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Cadherin Engagement Regulates Rho family GTPases*

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The formation of cell-cell adherens junctions is a cadherin-mediated process associated with reorganization of the actin cytoskeleton. Because Rho family GTPases regulate actin dynamics, we investigated whether cadherin-mediated adhesion regulates the activity of RhoA, Rac1, and Cdc42. Confluent epithelial cells were found to have elevated Rac1 and Cdc42 activity but decreased RhoA activity when compared with low density cultures. Using a calcium switch method to manipulate junction assembly, we found that induction of cell-cell junctions increased Rac1 activity, and this was inhibited by E-cadherin function-blocking antibodies. Using the same calcium switch procedure, we found little effect on RhoA activity during the first hour of junction assembly. However, over several hours, RhoA activity significantly decreased. To determine whether these effects are mediated directly through cadherins or indirectly through engagement of other surface proteins downstream from junction assembly, we used a model system in which cadherin engagement is induced without cell-cell contact. For these experiments, Chinese hamster ovary cells expressing C-cadherin were plated on the extracellular domain of C-cadherin immobilized on tissue culture plates. Whereas direct cadherin engagement did not stimulate Cdc42 activity, it strongly inhibited RhoA activity but increased Rac1 activity. Deletion of the C-cadherin cytoplasmic domain abolished these effects.

Cell-cell adhesion is essential for the establishment and maintenance of normal tissue architecture. Cadherins are a major class of cell adhesion molecules responsible for strong cell-cell adhesion. In epithelial cells, cadherins are concentrated within adherens junctions (AJs).[1] Here their extracellular domains interact homophilically with cadherins on adjacent cells, and their cytoplasmic domains provide attachment to the actin cytoskeleton via catenins and other cytoskeletal proteins (1, 2).

Structural components of AJs have been well characterized (3, 4), while more recently attention has been focused on signaling events at the cytoplasmic face of cell-cell junctions. The Rho family of low molecular weight GTP-binding proteins is important for cadherin-mediated adhesion. Several lines of evidence suggest that members of the Rho family are required for the establishment and maintenance of cadherin-based AJs (5–9). In fibroblasts, RhoA activation stimulates assembly of stress fibers and focal adhesions, whereas activation of Rac1 and Cdc42 promote lamellipodial and filopodial extensions, respectively (10). In epithelial cells, overexpression of constitutively active Rac1 or Cdc42 increases E-cadherin localization and actin accumulation at cell-cell junctions, whereas these events are inhibited by dominant negative mutants of Rac1 and Cdc42 (6, 7, 9, 11, 12).

When epithelial cells are grown at low density, such that they cannot develop AJs, these cells often appear more fibroblastic and exhibit stress fibers and focal adhesions. AJ development is accompanied by a decrease in stress fibers and focal adhesions. The idea that AJs may negatively regulate focal adhesions is further supported by the finding that localized stimulation of N-cadherin results in decreased vinculin labeling and tyrosine phosphorylation in focal adhesions (13, 14). The observations that RhoA regulates focal adhesions and stress fibers raise the possibility that formation of cell-cell junctions may depress RhoA activity, thereby decreasing focal adhesions. In this work we have used several strategies to investigate whether cell-cell adhesion and cadherin engagement affect the activities of RhoA, Rac1, and Cdc42.

EXPERIMENTAL PROCEDURES

Cell Culture—CHO, HEK293, and NIH3T3 cells were cultured as described previously (15). Madin-Darby canine kidney (MDCK) cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. CEC1–5 cells were cultured as described (16). To generate stable cell lines expressing C-cadherin (C-Cad) and C-cadherin with a cytoplasmic truncation (C-CT), CHO cells were transfected using LipofectAMINE Plus reagents (Life Technologies, Inc.), and transfected cells were selected using 800 μg/ml G418. G418-resistant cells were fluorescence-activated cell-sorted by labeling with an antibody against the extracellular domain of C-cadherin, and expressing clones were obtained. Several clonal cell lines were analyzed for each assay performed.

For density experiments on epithelial cells, MDCK cells were trypsinized, extensively triturated to generate a maximally dispersed cell suspension, and plated in the presence of serum. Suspended cells were plated at a 1:3 dilution for the high density cultures and at a 1:10 dilution for low density cultures. Cells were allowed to recover for 24 h before measurement of RhoA, Rac1, and Cdc42 activity. RhoA, Rac1, and Cdc42 activity assays were performed as described previously (15). Quantitation was performed by densitometric analysis of Western blots using Metamorph Image software. The relative amount of active

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[1] The abbreviations used are: AJs, adherens junctions; CHO cells, Chinese hamster ovary cells; C-Cad, C-cadherin expressing CHO cells; C-CT, C-cadherin cytoplasmic truncated expressing CHO cells; CEC, C-cadherin extracellular domain; MDCK cells, Madin-Darby canine kidney cells; p120ctn, p120 catenin; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione *S*-transferase; PI3-kinase, phosphatidylinositol 3-kinase.

GTPases was determined by measuring the amount of GTPases sedimented by glutathione *S*-transferase-Rac1/Cdc42-binding domain of PAK or -RhoA-binding domain of Rhotekin relative to the amount in whole cell lysates.

Calcium Switch Experiments—For calcium switch experiments, cells were incubated overnight in low calcium medium (10 μM). The medium was then switched to high calcium medium (1.8 mM) at various time points with or without E-cadherin function-blocking antibodies (DECMA clone-1 at 1:100 dilution) (Sigma-Aldrich), and Rac1 and Cdc42 activity levels were measured. For RhoA activity assays, cells were serum-starved overnight, and cell-cell junctions were disrupted by incubation with 4 mM EGTA for 30 min at 37 °C (17, 18). Cell-cell junction formation was then stimulated by the addition of serum-free DMEM. RhoA activity was measured in serum-free conditions to reduce background levels because of serum activation of RhoA. We also used this procedure for the Rac1 activity measurements and obtained identical results with both procedures.

CEC Experiments—The C-cadherin extracellular (CEC) domain was purified essentially as described previously (16) except the Mono Q step was omitted. The CEC domains were linked to tissue culture dishes by coating with 5% 3-aminopropyltrimethoxysilane (Pierce) in 1 mM acetic acid for 10 min. Dishes were washed and incubated with 0.5% glutaraldehyde in PBS for 30 min, washed, and then incubated with 1 μg/ml nitrilotriacetic acid ligand (NTA, Qiagen) for 20 min and charged with 100 mM nickel sulfate in water for 20 min. Dishes were then incubated with 20 μg/ml CEC overnight at 4 °C with rotation and then blocked in 1% bovine serum albumin in DMEM. In preliminary studies, we analyzed the binding of CEC to the 3-aminopropyltrimethoxysilane-coated dishes using radioiodinated CEC. Saturated binding was obtained at a concentration of 20 μg/ml. For CEC binding experiments, cells were starved overnight in 0.5% fetal bovine serum in the absence of G418. Cells were washed with PBS, suspended with 0.2 mM EDTA in PBS, washed twice in DMEM, and suspended for 0.5 h prior to plating in 0.5% fatty acid-free bovine serum albumin in DMEM.

RESULTS

To measure the effect of cell junction development on the activity of RhoA, we first determined whether the level of active RhoA differed in epithelial cells grown at high or low densities. MDCK cells were plated such that they were confluent or at a density at which they would develop few contacts with their neighbors. The cells were maintained in serum for ~24 h before the level of active, GTP-bound RhoA was measured using Rhotekin affinity precipitation (19). This revealed a decrease in active RhoA by ~95% in the confluent MDCK cells relative to the low density cultures ($p < 0.0001$) (Fig. 1*A*). The total level of RhoA expressed in MDCK cells was similar in both conditions. In addition, we examined the level of active RhoA in HEK293 and NIH3T3 cells at high and low density. HEK293 cells are epithelial in origin but express lower levels of E-cadherin than MDCK cells and do not assemble well developed AJs, whereas NIH3T3 cells are fibroblastic and lack E-cadherin. With the HEK293 cells, confluent cultures displayed a ~40% decrease in RhoA activity ($p < 0.04$). NIH3T3 cells displayed no significant change in the level of active RhoA under these conditions of differing cell densities ($p = 0.19$) (Fig. 1*A*).

The marked depression in RhoA activity in confluent cultures of MDCK cells prompted us to examine the effect of cell density on the activities of Rac1 and Cdc42 using affinity precipitation of the GTP-bound forms of these proteins (20, 21). With MDCK cells, there was a 3.7 ± 0.9-fold increase in Rac1 activity and a 3.3 ± 1.1-fold increase in Cdc42 activity in confluent cultures ($p < 0.05$ and 0.02; Fig. 1, *B* and *C*). The total levels of Rac1 and Cdc42 were comparable in confluent and subconfluent cultures. In confluent HEK293 cells there was a 1.9 ± 0.4-fold increase in Rac1 activity and a 1.4 ± 0.1-fold increase in Cdc42 activity compared with subconfluent cells ($p < 0.14$ and 0.04). There was no significant change in Rac1 or Cdc42 activity between subconfluent and confluent cultures of NIH3T3 fibroblasts ($p < 0.65$ and 0.69; Fig. 1, *B* and *C*).

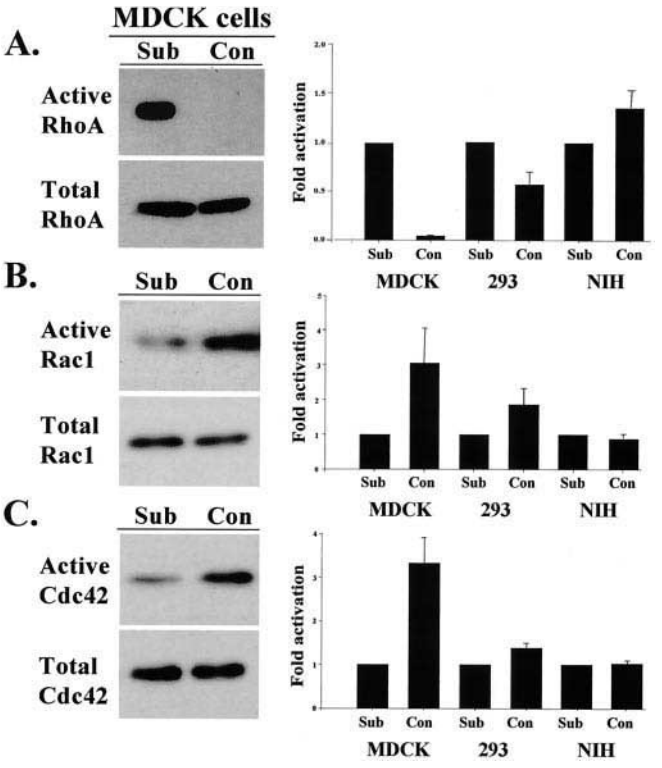

FIG. 1. **Cell density regulates RhoA, Rac1, and Cdc42 activity.** The levels of GTP-bound RhoA (*A*) Rac1 (*B*), and Cdc42 (C) were measured in subconfluent and confluent MDCK, NIH3T3, and HEK293 cells. Data are the means ± S.E. of at least three independent experiments.

To examine the role of cell-cell junctions in the depression of RhoA activity and elevation of Rac1 and Cdc42 that occur with high cell density, we manipulated cadherin-mediated junction formation by varying the level of extracellular calcium in the culture medium. Under conditions of low calcium, cadherin function is blocked, and epithelial cells fail to develop or maintain AJs (17). Restoration of calcium stimulates the rapid reformation of these adhesions. In low calcium conditions, the cells lacked AJs, as judged by immunofluorescence, and the E-cadherin was distributed diffusely over the surface of the cells (data not shown). Upon restoration of normal calcium levels, E-cadherin accumulated at sites of cell-cell contact within 5–10 min, and junctions appeared to be well developed within 1 h as determined by staining with antibodies against several junctional proteins (data not shown). In response to calcium addition, there was little or no change in RhoA activity for the first hour (Fig. 2*A*). Over longer time periods, however, RhoA activity decreased so that by 8 h it was very low. This slow response led us to question whether in addition to junctions depressing RhoA activity, the "calcium switch" manipulation itself may activate RhoA independently of junction assembly. To explore this possibility, the effect of the calcium switch manipulations on RhoA activity was determined using cells that lack cadherins (CHO cells) or that lack specifically E-cadherin (Rat1 cells). This approach revealed that RhoA activity was stimulated for at least 1 h in response to restoring normal extracellular calcium levels, suggesting that simply changing the exogenous calcium levels results in activation of RhoA through a cadherin-independent pathway (Fig. 2*A* and data not shown).

The level of active Rac1 was measured in MDCK cells in which AJs had been induced to form by the calcium switch procedure. Under these conditions, an increase in Rac1 activity was detected within 5 min (Fig. 2*B*). To determine whether this

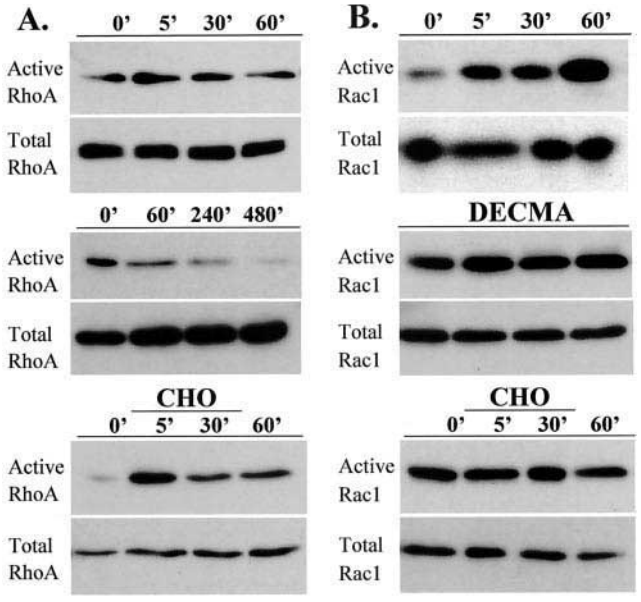

FIG. 2. **Cadherin-mediated adhesion regulates RhoA and Rac1 activity.** Cell-cell junctions were manipulated using a calcium switch procedure as described under "Experimental Procedures." *A*, at indicated time points after calcium restoration, cells were lysed, and the level of GTP-bound RhoA was measured for the first hour (*top panels*) and over several hours (*middle panels*). As a control, the same manipulations were performed using CHO cells (*bottom panels*). *B*, the level of GTP-bound Rac1 was measured at indicated time points after calcium restoration (*top panels*). Rac1 activity was measured using the same calcium switch protocol but in the presence of E-cadherin function-blocking antibodies (*middle panels*) and in CHO cells exposed to the calcium switch conditions (*bottom panels*).

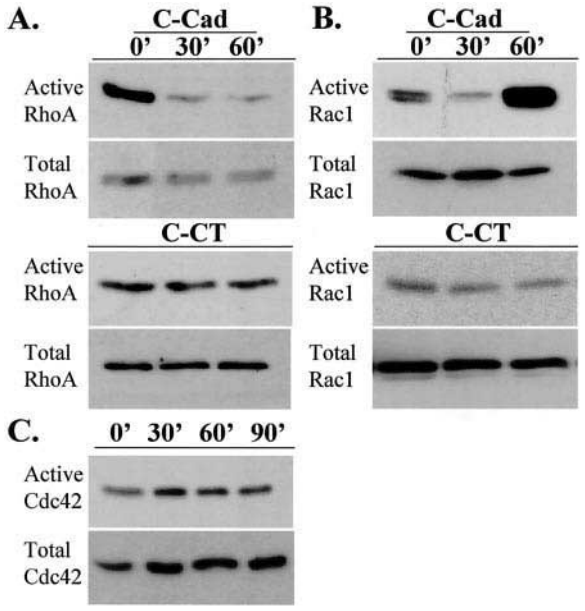

FIG. 3. **Regulation of Rho family GTPases by C-cadherin engagement.** Serum-starved C-Cad or C-CT cells were suspended for 0.5 h and plated on tissue culture dishes coated with the CEC domain (see "Experimental Procedures"). At the specified time points, cells were lysed and assayed for the levels of GTP-bound RhoA (*A*), Rac1 (*B*), and Cdc42 (*C*). Data are representative of multiple independent experiments.

increase in Rac1 activity required E-cadherin engagement, the restoration of calcium was performed in medium containing E-cadherin function-blocking antibodies (DECMA-1) (Fig. 2*B*). The increase in Rac1 activity induced by calcium restoration was blocked by DECMA-1, suggesting that E-cadherin is critical for Rac1 activation. We did not observe changes in Rac1 activity in CHO cells (Fig. 2*B*) or Rat1 cells (data not shown) following the calcium switch procedure. The level of active Cdc42 was also measured in MDCK cells developing junctions in response to calcium restoration. Cdc42 activity was stimulated under these conditions (data not shown) as reported by others (22).

The results detailed above do not distinguish whether the activation of Rac1 and Cdc42 and decrease in RhoA activity occurred directly via cadherins or indirectly as a result of adherens junction formation engaging other signaling components. To address the question of whether cadherins themselves signal directly to Rho family proteins, we examined cadherin engagement in the absence of cell-cell adhesion. For these experiments we generated CHO cell lines that stably express full-length *Xenopus* C-Cad or C-CT. C-cadherin is a classical cadherin closely related to E-cadherin. To engage the C-cadherin, a His-tagged construct of the CEC was purified from conditioned medium and bound to tissue culture plates via the His tag (see "Experimental Procedures"). We confirmed that adhesion to this surface was dependent on calcium in the extracellular medium (data not shown).

C-Cad cells were starved overnight in 0.5% serum and plated on surfaces coated with the CEC domain, and the levels of active RhoA, Rac1, and Cdc42 were measured at successive time points (Fig. 3). This revealed that within 30 min there was a marked drop in RhoA activity, which was sustained over longer time periods. At 30 min there was a slight dip in Rac1 activity, but by 1 h Rac1 activity was strongly stimulated. Little change in Cdc42 activity was detected under these conditions. The effect of the C-cadherin cytoplasmic domain truncation was examined with respect to the changes in activities of RhoA and Rac1. When C-CT cells were plated on surfaces coated with CEC, no changes in RhoA or Rac1 activity were detected (Fig. 3, *A* and *B*). Similarly, no change in the activity of Rac1 was detected when the parental CHO cells lacking C-cadherin expression were plated on CEC (data not shown). As positive controls, the C-Cad cells were plated on dishes coated with fibronectin. This resulted in a biphasic response in RhoA activity, with an initial dip being followed by a more prolonged elevation in activity (data not shown) as also reported by others (19, 23, 24). In response to adhesion to fibronectin, C-Cad cells demonstrated an increase in Rac1 and Cdc42 activity (data not shown) similar to that described in other cell types (24–27).

DISCUSSION

The observation that assembly of epithelial AJs is well known to be associated with reorganization of the actin cytoskeleton (28) led us to investigate whether members of the Rho family GTPases are regulated by the formation of AJs. In this study, we found that adherens junction assembly triggers a dramatic decrease in RhoA activity and a stimulation of Rac1 and Cdc42 activity. Kim and colleagues (22) recently used the calcium switch procedure to demonstrate that formation of AJs stimulates the activity of Cdc42. After completion of the current work, Nakagawa *et al.* (29) described the activation of Rac1 in response to assembly of AJs. We have extended these observations not only by examining RhoA activity following junction formation but also by determining whether the activation of Rac1 and Cdc42 and the decrease in RhoA activity is due to signaling directly via cadherins or indirectly via assembly of AJs. When AJs assemble, the plasma membranes of adjacent cells are brought into close proximity so that low affinity interactions may occur between signaling components on adjacent cells. To analyze whether the signaling was initiated directly by cadherins, we plated cells expressing C-cad-

herin on the extracellular domain of C-cadherin. Using this system, we found that cadherin engagement without cell-cell adhesion was sufficient to induce the decrease in RhoA activity and the increase in Rac1 activity. Significantly, these effects were not observed when the cytoplasmic domain of C-cadherin was deleted, implying that interactions mediated by the cytoplasmic domain are critical in these signaling pathways. Interestingly, although we and others (22) have observed activation of Cdc42 in the calcium switch paradigm, we did not observe significant activation of Cdc42 upon direct cadherin engagement using the C-cadherin system. This suggests that activation of Cdc42 is secondary to cadherin engagement, possibly occurring by AJs juxtaposing signaling molecules that interact between adjacent cells.

The decrease in RhoA activity we observed is in contrast to the recent results presented by Nakagawa *et al.* (29). Like us, they observed that the calcium switch did not induce a notable change in RhoA activity over the time course of their experiments. However, when we performed the same calcium manipulations on fibroblasts, these experiments indicated that RhoA was activated in these cells in response to calcium restoration. It seems likely that the initial calcium-induced stimulation of RhoA activity obscured any decrease in RhoA activity that results from cadherin engagement. These observations raise important concerns about drawing conclusions from the calcium switch paradigm when this is the only way in which cadherin engagement is manipulated.

What is the significance of the changes in activity of the Rho family of proteins as AJs develop? Multiple studies indicate that Rac1 and Cdc42 activities are needed for the assembly of AJs (6, 7, 9, 12). In addition, low levels of RhoA activity also appear necessary (5, 8, 12), although high RhoA activity is disruptive (8, 12). The stimulation of Rac1 and Cdc42 upon adherens junction assembly indicates a positive feedback loop that may contribute to the stability of junctions in epithelial sheets. On the other hand, the depression of RhoA activity may also be important so that myosin activity is kept low and excessive tension is not applied to the junctions.

Our current efforts are directed toward elucidating the pathways by which cadherin engagement depresses RhoA and elevates Rac1 activity. Multiple GTPase-activating proteins for RhoA have been identified. We are investigating known GTPase-activating proteins for RhoA that might be involved in this pathway. In previous work, we and others demonstrated that the cadherin-binding protein, p120 catenin (p120ctn), decreases RhoA activity and increases Rac1 and Cdc42 activity when over-expressed in cells (15, 30, 31). Additionally, we demonstrated that p120ctn binds to the guanine nucleotide exchange factor Vav2 (15). However, we consider it to be unlikely that p120ctn is involved in these pathways from cadherins to RhoA and Rac1, because during AJ formation p120ctn is recruited to the cadherin-catenin complex reducing cytosolic levels of p120ctn. Our previous data indicated that p120ctn only affected the activities of RhoA and Rac1 when it was not bound to cadherins but was in the cytosolic pool making it unlikely that regulation of Rho proteins by cadherin engagement is through p120ctn. In preliminary work, we have not detected activation of Vav2 by adherens junction formation.[2] One potential pathway to Rac1 activation is suggested by the finding that adherens junction formation stimulates PI3-kinase activity (32). In their recent work, Nakagawa and coworkers (29) found that inhibition of PI3-kinase blocked activation of Rac1 by cadherin-mediated adhesion. We have also made this observation.[2] A Rac1 exchange factor downstream of PI3-kinase is Tiam1 (33), and Tiam1 localizes to cell-cell junctions (6). We are currently analyzing the roles of Tiam1 and other exchange factors in response to cadherin engagement.

A large amount of information has been learned about the signaling pathways downstream from integrins. In contrast, cadherins have generally been considered to play a predominantly structural role, mediating strong cell-cell adhesion and providing a link for the actin cytoskeleton to the membrane at these sites of adhesion. Our finding here that cadherins themselves are receptors that signal to the Rho family of GTPases expands the repertoire of signaling pathways that cadherins initiate. It also contributes to the idea that many cell adhesion molecules signal to the Rho family of proteins, thereby relaying information from adhesion to the organization of the cytoskeleton.

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[2] N. K. Noren and K. Burridge, unpublished results.

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