Cadherin Engagement Inhibits RhoA via p190RhoGAP*

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Cadherins are transmembrane receptors that mediate cell-cell adhesion in epithelial cells. A number of changes occur during cadherin-mediated junction formation, one of which is a rearrangement of the actin cytoskeleton. Key regulators of actin cytoskeletal dynamics in cells are the Rho family of GTPases. We have demonstrated in previous studies that cadherin signaling suppresses RhoA activity and activates Rac1. The signaling events downstream of cadherins that modulate the activity of Rho family proteins remain unknown. Here we have identified a pathway by which RhoA becomes inactivated by cadherins. To determine whether cadherins regulate RhoA through activation of a GTPase-activating protein (GAP) for RhoA, we used constitutively active RhoA to isolate activated GAPs. Using this assay, we have identified the RhoA-specific GAP, p190RhoGAP, downstream from engaged cadherins. We found that cadherin engagement induced tyrosine phosphorylation of p190RhoGAP and increased its binding to p120RasGAP. The increased precipitation of p190RhoGAP with 63LRhoA was blocked by addition of PP2 suggesting that Src family kinases are required downstream from cadherin signaling. The inhibition of RhoA activity by cadherins was antagonized by expression of a dominant negative p190RhoGAP. Taken together, these data demonstrate that p190RhoGAP activity is critical for RhoA inactivation by cadherins.

Cell-cell adhesion is crucial during embryonic development and in the maintenance of normal tissue homeostasis. Cadherins are a major class of adhesion molecules responsible for the prominent adherens junctions (AJs) in epithelial cells. Cadherins bind homophilically to cadherins on neighboring cells in a calcium-dependent manner. In addition to their role as adhesion molecules, cadherins recently have been found to trigger signals that regulate the Rho family of GTPases (1).

Rho proteins are known regulators of cell morphology and the actin cytoskeleton. They cycle between an inactive GDP-bound state and an active GTP-bound state. The activation of these proteins is stimulated by guanine nucleotide exchange factors (GEFs) that exchange GDP for GTP and are inhibited by GTPase-activating proteins (GAPs) that hydrolyze the GTP to GDP (2). Rho family members are also negatively regulated by guanine nucleotide dissociation inhibitors (GDIs), which remove Rho proteins from the plasma membrane (2). In fibroblasts, Rac1 and Cdc42 activation result in lamellipodial and filopodial extension, respectively, and RhoA regulates stress fiber and focal adhesion formation (3). In epithelial cells, Rac1 and Cdc42 activity are required for cadherin-mediated adhesion (4–7). Additionally a basal level of RhoA activity is necessary for AJ formation, although high RhoA activity is disruptive (7–9).

In previous studies, we and other laboratories have found that cadherin-mediated adhesion activates Rac1 and Cdc42 and inhibits RhoA activation (1, 10–16). Moreover direct cadherin engagement or cadherin clustering was sufficient to activate Rac1 (11, 12, 15) and inhibit RhoA (12). Here we have explored the mechanism by which RhoA becomes inactivated by cadherins. Since Rho proteins are negatively regulated by GAPs, we examined whether cadherins regulate RhoA via activation of a GAP. To identify activated GAPs, we developed an assay that exploits the interaction of GAPs with the GTP-bound form of RhoA. This analysis identified p190RhoGAP downstream of cadherin engagement. We show that p190RhoGAP is necessary and sufficient for inactivating RhoA in response to cadherin engagement.

EXPERIMENTAL PROCEDURES

Plasmids—To generate pEGFP-p190RhoGAPR1283A the p190RhoGAP mutant was excised from pKH3-p190RhoGAP-R1283A (17) using BamHI and EcoRI and subcloned into pEGFP-C1 (Clontech) that had been digested with BglII and EcoRI. The bacterial expression vectors for GST-tagged nucleotide-free RhoA (17ARhoA), wild type RhoA (WTRhoA), and constitutively active RhoA (63LRhoA) have been described previously (18).

Cell Culture—CHO cells expressing C-cadherin (C-Cad) or C-cadherin with a cytoplasmic truncation (C-CT) were generated and cultured as described previously (12). For transfection experiments, cells were transiently transfected with pEGFP-C1 or pEGFP-p190RhoGAPR1283A using LipofectAMINE Plus reagents (InVitrogen) according to the manufacturer’s directions. RhoA activity assays were performed and quantified as described previously (19, 20).

GST Precipitations—For GST precipitations, C-Cad or C-CT cells were starved overnight in 0.5% fetal bovine calf serum. Cells were washed with phosphate-buffered saline, suspended with 0.2 mM EDTA in phosphate-buffered saline, washed twice with serum-free Dulbecco’s modified Eagle’s medium, and suspended for 1.0 h prior to plating in 0.5% fetal bovine serum albumin in Dulbecco’s modified Eagle’s medium. For PP2 experiments, suspended cells were treated for 30 min with MeSO or PP2 (0.5 μM, Calbiochem) and plated in the presence of the inhibitor. Cells were plated on fibronectin (25 μg/ml), polylysine (10 μg/ml), or C-cadherin extracellular domain 1–5 (20 μg/ml; CEC) for the indicated times. The CEC was purified as described previously (12).

After plating for the indicated times, cells were washed twice with Hanks’ balanced salt solution and lysed in 50 mM HEPES, 150 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 1 mM diithiothreitol, 10 μM each of aprotinin and leupeptin, and 0.5 mM sodium orthovanadate. Lysates

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Beads were pelleted, and the supernatants were incubated with ROCK antibodies against p120RasGAP, p190RhoGAP, phosphotyrosine were analyzed by SDS-PAGE followed by Western blotting with washed at least three times with lysis buffer, and bound proteins above lysis buffer for 10 min at 4°C incubated with glutathione-Sepharose beads equilibrated with the

were clarified by centrifugation for 10 min, and the supernatants were incubated with glutathione-Sepharose beads equilibrated with the above lysis buffer for 10 min at 4°C to reduce nonspecific binding. Beads were pelleted, and the supernatants were incubated with 10–30 μg of the appropriate GST fusion protein. The samples were washed at least three times with lysis buffer, and bound proteins were released from the beads by boiling in sample buffer. Samples were analyzed by SDS-PAGE followed by Western blotting with antibodies against p120RasGAP, p190RhoGAP, phosphotyrosine (PY20), ROCKα and β (all from BD Biosciences), Lsc, RhoGDI (Santa Cruz Biotechnology), and PSGAP (Dr. Wen-Cheng Xiong, University of Alabama, Birmingham, AL).

Immunoprecipitations—Immunoprecipitations were performed as described previously (20) and probed with monoclonal antibodies against p190RhoGAP or phosphotyrosine (PY20). Densitometric analysis of films was performed as described previously (20). The relative amount of phosphorylated p190RhoGAP was determined by measuring the amount of phosphorylated p190RhoGAP immunoprecipitated relative to the total amount of p190RhoGAP.

RESULTS

In previous work, we demonstrated that direct cadherin engagement suppresses RhoA activity (12). Since Rho family proteins are negatively regulated by GAPs, we wished to examine whether cadherin engagement regulates RhoA through activation of a RhoA-specific GAP. We developed an assay to detect activated GAPs in cell lysates. Because GAPs bind with high affinity to activated Rho proteins, we assessed whether a GST fusion with the constitutively active 63LRhoA is able to bind to GAPs. WTRhoA, nucleotide-free RhoA (17ARhoA), and 63LRhoA were expressed as GST fusion proteins, and affinity precipitations were performed on lysates of CHO cells. The 17ARhoA mutant was generated based on the analogous G15A mutation in Ras and has previously been shown to bind with high affinity to GEFs (18, 21). Pull-downs were immunoblotted with antibodies against the GAP p190RhoGAP, the effector ROCK (ROK or Rho kinase), a GEF Lsc, and RhoGDI to assess the ability of these GST fusion proteins and GST to bind to known regulators of Rho proteins. This analysis revealed that constitutively active RhoA selectively bind the RhoA-specific GAP p190RhoGAP, whereas it could not be detected in control precipitations (Fig. 1A). Additionally, the effector ROCK was detected in 63LRhoA precipitations. Although WTRhoA and 17ARhoA did not bind to p190RhoGAP, these fusion proteins bound to RhoGDI and GEFs, respectively, and as expected (Fig. 1A) (18).

The finding that 63LRhoA is able to bind GAPs led us to investigate whether we could use 63LRhoA to identify activated GAPs. To test this approach, we examined a pathway in which a GAP is known to be activated. Previous studies demonstrated that adhesion to fibronectin and integrin engagement inhibit RhoA by activating p190RhoGAP (22, 23). To establish whether increased quantities of GAPs are precipitated by 63LRhoA upon GAP activation, we investigated the binding of p190RhoGAP to 63LRhoA in response to fibronectin. We performed these experiments in C-Cad cells because these cells were to be used in experiments examining cadherin signaling (see Fig. 2) (12). Additionally these cells exhibit a transient inhibition of RhoA by integrin engagement (data not shown), similar to that previously described for other cell lines (16, 19, 22). In suspension, trace levels of p190RhoGAP were co-sedimented with 63LRhoA, whereas adhesion to fibronectin greatly increased the amount of p190RhoGAP recovered (Fig. 1B). As a control, 63LRhoA precipitations were probed with antibodies against PSGAP, another GAP for RhoA present in these cells (24). PSGAP, however, was not detected in 63LRhoA precipitations in response to fibronectin adhesion (Fig. 1B).

Recent evidence suggests that p190RhoGAP activity is regulated by tyrosine phosphorylation. In response to both epidermal growth factor stimulation and integrin engagement increased tyrosine phosphorylation of p190RhoGAP correlates with its activation (22, 25, 26). To examine whether the p190RhoGAP sedimented with 63LRhoA is tyrosine phosphorylated, we immunoblotted precipitations with antibodies against phosphotyrosine. This analysis revealed that the p190RhoGAP precipitated with 63LRhoA is tyrosine phosphorylated (Fig. 1C).

Active, tyrosine phosphorylated p190RhoGAP has been shown to bind to p120RasGAP (27, 28), and this binding is thought to be important in regulating cell adhesion and migration (29). Consequently we analyzed whether p120RasGAP could be detected together with p190RhoGAP in 63LRhoA precipitations. Increased amounts of p120RasGAP were detected...
in response to cell adhesion to fibronectin (Fig. 1C). Additionally the p120RasGAP precipitated was tyrosine phosphorylated (data not shown). Taken together, these results suggest that the p190RhoGAP sedimented with 63LRhoA is active.

The above results validate the use of 63LRhoA to bind active RhoA GAPs. We wanted to apply this technique to sediment RhoA GAPs activated by cadherin engagement. Src family kinases localize to and regulate AJs (30–33), and Src activates p190RhoGAP (22, 28, 34). These findings led us to examine whether p190RhoGAP is downstream from cadherin engagement. Previously we used a model system for studying direct cadherin engagement in the absence of cell-cell contact (12). Cadherin engagement was stimulated by placing C-Cad cells on the CEC immobilized on tissue culture plates. Using this system, we determined that cadherin signaling inhibits RhoA (12). Here we have exploited the same system to address whether cadherin engagement regulates p190RhoGAP. We found that direct cadherin engagement increased the amount of p190RhoGAP precipitated by 63LRhoA (Fig. 2). Moreover, the p190RhoGAP precipitated by 63LRhoA is tyrosine phosphorylated (Fig. 2A). Increased levels of p120RasGAP were also sedimented by 63LRhoA in response to cadherin engagement (Fig. 2A), and the p120RasGAP precipitated was tyrosine phosphorylated (data not shown). PSGAP could not be detected in 63LRhoA precipitations after cadherin engagement suggesting that this GAP is not regulated by cadherin engagement (data not shown).

To determine whether increased p190RhoGAP binding to 63LRhoA is specific to cadherin-mediated adhesion and not a general adhesion effect, we plated C-Cad cells on either polylysine or the CEC and analyzed the levels of p190RhoGAP that were pulled down by 63LRhoA. The engagement of cadherins stimulated the association of p190RhoGAP with 63LRhoA, whereas p190RhoGAP was not detected in precipitates from cells adhered to polylysine or in precipitates obtained with GST alone (Fig. 2B).

To determine whether cadherin engagement activates p190RhoGAP, we examined tyrosine phosphorylation levels of immunoprecipitated p190RhoGAP from C-Cad cells in response to cadherin engagement. Cadherin signaling induced a 2-fold increase in p190RhoGAP tyrosine phosphorylation relative to suspended cells (Fig. 2C). We observed no increase in p190RhoGAP tyrosine phosphorylation when cells expressing a cytoplasmic deleted C-cadherin were plated on the CEC suggesting that an intact cytoplasmic domain is required for p190RhoGAP phosphorylation and activation by cadherin engagement (data not shown).

Since p190RhoGAP is tyrosine phosphorylated and activated by Src (35–37), we examined whether Src family kinase activity is required for the increased precipitation of p190RhoGAP with 63LRhoA in response to cadherin engagement. C-Cad cells were treated with vehicle alone (Me2SO) or the Src inhibitor PP2 (0.5 μM). Cadherin engagement increased precipitation of p190RhoGAP with 63LRhoA, while PP2 treatment ablated this association (Fig. 2D). These data suggest that Src family kinases are required for activation of p190RhoGAP by cadherin engagement.

To establish whether p190RhoGAP mediates the depression of RhoA activity induced by cadherin engagement, we transiently expressed a GAP-deficient, GFP-tagged mutant of p190RhoGAP (p190RhoGAPR1283A), or GFP alone in C-Cad cells (17, 22, 23). This p190RhoGAP mutant lacks the arginine that is essential for its GAP activity (17), and expression of this mutant acts as a dominant negative toward endogenous p190RhoGAP and blocks inhibition of RhoA by integrin signaling (22). Blotting cell lysates with antibodies against p190RhoGAP revealed that the expression level of p190RA is typically 2–3-fold higher than that of endogenous p190RhoGAP (Fig. 3). Transient expression of this mutant antagonized RhoA inactivation in response to cadherin engagement (Fig. 3), consistent with p190RhoGAP mediating RhoA inhibition in response to cadherin engagement.

**DISCUSSION**

In this study we found that cadherins signal to RhoA through the GAP p190RhoGAP. Our previous work indicated that direct cadherin engagement was sufficient to activate RhoA and stimulate Rac1. At that time it was unclear how cadherins signal to Rho proteins. Our current results provide insight into the signaling downstream from cadherins. Additionally we describe here an assay to sediment active GAPs using constitutively active RhoA. We tested this assay on the pathway in which adhesion to fibronectin activates p190RhoGAP. At time points where p190RhoGAP is activated by integrins, more p190RhoGAP was precipitated with 63LRhoA. Using this assay to identify GAPs activated by cadherins, we found that cadherin engagement also increased the precipitation of p190RhoGAP with 63LRhoA. Expression of a dominant negative mutant of p190RhoGAP that inhibits endogenous p190RhoGAP activity blocks cadherin-mediated depression of RhoA activity. Together these results suggest that p190RhoGAP is downstream from cadherin engagement.

Several lines of evidence raised the possibility that p190RhoGAP might be responsible for depressing RhoA activity downstream from cadherins. Thus, p190RhoGAP is a substrate for and activated by Src family kinases (35–37), and these kinases localize to and regulate AJs (30–33). Moreover the depression of RhoA activity in response to integrin engagement has been shown to be due to Src-induced activation of p190RhoGAP (22). Additionally p120RasGAP was shown to become tyrosine phosphorylated in response to AJs (38), and p120RasGAP is a binding partner of activated p190RhoGAP. We have found that p190RhoGAP becomes tyrosine phosphorylated in response to cadherin engagement and that this is blocked by treatment of cells with the Src family kinase inhibitor PP2 (data not shown). This treatment also diminishes the binding of p190RhoGAP to 63LRhoA, consistent
with its activity being regulated by cadherin-mediated Src family phosphorylation. We have also found that p120RasGAP binding to p190RhoGAP is induced by cadherin engagement, further supporting a role for p190RhoGAP activation downstream from cadherins. Together these results suggest a pathway by which cadherin engagement stimulates activation of Src family kinases, which, in turn, phosphorylate and activate p190RhoGAP. The active p190RhoGAP, targeted in some manner to the AJs, promotes GTP hydrolysis by RhoA in this region. In future work, it will be interesting to explore how Src family kinases become activated downstream from cadherin engagement. However, our preliminary data indicate that the cadherin cytoplasmic domain is critical for this to occur.

In addition to tyrosine phosphorylation, specific localization of p190RhoGAP is thought to be important for its regulation. p190RhoGAP localizes to protrusive structures, such as the leading edge of migrating fibroblasts and to growth cones in neurons (26, 39, 40). Both of these regions are highly dynamic and protrusive. It has been proposed that Rho inhibition by p190RhoGAP is critical in the leading edge of fibroblasts to promote protrusion and thus cell migration (23). The formation of cell-cell AJs is also a dynamic process where the cells extend many protrusive structures to initiate cell-cell contact. We propose that p190RhoGAP inactivation of RhoA is needed to reduce tension and contractility, thereby promoting the formation of cell-cell AJs (16). Additionally Rac1 localizes to both the leading edge of fibroblasts and to initiating sites of cell-cell contacts in epithelial cells. Since Rac1 is important for E-cadherin localization and actin polymerization at cell-cell junctions, we can envision that local Rac1 activation is also critical for the formation of AJs. Interestingly we and others have found that cadherin engagement is sufficient to activate Rac1 (11, 12). Future studies will be aimed at determining how cadherins activate Rac1.

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