Sec14 Homology Domain Targets p50RhoGAP to Endosomes and Provides a Link between Rab and Rho GTPases*

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Sec14 protein was first identified in Saccharomyces cerevisiae, where it serves as a phosphatidylinositol transfer protein that is essential for the transport of secretory proteins from the Golgi complex. A protein domain homologous to Sec14 was identified in several mammalian proteins that regulates Rho GTPases, including exchange factors and GTPase activating proteins. P50RhoGAP, the first identified GTPase activating protein for Rho GTPases, is composed of a Sec14-like domain and a Rho–GTPase activating protein (GAP) domain. The biological function of its Sec14-like domain is still unknown. Here we show that p50RhoGAP is present on endosomal membranes, where it colocalizes with internalized transferrin receptor. We demonstrate that the Sec14-like domain of P50RhoGAP is responsible for the endosomal targeting of the protein. We also show that overexpression of p50RhoGAP or its Sec14-like domain inhibits transferrin uptake. Furthermore, both P50RhoGAP and its Sec14-like domain show colocalization with small GTPases Rab11 and Rab5. We measured bioluminescence resonance energy transfer between p50RhoGAP and Rab11, indicating that these proteins form molecular complex in vivo on endosomal membranes. The interaction was mediated by the Sec14-like domain of p50RhoGAP. Our results indicate that Sec14-like domain, which was previously considered as a phospholipid binding module, may have a role in the mediation of protein–protein interactions. We suggest that p50RhoGAP provides a link between Rab and Rho GTPases in the regulation of receptor-mediated endocytosis.

Endocytosis has a crucial role in the delivery of extracellular molecules to various intracellular compartments. The regulation of endocytosis is still poorly understood. Several routes and mechanisms of endocytosis were identified in eukaryotic cells (1). Among them, receptor-mediated endocytosis involves the internalization of receptors and their ligands by clathrin-coated pits. This process is essential for the efficient uptake of nutrients such as iron or lipids, and it is also involved in cellular signaling. Internalized receptors first enter the early endosome, from where they can either return to the plasma membrane or enter the endocytic recycling compartment (ERC). Through endocytic recycling, receptors are re-utilized several times, and recycling is also essential for maintaining the proper lipid composition of the plasma membrane. ERC is a complex network of vesicles and tubules that is often localized around the microtubule-organizing center (2, 3). Sorting in the ERC is a complex process where most molecules return to the plasma membrane, although transport also occurs to the trans-Golgi network.

Small GTPases, including members of the Rab and Rho families, are involved in the organization of receptor-mediated endocytosis. Rab GTPases regulate the budding, trafficking, and fusion of endocytic vesicles at the different compartments (4, 5). Rab5, one of the best characterized Rab GTPases, has a role in the formation of sorting endosomes. Rab5 together with early endosomal antigen 1 regulates fusion between endocytic vesicles and sorting endosomes (6). Besides participating in the regulation of fusion, Rab5 is also being recognized as an important link between microtubules and endosomes, thus having a role in the regulation of the movement of endosomes. Rab11 is present in the ERC and trans-Golgi-network membranes and has a role in the regulation of endocytic recycling (7). Several data suggest that members of the Rho family also participate in the regulation of receptor-mediated endocytosis. Rac1 and RhoA regulate the internalization of ligand-bound receptors (8), whereas RhoD and RhoB were described to associate with endocytic compartments (9, 10). The Cdc42 protein, which is a key regulator of cell polarity, was described to have a role in the endocytosis of EGF-receptors (11, 12), and it also regulates endocytosis in polarized epithelial cells (13).

The activity of small GTPases is controlled by guanine nucleotide exchange factors, GTPase activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (14). According to data base searches there are at least 60 different Rho GTPase activating proteins (15). The exact biological role of these regulatory proteins is poorly understood, and we know even less about how their activity is controlled. A common feature of small GTPase regulators is that besides their regulatory domains they also contain various conserved protein domains which represent potential sites for regulation and target these proteins to various intracellular compartments (16). Although much is known about the intracellular distribution of Rho GTPases, the subcellular localization and molecular interactions of their regulatory proteins remain elusive. We were interested in the intracellular localization and function of the p50RhoGAP protein.

P50RhoGAP (also called Cdc42GAP) was the first identified GTPase activating protein for Rho GTPases (17, 18). The protein was purified from human spleen and found to increase the endogenous GTPase activity of Rho, Rac, and Cdc42 (17, 18). P50RhoGAP is a 439-amino acid protein that contains an N-terminal domain homologous to the yeast Sec14 protein. The GAP domain is located at the C terminus, and a proline-rich sequence motif is present between the two domains. Crystallization studies revealed the structure of the GTPase activating domain of p50RhoGAP both alone and in complex with Cdc42 (19, 20). Importantly, a crystal structure of the yeast Sec14 protein, which shows substantial homology to the N-terminal part of p50RhoGAP, was also determined (21). Recent data suggest that p50RhoGAP exists in an
autoinhibited conformation stabilized by amino acids 1–48 and 169–197, and the prenyl group of small GTPases has a role in releasing the intramolecular inhibition (22). In contrast to the progress in structural analysis, we have little information about the biological function of this protein.

Here we describe that p50RhoGAP is localized to endosomal membranes via the N-terminal Sec14-like domain of the protein and colocalizes with internalized transferrin and EGF receptor during endocytosis. We show that overexpression of the Sec14-like domain or the full-length GAP protein inhibits transferrin uptake. We also demonstrate that the Sec14-like domain forms a molecular complex with Rab11, providing a link between Rab and Rho GTPases in the process of endocytosis.

EXPERIMENTAL PROCEDURES

Materials

Alexa568-transferrin, Alexa647-transferrin, Alexa488-EGF, Alexa488 anti-rabbit Fab, and Alexa568 anti-rabbit Fab were obtained from Molecular Probes (Carlsbad, CA). Cy3 anti-rabbit Fab was from Jackson ImmunoResearch (West Grove, PA). Anti-rabbit-horseradish peroxidase was from Amersham Biosciences. Brefeldin A was obtained from Calbiochem. Nocodazole, monensin, and all other chemicals were from Sigma. Glutathione S-transferase (GST)-p50RhoGAP full-length plasmid and GST-p50RhoGAP-(198–439) were generously provided by Alan Hall.

p50RhoGAP Antibody

RhoGAP antibody was purified from rabbit serum after intracutaneous injections of glutathione S-transferase-p50GAP-(198–439) fusion protein into rabbits. The serum was affinity-purified using Affi-Gel 10 beads (Bio-Rad) loaded with the antigen.

Cell Culture

HeLa cells were grown in Dulbecco’s modified Eagle’s medium with GlutaMAX I (Invitrogen) supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin in a 5% humidified CO2 incubator at 37 °C.

Transient Transfections

Transfections were carried out using FuGENE 6 (Roche Applied Science) or Lipofectamine 2000 (Invitrogen). siRNA was transfected at 100 nm concentrations 48 h before the experiments using Oligofectamine (Invitrogen).

siRNA Target Sequence

The target sequences on p50RhoGAP mRNA started at 875 nucleotides from the start codon (see Fig. 1) and at 127 nucleotides from the start codon (not shown). The siRNA was synthesized either by the Ambion Silencer siRNA construction kit (Ambion, Austin, TX) or with the manufacturer’s instructions. To verify mutations, mutant plasmids were sequenced. GFP-Rab5 and GFP-Rab11 used in this work were described earlier in Hunyady et al. (23). The RedFP-C1 plasmid used to create red fluorescent protein-Rab11 was described in Campbell et al. (24).

Transferrin Assays

Microscopic Analysis of Transferrin Uptake—HeLa cells on coverslips were incubated in extracellular medium (called H-medium, which contained 145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 0.8 mM CaCl2, 10 mM HEPES, 5 mM glucose, pH 7.4) for 10 min at 37 °C, then medium was replaced with H-medium containing 10 μg/ml Alexa568-transferrin for the indicated times.

Flow Cytometric Analysis of Transferrin Uptake—HeLa cells grown in 12-well plates were transfected with the Sec14-GFP or p50GFP constructs 24–30 h before the assay. After incubation in H-medium for 20 min, 10 μg/ml Alexa647-transferrin was added to the cells for the indicated times. Cells were then washed once with ice-cold H-medium then 2x with ice-cold PBS, and then trypsin-EDTA was added for 2 min at 37 °C. Dislodged cells were resuspended and washed once in ice-cold PBS and then fixed in 1% paraformaldehyde for 15 min. Finally, cells were washed 3x in PBS and kept at 4 °C until flow cytometric measurement was done. GFP intensity was measured in FL-1, and Alexa647 intensity of GFP-positive cells was measured in FL-4. The correct gating of GFP-positive cells was set using nontransfected cells as negative controls.

Flow Cytometric Analysis of Transferrin Recycling—HeLa cells grown in 12-well plates were transfected with the Sec14-GFP or p50GFP constructs 24–30 h before the assay. After loading the cells with 10 μg/ml Alexa647-transferrin in H-medium for 30 min at 37 °C, cells were washed 2x with ice-cold H-medium and then incubated in “chase” medium (H-medium containing 10% fetal calf serum, 0.1% bovine serum albumin, 100 μg/ml transferrin) for the indicated times at 37 °C. Finally, cells were washed once in ice-cold chase medium without transferrin and then in ice-cold PBS, trypsinized, and processed further for flow cytometric measurements as described above. Where indicated, 50 μM monensin was added 30 min before labeled transferrin and was present during the whole experiment.

Western Blot Experiments

Cells lysed on ice in Laemmli sample buffer were boiled and run on 10% polyacrylamide gels. After blotting onto nitrocellulose membranes, blocking was carried out in PBS, 5% milk, and 0.1% Tween 20 for 1 h at room temperature. After incubating the membranes with the first antibody for 1 h at room temperature, membranes were washed 5x in PBS 0.1% Tween 20, horseradish peroxidase-labeled anti-rabbit secondary antibody was used in a 1:5000 dilution, and signals were detected on Fuji Super RX films using the enhanced chemiluminescence method.

Immunofluorescent Labeling and Confocal Laser Microscopy—Cells grown on coverslips were fixed in 4% paraformaldehyde in PBS then rinsed 5x in PBS and incubated for 10 min in PBS containing 100 mM glycine. Coverslips were washed 2x in PBS and permeabilized in PBS containing 1% bovine serum albumin and 0.1% Triton X-100 for 20 min at room temperature. After 1 h of blocking in PBS containing 3% bovine serum albumin, cells were incubated with the primary antibody in PBS
p50RhoGAP on Endosomes

plus 2% bovine serum albumin, washed thoroughly 6 × in PBS, and incubated with the secondary antibody for 1 h and finally washed 6 × in PBS again. Coverslips were mounted using Mowiol 4–88 antifade reagent (prepared from polyvinyl alcohol 4–88, glycerol, H2O and Tris, pH 8.5).

Confocal images were collected on an LSM510 laser scanning confocal unit (Carl Zeiss) with a 63 × 1.4 numerical aperture plan Apochromat and a 40 × 1.3 numerical aperture plan Neofluar objective (Carl Zeiss). Excitation was with 25 milliwatt argon laser emitting 488 nm and a 1.0-milliwatt helium/neon laser emitting at 543 nm. Emissions were collected using a 500–530-nm band pass filter to collect Alexa488 and GFP and a 560-nm long pass filter to collect Alexa568 and red fluorescent protein emission. Usually images from optical slices of 1–2-µm thickness were acquired. Cross-talk of the fluorophores was negligible.

Biotin-labeled monoclonal antibody against the human p50RhoGAP protein, the p50RhoGAP-GFP fusion protein also showed colocalization with internalized, Alexa-labeled transferrin after 30 min of incubation (Fig. 3, A–C). Similarly to the endogenous protein, the p50RhoGAP-GFP fusion protein also showed colocalization with transferrin receptor (Fig. 2, E and F). The perinuclear localization of endosomal compartments is maintained by anchoring to microtubules (3). Therefore, we examined the effect of the microtubule-depolymerizing agent nocodazole on the distribution of p50RhoGAP. As is shown in Fig. 2, G and H, nocodazole treatment caused redistribution of p50RhoGAP from the perinuclear region to the entire cytoplasm. Next we studied the effect of low temperature (4 °C), a condition that also interferes with the polymerization of microtubules. Fig. 2I shows that after incubation at 4 °C, p50RhoGAP did not localize to the perinuclear region but became distributed throughout the cytoplasm.

In the next experiments we incubated the cells with Alexa-labeled transferrin and examined the relation of internalized transferrin to p50RhoGAP localization in time. At 0 min, p50RhoGAP showed dispersed staining due to previous incubation on ice that reversibly disturbs microtubules (Fig. 3, A–C). After 5 min of incubation, internalized transferrin localized to punctate structures in the cytosol, which did not show overlap with the p50RhoGAP signal (Fig. 3, D–F). These structures likely correspond to early endosomes. After 30 min of incubation at 37 °C, the bulk of internalized transferrin was localized to the perinuclear region where it showed strong colocalization with p50RhoGAP (Fig. 3, G–I). Transfected p50RhoGAP-GFP fusion protein also showed colocalization with internalized, Alexa-labeled transferrin after 30 min of incubation (Fig. 3, J–L). These results suggest that p50RhoGAP is present in the endocytic compartments.
The Golgi complex is also localized in the vicinity of the nucleus; therefore, we were interested if some portion of p50RhoGAP was localized to the Golgi apparatus. We treated HeLa cells with brefeldin A (BFA), which rapidly disrupts Golgi membranes (26). As is shown in Figs. 4, A and B, the localization of p50RhoGAP was not affected by the BFA treatment. On the contrary, the same treatment caused a rapid redistribution of Cdc42 from the perinuclear region to the cytosol (Fig. 4, C–D), indicating the effectivity of the BFA treatment. This result suggested that p50RhoGAP was not localized to the Golgi complex.

Based on its in vitro substrate preference toward Cdc42, p50RhoGAP was originally described as Cdc42GAP. Because the BFA treatment had a different effect on the localization of the two proteins, we became interested in knowing if the overexpression of p50RhoGAP has any effect on the distribution of endogenous Cdc42. When compared with untransfected cells, overexpression of p50RhoGAP-GFP had no effect on the distribution of Cdc42 (Fig. 4, E–G), and although both proteins localized to the vicinity of the nucleus, they did not show colocalization. This result suggested that Cdc42 was unlikely to represent the in vivo substrate of p50RhoGAP.

Although the transferrin receptor undergoes constitutive endocytosis, internalization of the EGF receptor is induced by the receptor-ligand binding. In experiments using Alexa-labeled EGF, we could show that p50RhoGAP also localized to endosomes when endocytosis was initiated by receptor ligand binding (data not shown).

**Sec14 Domain of p50RhoGAP Is Responsible for the Endosomal Targeting**—We went on to determine which part of the protein was responsible for the endosomal localization. p50RhoGAP is a 439-amino acid protein that contains an N-terminal domain homologous to the yeast Sec14 protein (27). We expressed the isolated Sec14-like domain as a GFP fusion protein and studied the intracellular localization of the protein. Similarly to the whole-length protein, Sec14-GFP localized to the perinuclear region where it co-localized with internalized transferrin (Fig. 5, A–C) and the transferrin receptor (data not shown). High level expression of the Sec14 domain induced the formation of enlarged, immobile vesicular and tubular structures, which were also present in the cell periphery (Fig. 5, D–E) and were positive for labeled transferrin.

In other experiments we introduced a point mutation into the Sec14-like domain. The Leu-173 residue is present in the yeast Sec14 protein at identical positions, and according to crystallographic studies, it contributes to the formation of the hydrophobic lipid binding surface in yeast Sec14 (21). HeLa cells expressed the mutant protein in similar amounts to the wild-type Sec14-like domain, but the protein showed diffuse cytoplasmic localization without enrichment in the perinuclear region (Fig. 5, F). The L173D mutation also inhibited the perinuclear localization of the p50RhoGAP protein (Fig. 5, G). We mutated the conserved amino acid Arg-282 in the GAP domain and Pro-235 in the proline-rich region of p50RhoGAP; however, these mutations failed to change the localization of the protein (data not shown). In summary, these experiments suggested that the Sec14-like domain localizes p50RhoGAP to endosomes.

When we studied the colocalization of Sec14-GFP with fluorescent transferrin, we noticed that cells which expressed the Sec14-like domain showed a diminished transferrin uptake (Fig. 6, A and B). To further characterize this effect, we used flow cytometry to measure transferrin uptake. In these experiments we also observed an inhibitory effect of the Sec14-like domain (Fig. 6, C), whereas overexpression of the L173D

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**FIGURE 2.** p50RhoGAP shows colocalization with endogenous transferrin receptor at the perinuclear region, and its localization is sensitive to disruption of microtubules. A–C, paraformaldehyde-fixed HeLa cells stained for p50RhoGAP (A) and transferrin (TIR) receptor (B) display perinuclear colocalization (C). D–F, cells transfected with p50RhoGAP-GFP exhibit similar colocalization with the endogenous transferrin receptor (see the arrows in the insets). Cells treated with 30 μM nocodazole for 1 h (H) or kept on ice for 30 min (I) show dispersed p50RhoGAP staining compared with control cells (G).
mutant Sec14-GFP did not affect transferrin uptake (Fig. 6D). In further experiments we examined if the overexpression of Sec14-like domain had any effect on endosomal recycling. To answer this question we preloaded the cells with Alexa-transferrin and measured its release in the presence of unlabeled transferrin. Figs. 6, E and F, show that the Sec14-like domain also interfered with endosomal recycling, and this inhibitory effect was not present when we used the L173D mutant form of the protein. Next we examined if the inhibitory effect on endosomal recycling was entirely responsible for the reduced transferrin uptake. In these experiments we preincubated the cells in the presence of monensin, an inhibitor of endosomal recycling (28). In our experiments the inhibitory effect of monensin was confirmed by measuring a complete inhibition of the chase of Alexa647-transferrin from loaded HeLa cells (not shown). Importantly in monensin-treated cells the Sec14-like domain still effectively inhibited the uptake of transferrin (Fig. 6, G and H), indicating that beside inhibiting endosomal recycling, the Sec14-like domain interferes with earlier steps of the endocytic process.

**Overexpression of p50RhoGAP Inhibits Transferrin Uptake—** After seeing the drastic effect of the Sec14-like domain on transferrin uptake, we sought to determine whether overexpression of the full-length p50RhoGAP protein has any effect on transferrin uptake. We overexpressed the p50RhoGAP as a GFP fusion protein and studied the transferrin uptake of the cells. Similarly to the Sec14-like domain, the full-length protein inhibited transferrin uptake (Fig. 7A). Interestingly the R282A mutant form of the protein, which has no intrinsic GAP activity, also inhibited transferrin uptake (Fig. 7B), whereas the L173D mutation abrogated the inhibition (Fig. 7C). These experiments highlighted the importance of the Sec14-like domain in the activity of the full-length protein as well.

**P50RhoGAP Interacts with Rab11 through Its Sec14-like Domain—** Members of the Rab family have an important role in endocytic trafficking. Among them Rab11 is considered to be a specific marker of the ERC, where it probably regulates traffic through this organelle. Because Rab11 is considered to be a specific marker of the ERC, we were interested if p50RhoGAP colocalized with Rab11. We expressed a Rab11-GFP fusion protein in HeLa cells and studied its relation to the endogenously expressed p50RhoGAP (Fig. 8, A–F). The two proteins showed high level of colocalization in the perinuclear region of the cell. Interestingly, in Rab11-expressing cells the localization of endogenous p50RhoGAP was characteristically changed in a way that p50RhoGAP was enriched in Rab11-positive vesicles and tubules (Fig. 8, D–F) and showed less intense staining in the proximity of the nucleus. A mutant form of Rab11 (7) that is locked in a GDP-bound state showed no colocalization with p50RhoGAP, suggesting that GTP binding is necessary for the colocalization (data not shown). Similarly a high level of colabeling was observed when Rab11-red fluorescent protein was expressed along with...
FIGURE 4. Localization of p50RhoGAP is resistant to brefeldin A and is different from the Cdc42 localization. Control cells (A and C), and cells treated with 5 μg/ml brefeldin A for 10 min (B and D) were fixed and stained for either p50RhoGAP (A and B) or Cdc42 (C and D), E–G, P50RhoGAP-GFP-transfected cells were stained for Cdc42 using rabbit polyclonal anti-Cdc42 antibody.

FIGURE 5. The N-terminal Sec14 domain determines the endosomal localization of p50RhoGAP. Sec14-GFP-expressing HeLa cells loaded with 10 μg/ml Alexa568-transferrin (Tf) for 30 min at 37 °C are shown in A–C. HeLa cell overexpressing Sec14GFP is displayed in D, and the boxed region with many enlarged vesicles is shown in E. The L173D mutant form of the Sec14 domain and of the full-length protein is shown in F and G, respectively.
Sec14-GFP (Fig. 8, G–I). Although Rab11 is mainly localized to the ERC, another member of the family, Rab5, is present on sorting endosomes (29). We were interested in the relation of p50RhoGAP to Rab5. In HeLa cells Rab5-GFP showed little perinuclear localization, but it was present in dispersed vesicles in the cell periphery (Fig. 8K). This pattern was in agreement with its localization to sorting endosomes. Although p50RhoGAP was not present in peripheral vesicles, colabeling was observed in more central Rab5-positive structures (Fig. 8L). This observation is in agreement with the previous reports, where partial overlap was observed between Rab5- and Rab11-positive endocytic compartments (30).

The colocalization of Rab proteins with p50RhoGAP was an exciting result since members of both Rab and Rho families were implicated in the regulation of endocytosis (4, 31); however, the connection between the two signaling networks is still unclear. We, therefore, sought to determine whether molecular interaction occurs between Rab11 and p50RhoGAP. We studied this question with the help of BRET, which allows the detection of molecular interactions in live cells (a detailed description of these experiments is provided under “Experimental Procedures”). In these experiments p50RhoGAP or its Sec14 domain were expressed as YFP fusion proteins, and Rab11 was expressed in fusion with Renilla luciferase protein. Juxtanuclear staining of the YFP fusion

[FIGURE 6. Overexpression of the Sec14 domain of p50RhoGAP results in marked inhibition of transferrin uptake. A and B, HeLa cells overexpressing Sec14-GFP (green) were loaded with 10 μg/ml Alexa568-transferrin (red) for 10 min at 37 °C. wt, wild type. C and D, Alexa647-transferrin uptake of cells expressing the wild type (C) or the L173D mutant form (D) of Sec14-GFP was measured by flow cytometry. E and F, recycling kinetics of Alexa647-transferrin in HeLa cells expressing wild type (E) or L173D mutant (F) of Sec14-GFP. G and H, kinetics of Alexa647-transferrin uptake in monensin-treated HeLa cells expressing either the wild-type (G) or the L173D mutant form (H) of Sec14-GFP. Results representative of three (C–F) or two similar experiments (G–H) are shown. □, curves display cells with low GFP intensity; ○, curves show cells with high GFP intensity (see also “Experimental Procedures”).]
p50RhoGAP on Endosomes

Overexpression of p50RhoGAP results in inhibition of transferrin uptake. Kinetics of Alexa647-transferrin uptake in HeLa cells expressing either the wild type (wt; A), the GAP Arg mutant R282A (B), or the Sec14 domain mutant L173D (C) form of full-length p50RhoGAP-GFP are shown. Results representative of four (A and B) and two (C) experiments are shown. ■, curves display cells with low GFP intensity; ○, curves show cells with high GFP intensity.

constructs was verified by confocal analysis (data not shown). The DNA constructs were expressed in HeLa cells, and we monitored the ratio of 530-nm emission and 480-nm emission after the addition of the membrane-permeable luciferase substrate, coelenterazine. We observed BRET between Rab11 and p50RhoGAP and also between Rab11 and the Sec14 domain mutant L173D (C) form of full-length p50RhoGAP-GFP (data not shown). We also tested if p50RhoGAP has a GTPase activating effect on Rab11, but GTP hydrolysis by Rab11 was not affected by p50RhoGAP (data not shown).

DISCUSSION

Rho GTPases were shown to participate in the regulation of receptor-mediated endocytosis (31). For proper functioning, Rho GTPases have to cycle between the GTP- and GDP-bound states. This process is actively regulated by GTPase exchange factor and GAP proteins. The importance of the GTPase cycle during endocytosis is well illustrated by the inhibitory effect of constitutively active Rho family mutants on different steps of receptor-mediated endocytosis. Constitutively active forms of RhoA and Rac1 inhibit transferrin and EGF receptor internalization (8), whereas a constitutively active form of RhoB inhibits traffic from the early endosomes to late endosomes (33). Another Rho GTPase, RhoD, seems to have a unique role in the regulation of endosome dynamics, since the active mutant form of RhoD effectively inhibits the motility of endosomal vesicles (9). It is obvious that regulator proteins have to be present in the same intracellular sites where small GTPases exert their regulatory effects. We have examined the intracellular localization and function of the p50RhoGAP protein. We found that p50RhoGAP was present on endosomal membranes, where it colocalized with transferrin receptor and Alexa-labeled transferrin molecules (Figs. 2 and 3). When endocytosis was induced by receptor-ligand binding, p50RhoGAP again showed colocalization with the internalized EGF receptor. To our knowledge p50RhoGAP is the first candidate molecule that can have a role in the regulation of Rho GTPase function in endosomal compartments.

Currently it is unclear which Rho family small GTPase would be the in vivo substrate of p50RhoGAP. Because we have not observed p50RhoGAP at the plasma membrane or on peripheral vesicles, it is unlikely that the protein has a role in the regulation of the GTPase activity of Rac1 and RhoA during the first steps of receptor internalization. Based on its in vitro activity, p50RhoGAP was previously described as Cdc42GAP. While this manuscript was in preparation, Wang et al. (34) showed that P50RhoGAP (Cdc42GAP)-deficient embryonic fibroblasts displayed increased activation of Cdc42. In HeLa cells, Cdc42 is probably not the physiological substrate of p50RhoGAP, since brefeldin A caused rapid redistribution of Cdc42 from the Golgi apparatus to the cytosol, whereas the localization of p50RhoGAP was resistant to BFA treatment (Fig. 4). Furthermore, overexpression of p50RhoGAP had no effect on the intracellular distribution of endogenous Cdc42, and the two proteins did not colocalize. Based on its localization to the ERC and to some extent to the early endosomes, it is more likely that RhoD and/or RhoB could be the physiological substrates of p50RhoGAP, since these small GTPases were described at similar intracellular locations (9, 10).

P50RhoGAP is a 439-amino acid protein that contains an N-terminal domain homologous to the yeast Sec14 protein. In our experiments we found that the Sec14-like domain of p50RhoGAP was responsible for the localization of p50RhoGAP to endosomal membranes. The Sec14 protein was originally identified in Saccharomyces cerevisiae. In the yeast, Sec14 functions as a phosphatidylinositol transfer protein that is essential for transport of proteins from the Golgi apparatus to the plasma membrane (33). Recently, it was shown that Sec14 interacts with the yeast PtdIns4P 5-kinase (24), which is involved in the PtdIns5P metabolism (25). In mammalian cells, the Sec14-like domain of p50RhoGAP may play a similar role in lipid transfer and a C-terminal lipid-interacting domain (21).

pg2103

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JOURNAL OF BIOLOGICAL CHEMISTRY 6103

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in several mammalian proteins, including regulators of small GTPases such as the GTPase exchange factor proteins Dbl, Dbs and Trio (35, 36). Among RhoGAP proteins, p50RhoGAP and its recently described homolog BPGAP1 contain a Sec14-like domain (37). The function of this protein motif in small GTPase regulators is largely unknown. In the GTPase exchange factor protein Dbs the Sec14-like domain has an inhibitory effect on the transforming potential of Dbs, and removal of this part of the protein results in redistribution of Dbs from its perinuclear localization to the plasma membrane (36). It is possible that Sec14-like domains of small GTPase regulators, similarly to their yeast homo-
log, also bind phospholipids. In fact, p50RhoGAP was isolated from neutrophil granulocyte cytosol through its binding to phosphatidylinositol 3,4,5-trisphosphate beads, although the binding was relatively weak (38). Furthermore, in phosphatidylinositol array experiments the Sec14-like domain of Dbs showed phosphatidylinositol binding with a preference toward phosphatidylinositol 4,5-bisphosphate (36). However, a using similar technique we were unable to detect phosphatidylinositol binding either by the isolated Sec14-like domain of p50RhoGAP or by the full-length protein (data not shown), although we cannot exclude that such binding can occur in vivo. In support of this possibility we found that a mutation of a highly conserved leucine (Leu-173), which is thought to contribute to a lipid binding pocket in a similar position in Sec14, completely abolished the characteristic localization to the ERC and the molecule became evenly distributed in the cytosol (Fig. 5). We frequently observed large, vesicular structures in cells overexpressing the Sec14-like domain that probably resulted from the expansion of endosomal compartments. The appearance of these structures indicated that the Sec14-like domain somehow interfered with the movements and/or fusion-fission process of endosomal vesicles. Cells with this phenotype also showed decreased transferrin internalization (Fig. 6). The reduced uptake of transferrin is only partially explained by inhibited endosomal recycling, since the Sec14-like domain inhibited transferrin uptake under conditions when endosomal recycling was blocked.

These observations suggest that Sec14-like domain interferes with the endocytic process at multiple steps. The importance of this domain was further strengthened by experiments where we showed that overexpression of the full-length protein also inhibited transferrin uptake in a Sec14-like domain-dependent way (Fig. 7).

In our experiments we found that both p50RhoGAP and its Sec14-like domain showed strong colocalization with Rab11 in the ERC (Fig. 8). This colocalization was dependent on the nucleotide binding state of Rab11 since a mutant form of the protein, which was locked in the GDP-bound state, showed no colocalization with p50RhoGAP. We also observed a partial colocalization with Rab5, a marker of early endosomes. Rab GTPases, including Rab4, Rab5, and Rab11, have an essential role in the regulation of several steps of receptor-mediated endocytosis. Importantly, beside their well-established role in membrane budding and fusion, Rab proteins also seem to regulate vesicle motility. For example, Rab6 was found to interact with a kinesin-like protein (39), and a regulatory role for Rab5 in the motility of early endosomes was described (32). The colocalization of p50RhoGAP and Rab proteins has urged us to examine if these proteins form a complex within the cells. Using the BRET technique, we could show that both p50RhoGAP and the isolated Sec14-like domain formed molecular complexes with Rab11. This finding suggests that the Sec14-like domain of p50RhoGAP was responsible for the binding of the full-length GAP protein to Rab11. The functional consequence of this complex formation is currently unknown. The p85α subunit of phosphatidylinositol 3-kinase was recently shown to have GTPase activating effect on Rab proteins, but in our experiments p50RhoGAP did not influence the GTPase activity of Rab11 (data not shown). The lack of a direct regulatory effect suggests that p50RhoGAP is more likely involved in connecting the function of Rab GTPases to Rho GTPases. To our knowledge this is the first demonstration of a potential link between the two signalling networks.

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