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Regulation of Cadherin Function by Rho and Rac: Modulation by Junction Maturation and Cellular Context

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Cadherins are cell–cell adhesion receptors whose adhesive function requires their association with the actin cytoskeleton via proteins called catenins. The small guanosine triphosphatases (GTPases), Rho and Rac, are intracellular proteins that regulate the formation of distinct actin structures in different cell types. In keratinocytes and in other epithelial cells, Rho and Rac activities are required for E-cadherin function. Here we show that the regulation of cadherin adhesiveness by the small GTPases is influenced by the maturation status of the junction and the cellular context. E-cadherin localization was disrupted in mature keratinocyte junctions after inhibition of Rho and Rac. However, an incubation of 2 h was required after GTPase inhibition, when compared with newly established E-cadherin contacts (30 min). Regarding other cadherin receptors, P-cadherin was effectively removed from mature keratinocytes junctions by blocking Rho or Rac. In contrast, VE-cadherin localization at endothelial junctions was independent of Rho/Rac activity. We demontrate that the insensitivity of VE-cadherin to inhibition of Rho and Rac was not due to the maturation status of endothelial junction, but rather the cellular background: when transfected into CHO cells, the localization of VE-cadherin was perturbed by inhibition of Rho proteins. Our results suggest that the same stimuli may have different activity in regulating the paracellular activity in endothelial and epithelial cells. In addition, we uncovered possible roles for the small GTPases during the establishment of E-cadherin–dependent contacts. In keratinocytes, Rac activation by itself cannot promote accumulation of actin at the cell periphery in the absence of cadherin-dependent contacts. Moreover, neither Rho nor Rac activation was sufficient to redistribute cadherin molecules to cell borders, indicating that redistribution results mostly from the homophilic binding of the receptors. Our results point out the complexity of the regulation of cadherin-mediated adhesion by the small GTPases, Rho and Rac.

INTRODUCTION

The importance of cell–cell adhesion in differentiation processes and in the maintenance of the differentiated phenotype is well established, particularly in epithelial cells. In simple and stratified epithelia, tight intercellular contacts are the determinant aspect of their characteristic morphology, linking tissue integrity with physiological functions such as polarized secretion and environmental barrier. Among the many cell–cell adhesion molecules that contribute to the maintenance of epithelial morphology, the cadherin super family of calcium-dependent adhesion receptors is the most well characterized. So far, more than 30 different

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types of cadherin have been identified, and they can be grouped into four subfamilies according to sequence similarities and structural aspects (Herrenknecht, 1996). The main features of the classical cadherin subfamily (E-, P-, N-cadherin, etc.) are the presence of cadherin repeats in the extracellular domain (calcium-binding sites) and a cytoplamic tail that is highly conserved among the different members of the subfamily. The spatial and temporal regulation of expression of the distinct cadherins during morphogenesis of muscle, neuronal, and epithelial tissues is consistent with their important role in differentiation processes (reviewed by Takeichi, 1995; Gumbiner, 1996). In support of this, there is evidence for the cadherin type specificity in the induction of gene expression, cellular differentiation, and the distribution of cytoplasmic proteins (Holt *et al.*, 1994; Marrs *et al.*, 1995; Larue *et al.*, 1996).

Adhesion mediated by cadherin receptors involves homophilic interactions in neighboring cells (reviewed by Kemler, 1993). The presence of calcium ions is required for adhesion, supposedly to stabilize the lateral stacking of the extracellular domain at the plane of the membrane (Shapiro *et al.*, 1995; Nagar *et al.*, 1996). On the intracellular side, the cadherin cytoplamic tail associates with either β-catenin or plakoglobin, and binding of α-catenin to this complex allows the interaction with the actin cytoskeleton. Although for 10 years now it has been demonstrated that the association with catenins and the actin cytoskeleton are essential for cadherin function, this is clearly not the whole story. For instance, it is known that the homophilic binding of the extracellular domains does not require an association with actin filaments (Wheelock *et al.*, 1987; Bixby and Zhang, 1990; Vestal and Ranscht, 1992; Kreft *et al.*, 1997). In addition, a region of the cadherin tail that does not contain the catenin-binding site has recently been shown to be important for clustering of cadherin receptors (Navarro *et al.*, 1995; Yap *et al.*, 1998). However, the cytoskeletal interaction via the catenins can provide strength to the adhesion by holding together the clustered receptors at sites of cell–cell contacts (Kemler, 1993; Brieher *et al.*, 1996; Yap *et al.*, 1997).

Although the association with the catenins is well documented, the process that leads to the formation of supramolecular cadherin complexes at points of cell–cell contacts is poorly understood. Cadherin receptors can be coprecipitated with growth factor receptors and phosphatases (Hoschuetzky *et al.*, 1994; reviewed by Brady-Kalnay and Tonks, 1995), and cadherin tail and catenins are constitutively phosphorylated in keratinocytes (Braga *et al.*, 1998). Phosphorylation events have also been implicated in the regulation of cell–cell adhesion and in the turnover of the catenin cytoplasmic pools (reviewed by Gumbiner, 1996; Miller and Moon, 1996). A step forward toward understanding the regulation of cell–cell contacts by intracellular proteins came with recent reports showing that the function of the small guanosine triphosphatases (GTPases), Rho and Rac, is necessary for cadherin-mediated adhesion (Braga *et al.*, 1997; Takaichi *et al.*, 1997; Zhong *et al.*, 1997).

The Rho subfamily of small GTPases (Rho, Rac, and Cdc42) regulates the assembly of specific actin structures in cells and the formation of cell-substratum adhesion plaques involving integrin receptors (reviewed by Machesky and Hall, 1996). More recently, they have also been implicated in processes such as the regulation of kinase cascades, cell growth, transformation, and gene expression (reviewed by Ridley, 1996; Hall, 1998). The relationships between these activities and the cytoskeletal rearrangements induced by Rho GTPases is not clear. What has become apparent is the complexity of the signaling pathways activated by small GTPases in terms of the number of effector proteins, target specificity, and cross-talk among the different Rho proteins (Hall, 1998).

In epithelial cells, Rho can induce the formation of stress fibers as it does in fibroblasts (Paterson *et al.*, 1990; Ridley and Hall, 1992; Ridley *et al.*, 1995). Activation of Rac in epithelium leads to an accumulation of actin at intercellular junctions in the characteristic cortical bundles of actin filaments (Eaton *et al.*, 1995; Harden *et al.*, 1995; Ridley *et al.*, 1995; Hordijk *et al.*, 1997). In addition, Rac also localizes at cell–cell contact sites in epithelial cells (Kuroda *et al.*, 1996; Hordijk *et al.*, 1997; Takaichi *et al.*, 1997). Regarding E-cadherin function, the clustering of receptors and the establishment of cadherin-mediated contacts depends simultaneously on endogenous Rho and Rac and the presence of calcium ions (to yield the receptors competent for binding) (Braga *et al.*, 1997).

How Rho and Rac can regulate cadherin-mediated adhesion in epithelial cells is an incipient matter. It is not known whether the function of the two small GTPases is coordinately required in the same or in distinct signaling pathways, but it seems that Rac activity alone is necessary for actin recruitment to clustered cadherin receptors (Braga *et al.*, 1997). In this article, we show that the appropriate positioning of cadherin receptors at the membrane is a prerequisite for the Rac-dependent redistribution of actin. We investigated whether inhibition of endogenous Rho or Rac can interfere with the function of other cadherin receptors in different cell types and showed that the cellular context and the maturation status of the junctions are important. Our results highlight the nuances and complexity of the regulation of cadherin-mediated adhesiveness, in spite of the extensive homology and functional conservation observed within the cadherin and small GTPase families.

MATERIALS AND METHODS

Cells

Normal human keratinocytes (strain kb, passage 3 to 6) were cultured on a mitomycin C-treated monolayer of 3T3 fibroblasts at 37°C and 5% CO_2. Cells were cultured in standard medium (1.8 mM calcium) consisting of a mixture of DMEM and Ham's F12 medium (1:3) (Imperial Laboratory) supplemented with 10% FCS, 1.8×10^{-4} M adenine, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 10^{-10} M cholera toxin, and 10 ng/ml epidermal growth factor as described previously (Hodivala and Watt, 1994; Rheinwald, 1989). For cultures grown in the absence of calcium-dependent cell–cell contacts, keratinocytes were seeded in standard medium, and 2–3 d later, cells were transferred to low calcium medium (0.1 mM calcium) and cultured until confluence (Hodivala and Watt, 1994). Reduction of the calcium levels in the medium was obtained by omitting calcium ions from the standard medium formulation described above and by chelation of divalent ions in the FCS using Chelex 100 resin (Bio-Rad, Richmond, CA; Hodivala and Watt, 1994). Keratinocytes were seeded on glass coverslips (13 mm diameter) at 3×10^4 cells/well.

Primary human umbilical cord endothelial cells (EC) were isolated, cultured in M199 medium supplemented with 20% newborn FCS, 50 μg/ml endothelial cell growth supplement, and 100 μg/ml heparin, and kept at 37°C in a 5% CO_2 incubator. EC to be microinjected were grown to confluence on glass coverslips (13 mm diameter) coated with 7 μg/ml of human fibronectin as described previously (Lampugnani *et al.*, 1992). VE-cadherin–transfected CHO cells were cultured as described (Navarro *et al.*, 1995).

L-cells expressing mouse E-cadherin full-length molecule, a kind gift from Dr. A. Nagafuchi (Nagafuchi *et al.*, 1994), were cultured in DMEM medium (Life Technologies, Paisley, United Kingdom) supplemented with 10% donor calf serum and 150 μg/ml G418 (Life Technologies). Swiss 3T3 fibroblasts were cultured as described previously (Nobes and Hall, 1995). Swiss cells were allowed to reach confluence and become quiescent for 7–10 d after seeding.

Antibodies

The following anti-cadherin antibodies were used: E-cadherin staining was performed using either ECCD-2 antibody (rat monoclonal; Hirai *et al.*, 1989a,b) or HECD-1 (mouse monoclonal; gift from M. Takeichi, Kyoto University, Japan; Shimoyama *et al.*, 1989). Antibody against P-cadherin was NCC-CAD-299 (mouse monoclonal; gift from S. Hirohashi, National Cancer Center Research Institute, Tokyo, Japan; Shimoyama *et al.*, 1989). Pan-cadherin antibody (mouse monoclonal; Sigma Chemical, St. Louis, MO) and anti–VE-cadherin antibody (TEA-1 mouse monoclonal; Leach *et al.*, 1993) were also used.

Other mouse monoclonals were anti-β1 integrin antibody (P5D2; Dittel *et al.*, 1993) and anti-desmoplakin (115F, gift from D. Garrod, Manchester University, Manchester, United Kingdom; Parrish *et al.*, 1987). Secondary antibodies were purchased from Jackson Immuno Research Laboratories (Stratech Scientific): indodicarbocyanine (Cy5)-conjugated donkey anti-mouse IgG; fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG and FITC-conjugated donkey anti-rat IgG. FITC-phalloidin was bought from Sigma.

Recombinant Proteins

Recombinant proteins were prepared as glutathione *S*-transferase fusion proteins in *Escherichia coli*, purified using glutathione Sepharose beads, thrombin cleaved, dialysed, and concentrated essentially as described previously (Ridley *et al.*, 1992). The activity of each batch of recombinant proteins was tested beforehand in fibroblasts and keratinocytes as described previously (Ridley *et al.*, 1992; Nobes and Hall, 1995; Braga *et al.*, 1997). Recombinant proteins used were: C3 transferase, dominant negative Rac (N17Rac), constitutively active Rac (L61Rac), constitutively active Rho (L63Rho), and RhoGDI (Ridley *et al.*, 1992; Hancock and Hall, 1993; Nobes and Hall, 1995). For comparison of the effects of Rho proteins on cadherin receptors among the different cell types, the same batch of recombinant proteins was used with keratinocytes, endothelial cells, CHO cells, and fibroblasts. At least two different batches of N17Rac and C3 were used throughout the experiments.

Microinjection

Microinjection was performed essentially as described (Braga *et al.*, 1997). Medium sized colonies of keratinocytes grown in standard medium were microinjected with recombinant proteins mixed with dextran-Texas Red (molecular weight, 10,000, Molecular Probes, Eugene, OR) to visualize the injected cells and incubated for 1 and 2 h. Confluent monolayers of L-cells transfected with E-cadherin receptor were microinjected in DMEM with 10% donor calf serum and incubated as above. CHO transfectants were microinjected as subconfluent colonies. Quiescent Swiss 3T3 cells were microinjected in serum-free medium, immediately replaced in their medium, and incubated for up to 2 h before fixation.

Primary human umbilical cord cells (EC) were microinjected as confluent or postconfluent monolayers. In experiments to analyze the role of small GTPases in newly formed VE-cadherin junctions, confluent monolayers were washed once in serum- and complement-free medium and transferred to medium containing 5 mM EGTA. Cells were incubated for up to 20 min to disrupt calcium-dependent contacts. Microinjection was performed in medium with 5 mM EGTA. Immediately after injection, coverslips were washed twice in serum and complement-free medium to remove the EGTA and transferred to complete medium for 1 h to restore junctions.

Cy3-labeled monomeric actin was prepared as previously described (Machesky and Hall, 1997). Cy3-actin (final concentration 15 μM) was mixed with L61Rac or L63Rho and injected into patches of keratinocytes grown in low calcium medium as reported (Braga *et al.*, 1997). Cells were incubated for 20 min either in low calcium medium or transferred to standard medium to induce cell–cell contacts. Quiescent Swiss 3T3 cells were serum-starved overnight (Nobes and Hall, 1995), microinjected in serum-free medium with a mixture of L61Rac and Cy3-labeled actin, and incubated for 20 min in the same medium.

Immunostaining

Cells were prepared for staining by fixing in 3% paraformaldehyde and subsequently permeabilizing with 0.1% Triton X-100 in 10% FCS for 10 min at room temperature each step. Staining was performed as described (Braga *et al.*, 1997).

Single labeling for E-cadherin in keratinocytes was detected using the mouse monoclonal HECD-1 followed by FITC-conjugated anti-mouse IgG. In L-cells, E-cadherin was labeled with the rat monoclonal ECCD-2 and FITC-conjugated anti-rat IgG. Swiss 3T3 fibroblasts were labeled with the pan-cadherin antibody followed by FITC-conjugated anti-mouse IgG. Endothelial cells and VE-cadherin–transfected CHO cells were stained with anti–VE-cadherin monoclonal (TEA-1) followed by FITC-conjugated anti-mouse IgG.

In keratinocytes, double labeling was performed by sequential incubations with anti-cadherin antibody (ECCD-2), FITC-conjugated anti-rat IgG, anti-β1 integrins (P5D2), and Cy5-conjugated anti-mouse IgG. The same protocol was used for double labeling of E-cadherin and P-cadherin (NCC-CAD-299) or anti-desmoplakin (115F). No cross-reactivity was observed between these conjugates.

Confocal images were obtained using a MRC 1024 laser scanning (Bio-Rad) attached to an Optiphot 2 microscope (Nikon, Garden City, NY). To avoid leakage between the different filters when double labeling experiments were analyzed, the laser was optimized for each fluorophore, and images were collected separately. Pictures were processed using Adobe Photoshop and printed in a Epson 600 color printer.

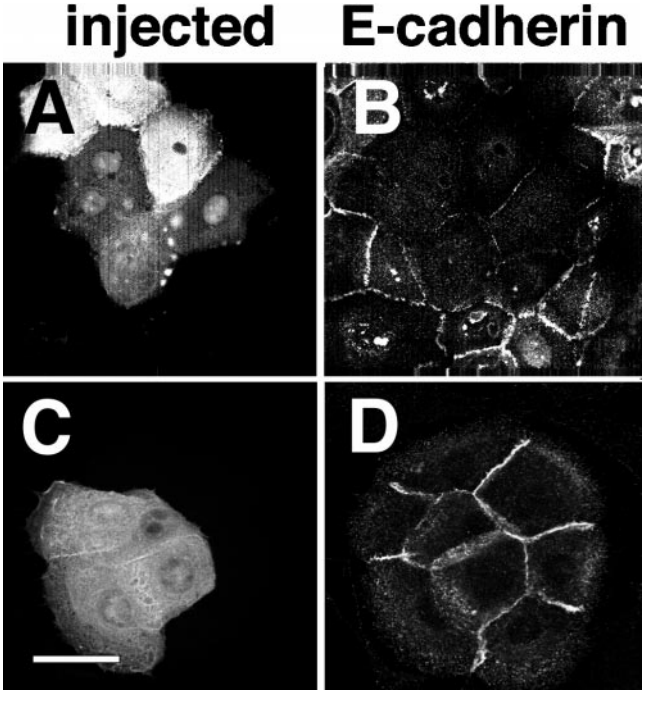

Figure 1. The effects of Rho and Rac on cadherin receptors is influenced by the maturation of the intercellular contacts. Keratinocytes grown in low calcium medium were transferred to standard medium to induce calcium-dependent cell–cell contacts for 3 h (newly formed junctions) (A and B) or alternatively, cells were grown in standard medium (mature junctions) (C and D). After microinjection of a dominant negative form of Rac (N17Rac) to block endogenous Rac, cells were incubated for further 30 min. E-cadherin was visualized by staining with HECD-1 followed by FITC-conjugated anti-mouse (B and D); injected cells are seen in panels A and C. Bar, 50 μm.

RESULTS

Effects of Small GTPases, Rho and Rac, on Cadherin Receptors Are Dependent on the Maturation Status

We have previously shown that, in newly formed junctions (up to 3 h in standard medium), cadherin molecules are removed from cell–cell contacts within 30 min after blocking Rho or Rac function (Braga *et al.*, 1997). However, junctions become more stable as they mature, i.e., with time after induction of contacts and confluence. We therefore investigated whether junction maturation would affect the responsiveness of cadherin receptors to inhibition of endogenous Rho and Rac.

Keratinocytes grown in the absence of cell–cell contacts were transferred to standard medium to induce calcium-dependent adhesion for 3 h, and subsequently injected with a dominant negative form of Rac (N17Rac) to block endogenous Rac (Figure 1, A and B; Braga *et al.*, 1997). Alternatively, cells that were grown in standard medium (favoring cell–cell contact formation) were also injected with N17Rac (Figure 1, C and D). Incubation for 30 min after the microinjection was sufficient to remove E-cadherin from cell–cell contacts induced for 3 h (Figure 1, A and B; Braga *et al.*, 1997), but not from mature junctions (Figure 1, C and D). The same results were observed after blocking endogenous Rho (our unpublished results).

We next investigated whether increasing the period of incubation after the microinjection would be effective in disrupting cadherin-mediated contacts. Keratinocytes grown in standard medium were microinjected with C3 transferase to inhibit Rho function (C3, Figure 2, A–F) or a dominant negative form of Rac (N17Rac, Figure 2, G–L) and incubated for up to 2 h. Cells were double labeled for E-cadherin (Figure 2, B, E, H, and K) and β1-integrins as control for other transmembrane proteins present at cell–cell contacts (Figure 2, C, F, I, and L). We observed that under these conditions, longer incubations resulted in removal of E-cadherin from intercellular junctions (Figure 2, B, E, H, and K). After 2 h incubation, integrins were still concentrated at contact sites (Figure 2, C, F, I, and L), and desmosomes were not affected (our unpublished results). In N17Rac-injected cells, the integrin-staining pattern seemed more punctate than in control surrounding cells, but, nevertheless, the integrin staining at cell–cell contacts was not significantly perturbed (Figure 2, I and L). Thus, as junctions mature, the localization of cadherin molecules become progressively insensitive to the inhibition of endogenous Rho and Rac. However, in spite of the longer period of time required, cadherin receptors were removed from the junctions before other transmembrane molecules linked to the actin cytoskeleton (i.e., integrins).

Effects of Rho and Rac on Other Members of the Cadherin Superfamily

We sought to investigate whether the regulation of E-cadherin function by the small GTPases would also occur with other types of cadherin receptors. Keratinocytes express P-cadherin in addition to E-cadherin (Hirai *et al.*, 1989b). We injected keratinocytes grown in the presence of cell–cell contacts with C3 (Figure 3, A–C) or N17Rac (Figure 3, D–F), and after 2 h incubation, the presence of P-cadherin at contact sites was evaluated (Figure 3, C and F) and compared with E-cadherin (Figure 3, B and E). We observed that both P- and E-cadherin were removed in a similar time course from intercellular junctions in response to inhibition of Rho or Rac activity.

We next investigated another type of cadherin, VE-cadherin, which is specifically expressed by endothelial cells (Lampugnani *et al.*, 1992; Leach *et al.*, 1993). Confluent endothelial cells (EC) were microinjected with different recombinant proteins and incubated for 1 or 2 h (Figure 4). To our surprise, the localization of VE-cadherin was largely unmodified by microinjec-

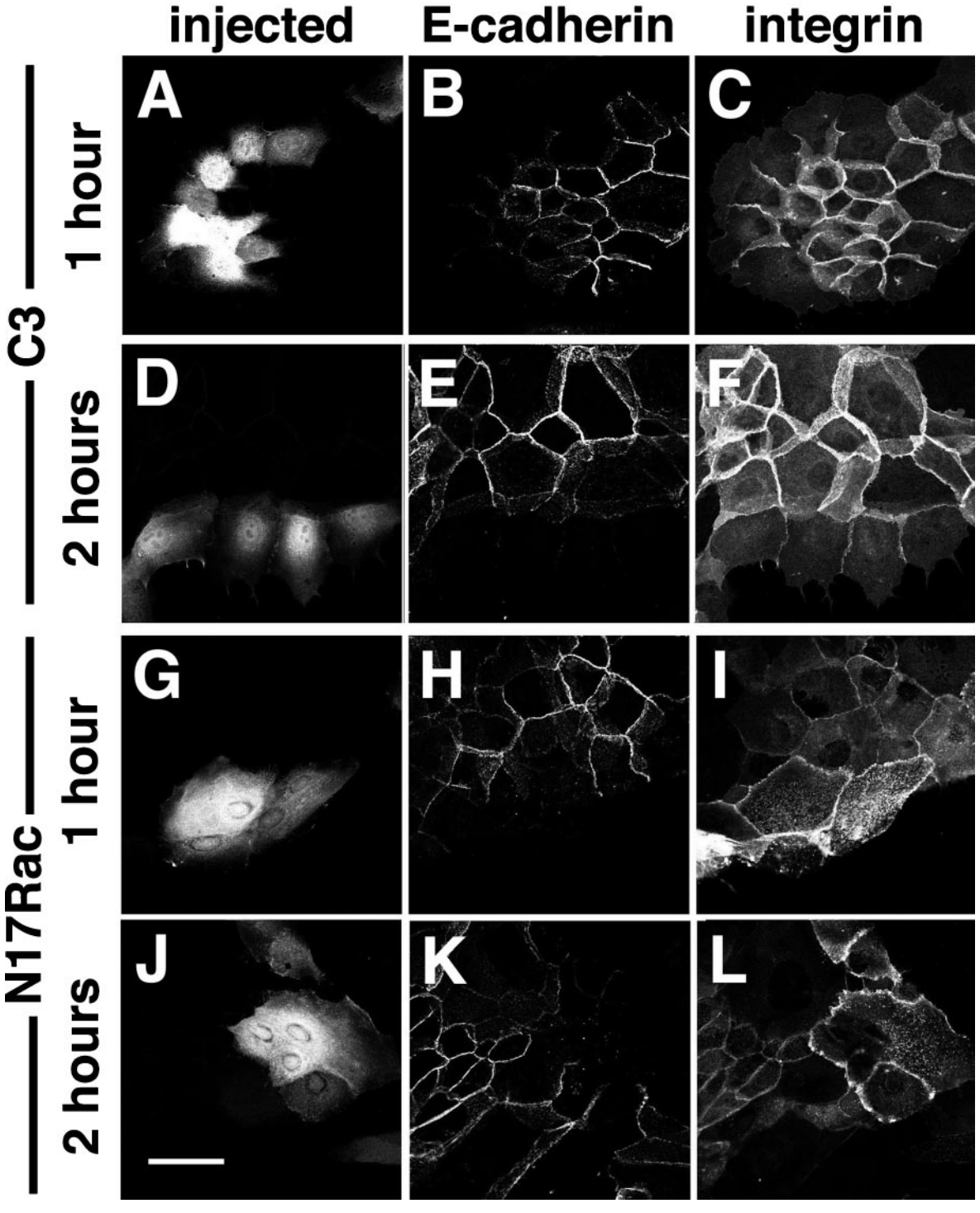

Figure 2. Mature keratinocyte cadherin junctions are disrupted by inhibition of endogenous Rho or Rac. Keratinocytes grown in the presence of cell–cell contacts (standard medium) were microinjected with C3 transferase to inhibit Rho (C3, A–F) or N17Rac (N17Rac, G–L) and subsequently incubated for 1 h (A–C and G–I) and 2 h (D–F and J–L). Cells were fixed, permeabilized, and double labeled with an antibody against E-cadherin (ECCD-2) followed by FITC-conjugated anti-rat IgG (B, E, H, and K), and an antibody against β1 integrins (P5D2) followed by Cy5-conjugated anti-mouse IgG (C, F, I, and L). Microinjected cells were visualized by coinjection of Dextran-Texas Red (A, D, G, and J). Bar, 50 μm.

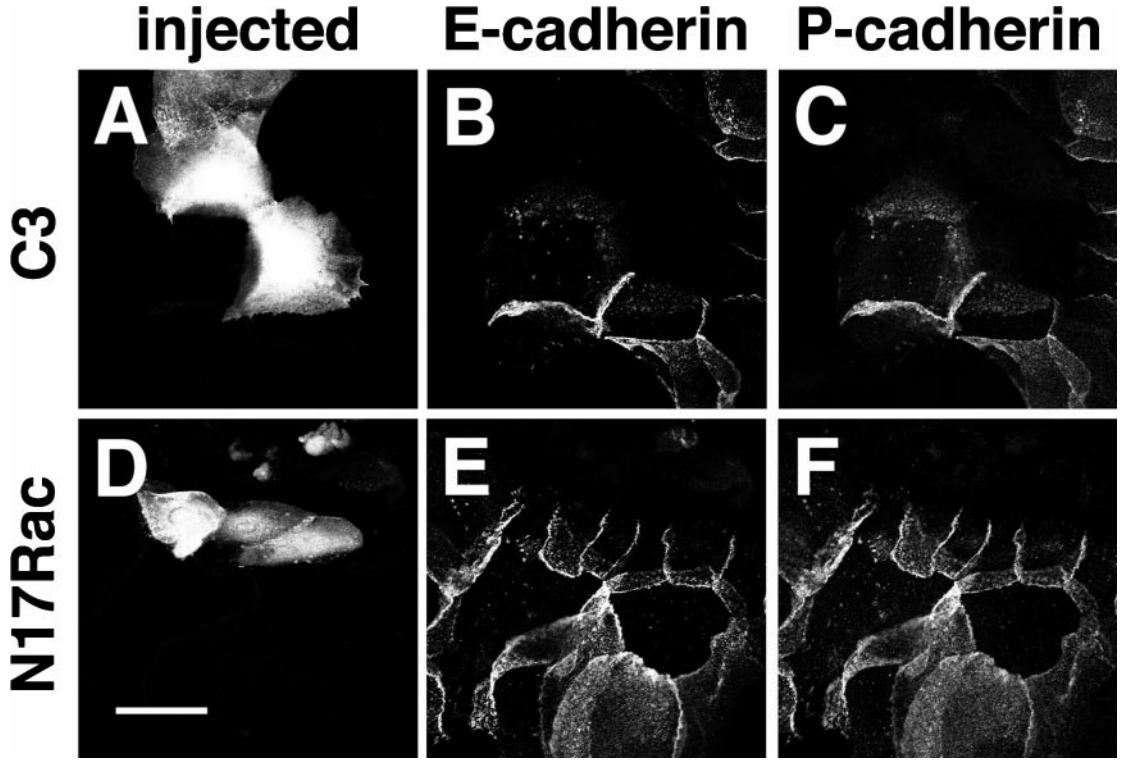

Figure 3. Rho and Rac function is required for the maintenance of both E- and P-cadherin–mediated cell–cell adhesion. Keratinocytes grown in standard calcium medium were injected with C3 transferase (A–C) or N17Rac (D–F). After 2 h incubation, cells were double stained for E-cadherin (ECCD-2) followed by FITC-conjugated anti-rat IgG (B and E) and P-cadherin (NCC-CAD-299) followed by Cy5-conjugated anti-mouse IgG (C and F). Microinjected cells were observed by coinjection of Dextran-Texas Red (A and D). Bar, 50 μm

tion of N17Rac (Figure 4, F and H), while blocking endogenous Rho function resulted in only qualitative changes in its staining pattern (Figure 4, B and D). Attempts to use RhoGDI, a general inhibitor of GTPases, showed that, after incubation for up to 60 min, there was no effect on the localization of VE-cadherin, while in keratinocytes this is sufficient to remove most of E-cadherin staining from junctions (our unpublished results). However, after 1 h incubation, the majority of RhoGDI-injected cells had detached from the dish, making it difficult to assess its effects on VE-cadherin junctions.

It is possible that the inability of VE-cadherin to be removed from intercellular junctions is due to the status of confluence of the EC. We observed that upon maturation of cell–cell contacts in keratinocytes, E-cadherin receptors become more resistant to blocking Rho or Rac function (Figures 1 and 2). We therefore asked whether newly formed VE-cadherin junctions were sensitive to the inhibition of Rho or Rac (Figure 5). Confluent EC cultures were briefly treated with EGTA to disrupt calcium-dependent adhesion and then were microinjected. Endothelial cells were retracted and showed very few contacts with neighboring cells. After microinjection of C3 or N17Rac, cell–cell contacts were induced by washing off the EGTA and changing to the medium with standard levels of calcium. Interestingly, injected cells were able to spread and reestablish contacts very similarly to the surrounding control cells (Figure 5, B and D). Our data suggested that the inability of VE-cadherin to be removed from intercellular contacts by inhibiting Rho or Rac is not related to the maturation of endothelial junctions. To test whether, in a different cellular context, VE-cadherin function could be modulated by the small GTPases, CHO cells transfected with full-length VE-cadherin were microinjected with C3 (Figure 5, E and F) or N17Rac (Figure 5, G and H). After 2 h incubation, the exogenous VE-cadherin was efficiently removed from cell–cell contacts.

Inhibition of E-Cadherin Function by Rac Is Cell Type Specific

The effects of Rho and Rac inhibition on exogenous cadherin function were also evaluated in L-cell transfectants expressing full-length E-cadherin molecule (Figure 6, A–D). Microinjection of C3 into E-cadherin–expressing L-cells perturbed the localization of the receptors at sites of cell–cell contacts (arrow, Figure

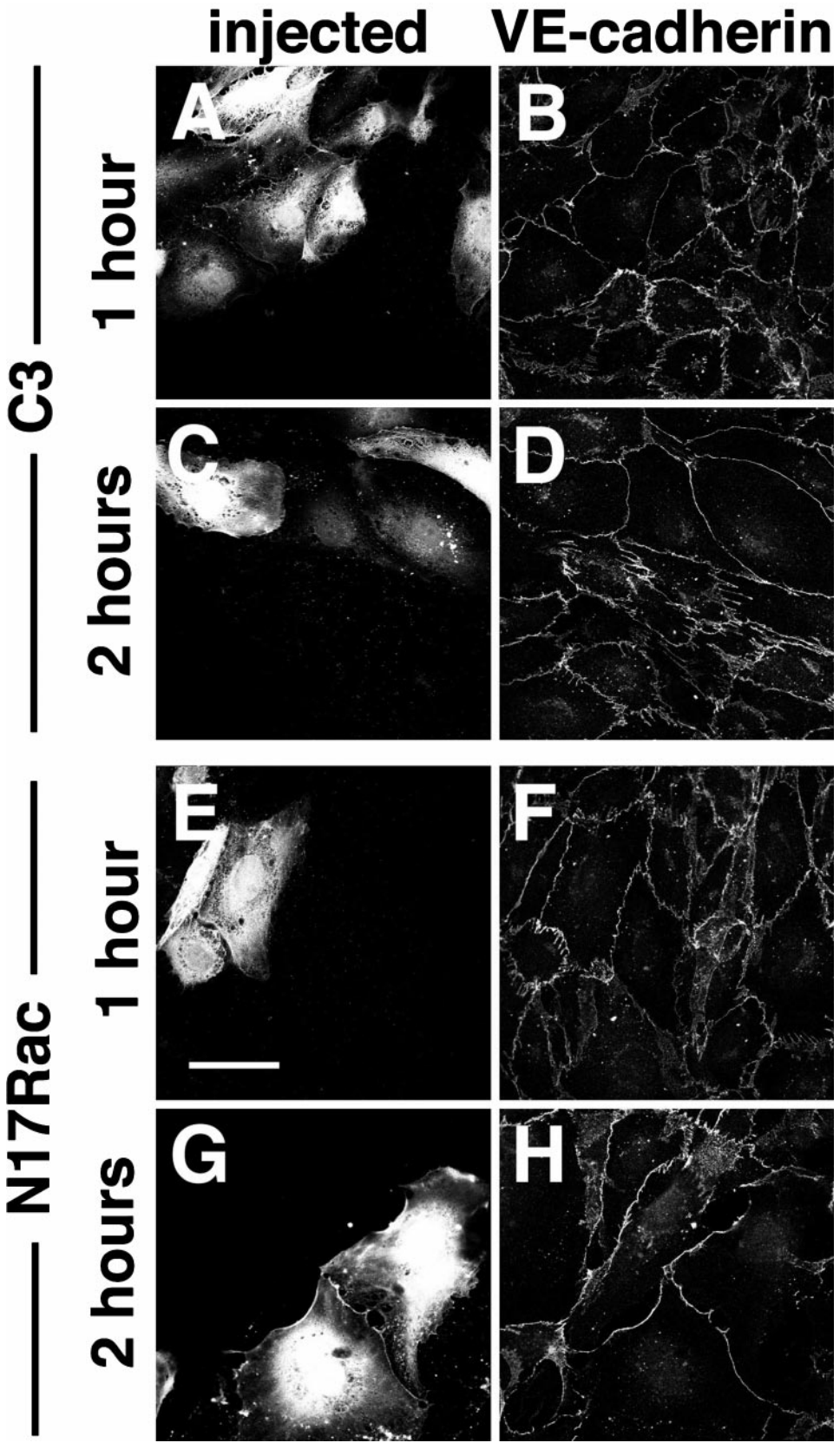

Figure 4. VE-cadherin–dependent cell–cell contacts are not perturbed by inhibiting Rho or Rac activity. Confluent endothelial cells were injected with C3 transferase to block endogenous Rho (A–D) or N17Rac to inhibit endogenous Rac (E–H) and subsequently incubated for 1 h (A, B, E, and F) or 2 h (C, D, G, and H). Cells were labeled with anti–VE-cadherin monoclonal antibody (TEA-1) followed by anti-mouse FITC. Injected cells are shown in panels A, C, E, and G, and VE-cadherin staining is shown in panels B, D, F, and H. Bar, 50 μm.

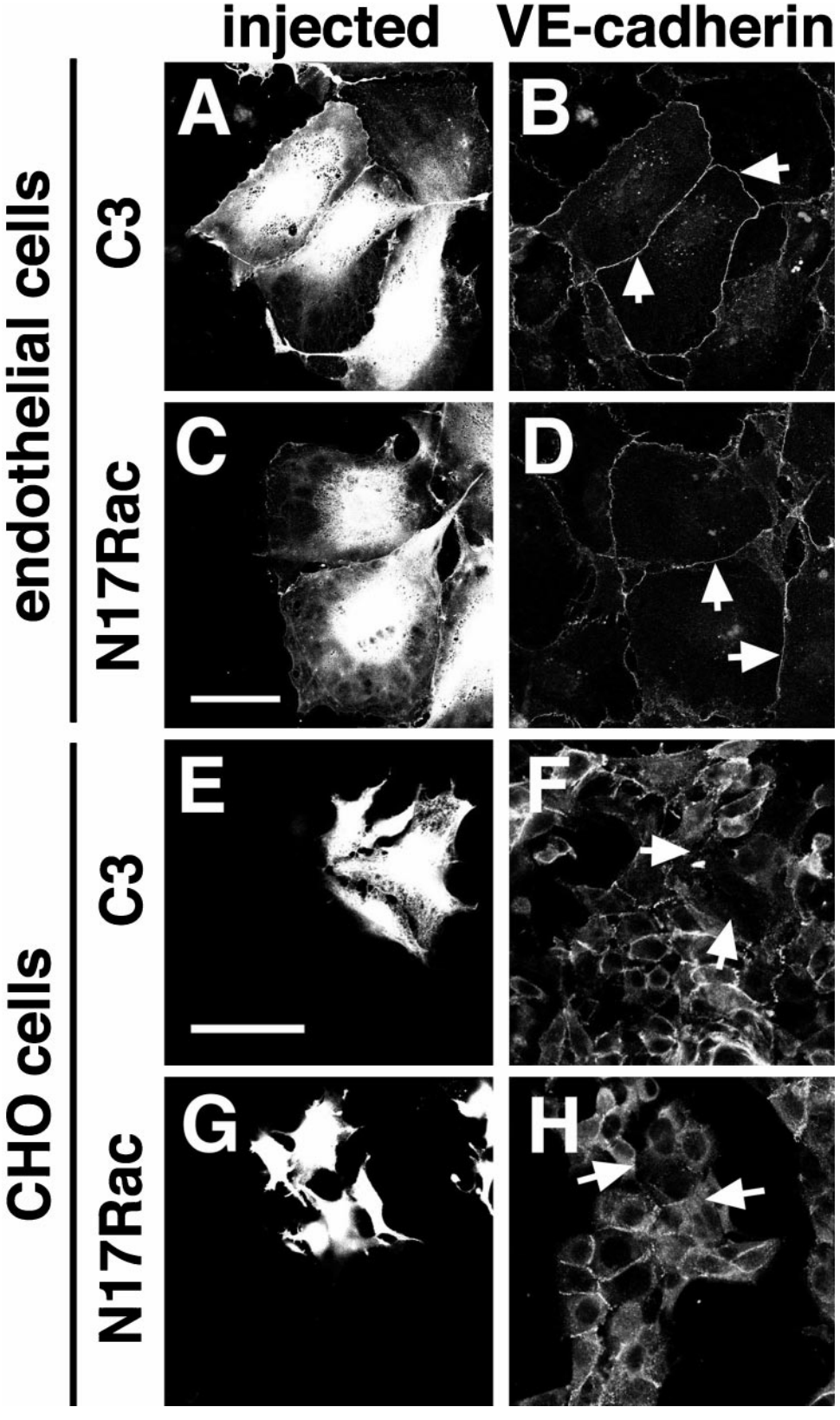

Figure 5. Formation of new VE-cadherin junctions in endothelial cells does not require the activity of Rho or Rac. Confluent endothelial cells (A–D) were incubated in medium without serum or complements and containing 5 mM EGTA to disrupt cadherin-dependent contacts for 5–20 min. After microinjection of C3 (A and B) or N17Rac (C and D), cells were washed to remove the EGTA and incubated in medium with serum and complements for 1 h to induce cell–cell contacts. Subconfluent CHO cells transfected with full-length VE-cadherin were also microinjected with C3 (E and F) or N17Rac (G and H) and incubated for up to 2 h. Staining of VE-cadherin was performed and visualized with anti-mouse FITC (B, D, F, and H); injected cells were identified with Dextran-Texas Red (A, C, E, and G). Arrows in panels B and D show the presence of VE-cadherin–dependent contacts; arrows in panels F and H show the dismantling of VE-cadherin contacts in CHO cells. Bar, 50 μm.

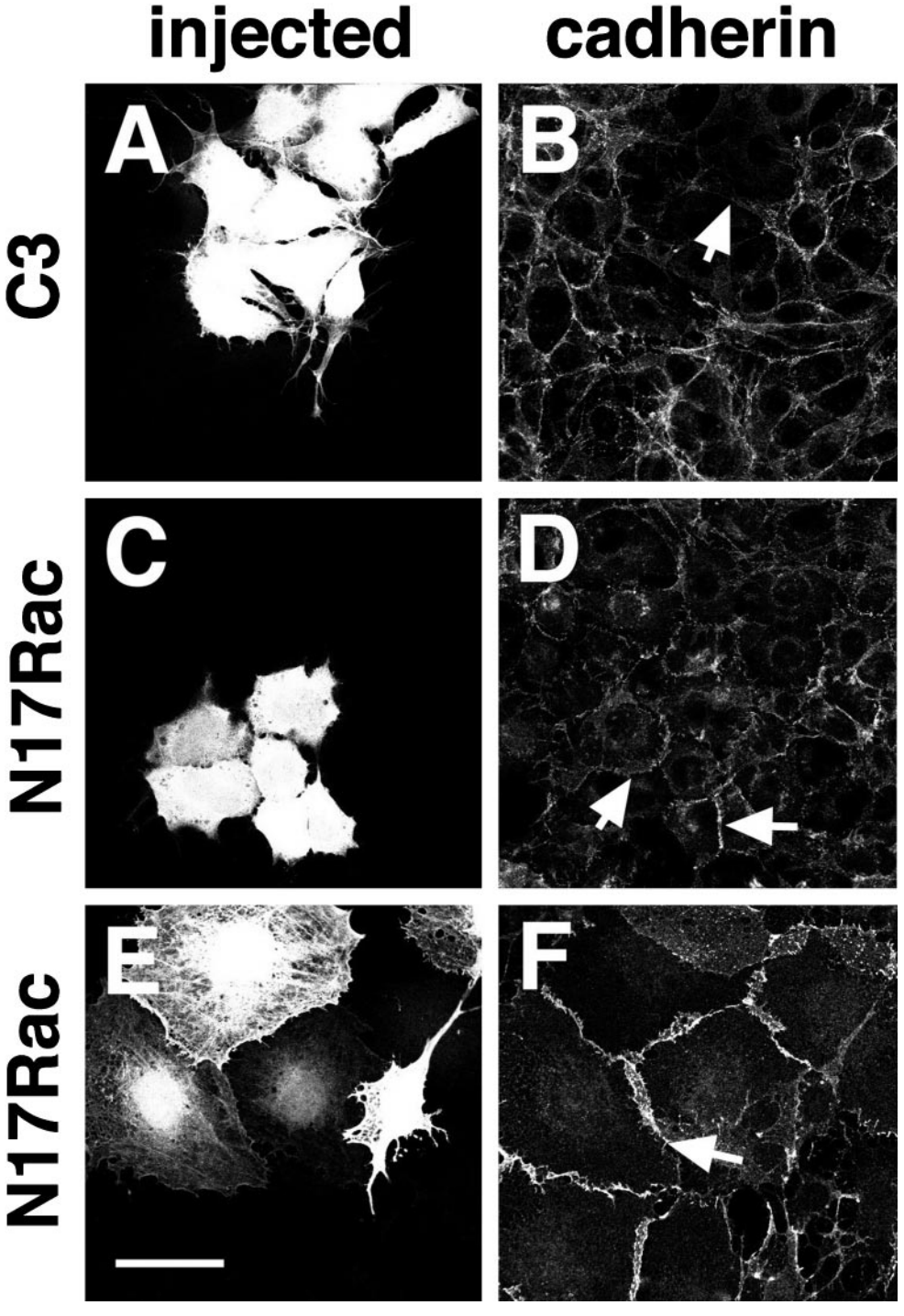

Figure 6. N17Rac is unable to disrupt cadherin-mediated adhesion in fibroblasts. L-cells expressing full-length E-cadherin were microinjected with C3 (A and B) or a dominant negative form of Rac (N17Rac; C and D), incubated for 1 or 2 h (A–D and our unpublished observations) and stained for E-cadherin (B and D). Quiescent Swiss 3T3 fibroblasts were also injected with N17Rac and incubated for up to 2 h (E and F and our unpublished observations) and stained with an anti–pan-cadherin antibody (F). Similar results were obtained after 1 or 2 h of incubation with either cell type. Injected cells are shown in panels A, C, and E. Arrows point to the absence (B) or presence (D and F) of cadherin at sites of cell–cell contacts. Bar, 50 μm.

6B). After microinjection of N17Rac, transfectant cells were incubated for up to 2 h, time sufficient for the removal of E-cadherin from the keratinocyte junctions (Figure 2). However, the localization of exogenous E-cadherin was not significantly affected when compared with control noninjected cells (Figure 6D, arrows). To test whether this effect was due to overexpression of E-cadherin by L-cells, injection of N17Rac into quiescent 3T3 cells was performed, and its effects on endogenous cadherin observed (Figure 6, E and F). Our results suggested that inhibition of Rac in either fibroblast cell line was unable to disrupt cadherin-

mediated adhesion (Figure 6, D and F, arrows). Unfortunately, a comparison with the response of the Swiss 3T3 endogenous cadherin to inhibition of Rho was not possible, as the cells retracted very quickly after microinjection (within 30 min, our unpublished results). Our results are consistent with the idea that the same type of receptor, E-cadherin, may be regulated differently by Rac, depending on the cell type context.

Activation of Rac Is Not Sufficient for the Redistribution of Actin and Cadherin Receptors

Inhibition of the small GTPases, Rho and Rac, in keratinocytes prevents the redistribution of cadherin to sites of cell–cell contacts, but only Rac function is required for the recruitment of actin to clustered cadherin receptors (Braga *et al.*, 1997). In fibroblasts, it has been shown that activation of Rac leads to a rapid accumulation of labeled actin at the cell periphery (Machesky and Hall, 1997; Figure 7A, arrow). We asked whether activation of Rac in epithelial cells is sufficient for the recruitment of actin to the cell borders, irrespective of the adhesive function mediated by cadherins.

Keratinocytes grown in the absence of cell–cell contacts were microinjected with an activated form of Rac (L61Rac) together with Cy3-labeled actin. Cells were incubated for 20 min either in the same medium (low calcium, Figure 7, E and F) or transferred to standard medium to induce intercellular contacts (std calcium, Figure 7, C and D), fixed and stained with an anti–E-cadherin antibody (Figure 7, D and F). Our results showed that actin accumulation at the cell periphery is dependent on cadherin-mediated cell–cell adhesion (Figure 7C, arrows; Braga *et al.*, 1997). In low calcium medium, Rac activation per se was not sufficient to drive the redistribution of actin or cadherin receptors to the cell periphery (Figure 7, E and F, arrows).

For comparison, the same experiment was performed using an activated form of Rho (L63Rho) and Cy3-labeled actin (Figure 7, G and H, and our unpublished results). Microinjection of L63Rho does not perturb the establishment of cadherin-dependent adhesion (Braga *et al.*, 1997) or the accumulation of labeled actin at cell–cell contacts (our unpublished results). However, constitutive activation of Rho was not sufficient to promote the accumulation of actin and cadherin receptors to the cell borders in the absence of calcium-dependent adhesion (Figure 7, G and H, arrows). Taken together, our results indicate that neither Rho nor Rac activation per se is sufficient to redistribute cadherin receptors to the cell periphery. In addition, in a process that differed from Rac effects in fibroblasts (Figure 7A, arrow), activation of Rac in keratinocytes was unable to promote the concentration of actin at the cell periphery in the absence of cadherin-mediated adhesion (Figure 7, E and F, arrows; Machesky and Hall, 1997).

DISCUSSION

We present evidence that the susceptibility of cadherin receptors to the inhibition of endogenous Rho and Rac is dependent on junction maturation and the cellular background. These results suggest that the regulation of cadherin function by the small GTPases might depend on the physiological context of the cells examined and might not be, therefore, a widespread phenomenon. Our results are summarized in Table 1 and are surprising for two reasons: first, the extensive homology and functional conservation observed within the cadherin and small GTPase family. Second, four different members of the cadherin family (E-, P-, N- and VE-cadherin) have been studied in detail in different cell types and shown to form similar intracellular complexes (Nagafuchi and Takeichi, 1989; Ozawa *et al.*, 1989; Knudsen and Wheelock, 1992; Johnson *et al.*, 1993; Butz and Kemler, 1994; Hinck *et al.*, 1994; Lampugnani *et al.*, 1995; Hertig *et al.*, 1996; Uchida *et al.*, 1996; Braga *et al.*, 1998).

Cadherin-dependent contacts are formed very quickly (within minutes) after addition of calcium ions, and junctions become mature with time in culture and upon confluence. This maturation is reflected, for instance, in the stabilization of the cadherin complexes at the surface, in the resistance of the receptors to detergent extraction from cell–cell contact sites, or in the development of tight junction-mediated permeability (Gumbiner *et al.*, 1988; Shore and Nelson, 1991; Marrs *et al.*, 1993; McNeill *et al.*, 1993; Braga *et al.*, 1995). Our results demonstrate that E-cadherin receptors present in mature keratinocyte junctions are sensitive to blocking endogenous Rho or Rac (Figure 2). Irrespective of the longer incubation time required after inhibition of the small GTPases, cadherin is removed from mature junctions before cell retraction and before desmosomes and other actin-binding receptors (i.e., integrins), indicating the specificity of the process. Although the biochemical modifications or interactions with the cytoskeleton that lead to junction maturation have not yet been defined, it seems that they also render the cadherin complexes more resistant to the inhibitory effects of C3 and N17Rac.

We next investigated the regulation of the function of different cadherin receptors by the small GTPases. E- and P-cadherin are coexpressed in keratinocytes, where they form independent complexes (Hirai *et al.*, 1989b; Johnson *et al.*, 1993). Both receptors are efficiently removed from junctions upon blocking Rho or Rac activity, suggesting that P-cadherin localization is regulated in a similar manner as E-cadherin in keratinocytes. Upon microinjection of C3 or N17Rac, E- and P-cadherin appear to be quickly degraded after

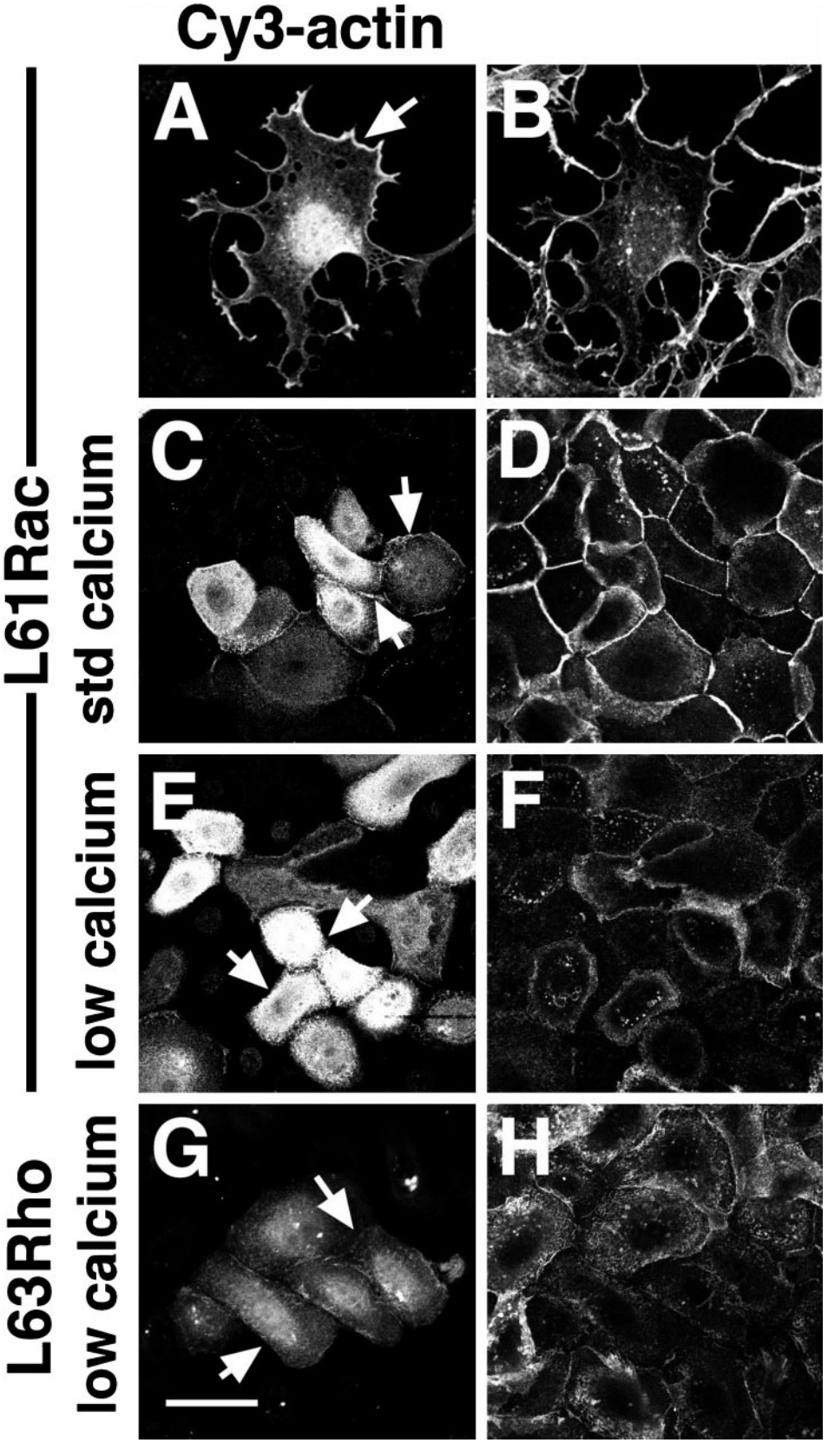

Figure 7. Activation of Rho or Rac is not sufficient to redistribute cadherin receptors and Cy3-labeled actin to the periphery of keratinocyte cells. Starved Swiss 3T3 fibroblasts were microinjected with L61Rac and Cy3-labeled actin (A) and after 20 min incubation, cells were fixed and stained with FITC-phalloidin (B). Keratinocytes grown in the absence of cell–cell contacts were injected with Cy3-actin and activated Rac (L61Rac, panels C–F) or activated Rho (L63Rho, panels G and H). Cells were incubated for 20 min in the same medium (low calcium, panels E–H) or immediately transferred to standard calcium medium to induce intercellular contacts (std calcium, panels C and D). Staining for E-cadherin receptors was performed (D, F, and H) and Cy3-actin pattern in injected cells was shown (A, C, E, and G). Arrows in panels A and C indicate accumulation of Cy3-actin at the cell periphery; arrows in E and G show diffuse localization of labeled actin. Bar, 50 μm.

Table 1. Summary of cadherin function regulation by the small GTPases: cellular context effect

Receptor	Cell type	Regulation by Rho	Regulation by Rac
E-cadherin	Keratinocytes	+	+
	L-cell	+	−
P-cadherin	Keratinocytes	+	+
VE-cadherin	Endothelial cells	−	−
	CHO	+	+

removal from cell–cell contacts, and this could be a specific effect of Rho or Rac inhibition that requires further investigation. However, it is known that in the absence of intercellular contacts the half-life of cadherin protein is reduced. Therefore, it is possible that the cadherin degradation seen after blocking Rho proteins is an indirect consequence of their destabilization from cell-cell contact sites.

In contrast to the effects observed with E- and P-cadherin in keratinocytes, the distribution of VE-cadherin is not perturbed by inhibition of Rho or Rac in unstimulated endothelial cells. The question is whether the maturation of endothelial junctions in a confluent monolayer is responsible for the resistance of VE-cadherin-dependent contacts to inactivation of Rho and Rac. However, the inhibiton of Rho or Rac have no effect on the localization of VE-cadherin in subconfluent or postconfluent cells (our unpublished results). Surprisingly, the formation of new VE-cadherin cell–cell contacts is also not perturbed when endogenous Rho or Rac is blocked. Nevertheless, VE-cadherin adhesion can be efficiently regulated by Rho proteins when the receptors are transfected into CHO cells. Our data indicate that the lack of inhibition of VE-cadherin function by small GTPases in endothelial cells does not correlate with the maturation status of the contacts, but rather the cellular context.

The above results are intriguing. We cannot formally exclude the possibility that, in endothelial cells, VE-cadherin adhesiveness is not regulated by the small GTPases in a similar way as E-cadherin in epithelia. However, we believe that the small GTPases are functional, as C3 microinjection results in qualitative changes in the pattern of VE-cadherin staining, even though the receptors are not released from intercellular junctions. In addition, endothelial cells are sensitive to microinjection of RhoGDI, a general inhibitor of Rho proteins (our unpublished results). Exogenous constitutively active Rho and Rac have been shown to induce the formation of stress fibers and lamellipodia in endothelial cells (Wojciak-Stothard *et al.*, 1998). This implies that endothelia contain all the cytoskeletal machinery to respond to the activation of small GTPases and produce similar actin structures as observed in fibroblasts and epithelial cells. However, it is unclear how these results fit with the different regulation of E- and P-cadherin versus VE-cadherin by Rho and Rac. It is possible that distinct targets of the small GTPases are expressed in different cells, and this may account for the cell type-specific activities of Rho proteins with respect to cadherin function. This is an important issue that requires further investigation. The fact that the cellular context determines the response to Rho and Rac may be relevant in terms of cell-specific response for permeability control in epithelial versus endothelial cells.

Consistent with these results, when E-cadherin receptors are expressed in fibroblast L-cells, their function is regulated by Rho but not Rac. Inactivation of Rho in fibroblasts leads to the removal of transfected E-cadherin from cell-cell contacts in a similar time course as in keratinocytes, suggesting that the same mechanism(s) might operate in either cell type. In contrast, even though Rac activation induces the formation of lamellipodia in both fibroblasts and epithelial cells, the regulation of cadherin adhesiveness via Rac differs between the two cell types.

We have previously shown that in keratinocytes actin redistribution to newly formed cell–cell contacts requires functional cadherin receptors and Rac activity (Braga *et al.*, 1997). Here we investigated further the effects of Rac on actin remodeling in keratinocytes and compared it with fibroblasts. Rac activation in fibroblasts results in a rapid accumulation of actin at the cell borders, in a process independent of adhesion to substratum via integrin receptors (Machesky and Hall, 1997). However, we demonstrate that Rac activation by itself cannot promote accumulation of actin at the keratinocyte periphery in the absence of cadherin-dependent contacts (Figure 7). It appears that, in epithelia, the appropriate clustering of cadherin receptors provides the spatial clues for actin accumulation.

One can envision a tripartite system, similar to the formation of integrin-dependent adhesion (Hotchin and Hall, 1995): a successful cadherin-mediated cell–cell contact involves functional cadherin complexes (in the presence of calcium ions and catenins), actin cytoskeleton, and the activity of endogenous Rho and Rac. Activation of Rho or Rac per se is not sufficient to promote the redistribution of cadherin receptors to the cell periphery, indicating that the correct positioning of cadherin receptors at cell borders requires homophilic binding. In addition, Rac participates in the recruitment of actin to the cell periphery but requires prior clustering of cadherin receptors at cell–cell contacts (Figure 7; Braga *et al.*, 1997).

In summary, our data provide insights on the possible roles that the small GTPases play in the regulation of cadherin-dependent adhesion. One important finding is that the modulation of the cadherin receptors adhesion by Rho or Rac depends on the matura-

tion of the junction and the cellular context. This implies that there is not a simple correlation between the presence of functional cadherin/catenin complexes and regulation of their function by the small GTPases. In addition, the same stimuli may have different activity in regulating the paracellular permeability in function of the cell type (i.e., endothelial or epithelial cells). The effects of Rho and Rac on cadherin-mediated adhesion would suggest a role during epithelial differentiation in the developing embryo. Appropriate levels of GTPase activity would be important to ensure junctional maturation and maintain tissue integrity after mesenchymal-epithelial transition and cytokinesis. Moreover, our results indicate that there are ways of strengthening cadherin-dependent cell–cell contacts, and understanding how it can be achieved will prove extremely useful in designing novel therapies. It will be important to dissect the elements that contribute to the differential effects of Rho and Rac in distinct cellular backgrounds, as it will provide insights into how to positively regulate cell–cell adhesion in tumors.

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