Interaction of PDZRhoGEF with Microtubule-associated Protein 1 Light Chains

LINK BETWEEN MICROTUBULES, ACTIN CYTOSKELETON, AND NEURONAL POLARITY

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Rat (r) PDZRhoGEF, initially identified as a glutamate transporter EAAT4-associated protein, is a member of a novel RhoGEF subfamily. The N terminus of the protein contains a PDZ and a proline-rich domain, two motifs known to be involved in protein-protein interactions. By using the yeast two-hybrid approach, we screened for proteins that interact with the N terminus of rPDZRhoGEF. The light chain 2 of microtubule-associated protein 1 (LC2) was the only protein identified from the screen that does not contain a type I PDZ-binding motif at its extreme C terminus (−(S/T)Xϕ–COOH, where ϕ is a hydrophobic amino acid). However, the C terminus does conform to a type II-binding motif (−XϕXϕ). We report here that rPDZRhoGEF interacts with LC2 via the PDZ domain, and the interaction is abolished by mutations in the carboxylate-binding loop. The specificity of the interaction was confirmed using GST fusion protein pull-down assays and coimmunoprecipitations. Expression of rPDZRhoGEF mutants that are unable to interact with proteins via the carboxylate-binding loop induced changes in cell morphology and actin organization. These mutants alter the activation of RhoGTPases, and coexpression of dominant-negative RhoGTPases prevent the morphological changes. Furthermore, in cells expressing wild type rPDZRhoGEF, drug-induced microtubule depolymerization produces changes in cell morphology that are similar to those induced by rPDZRhoGEF mutants. These results indicate that modulation of the guanine nucleotide exchange activity of rPDZRhoGEF through interaction with microtubule-associated protein light chains may coordinate microtubule integrity and the reorganization of actin cytoskeleton. This coordinated action of the actin and microtubular cytoskeletons is essential for the development and maintenance of neuronal polarity.

RhoGTPases play an important role in the regulation of several cellular processes, including cytoskeletal dynamics, gene transcription, and cell cycle progression. The best studied effect of RhoGTPases is the regulated reorganization of the actin cytoskeleton. This coordinated action of the actin and microtubular cytoskeletons is essential for the development and maintenance of neuronal polarity.

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§ The abbreviations used are: GEF, guanine nucleotide exchange factor; PDZ, PSD-95/DlgA/Zo-1-binding domain; MAP, microtubule-associated protein; DH, Dbl homology; RGS, regulator of G protein signaling; GST, glutathione S-transferase; Bt2cAMP, dibutyryl cyclic AMP; HA, hemagglutinin; PBS, phosphate-buffered saline; PAK, p21-activated kinase; WT, wild type; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate; RBD, Rho-binding domain; rPDZRhoGEF, rat PDZRhoGEF; AD, activation domain.

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GEF activity may be regulated, we sought to identify proteins that interact with the N terminus of rPDZRhoGEF.

Here we report that the PDZ domain of rPDZRhoGEF interacts with microtubule-associated light chain 2 and that the interaction can modulate the activation of RhoGTPases. Our results suggest that the rPDZRhoGEF/MAP1ALC2 interaction provides a link between microtubule dynamics and the reorganization of the actin cytoskeleton, which is essential for maintaining cell polarity and directed neurite outgrowth.

MATERIALS AND METHODS

Yeast Two-hybrid Assay—The N-terminal rPDZRhoGEF bait (amino acid residues 1–186) was constructed by PCR amplification using primers that introduced EcoRI restriction sites. The fragment was cloned into the EcoRI site of pGBK7T in-frame with the GAL4 DNA-binding domain (Clontech). The bait plasmid was introduced into yeast strain Y190 by the lithium acetate-mediated method. Yeast expressing the bait protein was sequentially transformed with an adult rat brain MATCHMAKER cDNA library constructed in pACT2 (Clontech), and plated on SD agar deficient in leucine, tryptophan, and histidine and containing 30 μg 3-amino-1,2,4-triazole. Colonies grew in 3–6 days and were picked and screened for β-galactosidase activity as per the manufacturer’s instructions. pGADT7-T and pGBK7-T5 were used as control prey and bait, respectively.

Plasmids—The GAL4(AD)-LC2ct deletions were constructed using GAL4(AD)-LC2ct145 as template and PCR primers that introduced EcoRI and XhoI restriction sites. Full-length LC2 and LC1 were amplified using Quickclone cDNA (Clontech) as template. The generated fragments were cloned into EcoRI/XhoI restriction sites of pACT in-frame with the GAL4 activation domain (AD). The GAL4(DB)-rPDZRhoGEF-(1–186) deletions were amplified using GAL4(DB)-rPDZRhoGEF-(1–186) as template and PCR primers that introduced EcoRI restriction sites. The resulting fragments were cloned into EcoRI restriction site of pGBK7T7. The GAL4(DB)-rPDZRhoGEF-(1–186) and rPK5-yc-f-rPDZRhoGEF mutants were constructed using the QuickChange site-directed mutagenesis kit (Stratagene) as directed. EcoRI/XhoI inserts of pACT-LC2ct145, -LC2ct111, and -LC2ct39 were subcloned into the bacterial expression vector pGEX-3T (Amersham Biosciences) to obtain plasmids for the expression of GST fusion proteins. Plasmids encoding HA-tagged domain in C-terminal rPDZRhoGEF (1–186) and rPDZRhoGEF-LC2 Interaction

Antibodies—The following primary antibodies were used: mouse anti-MAP1 clone HM-1, mouse monoclonal anti-β-tubulin TUB2B1, mouse monoclonal anti-MAP1 light chain clone E12 (LC1), anti-actin clone AC-40 (all from Sigma), goat polyclonal anti-MAP-1B (C-20) (Santa Cruz Biotechnology), rabbit anti-rPDZRhoGEF, mouse anti-c-Myc (Ab-1) (Calbiochem), monoclonal antibody HA.11 (Covance), mouse anti-RhoA (Pierce), and mouse anti-Cdc42 (Pierce). The secondary antibodies used were as follows: fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Cappel), TRITC-conjugated goat anti-mouse IgG (Southern Biotech), horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences), horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Biosciences), and horseradish peroxidase conjugated donkey anti-goat IgG (Santa Cruz Biotechnology). Immunoprecipitations—Adult mouse brains were rapidly dissected and homogenized in ice-cold homogenization buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Calbiochem)) with a Teflon glass homogenizer. Protein concentrations were determined using Coomassie-Plus Reagent and bovine serum albumin as standard (Pierce). Protein samples were resolved by denaturing SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). The membranes were blocked for 1 h at room temperature with 5% w/v nonfat dry milk in Tris-buffered saline/Tween 20 (TBS/T, 20 mM Tris, 15 mM NaCl, pH 7.6, with 0.1% v/v Tween 20). Blots were incubated overnight at 4 °C with affinity-purified rabbit anti-rPDZRhoGEF (1:200), anti-MAP1A (1:1000), anti-LC1 (1:1000), anti-MAP1B (1:200), anti-tubulin (1:1000), and anti-actin (1:500) in blocking buffer. After washing with TBS/T, the blots were incubated for 1 h at room temperature with either horseradish peroxidase-conjugated donkey anti-rabbit IgG, horseradish peroxidase-conjugated sheep anti-mouse IgG, or horseradish peroxidase-conjugated donkey anti-goat IgG in blocking buffer (1:3000). Immunoreactive proteins were visualized with enhanced chemiluminescence (ECL, Santa Cruz Biotechnology).

GST Binding Assays—GST or GST-LC2 fusion proteins were expressed and extracted from Escherichia coli strain BL21 and bound to glutathione-Sepharose 4B according to the manufacturer’s instructions (Amer sham Biosciences). Adult mouse brains were rapidly dissected and homogenized in ice-cold homogenization buffer. Triton X-100 was added to 1% (v/v) and homogenate rotated for 2 h at 4 °C before centrifugation at 15,000 × g for 20 min to remove cellular debris. The soluble material was added to GST fusion proteins bound to glutathione-Sepharose 4B and rotated overnight at 4 °C. Beads were washed three times with buffer, and bound proteins were separated by SDS-PAGE and detected by Western blotting.

Cell Culture and Transfection—For microscopic observation, cells were plated onto coverslips in 35-mm dishes and transfected with 1 μg (unless stated otherwise) of each DNA construct using FuGENE reagent (Roche Applied Science) in accordance with the manufacturer’s instructions. For GTPase pull-down assays, cells were grown in 90-mm dishes and transfected with 4 μg of each DNA construct. HeLa cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 10 mM glutamine, and antibiotics (penicillin and streptomycin). Neuro2a cells were grown in minimum essential media containing 10% fetal bovine serum, 10 mM glutamine, 1 mM nonessential amino acids, 1 mM sodium pyruvate, and antibiotics.

Fixation and Immunofluorescence Staining—At 24 h post-transfection HeLa cells were serum-starved for 12 h and fixed for 15 min in 4% paraformaldehyde. At 24 h post-transfection, Neuro2a cells were differentiated for 24–36 h by replacing media with Opti-MEM containing 300 mM Bt2cAMP, 10 mM glutamine, and antibiotics. Cells were fixed in 4% paraformaldehyde. Cells were permeabilized in PBS containing 0.4% v/v Triton X-100 for 15 min, blocked with 5% normal goat serum in PBS, and incubated with primary antibody (1:50) in PBS containing 2% normal goat serum, anti-c-Myc (Calbiochem), anti-HA (Covance), or anti-rPDZRhoGEF. Secondary antibodies used were FITC-conjugated goat anti-rabbit IgG (Cappel) and TRITC-conjugated goat anti-mouse IgG
FITC-conjugated phalloidin was used to stain F-actin (Sigma). Individual coverslips were treated with 3 mM nocodazole (Sigma) for 2 h before fixation. Data collected were compared using one-way analysis of variance with Bonferroni post-hoc analysis.

GTPase Pull-down Assay—pRK5-myc-tagged-rPDZRhoGEF constructs were expressed in HeLa cells. After 24 h of expression, cells were serum-starved for 12 h before being lysed according to the manufacturer’s instructions (EZ-Detect RhoGTPase activation assay; Pierce). Lysates were centrifuged to remove debris and incubated for 1 h at 4 °C with GST-Sepharose loaded with either 400 μg of GST-rhotekin-RBD (Rho-binding domain) or 20 μg of GST-PAK1-PBD (p21-binding domain). Beads were washed three times with lysis buffer, and bound proteins were separated by SDS-PAGE and Western-blotted using anti-RhoA and anti-Cdc42 antibody. Immunoreactive proteins were visualized with SuperSignal West Pico chemiluminescent substrate (Pierce).

RESULTS

rPDZRhoGEF-LC2 Interaction

A.

B.

C.

FIGURE 1. N terminus of rPDZRhoGEF interacts with MAP1 light chains. A, Amino acid sequence comparison of MAP1 light chains. Boxed sequence represents LC2ct145, the region of LC2 isolated from the yeast two-hybrid screen. B, N terminus of rPDZRhoGEF fused to GAL4 DNA-binding domain (DB) was tested for interaction with full-length LC2 and LC1 fused to GAL4(AD) using the yeast two-hybrid system. Protein interaction was measured by the ability to grow on dropout media and the ability to turn yeast colonies blue in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. β-Galactosidase activity was quantified with the o-nitrophenyl β-D-galactopyranoside liquid assay. C, cerebellar homogenates (20 μg of protein) prepared at the indicated developmental time points were analyzed by SDS-PAGE and immunoblotted with antibodies against rPDZRhoGEF, LC1 (anti-MAP1 light chain clone E12), LC2 (anti-MAP1B C-20), and MAP1A (anti-MAP1 clone HM-1). Antibodies against tubulin and actin were used as internal loading controls.

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RESULTS

rPDZRhoGEF Interacts with the Light Chains of Microtubule-associated Protein 1—To further elucidate the neuronal function of rPDZRhoGEF, we searched for N-terminal interacting proteins. We performed a yeast two-hybrid screen using an adult rat brain library and the first 186 amino acids of rPDZRhoGEF fused to the GAL4 DNA-binding domain (DB) as bait. We screened 1/100,000,000 clones and sequenced 30 of the 132 confirmed positive clones. The only clone that did not possess a type I PDZ-binding motif at the extreme C terminus (-(S/T)Xφ-COOH, where φ is a hydrophobic amino acid) corresponded to the last 145 residues of light chain 2 of microtubule-associated protein 1A (LC2). MAP1A, like MAP1B, is a multimeric protein that is translated as a polyprotein precursor and post-translationally modified to produce a heavy and a light chain (22). The light chains of MAP1 are interchangeable, with LC1 and LC2 being able to bind to both MAP1A and MAP1B heavy chains (23). Because the C terminus of LC2 (LC2ct145), the region pulled out in the yeast two-hybrid screen, shares the most extensive sequence identity with LC1 (Fig. 1A), we determined whether LC1 could also interact with rPDZRhoGEF in the yeast two-hybrid system. We found that full-length LC1 cDNA fused to the GAL4(AD) grew on dropout media, and quantification of β-galactosidase activity using the o-nitrophenyl β-D-galactopyranoside liquid assay revealed that both LC1 and LC2 interact strongly with the N terminus of rPDZRhoGEF (Fig. 1B). The specificity of the yeast interaction was confirmed as expression of T7 fused to GAL4(AD) yielded no growth on dropout media or β-galactosidase activity when coexpressed with GAL4(DB)-rPDZRhoGEF (Fig. 1B). The specificity of the yeast interaction was confirmed as expression of T7 fused to GAL4(AD) yielded no growth on dropout media or β-galactosidase activity when coexpressed with GAL4(DB)-rPDZRhoGEF (Fig. 1B). Similarly, no interaction was observed between the control bait pGBKT-53 and any MAP light chain construct (data not shown).

It is known that expression of MAP1B increases during early stages of neuronal development and then drops to lower levels (24, 25), whereas MAP1A exhibits a reciprocal pattern of expression, reaching its peak in the adult brain when neuronal differentiation is complete (23, 26). We determined the developmental expression pattern of rPDZRhoGEF in the cerebellum by Western blot analysis. We found that rPDZRhoGEF is expressed early in development, with levels increasing during the postnatal period and maintained into adulthood (Fig. 1C). Our results confirm the switch between LC1 and LC2 expression during develop-
ment. The antibody used to detect LC2 was in fact an antibody raised against MAP1B. However, the epitope lies within the conserved C terminus of the light chains, and by using transfected cells we confirmed that the antibody does recognize both LC1 and LC2 (supplemental Fig. 1). Therefore, it is possible that rPDZRhoGEF may switch interacting partners from LC1 to LC2 during development.

The C Terminus of LC2 Is Required for Interaction with rPDZRhoGEF but Is Not Sufficient—To map the region on LC2 required for interaction with rPDZRhoGEF-(1–186), we generated N- and C-terminal deletions (Fig. 2). Deletion of the last 39 residues of LC2ct145 abolished the interaction with GAL4(DB)-PDZRhoGEF-(1–186). Shortening the deleted region to just the last 18 residues did not restore the interaction, suggesting that the C terminus of LC2 is required for the interaction with rPDZRhoGEF. However, the C terminus is not sufficient for interaction as the last 39 amino acids fused to GAL4(AD) failed to show β-galactosidase activity. Deleting the N terminus of GAL4(AD)-LC2ct145 also resulted in less robust β-galactosidase activity, suggesting that a tertiary conformation of the C terminus, conferred by the N terminus, is required for a strong interaction.

Confirmation of the Interaction of LC2 with rPDZRhoGEF Using in Vitro and in Vivo Pull-down Assays—To confirm the specificity of the rPDZRhoGEF-LC2 interaction in systems other than yeast, we first performed GST pull-down assays. A GST fusion protein for LC2ct145 and GST alone were expressed and bound to glutathione-Sepharose beads. Lysates from adult mouse brain were applied to beads, and after extensive washing the bound proteins were eluted and resolved by SDS-PAGE. rPDZRhoGEF was found to bind to GST-LC2ct145 but not to GST alone (Fig. 3A). Subsequently a GST fusion protein for the N-terminally truncated construct LC2ct111, which was shown to interact with rPDZRhoGEF-(1–186) in the yeast system, was also found to pull down rPDZRhoGEF, but a GST fusion protein of the last 39 residues (GST-LC2ct39) did not (Fig. 3B).

We also determined whether rPDZRhoGEF interacts with MAP1A and LC2 in vivo. rPDZRhoGEF was immunoprecipitated from adult mouse brain lysate using a specific anti-MAP1A antibody, whereas a mouse IgG1 failed to immunoprecipitate rPDZRhoGEF (Fig. 3C). Similarly, antibodies against the HA epitope were able to immunoprecipitate HA-tagged LC2 and rPDZRhoGEF from transfected HeLa cells (Fig. 3D). Although in the yeast two-hybrid assay the N terminus rPDZRhoGEF was shown to interact with both LC2 and LC1, we have so far been unable to coimmunoprecipitate rPDZRhoGEF from P1 brain homogenates using the specific anti-LC1 antibody. Nevertheless, because of the poor efficiency of the anti-LC1 antibody in immunoprecipitating LC1 (data not shown), we cannot rule out the possibility that rPDZRhoGEF also interacts with LC1 in vivo.

LC2 Interacts with the PDZ Domain of rPDZRhoGEF—The yeast two-hybrid assay was used to identify the region of rPDZRhoGEF-(1–186) that interacts with LC2. Deletion of the last 26 residues, which encompass the proline-rich region, failed to abolish the interaction with GAL4(AD).LC2ct145 (Fig. 4A) as did site-directed mutagenesis of the multiple proline residues to alanines (data not shown). The initial 60 N-terminal residues were not able to interact and not required for interaction, but when the PDZ domain was expressed, strong β-galactosidase activity was observed. Therefore, although the extreme C terminus of LC2 does not contain a type I PDZ-binding motif, unlike all the other interacting clones pulled out in the yeast two-hybrid screen, LC2 does interact with the PDZ domain of rPDZRhoGEF.

This places the PDZ domain of rPDZRhoGEF into an expanding group of PDZ domains that can recognize more than one class of binding motif. It is known that some PDZ domains can interact with both type I- and type II-binding motifs (27, 28), with internal type I ligands (29), and with target sequences that adopt a β-hairpin structure (30). Sequence analysis revealed that the extreme C terminus of LC2 (-IEF) and LC1 (-IEL) conform to a type II consensus PDZ-binding motif (-dXκd). There are also several potential internal PDZ ligands (-STV, -TVV, and -VVM) within the last 18 residues that were identified previously as being essential for interaction (Fig. 2). Site-directed mutagenesis of the residues at positions P0 and P−2 of LC2ct145 to -AAA, -AEE, or EEE failed to abolish growth on dropout media (-L/T/H + 30 mM 3-amino-1,2,4-triazole), but the amino acid substitutions did drastically reduce β-galactosidase activity, indicating that the interaction was severely compromised (Fig. 4B). This is in contrast to clones possessing a type I-binding motif, as both the growth on dropout media and β-ga-
lactosidase activity were completely abolished when residues at positions P$_2$ and P$_{−3}$ were mutated to alanines (data not shown). No effect on protein interaction was observed when the internal PDZ ligands were mutated. These results show that the PDZ domain of rPDZRhoGEF recognizes both type I and type II-binding motifs, but for type II motifs a tertiary conformation appears to play a critical role.

**PDZ Carboxylate-binding Loop Required for Interaction with LC2**—PDZ domains are believed to function by binding the target protein in a structurally conserved groove, termed the carboxylate-binding loop. The first residue of a carboxylate-binding loop is almost always an arginine or a lysine, and a conserved glycine-rich loop is also critical for C-terminal peptide binding. Specificity for type I peptide binding is determined by interaction of the S/T hydroxyl side chain at the $−2$ position with the positively charged histidine side chain at the base of $ε_2$ helix. In the case of the PDZ domain of rPDZRhoGEF, the carboxylate-binding loop contains a lysine residue and a GFGF motif (Fig. 5A).

To identify residues that are critical for interaction with LC2, we mutated conserved residues within the PDZ domain of rPDZRhoGEF-(1–186) and examined the ability to interact with the expression in HeLa cells of full-length Myc-tagged rPDZRhoGEF (WT) induces the formation of actin stress fibers (Fig. 5C). This specific cellular distribution indicates why, due to localized changes in RhoGTPase activity, the active truncated protein to be found at leading edges, areas rich in dynamic actin assembly (Fig. 5C, arrows). This expression of full-length protein containing the H119G point mutation within the PDZ domain revealed that abolishing the ability to interact with proteins containing a type I PDZ ligand had no effect on cell morphology and actin organization, when compared with wild type protein. The majority of cells expressing either WT or H119G protein exhibited an elongated, polarized shape with actin stress fibers running along the long axis of the cell, similar to G14VRho. Cells expressing F76A showed a marked cell rounding and the appearance of membrane blebbing similar to cells expressing truncated $Δ1–186$ protein. Strong actin staining was evident in the rounded cells. Taken together, these results suggested that the N terminus of rPDZRhoGEF, via protein-protein interactions mediated by the carboxylate-binding loop of the PDZ domain, modulates the cellular localization and GEF activity of the protein. Western blot analysis confirmed that all proteins were expressed at the same level (data not shown).

**Activation of RhoGTPases Leads to Morphological Changes and Loss of Cell Polarity**—The results so far suggested that the changes in cell morphology were functionally connected to the nucleotide exchange activity of the mutant proteins. To examine whether expression of the rPDZRhoGEF mutants did alter RhoGTPase activity, we first measured the level of active RhoA in transfected cells. The Rho-binding domain (RBD) of rhotekin was used to specifically precipitate GTP-bound RhoA from cell extracts. When
cells were transfected with either WT or H119G, only a very low level of Rho activation was actually detected by Western blot analysis (Fig. 6A). However, expression of truncated rPDZrGEOF-(1–186) or mutant F76A protein led to a much greater level of Rho activation. No activation of Rho was observed when rPDZrGEOF-(1–186)/H11001Y893A was expressed, confirming that the mutation within the Dbl domain had abolished GEF activity.

**FIGURE 6. Activation of Rho by rPDZrGEOF mutant constructs in vivo.** A, HeLa cells transfected with different Myc-tagged rPDZrGEOF constructs were lysed, and GTP-bound RhoA was precipitated with GST-RBD. The amount of RhoA bound to RBD and the total level of RhoA in whole cell lysates were examined by Western blot analysis using an antibody against RhoA. Each value is the mean ± S.D. for 200–250 cells sampled from three independent experiments. B, HeLa cells cotransfected with F76A and the indicated HA-tagged dominant-negative (DN) RhoGTPase were fixed and stained with antibody against rPDZrGEOF and an anti-HA antibody to identify cells expressing both proteins. Bar, 20 μm. C, quantification of cell morphology. Each value is the mean ± S.D. for 150–200 cells sampled from three independent experiments. The asterisks indicate a significant difference when compared with WT (**, p < 0.01).
To determine whether this observed increase in Rho activation was responsible for the changes in cell morphology, we coexpressed dominant-negative (DN) RhoA-TN19, Rac1-TN17, and Cdc42-TN17 with mutant F76A protein (Fig. 6B). Analysis of the resulting cell morphology revealed that coexpression of DN-RhoA and DN-Cdc42 significantly reduced the number of cells that appeared rounded, whereas dominant-negative Rac1 did not (Fig. 6C). Western blot analysis demonstrated that the lack of an effect with DN-Rac1 was not because of different levels of protein expression as both DN-RhoA and DN-Rac1 were expressed at similar levels (Fig. 6D). In fact, it was the level of DN-Cdc42 that was consistently lower.

The discovery that coexpression of DN-Cdc42 inhibited the changes in cell morphology was unexpected, as using in vitro assays rPDZRhoGEF has only been shown to activate Rho and not Rac1 or Cdc42 (18). However, the in vitro activity of RhoGEFs has been shown not to reflect the complex regulation of RhoGTPases in vivo. We measured the level of active Cdc42 in transfected cells using the p21-binding domain of PAK1 to precipitate GTP-bound Cdc42. We found that expression of Δ1–186 and F76A did lead to an increase in activation of Cdc42 (Fig. 6E). When we abolished the GEF activity of mutant F76A, an increase in Cdc42 activation was still observed, supporting the in vitro findings that rPDZRhoGEF does not directly activate Cdc42. Instead the cross-talk observed here between RhoGTPase signaling pathways is likely to be mediated by their protein effectors and may result from their spatial distribution.

**Stable Microtubules Play a Role in Modulating Nucleotide Exchange Activity of rPDZRhoGEF**—We have shown that interaction of rPDZRhoGEF with MAP light chains is mediated by the carboxylate-binding loop of the PDZ domain. Furthermore, we have demonstrated that it is the interactions mediated by the carboxylate-binding loop that are important for maintaining cell shape and polarity. Therefore, we addressed whether it could be the interaction with microtubules via MAP1 light chains that modulates the GEF activity of the protein and the reorganization of the actin cytoskeleton.

We found that if we increased the level of WT rPDZRhoGEF expression (Fig. 7A), there was a concomitant increase in the number of cells that appeared rounded. However, if we coexpressed LC2, there was a significant reduction in rounded cells, supporting the idea that it is the interaction with LC2 that regulates the ability of rPDZRhoGEF to activate RhoGTPases. We have used the change in morphology as a read-out of GEF activity based on the previous findings (Fig. 6).

To demonstrate further a role for LC2 and association with microtubules in modulating rPDZRhoGEF activity, we expressed wild type rPDZRhoGEF in HeLa cells and treated the cells with nocodazole (3 μM) for 2 h to partially disrupt stable microtubules. We found that when microtubules were depolymerized, the expression of WT protein induced the same cell rounding and membrane blebbing (Fig. 7B) that was observed when truncated Δ1–186 and mutant F76A protein were expressed in nontreated cells.
(Fig. 5C). The coexpression of dominant-negative RhoA (T19NRhoA) with WT protein prevented the nocodazole-induced cell rounding. Furthermore, cells transfected with full-length protein deficient in GEF activity (Y893A) remained elongated when treated with nocodazole. These results support the previous findings that the cytoskeletal contraction/cell rounding is linked to the GEF activity of rPDZRhoGEF, but in addition they indicate that it is the cellular distribution of Rho activation (i.e. whether rPDZRhoGEF is microtubule bound) that plays a critical role in the reorganization of the actin cytoskeleton and the resulting cell morphology. In contrast, the nocodazole treatment of cells expressing constitutively active RhoA (G14VRhoA) did not induce cell rounding in the majority of cells, and actin stress fibers were still observed in both elongated and rounded cells (Fig. 7C).

However, we did notice that nocodazole-treated cells expressing Y893A often possessed the same membrane swellings along the edge of the cell that were observed when mutant and truncated forms of rPDZRhoGEF were expressed (Fig. 7B, arrows). This indicated that the formation of the membrane protrusions was not necessarily a direct consequence of the RhoGEF activity, which had been abolished by introducing the Y893A substitution within the Dbl homology domain. The fact that we did not observe protrusions in untransfected cells treated with nocodazole also suggested that microtubule depolymerization is not sufficient for their formation. Instead both microtubule depolymerization and the membrane localization of rPDZRhoGEF, regardless of its ability to directly activate RhoA at the cell periphery, appear to be required for the formation of membrane swellings. We examined the microtubule network in untransfected HeLa cells and cells transfected with WT and F76A constructs (Fig. 7D). We found that the extension of microtubules at the leading edge was still evident in cells expressing WT protein and was comparable with untransfected cells. However, in cells expressing F76A mutant protein no clear microtubule network was visible within the membrane protrusions, and the majority of anti-tubulin staining was punctate. The enhanced activation of RhoA by nonmicrotubule-bound rPDZRhoGEF may therefore result in the destabilization of microtubules at leading edges, in addition to stimulating cortical actin reorganization.

We cannot rule out the possibility that interaction with another protein, possessing a nontype I PDZ-binding motif, is responsible for regulating the cellular localization and GEF activity of rPDZRhoGEF. However, the fact that disruption of the microtubular network generates changes in cell morphology that are similar to those observed when the light chain interaction is prevented together with the observation that overexpression of LC2 prevents the morphological changes strongly supports a central role for the MAP1 light chain interaction in maintaining cell polarity. In addition, the observed loss of an elongated polarized cell shape and lack of extension of microtubules in cells expressing mutant constructs suggest that interaction of rPDZRhoGEF with MAP1 light chains may be required for the stabilization and orientation of microtubules at the leading edge.

Effect of rPDZRhoGEF Mutants on Neurite Extension in Neuro2a Cells—Because rPDZRhoGEF is predominantly expressed in the brain, we examined what effect expression of the mutant proteins had on the morphology of Neuro2a cells (Fig. 8A). Cells were transfected with Myctagged rPDZRhoGEF constructs, and after 24 h of expression, we differentiated the cells by replacing media with Opti-MEM containing 100 ng/ml of colcemid, a potent inducer of neuritogenesis, for a further 24 h. The majority of cells expressing WT and H119G protein appeared flattened and elongated (Fig. 8B), with rPDZRhoGEF present throughout the cell. However, leading edges and areas rich in actin were strongly labeled. Cells expressing Δ1–186 very rarely possessed extended neurites, instead the truncated protein was enriched in short filopodia-like extensions around the cell body (Fig. 8A, arrows). Expression of F76A also led to inhibition of neurite outgrowth. The observed absence of neurites in these cells is consistent with expression of Δ1–186 and F76A having enhanced activation of Rho and is in stark contrast to cells expressing WT and mutant forms that lack RhoGEF activity (Y893A, Δ1–186 + Y893A, and F76A/Y893A). These mutant proteins were often enriched at neurite tips (Fig. 8A, arrowheads). Here a large number of cells were observed to possess one or two processes that were greater in length than their soma, more than in untransfected cells, suggesting that the GEF-inactive proteins enhance neurite outgrowth. Expression of Δ1–186 + Y893A or F76A/Y893A also led to a greater number of cells with more than two neurites longer than their cell body. Taken together, these results indicate that rPDZRhoGEF plays a role in balancing Cdc42-mediated neurite outgrowth and plays an important role in correctly orienting and orchestrating protein complexes via interaction with microtubules, to maintain neuronal polarity.

The observed neuronal morphologies suggest that the regulated control of rPDZRhoGEF localization, imparted by interactions via the carboxylate-binding loop of PDZ domain, is critical for directed neurite outgrowth and the maintenance of neuronal polarity. Disruption of the balance leads either to inhibition of neurite outgrowth or overextension and loss of polarity. Therefore, rPDZRhoGEF may play an important role in neuronal development and plasticity.

**DISCUSSION**

We used the yeast two-hybrid assay to screen an adult rat brain cDNA library for proteins that interact with the N terminus of rat PDZRhoGEF. We revealed that the PDZ domain of rPDZRhoGEF possesses dual ligand specificity, interacting with a number of proteins that possess a C-terminal PDZ type I motif (S/T)X[4] COOH and with MAP1 light chains via type II motifs in the C terminus. Mutation of critical residues within the PDZ domain of rPDZRhoGEF revealed that interaction with the carboxylate-binding loop is essential for interaction with both types of ligand. An earlier study using a cDNA library from mouse embryo revealed that human PDZRhoGEF interacts with the C terminus of plexin-B family members through type I-binding motifs (22). The study revealed a link between plexin activation, PDZRhoGEF-mediated Rho activation, and growth cone morphology. A subsequent study also demonstrated that plexin-B associates directly with PDZ-RhoGEF (23). However, none of the clones sequenced in this study were identified as members of the plexin-B family. This is probably because of the fact that different cDNA libraries were used, adult versus embryonic tissue (23) and brain versus bone marrow tissue (24).

The identification of MAP1 light chains as interacting partners further highlights the role of PDZRhoGEF in neuronal morphogenesis. It has been extensively documented that the coordinated action of the actin and microtubular cytoskeletons is required for several types of cell movement and for the development and maintenance of cell structural polarity (25). The role of RhoGTPases in the remodeling of the actin cytoskeleton has been well established. Recent studies have also identified a link between RhoGTPases and microtubule dynamics. Microtubule growth induces activation of Rac (26), whereas the depolymerization of microtubules results in the activation of Rho (27).

We have reported here that MAP1 light chains interact with rPDZRhoGEF in several systems, yeast two-hybrid assay, in vitro pull-downs, and in vivo coimmunoprecipitations. We have shown that if the protein interaction is prevented by protein truncation/mutation or disruption of the microtubule network, then changes in cell morphology and actin organization are induced. Furthermore, we have shown that

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the GEF activity of rPDZRhoGEF and alterations in RhoGTPase activation are responsible for the morphological changes.

Recently, other RhoGEFs, notably GEF-H1 (32) and p190RhoGEF (38), have also been reported to localize to microtubules. In the case of GEF-H1 both the N and C termini are required for interaction with microtubules, whereas only the C terminus of p190RhoGEF is essential for microtubular association. However, in both cases it is not known whether the interaction with microtubules is direct or involves the interaction with MAPs. Similar to rPDZRhoGEF, loss of microtubule binding was shown to induce the activation of GEF-H1 and lead to the concomitant activation of RhoA. In contrast, no enhanced p190RhoGEF-mediated activation of RhoA was observed in microtubule-disrupted cells. Microtubule binding has also begun to emerge as a key mechanism to regulate other RhoGTPases. For example p120Ctn, an Armadillo protein, fails to activate Rac1 and induce a dendritic morphology when bound to microtubules (39).

Most interestingly, a study using human PDZRhoGEF/KIAA0380 revealed that the reorganization of the actin cytoskeleton differed depend-
ing on the cellular distribution of KIAA0380 and hence the localized activation of RhoA. The authors of this study (40) reported that the cytoplasmic activation of Rho induced actin stress fiber formation, whereas activation of Rho at the cell membrane induced cortical actin reorganization, and stimulated contractility but did not induce the formation of actin stress fibers. These results correlate with our findings as we observed actin stress fiber formation when rPDZRhoGEF was tethered to microtubules via MAP1 light chains (WT and H119G). However, when the majority of the protein is located at the cell membrane as a result of truncation (ΔH9004–1-186) or mutation (F76A), we observed marked cell rounding and no actin stress fiber formation. Other authors have also dissociated cytoskeletal contraction from stress fiber formation. They revealed that the translocation and activation of Rho at the cell periphery is essential for cell contractility, whereas actin stress fibers are still induced when active Rho cannot bind to the cell membrane (41). We also observed cell contractility and no actin stress fiber formation when microtubule depolymerization was drug-induced in cells expressing wild type rPDZRhoGEF. The fact that stress fiber formation was still observed in nocodazole-treated cells expressing constitutively active RhoA indicates that the changes in morphology induced by rPDZRhoGEF are not simply because of Rho activation but are also a result of changes in the cellular distribution of Rho activation by rPDZRhoGEF (i.e. when it is no longer tethered to microtubules). Therefore, the results support the idea that rPDZRhoGEF forms an important link between microtubule integrity and the cellular location of RhoA activation.

Furthermore, our results suggest that rPDZRhoGEF may mediate cross-talk between the RhoA and Cdc42 signaling pathways. We revealed that DN-Cdc42 blocks the morphological changes induced by F76A expression, and we also observed an increase in Cdc42 activation when mutant rPDZRhoGEF proteins were expressed. In fact, it has been reported that PAK4 (p21-activated kinase 4), a Cdc42 effector, interacts with the C terminus of human PDZRhoGEF (42). Based on the observed loss of actin stress fiber formation when active PAK4 was expressed, Barac et al. (42) proposed that the interaction of PAK4 with PDZ-RhoGEF inhibited the activation of Rho. However, another possibility for the loss of actin stress fiber formation when PAK4 is overexpressed is the redistribution of PDZRhoGEF and the subsequent activation of RhoA at the cell membrane. Interestingly, PAK4 has also been shown to interact with GEF-H1, another RhoGEF associated with microtubules (32), and PAK4 phosphorylation prevents GEF-H1 microtubule binding and results in loss of actin stress fibers (43).

Given that RhoA and Cdc42 signaling pathways have antagonistic effects on the actin cytoskeleton, it may be that activation of an effector of the Rho signaling pathway by a Cdc42 effector such as PAK4 curtails excessive growth. The increased levels of rPDZRhoGEF at sites of dynamic actin and active PAK4 may explain the observed membrane blebbing. Membrane protrusions would be initiated by active Cdc42, but in the absence of stable microtubules to enter and reinforce the protrusions, they are not extended. Instead, because of the increased Rho-dependent contractility, they collapse giving rise to the multiple cell swellings. However, further work is required to elucidate the pathways that link Cdc42, RhoA, and rPDZRhoGEF to changes in cell morphology, and additional studies are required to determine whether in fact PAK4 can modulate rPDZRhoGEF activity when located at the cell membrane.

In summary, the interaction of rPDZRhoGEF with MAP1 light chains appears to provide an important mechanism to maintain cell polarity by controlling the cellular localization and degree of Rho activation (Fig. 9). At the leading edge, Rho activation is curtailed through interaction of rPDZRhoGEF with MAP1 light chains, and a balance between Rho and Cdc42 activation is maintained. The interaction may also result in the stabilization or possible capture and orientation of microtubules at the

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**FIGURE 9. Proposed model for rPDZRhoGEF-mediated neurite outgrowth.**

RhoGTPase activity is modulated by cellular localization of rPDZRhoGEF. A, shuttling between membrane-bound protein partners and being bound to microtubules via MAP1 light chains (LCs) rPDZRhoGEF control the level and location of Rho/Cdc42 activation. This leads to directed and regulated neurite outgrowth, B, loss of balance in RhoGTPase activation by rPDZRhoGEF leads to microtubule instability, cortical actin reorganization, and growth inhibition following excessive Rho activation. Loss of polarity and overextension of neurites occur when rPDZRhoGEF-mediated Rho activation is abolished.
leading edge, thus facilitating directional outgrowth. When rPDZRhoGEF can no longer bind to microtubules via an interaction with MAP1 light chains, the inhibition of GEF activity is lost, and enhanced activation of Rho occurs at the cell surface. This results in the depolymerization of microtubules and the reorganization of cortical actin, ultimately leading to cell rounding and growth inhibition. The membrane localization of rPDZRhoGEF interact with membrane-associated signaling proteins and with transmembrane proteins. We have already shown that the C terminus of rPDZRhoGEF interacts with the intracellular C terminus of the neuronal glutamate transporter EAAT4. Future studies are required to determine how the proteins that bind rPDZRhoGEF interact with each other, and whether they are involved in the same signaling pathway. However, like a variety of PDZ domain-containing neuronal proteins, rPDZRhoGEF appears to bring proteins from the Rho and Cdc42 signaling pathways together at neurite tips and plays an important role in coordinating neurite outgrowth.

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Interaction of PDZRhoGEF with Microtubule-associated Protein 1 Light Chains: LINK BETWEEN MICROTUBULES, ACTIN CYTOSKELETON, AND NEURONAL POLARITY

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