Binding of GEF-H1 to the Tight Junction-Associated Adaptor Cingulin Results in Inhibition of Rho Signaling and G1/S Phase Transition

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Abstract

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Reference


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Binding of GEF-H1 to the Tight Junction-Associated Adaptor Cingulin Results in Inhibition of Rho Signaling and G1/S Phase Transition

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Summary

The activity of Rho GTPases is carefully timed to control epithelial proliferation and differentiation. RhoA is downregulated when epithelial cells reach confluence, resulting in inhibition of signaling pathways that stimulate proliferation. Here we show that GEF-H1/Lfc, a guanine nucleotide exchange factor for RhoA, directly interacts with cingulin, a junctional adaptor. Cingulin binding inhibits RhoA activation and signaling, suggesting that the increase in cingulin expression in confluent cells causes downregulation of RhoA by inhibiting GEF-H1/Lfc. In agreement, RNA interference of GEF-H1 or transfection of GEF-H1 binding cingulin mutants inhibit G1/S phase transition of MDCK cells, and depletion of cingulin by regulated RNA interference results in irregular monolayers and RhoA activation. These results indicate that forming epithelial tight junctions contribute to the downregulation of RhoA in epithelia by inactivating GEF-H1 in a cingulin-dependent manner, providing a molecular mechanism whereby tight junction formation is linked to inhibition of RhoA signaling.

Introduction

The epithelial junctional complex mediates adhesion and regulates cell proliferation and differentiation (Balda and Matter, 2003). Tight junctions are the most apical component of the junctional complex and separate the apical from the basolateral membrane (Cereijido et al., 2000; Schneeberger and Lynch, 2004; Tsukita et al., 2001). They regulate paracellular permeability and restrict apical/basolateral intramembrane diffusion of lipids. Multiple signaling components have been localized to epithelial tight junctions, some of which function in the regulation of epithelial polarization, differentiation, and growth control (Matter and Balda, 2003). These signaling components interact with different types of adaptor proteins that also bind junctional membrane components and the cytoskeleton (Anderson et al., 2004; Gonzalez-Mariscal et al., 2003).

Rho GTPases are molecular switches that are important components of many subcellular signaling processes that govern cell proliferation and differentiation (Etienne-Manneville and Hall, 2002; Ridley, 2004). In their GTP-bound state, they can bind effector molecules that activate downstream components; in their GDP-bound state, they are inactive. Activation is catalyzed by guanine nucleotide exchange factors (GEFs) that stimulate the exchange of GDP by GTP and inactivation by GTPase-activating proteins that promote GTP hydrolysis. The spatial and temporal control of signaling by Rho GTPases is thought to be determined by regulating the localization and activation of these regulators at specific subcellular sites, but our knowledge about these processes is still very limited (Etienne-Manneville and Hall, 2002).

In epithelial cells, confluence is paralleled by a reduction of active RhoA levels and activation of Rac1 and Cdc42 (Braga, 2002; Fukata and Kaibuchi, 2001; Noren et al., 2001). Activation of Rac1 and Cdc42 is triggered by E-cadherin engagement and promotes formation of the junctional complex. Downregulation of RhoA at cell confluence is observed in different cell types, resulting in inhibition of G1/S phase progression (Coleman et al., 2004). One way of inhibiting RhoA signaling is activation of p190RhoGAP, which has been observed upon cadherin engagement by immobilized ligands in transfected CHO cells, which do not form a junctional complex (Noren et al., 2003). The relevance of GEFs for the confluence-dependent regulation of RhoA and the contribution of mechanisms not associated with cadherins is unknown.

Here we focus on GEF-H1/Lfc, a GEF for Rho that associates with tight junctions in epithelial cells and regulates paracellular permeability (Benais-Pont et al., 2003). GEF-H1, originally cloned in mice and called Lfc, is an oncoprotein of the Dbl family that activates RhoA but not Rac1 or Cdc42 (Glaven et al., 1996; Krendel et al., 2002; Ren et al., 1998). GEF-H1 can associate with different cytoskeletal structures, microtubules as well as the actin cytoskeleton, and has been proposed to mediate crosstalk between the two types of filaments (Benais-Pont et al., 2003; Glaven et al., 1999; Krendel et al., 2002; Ren et al., 1998). The molecular basis for the association of GEF-H1 with different cytoskeletal structures is unknown. We now show that GEF-H1 binds to the F-actin binding junctional adaptor cingulin. Cingulin binding inhibits GEF-H1 and, hence, results in downregulation of RhoA and inhibition of G1/S phase transition, providing a molecular mechanism whereby tight junction formation is linked to RhoA inactivation.

Results

GEF-H1 Interacts with the F-Actin Binding Protein Cingulin

GEF-H1 can associate with two different types of actin-based structures in different cell types: tight junctions
in epithelial cells and stress fibers in fibroblasts (Benais-Pont et al., 2003). We therefore tested whether tight junction-associated actin binding proteins coprecipitate with endogenous GEF-H1. Figure 1A shows that GEF-H1 was precipitated by monoclonal antibody (mAb) B4/7. We then blotted the same samples with antibodies specific for cingulin, a tight junction-associated F-actin binding protein (Citi et al., 1988; D’Atri and Citi, 2001). Anti-cingulin antibodies detected a band of approximately 150 kDa in B4/7 immunoprecipitates, suggesting that cingulin exists in a complex with GEF-H1.

We next determined the domain of GEF-H1 required for complex formation with cingulin using GEF-H1/GST fusion proteins (Figure 1B). Glutathione beads loaded with equal amounts of GEF-H1/GST fusion proteins were incubated with MDCK cell extracts. Specific precipitation of full-length cingulin was only observed with constructs containing the PH domain (Figure 1C). The anti-cingulin antibody also recognized a band of approximately 70 kDa that was only present in pull-downs with fusion proteins containing the PH domain. Since this antibody had been generated against a recombinant protein containing the rod and tail domains (Figure 1D), this suggests that the PH domain of GEF-H1 binds to either one of these two cingulin domains. This was confirmed with experiments with recombinant GST fusion proteins containing different regions of cingulin that were tested for pull-down of recombinant His<sup>6</sup>-tagged PH domain. The PH domain of GEF-H1 was efficiently precipitated by a cingulin fusion protein containing residues 782 to 1025, suggesting that the GEF-H1/cingulin interaction is due to direct binding of GEF-H1’s PH domain to the cingulin rod domain (Figures 1E and 1F). Because GEF-H1 and cingulin colocalize at intercellular junctions (Figure 1G), GEF-H1/cingulin complexes are likely to be primarily associated with tight junctions.

We next tested whether cingulin can influence the distribution of GEF-H1. When MDCK cells were transfected with myc-tagged wild-type cingulin, the transfected protein was found to colocalize with GEF-H1 at cell-cell junctions (Figure 2A). In cells that stained more brightly, myc-cingulin was not only closely associated with cell-cell junctions but could also be seen in the cytosol. This behavior was enhanced by removal of the head domain (myc-cingulin<sup>R+T</sup>), a mutation known to inhibit junctional recruitment of cingulin and to induce cytosolic aggregates (D’Atri et al., 2002). Endogenous GEF-H1 colocalized with both cingulin constructs. Expression of a construct containing cingulin residues 782-1025, which include the GEF-H1 binding site, yielded a diffuse GEF-H1 distribution. Transfection of the cingulin head domain did not affect the distribution of GEF-H1. Staining for GEF-H1 generally appeared to be brighter in cells expressing a GEF-H1 binding cingulin fragment. This was not due to a crossreaction of the secondary antibodies since it was not observed when the anti-GEF-H1 antibody was omitted (not shown). Immunoblotting did not reveal upregulation of GEF-H1 expression. Although this might be due to a low transfection efficiency, it is likely that cytosolic GEF-H1 was more efficiently labeled. ZO-1, another tight junction protein known to associate with cingulin,
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Cingulin can thus interact in vivo and this interaction is sufficiently strong to affect the distribution of GEF-H1. Because the PH domain of GEF-H1 interacts with cingulin, it might be sufficient for junctional recruitment. Although a VSV-tagged PH domain was partially targeted to intercellular junctions, the transfected protein also localized to cytoplasmic aggregates (Figure 2D). Mutation of tryptophane-563 (PHW563A-VSV), a residue that is conserved in PH domains, or deletion of the PH domain in full-length GEF-H1 resulted in proteins that accumulated in the cytosol (Figure 2E), suggesting that the PH domain is important for junctional recruitment.

The N-Terminal Domains of GEF-H1 Regulate Junctional Recruitment

Since overexpressed GEF-H1 is not targeted efficiently to junctions, we expressed N- and C-terminally truncated GEF-H1-VSV to determine whether a specific domain inhibits junctional recruitment. Removal of the C-terminal domain (GEF-H9004CTD-VSV) did not affect the distribution of the protein: This mutant protein was still associated with filaments. Cells expressing GEF-H9004CTD-VSV appeared more spread out and flatter; this appearance was paralleled by increased RhoA activation (not shown) as in other cell lines (Glaven et al., 1996; Krendel et al., 2002). In contrast, removal of the N-terminal domain (GEF-H9004NTD-VSV), which contains the C1 domain, eliminated the filamentous staining, and a significant fraction of the protein colocalized with cingulin at junctions. Additionally, a nonfilamentous cytoplasmic pool was observed. These data indicate that the N terminus regulates junctional recruitment of GEF-H1, which is in agreement with the observation that mutation of the Zinc-fingers in the C1 domain impedes microtubule binding (Krendel et al., 2002).

We next tested whether the N terminus is sufficient for microtubule binding. A construct containing the C1 and the intervening domain (C1/ID-VSV) was recruited to microtubules and induced microtubule bundling; the latter was not observed with the full-length construct (Figure 3B). Constructs containing either one of these domains alone remained cytosolic and often aggregated (not shown).

Since the N-terminal domain is sufficient and required for microtubule binding but a construct lacking the PH domain does not bind microtubules (Figure 2E), it is possible that the two domains interact with each other and thereby modulate targeting to microtubules and junctions. A recombinant His6-tagged PH domain construct was indeed pulled down by a GST fusion protein containing the C1 domain but not by GST alone (Figure 3C). Binding of the PH domain to a GST fusion protein containing the C-terminal domain was not observed (not shown). Thus, the C1 and PH domains of GEF-H1 can interact, providing a possible explanation for the observed effects on each other's targeting activities.

Cingulin Inhibits RhoA Activation by GEF-H1

RhoA is downregulated when cells reach confluence and cease to proliferate (Coleman et al., 2004). Since cingulin expression increases with cell density whereas...
expression of GEF-H1 remains comparatively constant (Figure 4A), the interaction between cingulin and GEF-H1 might be part of a mechanism that inactivates RhoA when epithelial cells reach confluence. Indeed, transfection of a GEF-H1 binding cingulin fragment resulted in a reduction of stress fibers, suggesting inhibition of RhoA (Figure 4B). To test whether increased expression of cingulin affects GEF-H1 activity, we measured transcriptional activity of SRF (Hill et al., 1995). Because MDCK cells were found not to stimulate SRE-driven transcription in response to RhoA activation, we used the retinal pigment epithelium cell line ARPE-19 as a reporter system. Transfection of myc-cingulin and of the GEF-H1 binding mutant myc-cingulinR+T inhibited SRE-driven transcription (Figure 4C). Transfection of the cingulin head domain stimulated the response whereas expression of the GEF-H1 binding myc-cingulin 782-1025 fragment was sufficient to inhibit transcription. Importantly, expression of GEF-H1 stimulated luciferase expression, and cotransfection of full-length cingulin or mutants that contained the GEF-H1 binding site inhibited, indicating that cingulin can inhibit GEF-H1 function.

To monitor Rho activation directly, we used a FRET-based assay that makes use of a fusion protein in which YFP is separated from CFP by a Rho binding domain (Yoshizaki et al., 2003). Binding of Rho-GTP results in a loss of FRET and, hence, reduced YFP emission. Coexpression of full-length cingulin with the FRET probe resulted in increased YFP emission, indicating reduced Rho-GTP levels (Figure 4D). Only mutants containing the GEF-H1 binding site (myc-cingulin R+T, myc-cingulin 782-1025) increased YFP emission. Although Rho inhibition was significant, the increase in YFP emission was more pronounced when Rho was inhibited by adding TAT-C3, a membrane permeable version of C3 transferase (Coleman et al., 2001). In contrast, transfection of GEF-H1 reduced YFP emission indicative of Rho activation. Cotransfection of full-length, myc-cingulin R+T and myc-cingulin 782-1025 again counteracted Rho activation by GEF-H1, supporting the conclusion that cingulin inhibits GEF-H1.

To test whether inhibition of Rho activation depends on GEF-H1, we made use of a cell line permitting the tetracycline-regulated depletion of the exchange factor by RNA interference (Figure 4E). In GEF-H1-depleted cells, transfection of cingulin no longer affected the YFP/CFP ratio, suggesting that inhibition required the presence of GEF-H1 (Figure 4F).

Since the above-used FRET probe can respond to other Rho GTPases than RhoA, we used a similar probe consisting of YFP separated from CFP by RhoA fused to a Rho binding domain, resulting in FRET when the GTPase is in the GTP-bound form (Yoshizaki et al., 2003). Analogous probes specific for Rac1 and Cdc42 were used for comparison. RhoA inactivation was observed when cingulin constructs containing the GEF-H1 binding site were cotransfected (Figure 4G). No effects on the Rac1 probe were observed, and the Cdc42 probe was only affected by the head domain, which stimulated Cdc42 activation, suggesting that the stimulation of SRE-driven transcription (Figure 4C) might have been mediated by Cdc42.

GEF-H1 Regulates G1/S Phase Transition

In fibroblasts as well as epithelial cells, RhoA signaling regulates G1/S phase transition (Auer et al., 1998; Coleman et al., 2004; Liberto et al., 2002; Olson et al., 1998). Since tight junctions regulate this step of the cell cycle (Balda et al., 2003), we tested whether GEF-H1 is important for G1/S phase transition in MDCK cells using tetracycline-regulated depletion of GEF-H1 by RNA interference. Cells were plated at low density and synchronized in G1 phase in medium with low serum, a treatment that results in efficient accumulation of MDCK cells in G1 phase (Balda et al., 2003). G1/S phase transition was then stimulated by the addition of serum and cells synthesizing DNA were visualized by bromodeoxyuridine labeling. More than 70% of cells entered S phase in wild-type MDCK cells (Figure 5A). In contrast, GEF-H1 depletion inhibited G1/S transition as only about 30% of the cells incorporated bromodeoxyuridine. GEF-H1 is thus required for efficient serum-induced G1/S phase transition in MDCK cells.

We next tested whether inhibition of GEF-H1 by cingulin is sufficient to inhibit G1/S phase transition. Transfected MDCK cells were synchronized and labeled with bromodeoxyuridine. The cells were then double labeled for transfected myc-tagged cingulin and bromodeoxyuridine, and the fractions of cells stained with either
Figure 4. Inhibition of GEF-H1 and Rho Activation by Cingulin

(A) MDCK cells grown to 20%, 50%, or 100% confluence were lysed and equal amounts of protein were analyzed by immunoblotting for the expression of GEF-H1, cingulin, and, as a loading control, α-tubulin. Note the pronounced upregulation of cingulin with cell confluence.

(B) MDCK cells were transiently transfected with a truncated cingulin construct lacking the head domain (myc-cingulinR+T). Cells were stained with antibodies against myc, GEF-H1, and fluorescent phalloidin. Note the reduced appearance of stress fibers in transfected cells.

(C) Inhibition of SRE-driven transcription by cingulin. ARPE-19 cells were cotransfected with a plasmid containing a SRE driving firefly luciferase expression, one with a control promoter regulating renilla luciferase expression, and the indicated expression vectors. After 30 hr, the luciferases were assayed and the ratios of the values for firefly divided by those from renilla luciferase calculated. Shown are the means ± 1 SD of a typical experiment performed in triplicates. Both panels were normalized to plasmid controls without GEF-H1 cotransfection. Asterisks indicate p values smaller than 0.05 that were calculated with two-tailed t tests comparing the single transfections to plasmid controls and the double transfections to GEF-H1 transfections.

(D) Inhibition of RhoA activation by cingulin. MDCK cells were transfected with pRaichu-RBD, a Rho-specific FRET probe, and the indicated expression vectors. TAT-C3 labels cells that were incubated with TAT-modified C3 between the transfection and cell lysis. After cell lysis, the emission for YFP (530 nm) and CFP (475 nm) was measured with an excitation wavelength of 430 nm, and the ratios were calculated. Shown are the means ± 1 SD of a typical experiment (n = 4). Asterisks label p values smaller than 0.05 that refer to comparisons of single transfections with plasmid controls and double transfections with GEF-H1-transfected samples. Note, increased YFP emission indicates Rho inactivation.

(E) Downregulation of GEF-H1 in MDCK cells by tetracycline-regulated RNA interference. Confluent MDCK cells expressing control RNA duplexes or GEF-H1-directed RNA duplexes were treated with tetracycline for 3 days. Expression of GEF-H1 and α-tubulin was then analyzed by immunoblotting with monoclonal antibodies B4/7 and 1A2, respectively.

(F) The Rho-specific FRET probe pRaichu-RBD was transfected into tetracycline treated control or GEF-H1 RNAi cells together with empty expression vector or full-length or truncated cingulin. The extracts were then analyzed as in (D) (asterisks, p < 0.05).

(G) FRET probes specific for RhoA, Rac1, or Cdc42 were transfected into MDCK cells together with the indicated cingulin constructs (asterisks, p < 0.05). Note, decreased YFP emission indicates inactivation.

These observations suggest that increased expression of cingulin inhibits G1/S phase transition and that this inhibitory function maps to the GEF-H1 binding site.

Cingulin Depletion Activates RhoA Signaling

To test whether endogenous cingulin is indeed important for the regulation of RhoA signaling, we depleted one or both antibodies were determined. Myc-cingulin efficiently inhibited G1/S phase transition and so did the expression of the construct containing the rod and the tail domain (Figure 5B). Whereas expression of the head domain alone did not have an effect, transfection of myc-cingulin 782-1025, which binds and inhibits GEF-H1, inhibited bromodeoxyuridine incorporation.

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We present evidence that the RhoA exchange factor GEF-H1 interacts with cingulin, resulting in inhibition of the GEF. Because cingulin expression increases with increasing cell confluence (Figure 4), the cingulin/GEF-H1 interaction provides a mechanism that links regulation of RhoA signaling to cell confluence.

Regulation of GEF-H1 and RhoA Signaling by Cingulin

Cingulin binds to the PH domain of GEF-H1, a domain important for GEF-H1’s transforming activity (Whitehead et al., 1995). PH domain-mediated interactions are important for the targeting of several Dbl family members to specific subcellular sites, including the actin cytoskeleton, and mediate interactions with proteins and lipids (Bellanger et al., 2000; Hoffman and Cerione, 2002; Olson et al., 1997; Zheng, 2001). PH domains of some GEFs bind phosphoinositides; however, binding affinities are relatively low and phosphoinositides may not be required for membrane targeting (Baumeister et al., 2003; Snyder et al., 2001). The PH domain of GEF-H1 does not bind to the main plasma membrane phosphoinositides in vitro (not shown).

Junctional targeting of GEF-H1 is regulated by the N-terminal domain, which binds microtubules as well as the PH domain. In the full-length protein, the PH domain is required for microtubule binding, suggesting that the interaction between the PH domain and the C1 domain is part of a mechanism that regulates recruitment to different cytoskeletal structures. The C1 domain might also have other interaction partners such as diacylglycerol. However, we have not been able to detect binding of this lipid (not shown).

Binding to cingulin as well as microtubules inhibits GEF-H1 function (Krendel et al., 2002). It will thus be important to understand how the C1/PH domain interaction is modulated to determine the sizes of these pools as well as the amount of free GEF. Our data indicate that the level of cingulin expression is an important determinant of GEF-H1-mediated Rho regulation. It is possible that other parameters affect GEF-H1 activity as well. GEF-H1 can be phosphorylated by Pak1, resulting in binding to 14-3-3 (Zenke et al., 2004). However, Pak1 phosphorylation and 14-3-3 binding do not appear to affect the activity or localization of GEF-H1 (Zenke et al., 2004).

Cingulin binds to several TJ proteins and to F-actin (Cordenonsi et al., 1999; D’Atri and Citi, 2001). Cingulin expression in several cell types increases upon inhibition of histone deacetylase, a treatment that can induce differentiation (Bordin et al., 2004). This is supported by the observation that cingulin expression increases with cell density (Figure 4). Targeted deletion of the head domain of cingulin results in expression of a truncated form of cingulin and altered expression of genes regulating endodermal differentiation (Guillemot et al., 2004). Although this was paralleled by altered expression of GEF-H1, it is not clear whether this was caused
by the expression of the truncated form of cingulin, which contains the GEF-H1 binding site, or an adaptive response that occurred during the selection. However, the present results suggest that the effects of cingulin mutation on gene expression may in part be due to effects on Rho signaling.

Junction Formation and RhoA Inactivation

Increased expression of cingulin results in lower levels of active RhoA, whereas reduced cingulin expression causes higher levels of active RhoA. These observations suggest a molecular mechanism for the inhibition of RhoA signaling with cell confluence that is linked to the formation of tight junctions: in low confluent cells expression of cingulin is low and GEF-H1 is primarily cytoplasmic; with increasing cell density, cingulin accumulates at forming tight junctions resulting in sequestration of free GEF-H1 at tight junctions and inhibition of RhoA signaling.

Engaging cadherins by plating transfected CHO cells on immobilized ligands stimulates the activity of p190RhoGAP (Noren et al., 2003). Although CHO cells do not form a junctional complex, this suggests that different types of intercellular junctions contribute to the regulation of RhoA signaling: adherens junctions by activating a Rho GAP and tight junctions by inhibiting a Rho GEF. Our results indicate that the regulation of GEF-H1 by cingulin makes a significant contribution to the regulation of RhoA in epithelial cells because total cellular levels of active RhoA were found to change significantly in response to changes in cingulin expression.

When epithelial cells reach confluence, RhoA is inactivated, whereas Rac1 and Cdc42 are activated (Braga, 2002). Strikingly, both processes appear to be mediated by GEFs that are recruited to the forming junctional complex. Adherens junctions recruit GEFs for Cdc42 and Rac1, resulting in stimulation of Rac1 and Cdc42, and stabilization of the junctional complex (Fu-kuyama et al., 2004; Irie et al., 2004; Kawakatsu et al., 2005; Sander et al., 1998). Our observations now link tight-junction formation with inactivation of GEF-H1 and inhibition of RhoA signaling, suggesting that the changes in Rho GTPase signaling that occur at confluence are orchestrated by the recruitment of different GEFs to the forming junctional complex.

Inactivation of RhoA signaling is thought to be important for the inhibition of signaling pathways that promote proliferation in various cell types including epithelial cells (Coleman et al., 2004). Our results indicate that GEF-H1 promotes G1/S phase transition and that its inhibition by cingulin binding regulates cell-cycle progression. Tight junctions recruit other signaling components that have been linked to the downregulation of signaling pathways that promote proliferation and, in particular, G1/S phase transition. These include the tumor suppressor PTEN and a complex formed by the transcription factor ZONAB and CDK4 (Balda et al., 2003; Matter and Balda, 2003; Wu et al., 2000). It is not known whether these signaling systems are regulated by RhoA. Nevertheless, the data described here link formation of tight junctions to inactivation of RhoA signaling and inhibition of cell-cycle progression, supporting a model according to which tight-junction assembly serves as an indicator of epithelial cell density that progressively inhibits different proliferation promoting signaling pathways with increasing cell density.

Experimental Procedures

cDNA Constructs, RNA Interference, and Cell Lines

The canine GEF-H1 sequence was used for all cDNA constructs (Benais-Pont et al., 2003). A C-terminally VSV-tagged full-length protein was cloned into pcDNA4/TO (Invitrogen). All mutants were generated by PCR and confirmed by sequencing. GEF-H1CTD-VSV was generated by inserting the VSV-tag after the codon for amino acid residue 600 and GEF-H1NTD-VSV by deleting the sequence coding for residues 2 to 216. Amino acids 447 to 575 were removed for the GEF-H1PH construct. For regulated expression, pcDNA8/
Figure 7. Depletion of Cingulin and RhoA Activation

(A) MDCK cells were transfected with the Rho-specific FRET probe 1A2; the VSV-epitope, mAb P5D4 or a rabbit anti-peptide antibody; M GST-rhotekin. Samples of total cell extracts and pellets were analyzed by immunoblotting with GST-RBD and -PH domain monomers. For GST pull-down assays with GEF-H1 fusion proteins, cells were extracts were then incubated with inactive beads for 15 min prior to incubation with glutathione-Sepharose beads coated with 15 μg of fusion proteins. To monitor interactions between recombinant proteins, 0.5 μg GST-cingulin proteins were bound to beads, washed three times with buffer S (PBS, 1% Triton X-100, 1 mM DTT, and protease inhibitors), incubated at 4°C for 4 hr with glutathione-Sepharose beads coated with 15 μg of fusion proteins. To monitor interactions between recombinant proteins, 0.5 μg GST-cingulin proteins were bound to beads, washed three times with buffer S (PBS, 1% Triton X-100, 1 mM DTT, and protease inhibitors), incubated with 0.5 μg His6-PH domain for 3 hr at 4°C in the presence of 3% BSA, and then washed twice with buffer S and once with PBS. Saturation of binding was tested using 0.5, 1, and 1.5 μg His6-PH domain. For immunoblotting, samples were separated on 6 to 15% gradient gels and then transferred to nitrocellulose membranes (Balda et al., 1996). GEF-H1 was detected with mAb B4/7, anti-RhoA antibodies. The following antibodies were used for immunofluorescence: GEF-H1, mAb B4/7; cingulin, rabbit polyclonal antibody; α-tubulin with mAb 1A2, cingulin with a rabbit anti-cingulin antibody, the VSV-epitope with mAb P5D4, the myc-epitope with mAb 9E10 (Benais-Pont et al., 2003). Antibodies specific for myosin light chain phosphatase and phosphorylated (T696) MYPT-1 were obtained from Upstate Biotechnology (Lake Placid, NY).

Immunofluorescence and Bromodeoxyuridine Incorporation

Cells were fixed with methanol at −20°C or 3% paraformaldehyde and then processed for double immunofluorescence using FITC- and Cy3-conjugated donkey secondary antibodies (Balda et al., 1995). For triple labeling with phalloidin, FITC-phalloidin was used together with Cy3- and Cy5-conjugated secondary antibodies. The following antibodies were used for immunofluorescence: GEF-H1, mAb B4/7; cingulin, rabbit polyclonal antibody; α-tubulin, mAb 1A2; the VSV-epitope with mAb P5D4, the myc-epitope with mAb 9E10 (Benais-Pont et al., 2003; Cordenonsi et al., 1999; Kreis, 1987). RhoA pull-down assays were performed as described (Balda et al., 2003). Antibodies specific for myosin light chain phosphatase and phosphorylated (T696) MYPT-1 were obtained from Upstate Biotechnology (Lake Placid, NY).

SRE Reporter and FRET Assays

Reporter assays were done by cotransfecting ARPE-19 cells with a plasmid containing a SRE-containing promoter driving firefly luciferase expression (Clonetech), a control plasmid for renilla luciferase expression, and the indicated expression vectors (Balda and Matter, 2000). After 30 hr, firefly luciferase was measured and standardized using the renilla luciferase values. For FRET assays, pRaichu-RBD, pRaichu-RhoA, pRaichu-Rac1, and pRaichu-Cdc42 were cotransfected with the indicated expression and RNAi plas-
mids into 50% confluent MDCK cells cultured in 12-well plates (Itoh et al., 2002; Yoshizaki et al., 2003). 30 hr after the transfection, the cells were washed with cold PBS and lysed with 300 μl/well of 20 mM Tris (pH 7.5), 100 mM NaCl, 5 mM MgCl2, 0.5% Triton X-100, and 20 μg/ml PMSF. The lysates were transferred to 96-well plates for centrifugation at 2000 g for 5 min. Fluorescence was measured with a Perkin Elmer LS 50B fluorometer (excitation, 430 nm; emission, 475 and 530 nm). The ratios of emission at 530 nm/475 nm were calculated. Significance of the results in both types of assay was determined with two-tailed t tests.

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References


