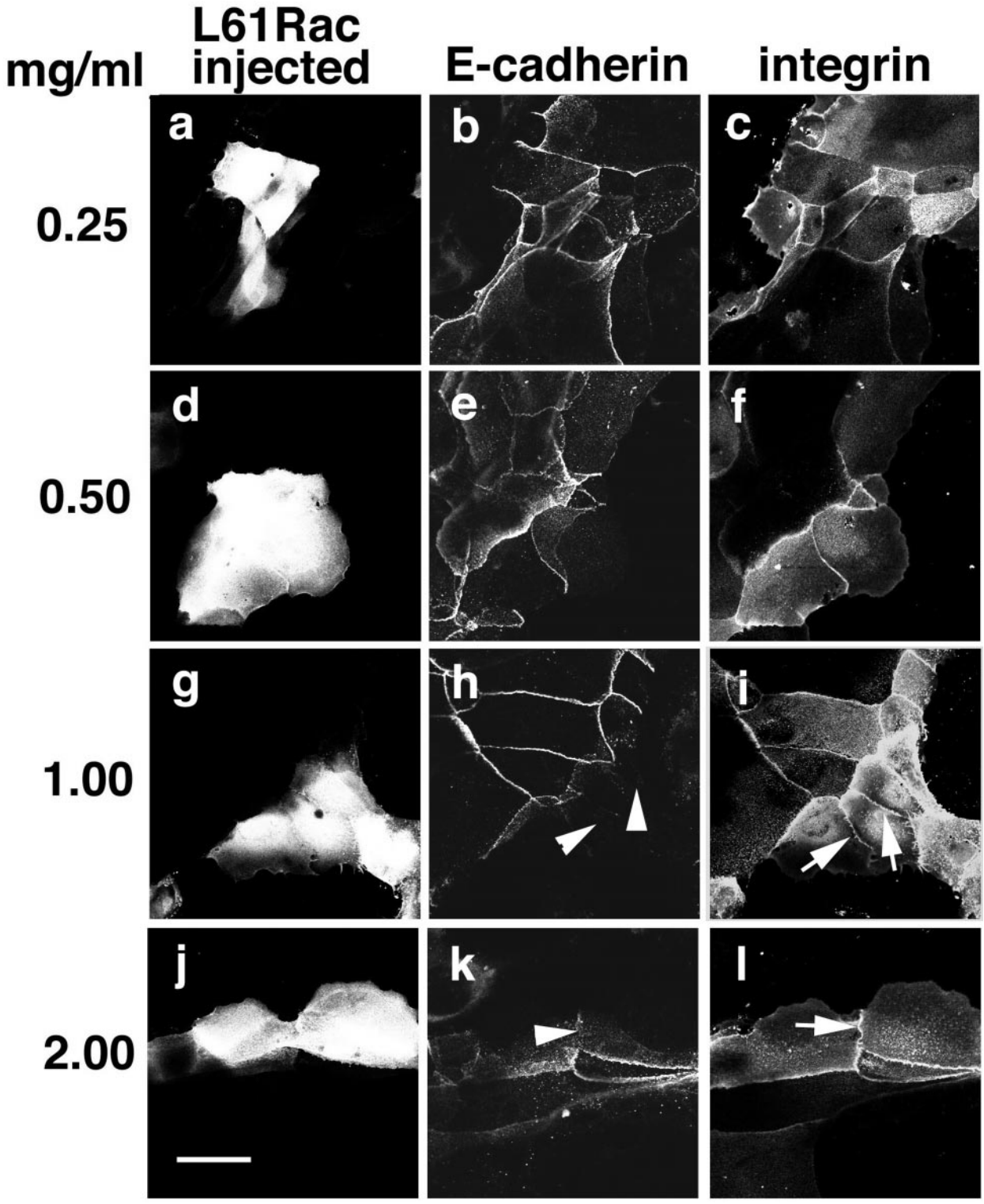


Figure 3. Increased Rac activation perturbs the stability of cadherin receptors in mature junctions. Keratinocytes grown in standard medium (mature cell-cell contacts) were microinjected with different concentrations of constitutively active Rac (L61Rac) as follows: 0.25 mg/ml (a–c), 0.5 mg/ml (d–f), 1.0 mg/ml (g–i), and 2.0 mg/ml (j–l). After a 2-h incubation in the same medium, cell were fixed and double labeled for E-cadherin (b, e, h, and k) and integrins (c, f, i, and l). Injected cells are seen in a, d, g, and j. Arrows (i and l) show integrin staining at junctions; arrowheads (h and k) show absence of cadherin receptors at junctions. Bar, 50 μm.

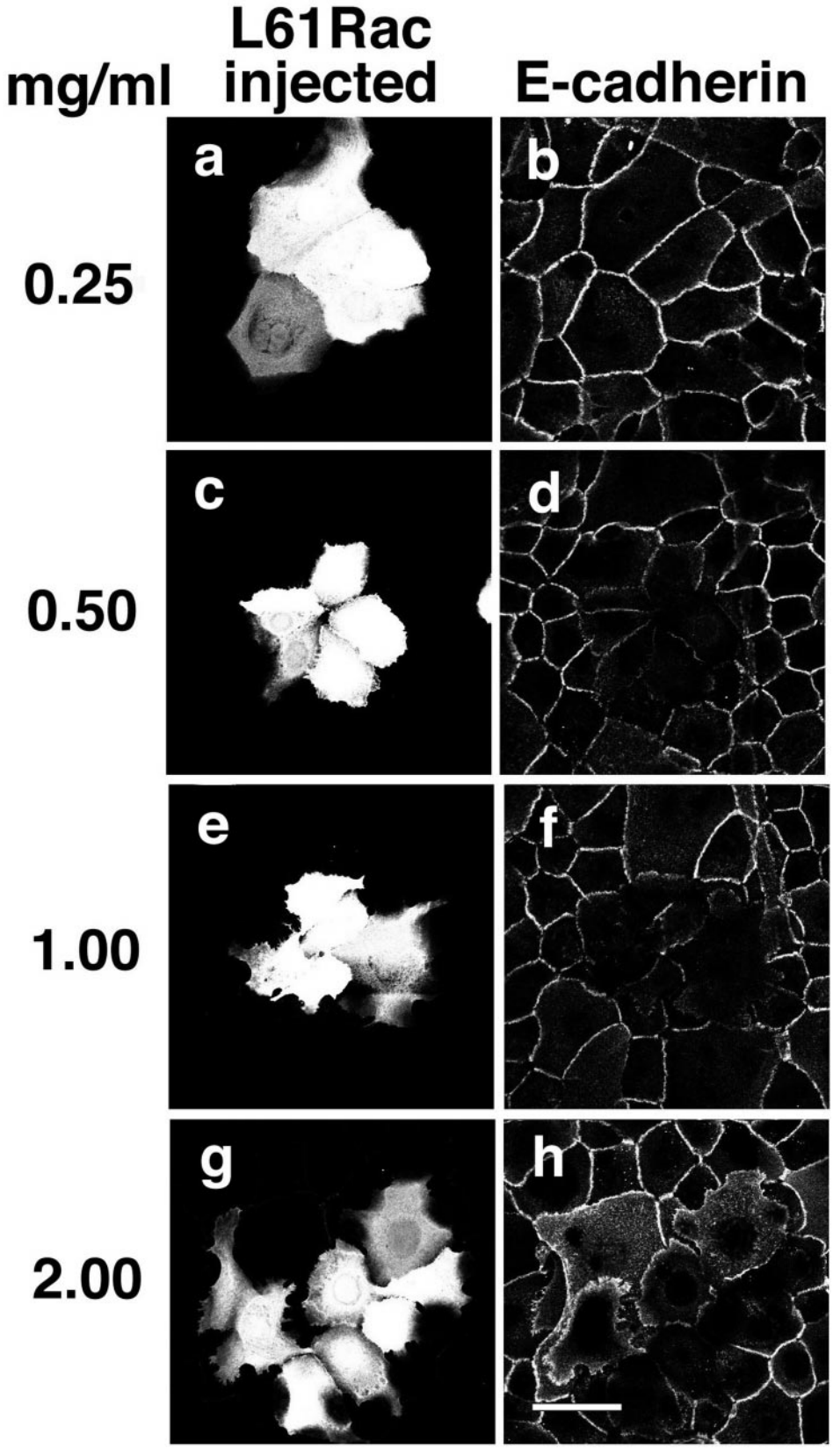


Figure 4. Increasing concentrations of constitutively active Rac (L61Rac) perturb newly formed cell-cell adhesion and epithelial cell shape. Different concentrations of activated Rac (L61Rac) were microinjected into keratinocytes in the absence of intercellular contacts as follows: 0.25 mg/ml (a and b), 0.5 mg/ml (c and d), 1.0 mg/ml (e and f), and 2.0 mg/ml (g and h). Calcium-dependent cell-cell adhesion was induced for 2 h; cells were then fixed and labeled for E-cadherin, followed by an FITC-conjugated anti-mouse IgG (b, d, f, and h). Injected cells are seen in a, c, e, and g. Bar, 50 μm.

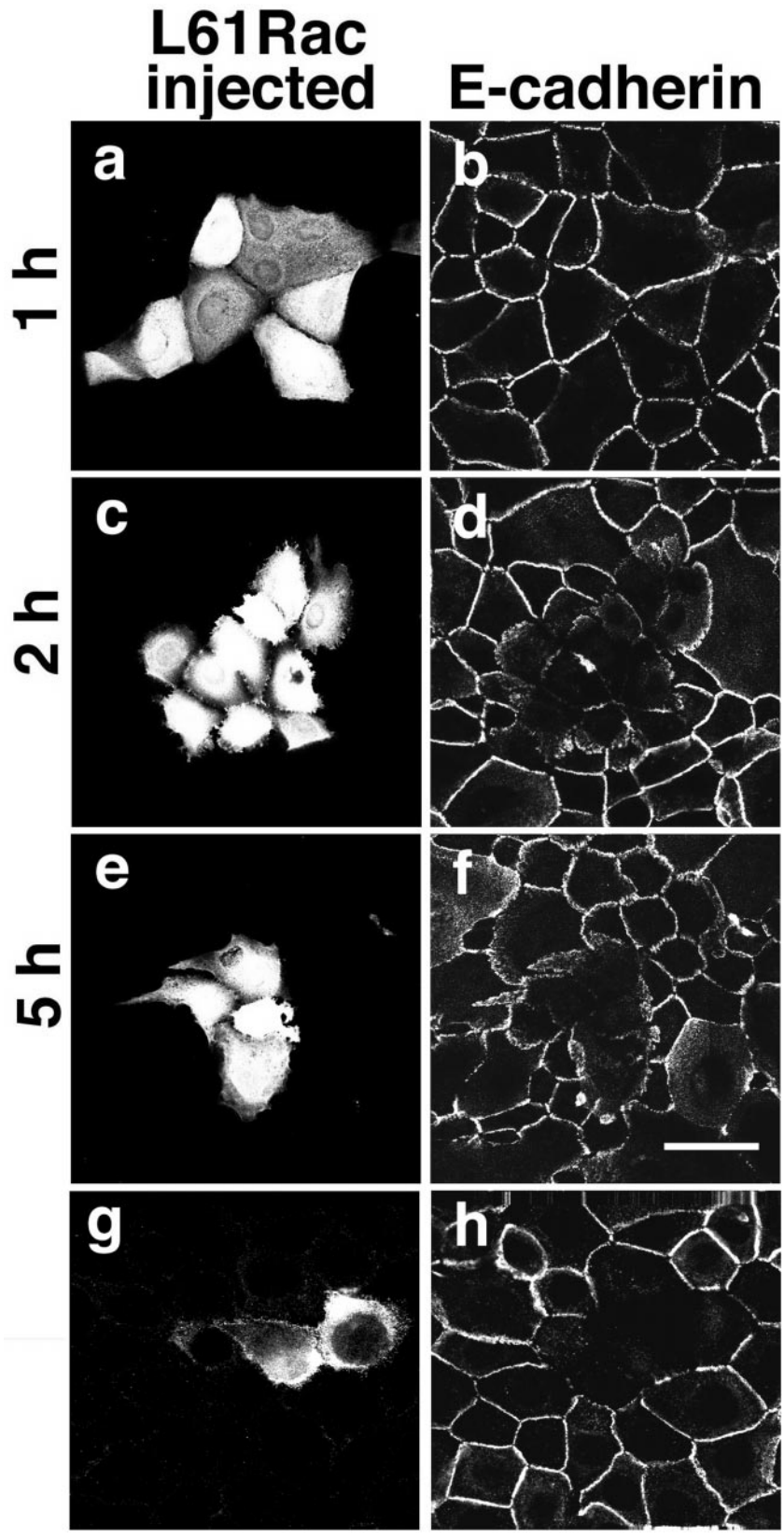


Figure 5. Sustained Rac activation interferes with the stability of newly formed cadherin-dependent contacts. Constitutively active Rac (L61Rac, 0.5 mg/ml) was microinjected into cells grown without contacts, and cadherin-dependent cell-cell adhesion was induced for 1 h (a and b), 2 h (c and d), or 5 h (e and f). Alternatively, L61Rac-pRK5myc expression vector was injected into the nucleus of HaCat cells (g and h), and after a 2-h expression, cell-cell contacts were induced for further 4 h. Cells were fixed and labeled for E-cadherin (b, d, f, and h) as stated in Figure 2; injected patches of cells are visualized in a, c, e, and g. In g, cells are labeled for the myc-tag epitope. Bar, 75 μm for g and h; 50 μm for all the other images.

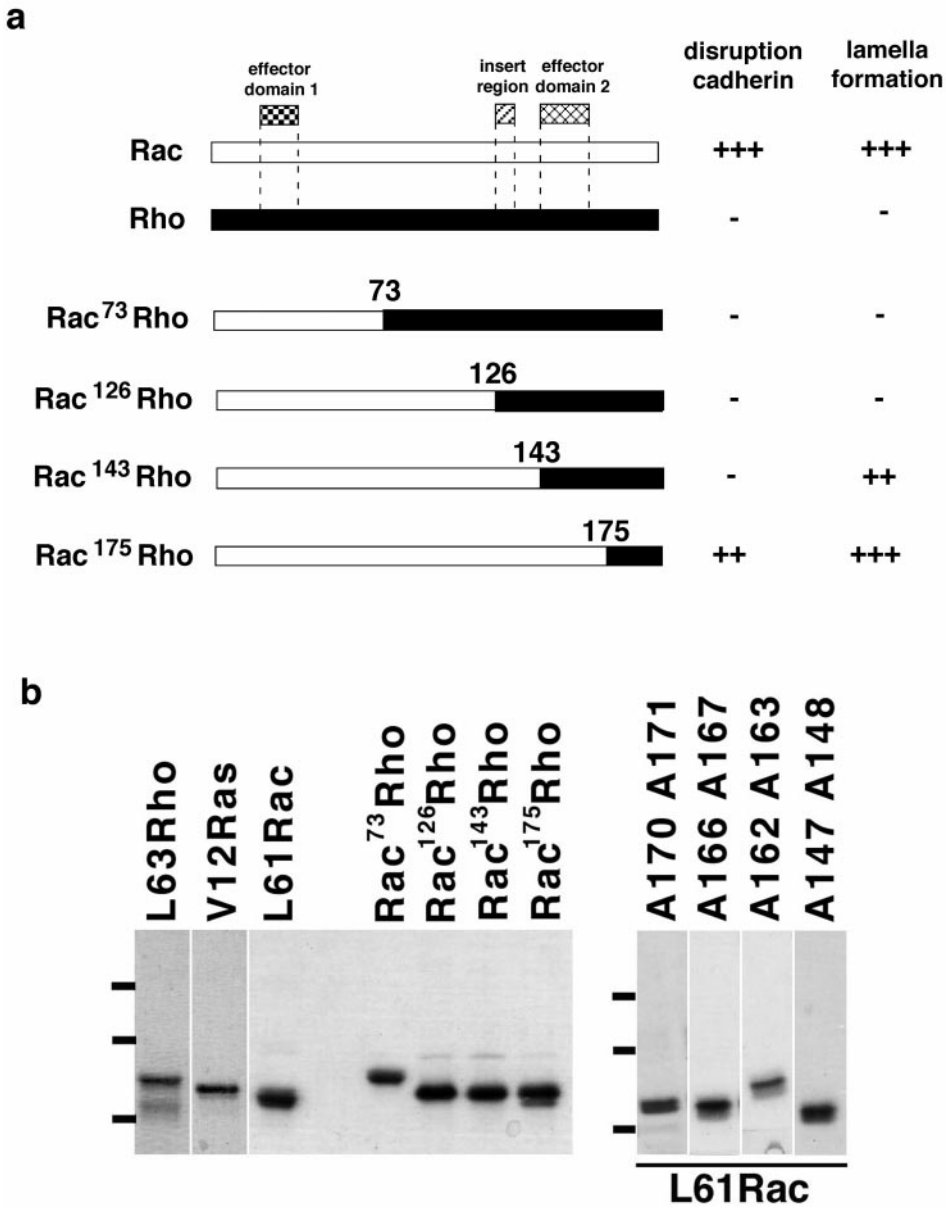


Figure 6. Mapping the Rac domain important for the disruption of cadherin-dependent adhesion. (a) Comparison of the Rac domains relevant for disruption of cadherin-mediated contacts in keratinocytes and lamella formation in Swiss 3T3 cells (Diekmann *et al.*, 1995; data not shown). Rac protein (□), Rho protein (■), and the small GTPase functional domains are represented: N-terminal effector domain 1, insert region, and the C-terminal effector domain 2. Chimeras containing portions of the Rac sequence (□) and Rho sequence (■) are also shown: Rac^{73}Rho (3.35 mg/ml); Rac^{126}Rho (0.53 mg/ml); Rac^{143}Rho (0.43 mg/ml); Rac^{175}Rho (2.39 mg/ml) (see text for details). Activated Rac and Rho were tested at 0.5 mg/ml. (b) Recombinant proteins used in this study were separated in SDS-PAGE and visualized by Coomassie blue staining. Molecular weight markers are shown on the left (top to bottom): 36.5, 26, and 20 kDa.

observations). The reasons for the distinct effects of Rac on mature junctions versus newly formed junctions are not clear.

Rac Domain Important for Disruption of Cadherin-dependent Contacts

We next determined which domain in the Rac protein is required for its inhibitory activity on cadherin function. Different chimeric molecules containing an activated Rac N-terminal domain and Rho C-terminal domain were microinjected into keratinocytes or Swiss 3T3 fibroblasts (summarized in Figure 6 a; Diekmann *et al.*, 1995; Kwong *et al.*, 1995; Nisimoto *et al.*, 1997). To demonstrate the purity of the microinjected proteins used in this study, the different recombinant proteins are shown in Figure 6b. After injection into keratinocytes without cell-cell contacts, intercellular junctions were induced for 4 h, and cells stained for E-cadherin (Figure 7, b, d, and f). As opposed to full-length activated Rac (0.5 mg/ml, Figure 5, e–h), activated Rho did not cause significant disruption of intercellular junctions at the concentration tested (0.5 mg/ml, our unpublished results). Rac^{73}Rho (3.35 mg/ml, our unpublished results), Rac^{126}Rho (0.53 mg/ml, Figure 7, a and b) and Rac^{143}Rho (0.43 mg/ml; Figure 7, c and d) had no significant effect on cell shape or the localization of cadherin receptors. In contrast, Rac^{175}Rho (2.39 mg/ml, Figure 7, e and f) interfered with cadherin stability and induced formation of lamellae in a similar manner to constitutively active, full-length Rac (0.5 mg/ml, Figure 5, e–h).

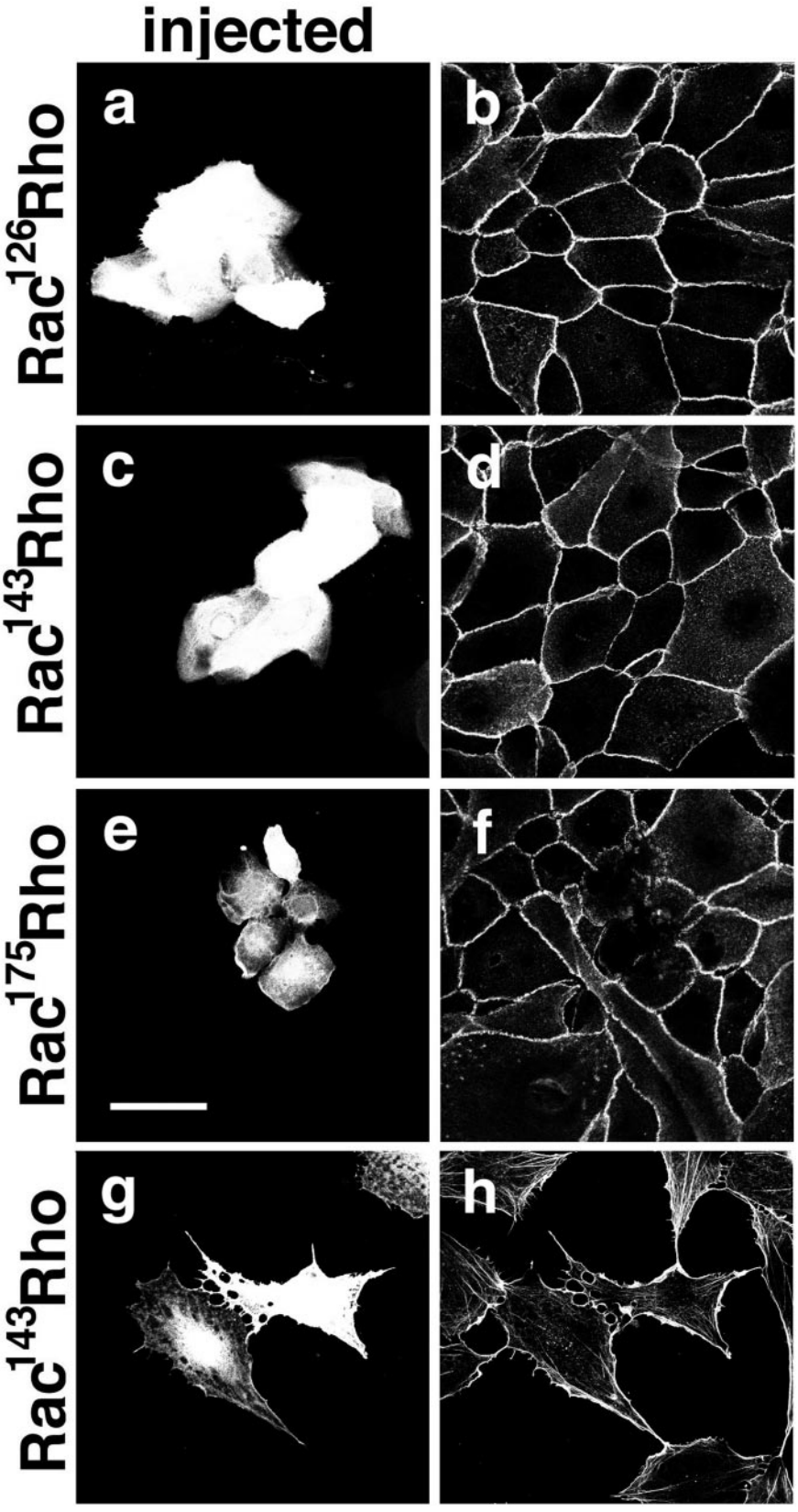


Figure 7. Different chimeric RacRho molecules were injected into keratinocytes grown in the absence of cell-cell contacts, and calcium-dependent adhesion was induced for 4 h (a–f). The following proteins were injected: $Rac^{126}Rho$ (a and b); $Rac^{143}Rho$ (c, d, g, and h); and $Rac^{175}Rho$ (e and f). Injected cells (a, c, e, and g) and E-cadherin staining (b, d, and f) are visualized. In g and h, $Rac^{143}Rho$ was injected into Swiss 3T3 fibroblasts and cells were stained for filamentous actin (h). Bar, 50 μm.

$Rac^{175}Rho$ diluted to 1 mg/ml showed the same disruptive effect (our unpublished results). These results suggest that the Rac sequence between amino acid residues 143 and 175 was necessary to perturb cell-cell contacts.

The chimeric molecules were also evaluated for their ability to induce ruffles by injection into serum starved Swiss 3T3 cells seeded onto fibronectin coverslips for 2 days (Puls *et al.*, 1999). Under these conditions, $Rac^{175}Rho$ induced ruffles and lamellipodia as previously reported (Diekmann *et al.*, 1995). However $Rac^{143}Rho$ was also able to promote lamellipodia formation (Figure 7, g and h; our unpublished results). A previous study did not demonstrate ruffling activity for $Rac^{143}Rho$ (Diekmann *et al.*, 1995). It is not clear why, but we attribute this difference to the culturing conditions, which may affect the nature of the response. In the former study, microinjection was performed on freshly plated, serum-starved fibroblasts (2 h, Diekmann *et al.*, 1995). In our study, we used cells seeded 48 h before in serum-free medium supplemented with 1/50 dilution of conditioned medium (Puls *et al.*, 1999). It is also possible that the protein batch used in our study is more active than the batch used before. The data are summarized in Figure 6a and, taken together, indicate that the Rac domains responsible for lamella formation and cadherin disruption did not overlap completely.

Two Distinct Pathways?

We confirmed the above-mentioned results by an alternative approach, site-directed mutagenesis. This approach allowed us 1) to dissociate between the Rac-induced lamellipodia activity and the Rac-dependent perturbation of cadherin adhesion, and 2) to map the important domain more precisely between amino acids 143 and 175. Mutations were introduced into L61Rac at different positions: A147 A148 (single letter code KE to AA); A162 A163 (QR to AA); A166 A167 (KT to AA); and A170 A171 (DE to AA) (see MATERIALS AND METHODS for details). These mutants were tested for their ability to disrupt cadherin-dependent contacts in keratinocytes or to induce lamella formation in Swiss 3T3 cells and representative pictures are shown in Figure 8. During formation of intercellular adhesion in keratinocytes, all the mutants showed qualitatively the same phenotype (but see below): no changes in cell morphology or significant decrease in cadherin staining at cell-cell borders in keratinocytes (Figure 8, a–d, and our unpublished results). Interestingly, induction of lamellipodia was not impaired in any of the mutants (as assessed by actin staining in fibroblasts, Figure 8, e–h; our unpublished results).

The quantification of these effects is shown in Figure 9. Control L61Rac at 0.5 mg/ml disrupted newly formed junctions in ~85% of the injected patches after 4–5 h of incubation (Figure 9a). On the other hand, when L61Rac second effector domain mutants were injected at concentrations between 0.9 to 2.9 mg/ml, cadherin-dependent contacts were perturbed in only 10% of injected patches (Student's *t* test, $p < 0.005$). The mutant A170 A171 (3.5 mg/ml) was the exception, showing perturbed junctions in 35% of the patches (Student's *t* test, $p < 0.01$; Figure 9a). On the other hand, all mutants were able to induce lamellipodia to a similar extent as L61Rac (at 2 mg/ml), and no significant difference was detected (Figure 9b; Student's *t* test). Further experiments using dilutions of two of the mutants, A162 A163 and A170 A171, revealed that their ruffling activity could be titrated down in a similar pattern to L61Rac (Figure 9c). These results are summarized in Figure 9d and taken together suggest that disruption of cadherin adhesion and lamella formation are two independent activities triggered by Rac.

To assess whether the Rac mutants could interact with known Rac targets, in vitro binding assays were performed using recombinant proteins. Fusion proteins containing the GTPase binding domain of known Rac targets were immobilized onto membranes and probed with radioactively labeled Rac or the second effector domain mutants (Figure 10 a; Burbelo *et al.*, 1995; reviewed by Van Aelst and D'Souza Schorey, 1997). In addition, interactions were tested by yeast two-hybrid technique and evaluated by growth on 3AT plates (Figure 10b and Table 1) or β-galactosidase filter assay (our unpublished results). Both techniques produced similar results: no binding was detected to the negative controls (GST, Figure 10a; empty vector, Figure 10b). All GTPases interacted similarly with RhoGAP, PAK, ROK-α, and IQGAP2 (Figure 10; our unpublished observation). Two Rac mutants (A147 A148 and A162 A163) were able to interact with POSH and MLK2. The only target that showed limited binding to all 4-s effector mutants is MLK3 (Table 1). In addition, with the exception of A170 A171, all other Rac mutants were able to activate the JNK kinase pathway (Table 1; our unpublished results). Because of the similar properties of L61Rac and second effector mutants, we concluded that these mutations did not affect the overall shape and activity of the mutants. Instead, the double alanine mutations interfered with the interaction with a particular subset of target(s) (among them MLK3).

DISCUSSION

In this article, we identified Rac as a key regulator of cadherin-mediated adhesion in human keratinocytes. Our major findings are as follows: Rac-signaling pathways contribute to the destabilization of cadherin receptors at junctions during Ras transformation in keratinocytes. During epithelial tumorigenesis, sustained levels of Rac activation can be achieved in vivo (Mira *et al.*, 2000) and we demonstrate that Rac activation is sufficient to specifically disrupt cadherin-dependent adhesion. In addition, we demonstrate that perturbation of cell-cell contacts is a new Rac activity, distinct from lamellipodia formation. We mapped the putative second effector domain of Rac as an important domain for disruption of cadherin receptor localization, and produced mutants that can be useful tools to identify putative Rac targets. These results are discussed below.

Transfection of activated Rac in MDCK cells induces enhanced levels of cadherin and actin at junctions (Hordijk *et al.*, 1997; Takaishi *et al.*, 1997). In addition, transfection of Tiam-1, a Rac activator, can cause a reversion of the fibroblastoid morphology of Ras-transformed in MDCK (Hordijk *et al.*, 1997; Sander *et al.*, 1998). Contrary to the above-mentioned reports, Rac activation in normal human keratinocytes does not result in an increased localization or stability of cadherin receptors at cell-cell contacts. Our results are consistent with published data that Rac activation promotes cell-cell adhesion breakdown and migration of different carcinoma cells (Keely *et al.*, 1997). In other epithelial cell lines, Rac activation also plays a role in scattering after distinct

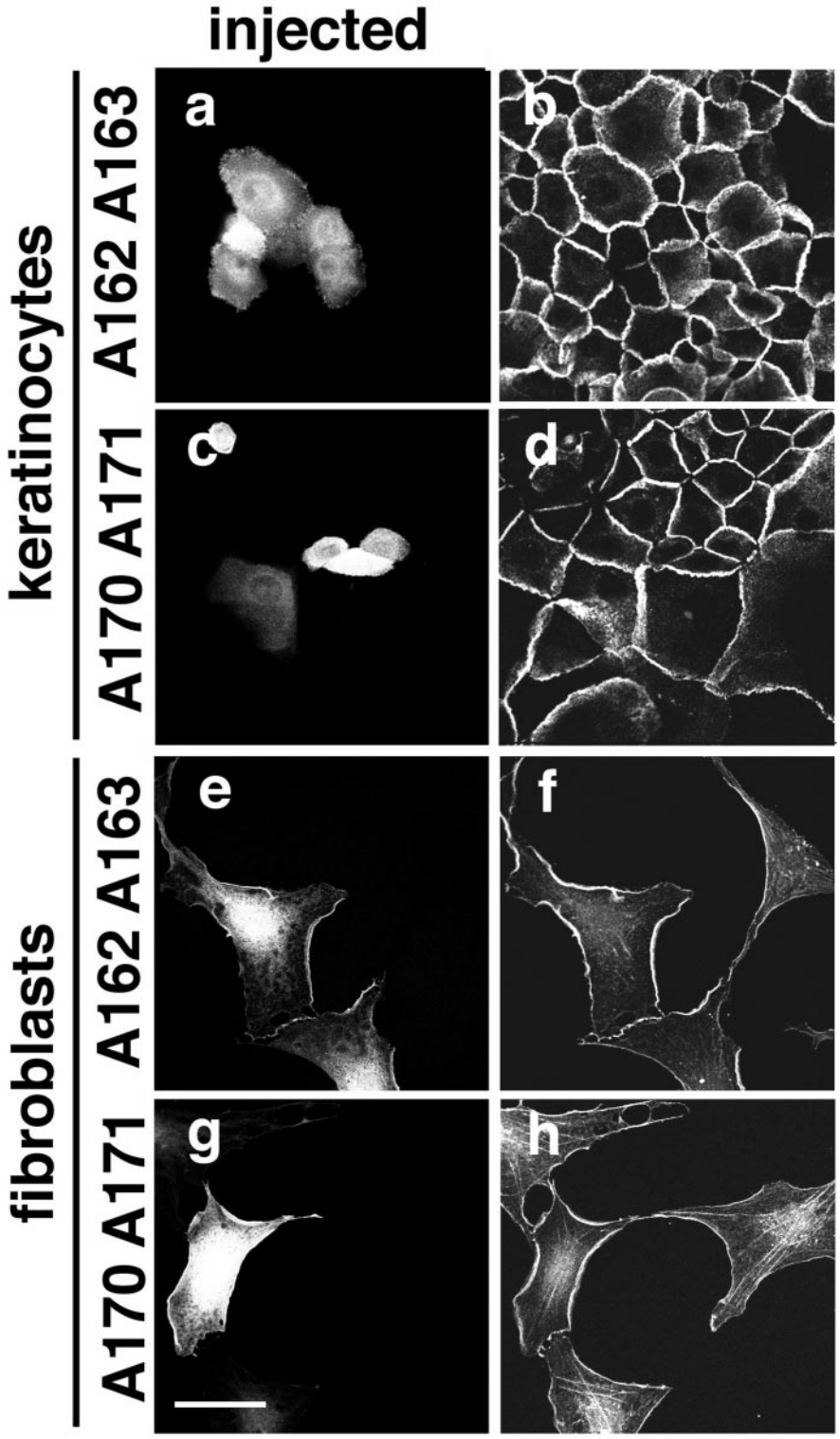


Figure 8. Second effector domain analysis with respect to disruption of cadherin-dependent adhesion and lamella formation. Different positions within the putative second effector domain were mutated to alanine to generate four mutants in a constitutively active Rac background. Similar results were obtained for all mutants, and representative pictures are shown for the mutants A162 A163 (a, b, e, and f) and A170 A171 (c, d, g, and h). To evaluate disruption of cadherin function in keratinocytes (a–d), cell-cell contacts were induced for 4–5 h after microinjection, and cells were then fixed and stained for E-cadherin (b and d). The same mutants were also analyzed for their ability to induce formation of lamellae/ruffles in Swiss 3T3 cells, after staining with FITC-phalloidin (e–h). Bar, 50 μm.

stimuli such as growth factor stimulation or integrin engagement (Takaishi *et al.*, 1994; Ridley *et al.*, 1995; Shaw *et al.*, 1997; Potempa and Ridley, 1998; Gimond *et al.*, 1999).

This controversy found in the literature might reflect the distinct cellular context, methodology used or different levels of Rac activation achieved. It is also conceivable that

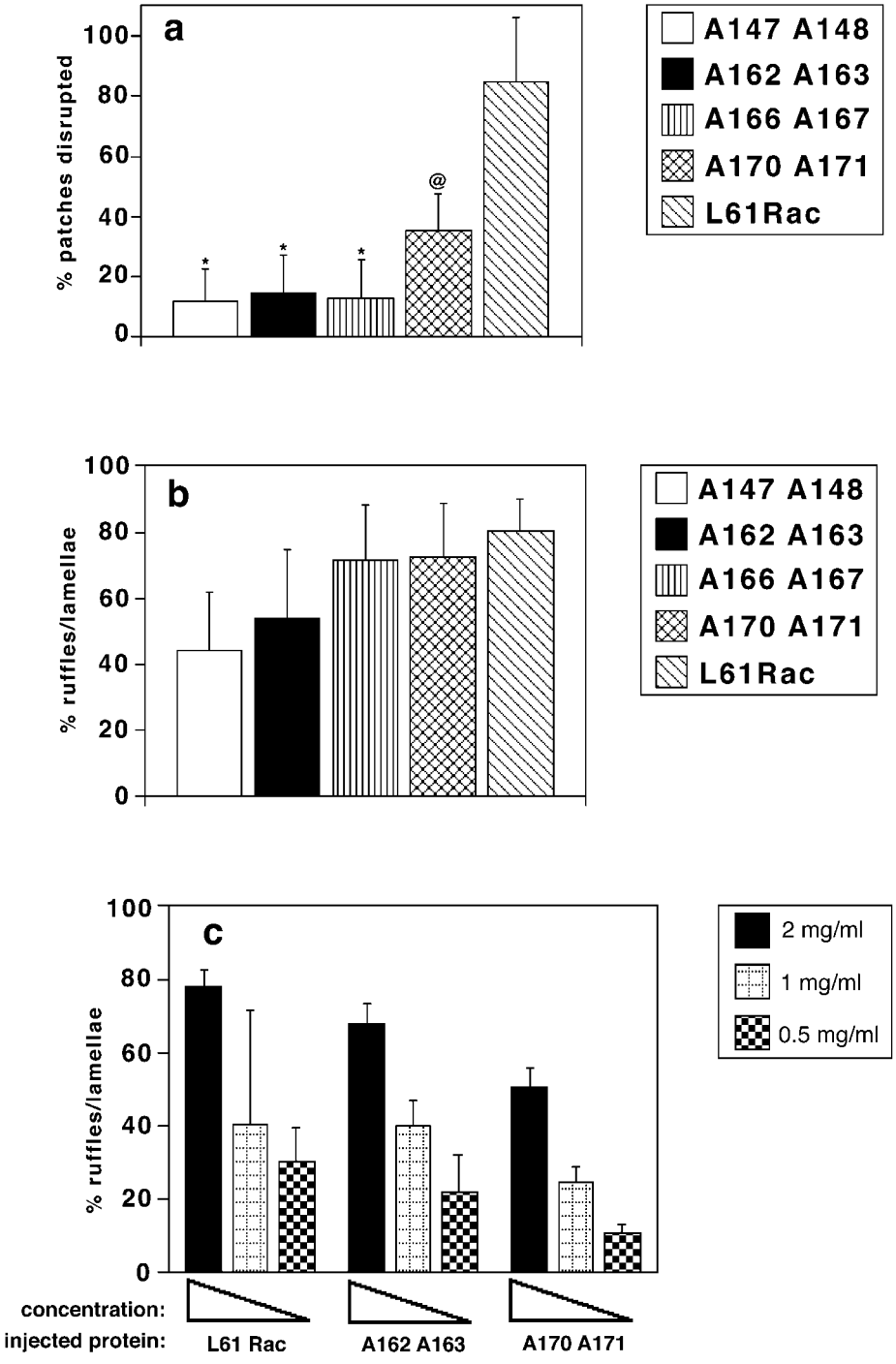


Figure 9. Characterization of the Rac second effector domain mutants. (a) Quantification of the effects of the Rac mutants on cadherin-dependent adhesion. Patches of keratinocytes microinjected with the different mutants were scored for the presence of perturbed cadherin staining at intercellular junctions and expressed as a percentage of the total number of patches (see MATERIALS AND METHODS). (b) Quantification of the lamella-inducing activity of Rac mutants. Swiss 3T3 cells were injected with the different proteins and scored as a percentage of cells showing ruffles/lamellae. (c) Titration of lamellipodia formation induced by L61Rac, A162 A163, and A170 A171. The same amount of recombinant protein (2, 1, or 0.5 mg/ml) was injected into Swiss 3T3 cells and the percentage of injected cells with ruffles/lamellae were scored. (d) Summary of Rac mutants' ability to perturb cadherin adhesion in keratinocytes or induce lamellipodia in fibroblasts. A diagram representing constitutively active Rac containing the relevant domains (effector domains 1 and 2, insert domain), and the different mutants generated in the second effector domain. Unless otherwise stated, in the microinjection experiments mutants were used at the following concentrations: A147 A148, 0.89 mg/ml; A162 A163, 2.26 mg/ml; A166 A167, 2.91 mg/ml; and A170 A171, 3.57 mg/ml. L61Rac was tested at 0.5 mg/ml in keratinocytes and at 2 mg/ml in Swiss 3T3 cells. $*p < 0.005$; $^{@}p < 0.01$ (Student's *t* test). Results are the mean of at least three independent experiments; Figure 9c shows the mean of at least two experiments for each concentration of distinct mutants. Error bars represent SD.

activation of Rac-dependent pathways by a constitutively active form (L61Rac) or via Tiam-1 might differ, because the latter also provides a localization signal (Sander *et al.*, 1998; this work). More experimental work is necessary to understand and reconcile the distinct phenotypes produced by Rac activation in different cell types.

Nevertheless, it is clear that in keratinocytes, sustained Rac activation does not alter the detergent solubility of the receptors nor the amount of actin recruited to junctions (our unpublished results). If Rac activation is not promoting the localization of cadherin receptors to junctions, what are the consequences of Rac activity? In keratinocytes, when junc-

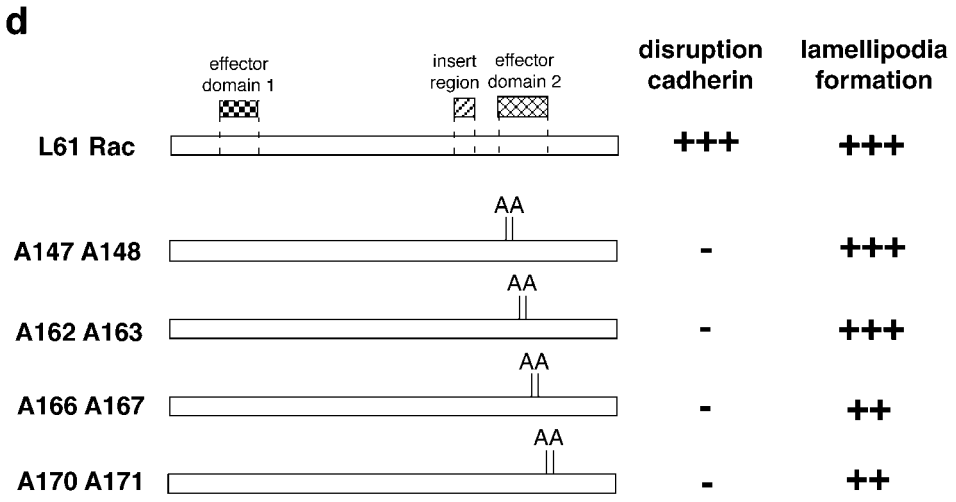
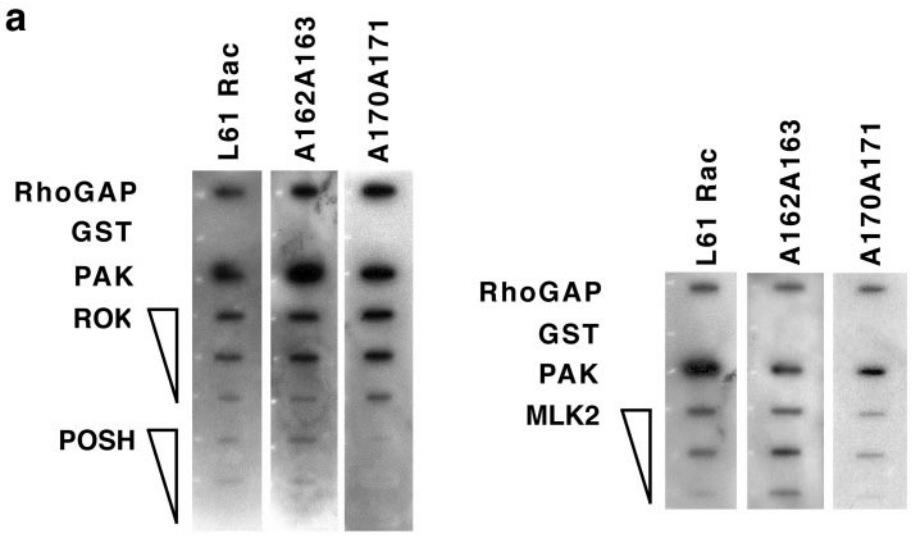


Figure 9. Continued



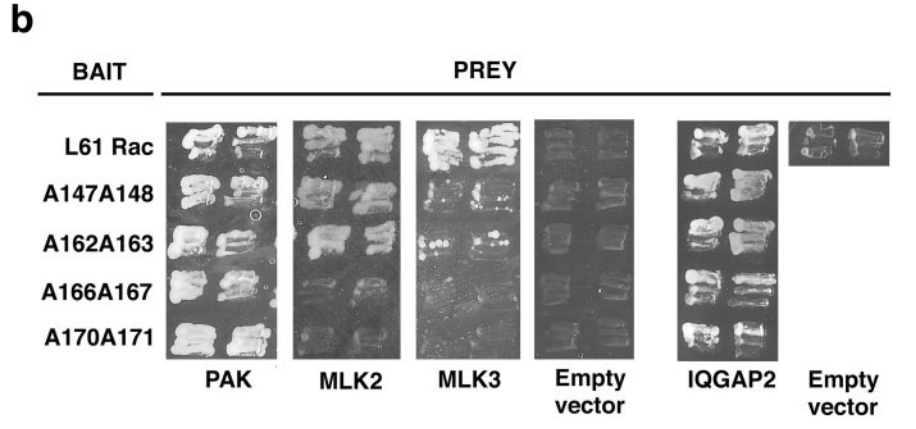


Figure 10. Binding properties of Rac mutants with known Rac targets. (a) In vitro interaction: L61Rac, A162 A163, and A170 A171 were labeled with radioactive GTP and allowed to interact with different Rac-binding proteins: GST-RhoGAP, GST-PAK, GST-ROK-α, GST-POSH, and GST-MLK2. Different amounts of recombinant protein were spotted onto the membranes (10, 5, or 1 μg). GST was used as a negative control; positive (RhoGAP and PAK) and negative controls were used at 10 μg. (b) Yeast two-hybrid interaction by using L61Rac or the second effector domain mutants as baits and the following targets as prey: PAK, MLK2, MLK3, and IQGAP2. Empty vector was used as a negative control. *, IQGAP interaction was tested at lower concentration of 3AT (10 mM) because the association with activated Rac was barely detectable with the standard concentration used for the other targets (25 mM). A summary of the yeast two-hybrid results with all the targets tested is shown in Table 1.

Table 1. Characterization of the Rac second effector mutants

		Mutants			
Assay	L61Rac	A147 A148	A162 A163	A166 A167	A170 A171
JNK activation	+	+	+	+	−
Target binding					
RhoGAP	+	+	+	+	+
PAK	+	+	+	+	+
ROK-α	+	+	+	+	+
MLK2	+	+	+	−	−
MLK3	+	+/−	+/−	−	−
IQGAP2	+	+	+	+	+
Empty vector	−	−	−	−	−

L61Rac and the double alanine Rac mutants were evaluated for activation of the JNK pathway and their binding with distinct targets in the yeast two-hybrid assay. The Rac targets tested are RhoGAP, PAK, ROK-α, MLK2, MLK3 and IQGAP2: (−), negative; (+), positive; (+/−), weakly positive as revealed by growth in 3AT plates (see MATERIALS AND METHODS for details). Empty vector was used as a negative control.

tion stability is challenged by either Rho inhibition (our unpublished results) or H-Ras activation, coinjection of actived Rac cannot protect cadherin receptors from these destabilizing stimuli (Braga *et al.*, 1997). Indeed, Rac activation is necessary for the disassembly of cadherin contacts induced by the oncogene H-Ras. These results are also in agreement with published data, in which Rac activation contributes to the H-Ras-dependent perturbation of cell-cell contacts in breast cancer cell lines (Quillan, 1999). In keratinocytes, we demonstrate that inhibition of Rac signaling pathways prevents the Ras-dependent perturbation of cell-cell adhesion, whereas blocking PI3 kinase and mitogen-activated protein kinase pathways have only a partial effect (our unpublished results).

Rac Can Specifically Destabilize Cadherin-dependent Adhesion

A previous report has shown activation of Rac 3 in breast cancer cells, suggesting that Rac activation during tumorigenesis might be a widespread process (Mira *et al.*, 2000). Together with our results, the above-mentioned results demonstrate that activation of Rac can occur after transformation and that this pathway may contribute to the Ras-dependent disassembly of junctions. Moreover, our data show that Rac activation is sufficient to disassemble cadherin-dependent contacts in a time- and concentration-dependent manner in human keratinocytes. Activated Rac specifically interferes with the localization of cadherin but not integrin receptors in the time frame examined. Disruption of cadherin adhesion is observed in both newly formed and mature junctions, but it is more clearly seen during the induction of cell-cell adhesion (see RESULTS). However, formation of new cell-cell contacts is not prevented by activated Rac, but rather the stability of cadherin receptors at intercellular contacts is compromised (after 1 h).

Our results that Rac activation may promote junction disassembly are intriguing. It is possible that, as for other biological stimuli, the cellular response to Rac activation may follow a bell-shaped curve: too little Rac is inhibitory to junctions as is too much Rac activity. We think that the Rac destabilization of junctions is unlikely to be a nonspecific effect of bacterial contaminants or overexpression for the following reasons: 1) the same effect is obtained by expression of activated Rac DNA in HaCat cells; 2) microinjection of distinct proteins at much higher concentration cannot disrupt cell-cell contacts; and 3) injected keratinocytes do not show any signs of apoptosis (i.e. annexin V staining, our unpublished results). In addition, there is specificity in the response because cadherin molecules are removed from junctions before other cell-cell adhesion receptors.

Rac activation perturbs cadherin contacts with a concomitant change in cell shape, including formation of lamellae/protusions and a clear conversion to a more fibroblastic morphology. Although lamellae are also present upon Rac activation in keratinocytes containing mature junctions, the change in cell shape is not observed over a 2-h incubation. Interestingly, levels of Rac activation that disrupt newly formed cadherin contacts very efficiently (0.5 mg/ml, 85% of injected patches) can only induce ruffling in 30% of injected fibroblasts.

Both lamella formation and cadherin adhesion require actin polymerization dependent on Rac activity (Machesky and Hall, 1997; Braga *et al.*, 1999). Because our data suggest that lamella formation may antagonize cadherin-mediated adhesion, two possibilities can be envisaged. First, induction of lamellipodia may cause the destabilization of cadherin receptors at junctions, or second, lamella formation and perturbation of cadherin adhesion may be two independent activities triggered by Rac. Our results support the latter possibility because we are able to dissect these two Rac activities.

Important Domain in the Rac Molecule for Interfering with Cadherin-dependent Contacts

Previous work has identified three functional domains in the Rho subfamily of small GTPases: the N-terminal effector domain, an insert region, and a putative C-terminal effector domain (Diekmann *et al.*, 1995; Joseph and Pick, 1995; Kwong *et al.*, 1995; Nisimoto *et al.*, 1997). We restricted an important region for destabilizing intercellular junctions to the putative second effector domain of Rac, between amino acids 143 and 175. This domain overlaps with, but is not identical to, the domain necessary to induce lamellae (Diekmann *et al.*, 1995; our unpublished results). In addition, by mutating specific amino acids within residues 143 and 175 of activated Rac, we obtained mutants that are impaired in their ability to perturb cadherin adhesion in keratinocytes, but are still able to promote ruffling and lamellipodia in Swiss 3T3 cells.

Characterization and quantification of these effects indicate that the second effector domain mutants (A147 A148, A162 A163, A166 A167, and A170 A171) showed around fourfold reduction in the destabilization of cell-cell adhesion compared with L61Rac, in spite of being microinjected at much higher concentration (2- to 8-fold more concentrated). We think it unlikely that the double alanine mutations interfere with the overall stability or activity of the Rac molecule for the following reasons. First, like constitutively active Rac, all second effector domain mutants were able to induce

lamellae. Normalization of their concentrations and microinjection of dilutions produced a proportional decline in their ability to induce lamellae, as for L61Rac. Second, their GTP binding ability was not significantly perturbed (our unpublished results). Finally, the mutants can associate with RhoGAP and other Rac targets such as PAK, ROK-α, and IQGAP2 to a similar extent as activated Rac (reviewed by Van Aelst and D'Souza-Schorey, 1997).

To our knowledge, this is the first report showing extensive mutagenesis analysis of the putative second effector domain. This domain forms an exposed loop in the Rac three-dimensional structure, suggesting good access for target interaction (Hirshberg *et al.*, 1997). Interestingly, the binding of the small GTPases Rac and Cdc42 to distinct targets also requires and is stabilized by residues within the second effector domain (Abdul-Manan *et al.*, 1999, Mott *et al.*, 1999; Tolias *et al.*, 2000).

In an attempt to investigate the mechanism by which Rac activation may disturb cadherin function, preliminary results show that new protein synthesis is not required. The process does not involve down-regulation of Rho (Izawa *et al.*, 1998, Rottner *et al.*, 1999; Sander *et al.*, 1999; van Leeuwen *et al.*, 1999; Zondag *et al.*, 2000) nor does Rac activation interfere with the recycling compartment in keratinocytes (our unpublished results; Lamaze *et al.*, 1996). Together with our analyses of putative Rac targets, these results suggest that Rac is able to activate specific pathways that perturb the stability of cadherin receptors at the keratinocyte junction.

If this hypothesis is true, the Rac second effector domain mutants can be useful tools to identify which pathway is important for junction disassembly. Two possibilities can be envisaged. First, the mutants may display a reduced binding to specific targets. Alternatively, the binding interactions may be the same, but the ability to activate the target is compromised. We began to test these two possibilities with known Rac targets. We found that at least activation of the JNK pathway is not affected by the double alanine mutations in the second effector domain. By screening Rac targets for their ability to differentially interact with activated Rac and the second effector mutants, we identified MLK3 (mixed lineage kinase 3) as a putative effector candidate (Burbelo *et al.*, 1995; Nagata *et al.*, 1998; Hartkamp *et al.*, 1999) because it shows reduced binding to all mutants tested. We are currently performing experiments to address the question of whether MLK3 activation per se is sufficient to disturb cadherin-dependent adhesion.

In summary, we demonstrate that Rac is a key regulator of cadherin-dependent cell-cell contacts as sustained Rac activation is sufficient to destabilize normal keratinocyte junctions. An important question that remains to be addressed experimentally is the threshold level of Rac activation that is necessary to perturb cell adhesion during tumor progression. However, it is conceivable other pathways triggered by oncogenes may cooperate with Rac to promote cytoskeletal changes and junction breakdown. Because Rac plays an important role in cell migration, our study sheds light on the biological problem of how cells are able to integrate cell-cell and cell-substratum adhesion during tumorigenesis. Moreover, our data suggest that downstream signaling pathways activated by Rac could be potential therapeutical targets for preventing cell-cell disassembly.

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