Rho GTPases have been implicated in the control of several cellular functions, including regulation of the actin cytoskeleton, cell proliferation, and oncogenesis. Unlike RhoA and RhoC, RhoB localizes in parts to endosomes and controls endocytic trafficking. Using a yeast two-hybrid screen and a glutathione S-transferase pulldown assay, we identified LC2, the light chain of the microtubule-associated protein MAP1A, as a novel binding partner for RhoB. GTP binding and the 18-amino acid C-terminal hypervariable domain of RhoB are critical for its binding to MAP1A/LC2. Coimmunoprecipitation and immunofluorescence experiments showed that this interaction occurs in U87 cells. Down-regulation of MAP1A/LC2 expression decreased epidermal growth factor (EGF) receptor expression and modified the signaling response to EGF treatment. We concluded that MAP1A/LC2 is critical for RhoB function in EGF-induced EGF receptor regulation. Because MAP1A/LC2 is thought to function as an adaptor between microtubules and other molecules, we postulate that the RhoB and MAP1A/LC2 interactions facilitate endocytic vesicle trafficking and regulate the trafficking of signaling molecules.

Rho GTPases belong to the Ras superfamily of small GTP-binding proteins and act as molecular switches by cycling between an inactive GDP-bound state and an active GTP-bound state. They are involved in the regulation of some of the most fundamental processes of cell biology common to all eukaryotes, including the dynamic organization of the actin cytoskeleton and the regulation of signaling pathways that control cell morphology, polarity, adhesion, and movement (1). The Rho GTPases mediate a diverse set of cellular functions, including cell cycle progression, gene transcription, and intracellular traffic. As each of these functions is important for the initiation and progression of cancer, Rho GTPases appear to be promising targets for anti-tumoral drugs (2). RhoB, in contrast with its close relatives RhoA and RhoC, plays a negative role in oncogenesis and may thus act as a tumor suppressor (3). Moreover, expression of RhoB decreases with increasing tumor aggressiveness in lung cancers, head and neck carcinomas, and glioblastomas (4–7). In response to cellular stress, RhoB appears to play a regulatory role in sensing the level of damage in cells and quickly triggering either subsequent protective responses (8–10) or cell death induction (11–15).

At the molecular and cellular level, RhoB is different from RhoA and RhoC in several ways. First, unlike most of the small GTPases, RhoB has a short half-life (16). RhoB is an immediately early response gene that is induced by a variety of stimuli, including growth factors such as EGF,2 platelet-derived growth factor, and transforming growth factor-β (17–20), as well as by cellular stresses such as DNA-damaging agents (9, 21–23) or hypoxia (24). The RhoB protein is highly regulated at the promoter level, by modification of mRNA or protein stability, and by modification of the RhoB GTP/GDP ratio (9, 18, 19, 21, 25, 26). Second, compared with other prenylated Rho GTPases that are exclusively farnesylated or geranylgeranylated, RhoB has been reported to be both farnesylated and geranylgeranylated in vivo (27, 28). Third, RhoB is associated with the plasma membrane and with early endosomes and pre-lysosomal compartments, whereas RhoA and RhoC appear to be mainly cytoplasmic (10, 27, 29). Consistent with its endosomal location, RhoB modulates the traffic of several receptors through the endocytic system (30–32).

RhoB is activated as internalized EGF receptor passes through the endosomal compartment (33). Constitutively active RhoB prevents intracellular trafficking of the EGF receptor between endocytic compartments (20, 34, 35) in a process that may involve PRK1 (36), the exchange factor Vav2 (33), and Dia1, which controls the movement of endosomes along the actin cytoskeleton (37). Moreover, RhoB has also been implicated in the intracellular trafficking of AKT (38) and in controlling the outward movement of Src to the plasma membrane (39). Taken together, these findings highlight the important

2 The abbreviations used are: EGF, epidermal growth factor; GST, glutathione S-transferase; EGF-R, EGF receptor; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HA, hemagglutinin; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; siRNA, short interfering RNA; BD, binding domain; Pipes, 1,4-piperazinediethanesulfonic acid; GTPyS, guanosine 5′-O-(thio)triphosphate.

* This work was supported by institutional funding from INSERM, Université Paul Sabatier, Ligue Contre le Cancer, Association pour la Recherche sur le Cancer, and Groupe de Recherche de l’Institut Claudius Regaud. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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role that RhoB plays in the subcellular targeting of signaling molecules.

However, the molecular mechanisms by which RhoB modulates endosomal trafficking and exerts such myriad effects remain largely unclear. To elucidate the role of RhoB in these intracellular processes, we performed a yeast two-hybrid screen to identify effector proteins that interact specifically with RhoB. This screen identified LC2, the light chain associated with the microtubule-associated protein MAP1A, as a novel binding partner for RhoB. LC2 was characterized as a subunit of MAP1A/LC2 and has been determined to be an adaptor protein that facilitates the interaction of MAP1A with other signal transduction components or structural proteins (40). The ability of MAP1A/LC2 to interact with RhoB provides new insights into the involvement of RhoB and the microtubule cytoskeleton in the mechanisms underlying the controlled subcellular targeting of signaling molecules.

MATERIALS AND METHODS

Antibodies

The following primary antibodies were used: monoclonal anti-α-tubulin (clone B-5-1-2) from Sigma; monoclonal anti-Myc antibody (clone 9E10) from Oncogene; monoclonal anti-RhoB (sc-8048), polyclonal anti-RhoB (sc-180), polyclonal anti-MAP1A (sc-25728), monoclonal anti-HA probe (sc-7392), and polyclonal anti-p-EGF-R (sc-12351) from Santa Cruz Biotechnology Inc.; monoclonal anti-EGF-R (2232) and polyclonal anti-p-AKT (9271) from Cell Signaling Technology; polyclonal anti-AKT (559028) from Pharmingen; monoclonal anti-actin (MAB1501) from Chemicon; monoclonal anti-FAK (F15020) from BD Transduction Laboratories. The following secondary antibodies were used: Alexa Fluor 488- and Alexa Fluor 633-labeled secondary antibodies were purchased from Molecular Probes, and peroxidase-coupled secondary antibody was from Bio-Rad.

Plasmids

Standard PCR procedures were used to insert restriction sites into plasmids for cloning. Truncated cDNAs were generated using oligonucleotide-site-directed mutagenesis (QuickChange® II site-directed mutagenesis kit from Stratagene) according to the manufacturer’s instructions. All constructs were confirmed by sequencing.

Yeast Two-hybrid Constructs—The cDNA encoding the bait RhoBv14Δ4 was inserted into pGBK7T vector (Clontech) in-frame at the 3’ end of a Gal4 DNA-binding domain; the cDNAs encoding RhoBv14Δ4, RhoBwtΔ4, or RhoBv14Δ18 were inserted into the pLex12Tetra plasmid (a generous gift of Dr. J. Camonis, Institut Curie, France). Plasmids encoding CAAX (where A is an aliphatic amino acid) box deleted, constitutively active Rho GTPases (RhoA, RhoC, RhoG, Rac1, Cdc42, TC10, and TCT) fused to LexA-BD in pBTM116 were kindly provided by Dr. A. Blangy (CRBM-CNRS, France).

Bacterial Expression Vectors—The cDNAs encoding RhoAv14 or RhoBv14 were inserted into the pGSTMparallel2 vector from Dr. P. Sheffield (41), in-frame at the 3’ of glutathione S-transferase. RhoB C-terminal hypervariable coding regions were inserted after PCR amplification into pGSTparallel2 vector.

Mammalian Expression Vectors—Myc-tagged LC2 cDNA and HA-tagged RhoB cDNAs were inserted into pIRESpuro2 (Clontech).

Yeast Two-hybrid Assay

The bait plasmid pGBK7-T-RhoBv14Δ4 was introduced into yeast strain AH109 by the lithium acetate-mediated method, and concentrated overnight culture of yeast expressing the bait protein was mated to a yeast culture of strain Y187 containing a pretransformed human brain MATCHMAKER cDNA library constructed in pACT2 according to the manufacturer’s instructions (Clontech). Growth on medium lacking Trp, Leu, His, and Ade, and a β-galactosidase colony-lift filter assay were used to isolate cDNAs encoding candidate interacting proteins, following the Yeast Protocols Handbook instructions (Clontech). Gal4-BD or Gal4-BD-lamin fusions were used as negative control baits. For the yeast two-hybrid binary assays performed using the LexA-based two-hybrid system, the prey vector pACT2/LC2 was cotransformed with pBTM116-RhoGTPase constructs into the L40 yeast strain, using the lithium acetate method. Transformants were plated onto a Trp- and Leu-deficient medium and incubated for 3 days at 30 °C. A colony-lift filter assay was used to detect β-galactosidase activity, according to the Yeast Protocols Handbook instructions (Clontech). Observation of a blue color within 4 h was considered a positive result.

Mammalian Cell Culture and Transfection Experiments

HeLa (human carcinoma ATCC CCL-2) and U87-MG cells (human glioblastoma ATCC HTB-14) were routinely cultured in Dulbecco’s modified Eagle’s medium (Cambrex Lonza) supplemented with 10% fetal calf serum. Cells were incubated in either growth medium or serum-free media for 24 h before experiments with additions of EGF (Sigma) or GGTI-298 (Calbiochem), lovastatin (Sigma), or R115777 (Janssen). Transfection experiments were performed using jetPEI (PolyPlus) for plasmids and Oligofectamine (Invitrogen) for siRNA, according to the manufacturer’s instructions. siRNAs against MAP1A (siMAP1A-1 and siMAP1A-2, respectively, 5’-ACUCUAUCUGACAGUGACUUUATT-3’ and 5’-CGUGAGGUUGCUUGACGCUUATT-3’) were designed and synthesized as oligonucleotides by Qiagen. siRNA against RhoB (siRhoB) was described previously (24). For control siRNA (siControl) a non-specific sequence (5’-ACUCUAUCUGACAGUGACCUU-3’) synthesized by Eurogentec was used as a control.

Recombinant Proteins and GST Pulldown Experiments

For the GST pulldown assays, the GST fusion protein was induced overnight in 1 liter of Escherichia coli BL21 cells by addition of 100 μM isopropyl β-D-thiogalactopyranoside. After centrifugation and freezing, the bacterial pellet was resuspended in 50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 5 mM MgCl2, 1% Triton X-100, 10 mM dithiothreitol, 0.1 mg/ml DNase I, and protease inhibitors (Sigma). After centrifugation, the supernatant fraction was bound to 500 μl of pre-washed glutathione-Sepharose beads (GE Healthcare) for 2 h at 4 °C. The beads
were extensively washed with lysis buffer, and the purity of the bound GST fusion protein was analyzed by SDS-PAGE. Transfected myc-LC2 HeLa cell lysate (1 mg) or brain extract (100 μg) was added to 10 μg of GST-Rho beads in 500 μl of bacterial lysis buffer for 2 h at 4 °C. Beads were then washed twice with interaction buffer, resuspended directly in Laemmli buffer, and analyzed by SDS electrophoresis and Western blot with anti-Myc or anti-MAP1A antibodies. For GTP or GDP loading, GST-Rho beads were preincubated with 10 μM GDP or GTPγS in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA at 30 °C and then washed with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2 before incubation with the cell lysate used for GST pulldown assays.

For analysis of the level of activated RhoB, GTP-bound RhoB protein was measured using the method initially described by Ren et al. (42) using the GST fusion protein containing the Rho binding domain of the downstream effector rhotekin and adapted to RhoB (33). The amount of GTP-bound RhoB and the total amount of RhoB in cell lysates was determined by Western blot using polyclonal anti-RhoB antibodies.

**Cell Extracts, Immunoprecipitation, and Cell Fractionation Experiments**

For brain extract, adult mouse brains were homogenized in 100 mM Pipes, pH 6.9, 1 mM EGTA, 1 mM MgCl2, and protease/phosphatase inhibitors (Sigma). For cell extracts, cells were harvested 48 h after transfection in cell lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2, 0.5% Triton X-100, 10 mM dithiothreitol, and protease/phosphatase inhibitors). After centrifugation, the supernatant was recovered, and protein concentration was determined by Bradford assays (Bio-Rad). For immunoprecipitation experiments, cell extract (500 μg) from cells transfected with myc-LC2 or 2.5 mg of U87 cell lysate was incubated with monoclonal anti-RhoB antibody (4 μg) and with 50 μl of magnetic anti-mouse IgG (Dynal) for 4 h at 4 °C. After washing, the bound proteins were eluted with SDS sample buffer and analyzed by SDS-PAGE and Western blot using anti-RhoB or anti-Myc antibodies. For U87 experiments, NuPAGE™ 4–12% BisTris gels (Invitrogen) were used and immunoblotted with anti-MAP1A and anti-RhoB antibodies. For cell fractionation, transfected cells (4 × 10^7) were resuspended in homogenization buffer (8% sucrose, 3 mM imidazole, and protease/phosphatase inhibitors (Sigma)), and endosomal fractions were prepared on discontinuous sucrose gradient as described previously (43).

For visualization of proteins on Western blots, the ECL Western blotting substrate detection system was used (Pierce). Data shown were representative at least of three separate experiments.

**Immunofluorescence and Confocal Microscopy**

U87 or HeLa myc-LC2 transfected cells were plated onto acid-washed glass coverslips and fixed 24 h later for 15 min in 3.7% formaldehyde in PBS and then washed with PBS and permeabilized in 0.2% Triton X-100 in PBS for 5 min. The cells were then washed again in PBS, incubated with 0.1% sodium borohydride for 10 min, and then with PBS containing 3% BSA for 30 min. The cells were rinsed and incubated for 1 h at room temperature with primary antibodies in PBS plus 1% BSA and then with secondary antibodies for 30 min at room temperature in PBS, 1% BSA. After rinsing, cells were mounted on slides with Mowiol (Calbiochem). Images were taken with an LSM 510 confocal microscope (Zeiss, Yena, Germany) using a sequential mode of acquisition with a 63 × 1.4 numerical aperture oil immersion lens. The green channel was acquired using the 488 Ar laser line and detected between 505 and 545 nm; the red channel was acquired using the 633 nm HeNe laser line and detected between 645 and 760 nm. Using the sequential mode and spectral separation allowed us to avoid any bleed through in the red channel and ensured correct colocalization. Colocalization of RhoB with MAP1A or α-tubulin was quantified using Metamorph Imaging System software package (Molecular Devices Corp., Sunnyvale, CA) At least 10 fields were quantified for each staining. The percent colocalization is indicative of the area of RhoB-stained fluorescent pixels overlapping that of cytoskeleton markers. Visualization of spatial correlation was done by graphing the intensity values of those markers along a given line with separate traces for red and green components, using Metamorph software.

**RESULTS**

**Identification of LC2 as a Specific RhoB Partner Using the Yeast Two-hybrid System**—In an attempt to identify RhoB effector proteins, we employed a two-hybrid screen of a human brain cDNA library with RhoBv14Δ4 as bait; this construct is equivalent to the constitutively active Val-12 mutation in Ras and lacks the C-terminal CAAX motif to prevent prenylation modification in yeast cells during the experiments. RhoBv14Δ4 was fused to a Gal4 DNA-binding domain (Gal4-BD) and used as a bait to screen a human brain cDNA library fused to a Gal4-activating domain. Approximately, 2 × 10^8 independent clones were screened. Of the 43 colonies that grew on selective medium, 23 clones were positive in the β-galactosidase selection assay. These 23 clones were tested in binary two-hybrid experiments against Gal4-BD-RhoBv14Δ4 as well as against Gal4-BD or Gal4-BD-lamin as control baits. We obtained six confirmed positive clones; after sequencing, four independent clones were found to have the identical cDNA sequence. The cDNA sequence was 2133 nucleotides in length with an open reading frame of 729 nucleotides, coding for 241 amino acids (Fig. 1). A computer BLAST search in the human GenBank™ database revealed that this cDNA sequence encoded the 241 C-terminal amino acids of microtubule-associated protein 1A (MAP1A), and corresponded to its associated light chain (LC2). MAP1A/LC2 is translated as a polypeptide precursor (44), which is proteolytically cleaved into the heavy and the light chains (45).

To test the specificity of the interaction of the constitutively active form of RhoB with LC2, we performed a binary two-hybrid association assay using wild-type RhoBΔ4 fused with the Gal4 DNA-binding domain. We found that LC2 interacted more strongly with the constitutively active form of RhoB (Gal4-BD-RhoBv14Δ4) than with wild-type RhoB (Gal4-BD-RhoBwtΔ4) (Table 1). Because the primary structure of Rho GTPases is highly conserved and the proteins are at least 50–55% homologous to each other, we performed yeast two-
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A

Yeast two-hybrid interactions

Yeast two-hybrid interactions between RhoB and LC2. Yeast AH109 or L40 cells were cotransformed with expression vectors encoding Gal-4 or the LexA DNA-binding and activation domains fused with indicated protein. Each transformation mixture was plated on selective medium, and growth and β-galactosidase activity were estimated. AD, activation domain.

| DNA-binding hybrid | Activation hybrid | Colony color | Growth |
|--------------------|------------------|--------------|--------|
| Gal4-BD-RhoBv14D4  | Gal4-AD-LC2      | Blue         | ++     |
| Gal4-BD-RhoBv14A18 | Gal4-AD-LC2      | Blue         | ++     |
| LexA-BD-RhoBv14    | LexA/AD-LC2      | Blue         | ++     |
| LexA-BD-RhoBv14A18 | LexA/AD-LC2      | White        | -      |
| LexA-BD-RhoAv14    | LexA/AD-LC2      | White        | -      |
| LexA-BD-RhoCv14    | LexA/AD-LC2      | White        | -      |
| LexA-BD-RhoGv12    | LexA/AD-LC2      | White        | -      |
| LexA-BD-Racv12     | LexA/AD-LC2      | White        | -      |
| LexA-BD-cdc42v12   | LexA/AD-LC2      | White        | -      |
| LexA-BD-TC1Q75L    | LexA/AD-LC2      | White        | -      |
| LexA-BD-TCLQ94L    | LexA/AD-LC2      | White        | -      |

B

FIGURE 1. The MAP1A/LC2 protein. A, amino acid sequence of the LC2 clone identified by yeast two-hybrid screening. B, schematic representation of the MAP1A/LC2 polyprotein. Amino acid and cDNA sequencing has shown that LC2 is encoded within the 3'-terminal light chain, which is proteolytically cleaved into heavy and light chains. The LC2 clone identified by yeast two-hybrid screening is indicated (amino acids 2565–2805).

TABLE 1

Yeast two-hybrid interactions

Yeast two-hybrid interactions between RhoB and LC2. Yeast AH109 or L40 cells were cotransformed with expression vectors encoding Gal-4 or the LexA DNA-binding and activation domains fused with indicated protein. Each transformation mixture was plated on selective medium, and growth and β-galactosidase activity were estimated. AD, activation domain.

| DNA-binding hybrid | Activation hybrid | Colony color | Growth |
|--------------------|------------------|--------------|--------|
| Gal4-BD-RhoBv14D4  | Gal4-AD-LC2      | Blue         | ++     |
| Gal4-BD-RhoBv14A18 | Gal4-AD-LC2      | Blue         | ++     |
| LexA-BD-RhoBv14    | LexA/AD-LC2      | Blue         | ++     |
| LexA-BD-RhoBv14A18 | LexA/AD-LC2      | White        | -      |
| LexA-BD-RhoAv14    | LexA/AD-LC2      | White        | -      |
| LexA-BD-RhoCv14    | LexA/AD-LC2      | White        | -      |
| LexA-BD-RhoGv12    | LexA/AD-LC2      | White        | -      |
| LexA-BD-Racv12     | LexA/AD-LC2      | White        | -      |
| LexA-BD-cdc42v12   | LexA/AD-LC2      | White        | -      |
| LexA-BD-TC1Q75L    | LexA/AD-LC2      | White        | -      |
| LexA-BD-TCLQ94L    | LexA/AD-LC2      | White        | -      |

hybrid binary association assays using several Rho family members as baits cloned into a LexA-BS plasmid, which is known to be more specific than the Gal4-BS system. Despite the high degree of amino acid sequence homology between different Rho family members, only the constitutively active form of RhoB (LexA-BS-RhoBv14D4) interacted with LC2 in the yeast two-hybrid system, whereas no interaction was detected with the constitutively active mutants of CAAX-deleted RhoA, RhoC, RhoG, Rac1, Cdc42, TC10, and TCL (Table 1). RhoA, RhoB, and RhoC have around 90% amino acid homology, with the most divergence in the hypervariable domain that includes the 18 C-terminal amino acids. We thus tested the hypothesis that this C-terminal region could be involved in interaction of LC2 with RhoB. Deletion of this region completely abolished the interaction with LC2 in the two-hybrid assay (Table 1), demonstrating the critical role these 18 C-terminal amino acids play in the specificity of the LC2/RhoB interaction.

GTP-bound RhoB interacts with MAP1A/LC2 in an in Vitro GST Pulldown Assay—We next tested whether RhoB and LC2 could interact in GST pulldown assays. To this end, a Myc tag was added at the N terminus of LC2, and the fusion protein was transiently expressed in HeLa cells. Recombinant GST-RhoBv14 protein was produced in E. coli and then bound to glutathione-Sepharose beads and incubated with myc-LC2-expressing HeLa cell lysate. As shown in Fig. 2A, immunoblotting with anti-Myc (labeled HeLa-myLC2) showed that LC2 interacted strongly with GST-RhoBv14 beads, weakly with GST-RhoAv14 beads, and did not interact with the GST beads negative control.

Because LC2 is associated with the MAP1A heavy chain in vivo (46), we wished to determine whether RhoB also interacts with LC2 in its MAP1A heavy chain-associated form. To this end, mouse brain extracts containing large amounts of MAP1A (47) were incubated with GST-RhoBv14 beads in GST pull-down experiments. As shown in Fig. 2A (labeled Brain), immunoblotting with anti-MAP1A antibody revealed a specific band corresponding to endogenous MAP1A when brain extracts were incubated with GST-RhoBv14 beads. No signal was detected when brain extracts were incubated with GST-RhoAv14 beads or control GST beads.

To verify that the interaction between MAP1A/LC2 and RhoB was dependent on the GTP loading of RhoB, we preincubated GST-RhoB beads with 10 μM GDP or GTPγS before incubation with cell lysate. Fig. 2B shows that the GTP-bound form, but not the GDP-bound form, of RhoB interacted with LC2 in myc-LC2-expressing HeLa cell lysate or MAP1A in brain extracts. This indicates that the interaction was highly dependent on GTP loading.

To analyze the role of the RhoB C-terminal domain in the RhoB/LC2 interaction using a GST pulldown assay, we generated a C-terminal deletion of GST-RhoBv14. Recombinant GST-RhoBv14Δ18 protein was produced and bound to glutathione-Sepharose beads. We showed that the deletion of the 18 C-terminal amino acids of RhoB resulted in a weak interaction with LC2 in HeLa-myc LC2 extract and with MAP1A in brain extract (Fig. 2C), suggesting that the C terminus is required for the interaction with MAP1A/LC2. As this deletion could affect the tridimensional structure of RhoB, we generated a fusion of GST protein with 15 C-terminal amino acids (corresponding to the C-terminal part of RhoB after KVL proteolysis) or 37 C-terminal amino acids of RhoB (corresponding to the last conserved α-helix and the hypervariable region of RhoB) to analyze the role of the RhoB C-terminal domain in the RhoB/LC2 interaction. According to the tridimensional structure of RhoB, only the GST fusion with the 37 C-terminal amino acids of RhoB was able to interact with LC2, as shown in Fig. 2D. These results indicate that the conformation of RhoB C-terminal is critical for its interaction with LC2.

All these results indicate that GTP-binding and the C-terminal hypervariable domain of RhoB are critical for binding of RhoB to LC2 or MAP1A/LC2. Taken together, these data indicate that LC2 is a specific binding partner of RhoB.

RhoB interacts with MAP1A/LC2 in Cell Extract—To investigate whether the interaction between RhoB and LC2 occurs in cell extract, coimmunoprecipitation experiments were performed in three mammalian cell lines. First, Ras-transformed NIH3T3 cells stably expressing RhoBv14 (previously used in our laboratory (15)) were transiently transfected with plasmids expressing Myc-tagged LC2 protein. Immunoprecipitation
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FIGURE 2. RhoB interacts with LC2 and MAP1A/LC2 in vitro. The specificity of the RhoB and LC2 interaction was analyzed in vitro by GST pulldown assays with the following: GST, GST-RhoAv14, and GST-RhoBv14 protein beads (A); GST or GST-RhoB loaded with either GTPγS or GDP (B); GST-RhoBv14 and GST-RhoBv14Δ18 proteins beads (C); or GST, GST-C15, GST-C37, and GST-RhoBv14ΔD. In each case, GST proteins were incubated with cell extracts from Myc-tagged LC2-transfected HeLa cells (HeLa-myc-LC2) or with brain extract (Brain). Proteins eluted from beads were analyzed by immunoblotting with antibodies to the Myc tag or to the MAP1A protein. After immunoblotting, membranes were stained by Amido Black to ensure the presence of the GST protein in the pulldown assays.

FIGURE 3. Interaction between RhoB and MAP1A/LC2 in mammalian cells. The RhoB and MAP1A/LC2 interaction was analyzed by coimmunoprecipitation experiments with cell lysate from Ras-transformed NIH3T3 cells stably transfected with RhoBv14 (3T3Ras-RhoBv14) and transiently transfected with Myc-tagged LC2 plasmids or empty vector (A) and cell lysate from HeLa cells transiently transfected with myc-tagged LC2 plasmids or empty vector (B). Cells extracts were immunoprecipitated (IP) with anti-RhoB antibodies or with anti-mouse IgG and analyzed by Western blotting using anti-Myc and anti-RhoB antibodies. Total extract used for immunoprecipitation is indicated as lysate and examined by Western blotting using anti-Myc and anti-RhoB antibodies.

with anti-RhoB antibodies was then performed. Western blotting of the immunoprecipitates with anti-Myc antibodies revealed a specific band corresponding to Myc-tagged LC2 in myc-LC2 transfected cells, whereas no band was detected in the mock-transfected cell lysate (Fig. 3A). We next analyzed the interaction between RhoB and LC2 HeLa cells that transiently expressed Myc-tagged LC2. Immunoprecipitates of myc-LC2 expressing HeLa cell extracts with RhoB antibodies were blotted with anti-Myc antibody. The Western blot revealed a specific band corresponding to Myc-tagged LC2, whereas no band was detected in the mock-transfected HeLa cells or in mouse IgG immunoprecipitates (Fig. 3B).

FIGURE 4. Interaction between RhoB and MAP1A/LC2 in U87 cells. A, activation of RhoB was analyzed in U87 cells serum-starved for 24 h, treated with EGF (10 ng/ml), and lysed at different times. GTP-bound RhoB was precipitated with GST-RBD beads. GTP-bound RhoB in precipitate and RhoB in lysate were examined by Western blotting using an anti-RhoB antibody in comparison with an irrelevant protein (IR). B, U87 cells were serum-starved for 24 h and treated with EGF (10 ng/ml) for the indicated time. Cell extracts were then immunoprecipitated (IP) with anti-RhoB antibodies. Lysate and immunoprecipitates were analyzed by Western blotting using anti-MAP1A and anti-RhoB antibodies. Control immunoprecipitates were performed without extract (lg) or without antibodies (Cont). C, U87 cells were treated with different inhibitors as follows: 10 μM Lovastatin (Lova), 10 μM GGTTI-298 (GGTI), 4 mM R115777 (FTI), or no inhibitor (Cont) and serum-starved for 16 h, then stimulated for 60 min with EGF (10 ng/ml), and lysed. Lysates were immunoprecipitated with anti-RhoB antibodies. Immunoprecipitated proteins were analyzed by Western blotting using anti-MAP1A and anti-RhoB antibodies. Cells lysates were analyzed by Western blot with anti-MAP1A, anti-RhoB, anti-HDJ-2, and anti-unprenylated Rap1a. HDJ-2 and unprenylated Rap1A were shown as controls of GGTase and FTase inhibition by lovastatin (Lova), GGTI, or FTI.
analyze the interaction between an unprenylated form of RhoB and MAP1A, which is known to impair protein isoprenylation, to evaluate whether lovastatin, a competitive inhibitor of hydroxymethylgeranylated CoA reductase, alters the level of RhoB expression and consequently inactivates MAP1A (49). We used lovastatin, a competitive inhibitor of hydroxymethylgeranylated CoA reductase, to determine whether the MAP1A/RhoB interaction is affected by this compound. We then tested whether the interaction between RhoB and MAP1A is dependent on the activation state of RhoB. Our results suggest that the activation state of RhoB can be either farnesylated or geranylgeranylated in vivo (28), and the type of prenylation determines the subcellular localization of the protein. Geranylgeranylated RhoB (RhoB-GG) localizes to endosomes, and farnesylated RhoB (RhoB-F) localizes to the plasma membrane (10, 35). The farnesyltransferase inhibitor R115777 and the geranylgeranyltransferase inhibitor GGTTI-298 were used to explore the respective role of RhoB-F and RhoB-GG in MAP1A binding (Fig. 4C). Western blot analysis of Rap-1A, an exclusively geranyl-geranylated small GTPase and HDJ-2, exclusively farnesylated, were used, respectively, as a marker of geranylgeranyltransferase and farnesyltransferase inhibition after treatment with 10 μM lovastatin, 10 μM GGTTI-298, or 4 nM R115777. As with lovastatin, GGTTI-298 increased the expression of RhoB, as well as the level of RhoB in immunoprecipitates, and impaired the binding of MAP1A to RhoB (Fig. 4C, lane GGTTI). In contrast, R115777 did not modify RhoB expression but slightly decreased the RhoB/MAP1A interaction (Fig. 4C, lane FTTI). These results suggest that geranylgeranylation of RhoB is of particular importance for the RhoB/MAP1A interaction.

RhoB and MAP1A/LC2 Are Colocalized in the Cell—Our next step was to analyze the intracellular localization of MAP1A/LC2 and RhoB in U87 cells. Therefore, we first used HeLa cells transiently transfected with myc-LC2. Localization of RhoB and myc-LC2 was evaluated by immunofluorescence staining and confocal microscopy in transfected cells (Fig. 5A). In agreement with previous reports, in these cells, RhoB exhibited a granular cytoplasmic staining consistent with its known endosomal vesicle localization (10, 27, 35). It should be noticed that a significant staining at the plasma membrane could be observed when the cell are confluent (not shown). As reported previously, myc-LC2 staining was cytoplasmic and at nuclear levels (50). To evaluate colocalization in transfected cells, we used the MetaMorph Imaging system. We observed that myc-LC2 was colocalized with microtubules (93.9 ± 3.57%), and a partial colocalization of myc-LC2 and RhoB was observed at the endosomal vesicles (83.56 ± 3.36%).
Furthermore, endosomes were isolated by cellular fractionation on sucrose gradient as previously described by Gorvel et al. (43). Thus, we examined by Western blot the distribution of RhoB, myc-LC2 throughout the gradient in comparison with the endosomal marker EEA1 (Fig. 5B). In agreement with immunochemistry experiments, both RhoB and myc-LC2 were found in the endosomal fraction.

We next evaluated the localization of endogenous RhoB and endogenous MAP1A in U87 cells (Fig. 6A), and quantification of colocalization of RhoB with MAP1A or α-tubulin was performed (Fig. 6B). We found that a substantial amount of RhoB colocalized with MAP1A (90.37 ± 8.34%) at the cytoplasm. Consistent with MAP1A binding to microtubules, we found that a part of RhoB was colocalized with α-tubulin (69.26 ± 9.75%). To visualize the spatial colocalization between RhoB and MAP1A, fluorescence intensity values were graphed along a selected line designed into the merged image, using separate traces for the red (MAP1A) and the green (RhoB) components (Fig. 6C). As expected, we observed a complete colocalization between the RhoB and MAP1A but only a partial colocalization of MAP1A with RhoB consistent with cytoplasmic expression of MAP1A. Taken together, these data indicate that localization of RhoB and MAP1A/LC2 is spatially correlated and that these proteins are able to interact at the cellular level.

**MAP1A Is Critical for the Effects of RhoB on EGF-R Signaling**—To investigate the functional relationship between RhoB and MAP1A, we used RNA interference to down-regulate MAP1A expression in U87 cells. We used two different short interfering RNAs against the MAP1A sequence (siMAP1A) and checked their ability to down-regulate MAP1A expression in U87 cells. Fig. 7, A and B, presents the results obtained with the two most efficient siRNA sequences. Transfection of U87 cells with siRNA suppressed MAP1A protein expression level by 80% compared with control within 72 h of treatment (Fig. 7, A and B). At the cellular level, we observed that inhibition of MAP1A expression leads to a spreading of U87 cells (data not shown). Consistent with MAP1A binding to microtubules and to actin microfilaments, and with the suggested involvement of MAP1A in the cross-bridging between microtubules and other cytoskeletal elements (51, 52), down-regulation of MAP1A expression resulted in a modification of microtubule and microfilament organization in U87 cells (Fig. 7D).

It has been shown that RhoB causes a delay in EGF-induced EGF-R degradation by inhibiting the transfer from late endosomes to lysosomes (35). Moreover, MAP1A is thought to stabilize microtubules by altering their dynamic behavior (53), and both microtubules and actin cables are required for efficient addressing of proteins to late endosomes or lysosomes (54). To determine whether the presence of MAP1A affects the function of RhoB in EGF-R degradation and vesicular trafficking, we analyzed the level of endogenous EGF-R after EGF stimulation in U87 cells in the presence or absence of siMAP1A-1 or siMAP1A-2 (Fig. 7C). Western blotting using an antibody to EGF-R showed that U87 cells transfected with siMAP1A had lower levels of total EGF-R after EGF treatment compared with cells transfected with control siRNA. Similar results were obtained with U87 cells transfected with siRNA against RhoB (Fig. 8A, siRhoB) in agreement with previous results that demonstrated that cells overexpressing active RhoB had delayed EGF-R degradation (20, 35). Taken together, these results suggest that both MAP1A and RhoB are involved in EGF-induced EGF-R degradation.

To further characterize the functional link between RhoB and MAP1A, we studied the effect of down-regulation of MAP1A protein expression on the EGF-R signaling (Fig. 8A). To determine whether RhoB or MAP1A silencing modified EGF-R activation and signaling, we used U87 cells transfected with either siRhoB or siMAP1A. After these cells were stimulated with EGF, we analyzed the level of phosphorylation of Tyr-1173 of EGF-R, which is the major site of autophosphorylation resulting from EGF binding, as well as the level of phosphorylation of AKT’s Ser-473, which is representative of AKT activation. We found that suppression of RhoB or MAP1A expression decreased the level of EGF-R phosphorylation concomitantly with the level of the total EGF-R protein (Fig. 8A), suggesting acceleration of signaling attenuation of the receptor. Consistent with these data, the silencing of RhoB and MAP1A resulted in a decrease of Ser-473 phosphorylated AKT. Furthermore, we studied the effect of down-regulation of MAP1A expression on EGF-R regulation in RhoB overexpressed U87 cells transfected with MAP1A antisense RNA. Western blotting using an antibody to EGF-R showed a decrease of EGF-R expression in U87 cells transfected with either siRhoB or siMAP1A. Consistent with these results, we observed that inhibition of MAP1A expression decreased the level of total EGF-R protein (Fig. 8A, siMAP1A) suggesting down-regulation of MAP1A expression decreases the level of EGF-R expression.
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![Image](86x660 to 145x673)

**FIGURE 7. Inhibition of MAP1A expression modifies the microtubule and actin cytoskeletons and EGF-R expression.** A, U87 cells were transfected with two different siRNAs against MAP1A (siMAP1A-1 or -2) or siRNA control (siControl). 48 h after transfection, cells were lysed, and MAP1A or RhoB expression was analyzed by Western blotting with anti-MAP1A or anti-Fak antibodies. B, histogram represents quantification of MA1PA level at different concentration of siMAP1A used as determined by densitometry analysis of Western blots after normalization with an anti-Fak antibodies. C, 24 h after transfection with siMAP1A-1 or siMAP1A-2 as indicated, U87 cells were serum-starved for 24 h and then stimulated with EGF (10 ng/ml) for the indicated time. Cells lysates were examined by Western blot with antibodies against EGF-R, phosphorylated EGF-R, AKT, phosphorylated AKT, and actin in comparison with an irrelevant protein (IR). D, U87 cells were transfected with siControl or siMAP1A. 48 h later, cells were transfected with HA-RhoB expression vector and serum-starved overnight. Cells were then stimulated with EGF (10 ng/ml) for the indicated times, and cells lysates were analyzed by Western blot with antibodies against EGF-R, actin, or HA tag.

Cells (Fig. 8B). We found that forced expression of RhoB was not able to restore the level of EGF-R in siMAP1A transfected U87 cells, in agreement with interaction between MAP1A and RhoB. Overall, these results showed that RhoB and MAP1A are able to regulate EGF-R expression and subsequent signaling after EGF stimulation.

**DISCUSSION**

It is well established that different Rho proteins are not functionally redundant in the cell but play different roles in cell physiology (1). Despite the high amino acid sequence similarity between RhoA, RhoB, and RhoC, some regulators and effectors preferentially interact with one of the members of the Rho family over the others (55). The differences in the cellular function of these three Rho proteins are likely to be generated by a combination of their specific subcellular localization and their selective interaction with activators or effectors (56). These selective interactions could be the consequence of differences in the C-terminal hypervariable domain, which is the most divergent region of Rho. The C-terminal sequence includes a consensus sequence for protein isoprenylation that is essential for correct cellular localization (27, 29). RhoB is an atypical Rho protein in that it can be either farnesylated or geranylgeranylated, and the nature of the isoprenyl modification is critical for determining the subcellular localization of the protein (28, 29). RhoB-F is reported to be localized at the plasma membrane and RhoB-GG at the endosomal membrane (10, 35). This precise localization of RhoB at the endosomal compartment is thought to be related to its specific role in endosomal trafficking (18), implying that RhoB should have specific partners at the endosomal level. Here we characterized MAP1A/LC2 as a novel RhoB-specific effector that interacts with RhoB, which is required for RhoB-dependent EGF-R trafficking.

Using prenyltransferase inhibitors, we showed that the RhoB-MAP1A/LC2 interaction requires RhoB geranylgeranylation, strongly suggesting that correct cellular localization of RhoB is essential for this interaction. We excluded a direct interaction of MAP1A/LC2 through the isoprenyl domain, because the yeast two-hybrid experiments were performed with an unprenylated (ΔCAAX) protein. Moreover, GGTI treatment, which inhibits the localization of RhoB to endosomes, prevented the interaction between RhoB and MAP1A, whereas FTI treatment, which localizes a large amount of RhoB to the endosome, had a weaker effect. These
data reinforce the direct demonstration by confocal analysis that RhoB interacts with MAP1A/LC2 at the endosomal level.

Only 11 proteins are known to interact with RhoA, -B, and -C with different affinities (55). Among these proteins, only three have been described to be more specific for RhoB. One of these proteins is DB1, a transcription factor that interacts strongly with prenylated RhoB at the nuclear membrane; the transcriptional activity of DB1 is regulated by RhoB, apparently through a sequestration mechanism (57). A second protein that specifically interacts with RhoB is p76RBE, a protein implicated in the cyclic AMP signaling pathway (58); the third protein is the PRK1 kinase (36). p76RBE and PRK1 have been identified as RhoB effectors at the endosomal level. Moreover, p76RBE and PRK1 are recruited to an endosomal compartment when coexpressed with the activated form of RhoB, and in both cases, a Rho-binding domain (HR-1) has been found in their sequence, which is sufficient for endosomal targeting (36, 59). We did not identify a Rho-binding domain on MAP1A, and the protein domains involved in RhoB recognition remain to be discovered. However, we showed that the 18 C-terminal amino acids of RhoB are critical for the RhoB-MAP1A/LC2 interaction and that GTP binding is required for maximal interaction between these two proteins. These data suggest that two different domains of RhoB are involved in the interaction with MAP1A/LC2. This is the first demonstration that the C-terminal membrane targeting domain is critical for the interaction between an effector protein and a Rho protein. All together, these data shed light on the mechanism by which effectors can associate with Rho proteins at different subcellular levels.

Rho GTPases are key regulators of the actin and microtubule cytoskeletons and are involved in the control of endocytosis (54, 60). However, the molecular mechanisms that link the function of Rho on the cytoskeleton to endocytosis are only partially understood. Here we propose that MAP1A is a potential candidate for mediating the effect of RhoB on EGF-R trafficking through the actin and microtubule cytoskeletons. MAP1A/LC2 can bind microtubules as well as actin microfilaments, suggesting that MAP1A/LC2 is involved in the cross-bridging between microtubules and other cytoskeletal elements (52). Whether MAP1A/LC2 is a linker between the actin and microtubule cytoskeletons has not been determined. It has been proposed that MAP1A regulates the distribution of associated molecules as a linker between the molecules and microtubules or the actin cytoskeleton. Indeed, it has been shown that MAP1A/LC2 is a component of the cytoplasmic RNA-binding protein complex (61) and can interact with several partners as follows: DISC-1, a centrosome-associated protein implicated in intracellular transport (62); PSD-93, a membrane-associated guanylate kinase (47); BKC, a potassium channel (63); EPAC, an exchange protein directly activated by cAMP (64); and Stargazin, a protein involved in synaptic targeting (65). Moreover, it has recently been shown that LC2 interacts with PDZRhoGEF, a guanosine exchange factor implicated in the regulation of a neuronal glutamate transporter on microtubules (66). Taken together, these findings led to the hypothesis that MAP1A/LC2 functions as an adaptor molecule that regulates cellular trafficking via actin and microtubules.

It has been shown that RhoB regulates endosome transport to lysosomes for degradation and that it causes the accumulation of endocytosed EGF in peripheral vesicles, delaying EGF receptor trafficking (35). Moreover, RhoB induces the retention of endosomes on actin fibers and then prevents their transfer onto microtubules, thus preventing the further transport of endosomal cargo to lysosomes (37). This demonstrated the critical role of RhoB in the control of endosomal trafficking at different levels of the endocytosis process. MAP1A/LC2 is the first identified microtubule cytoskeleton component that interacts with RhoB. This strongly suggests that RhoB can interact with the microtubule cytoskeleton through its interaction with MAP1A, resulting in its control of endosomal trafficking. The interaction of MAP1A/LC2 and RhoB can provide an explanation of how EGF-R trafficking is regulated at the endosomal level by RhoB.

Removal of receptor molecules from the cell surface by distributing them to the endocytic pathway modifies the amplitude and kinetics of signal transduction (37). Here we demonstrated that MAP1A/LC2 expression was critical for the RhoB-dependent control of EGF-R expression and that this expression modified the duration of EGF-R signaling. It has also been reported that RhoB mediates the vesicle trafficking that contributes to regulation of AKT (38) and Src (67). Moreover, RhoB has been described as modulating the exit of cargo from endosomes in polarized Madin-Darby canine kidney cells (31). All these data suggest a general role for RhoB in addressing signal transduction molecules to specific cellular locations and in controlling subsequent signaling.

Studies are underway to elucidate how the MAP1A-LC2 complex results in delay of EGF-R trafficking and how RhoB is activated in this context. As MAP1A/LC2 was shown to bind and to stabilize microtubules (53, 68), we propose that the RhoB MAP1A/LC2 interaction could result in the stabilization of microtubules, thus facilitating the trafficking of endocytic vesicles and then modifying the activation of different signaling pathways.

Acknowledgments—We are grateful to Dr. A. Blangy and Dr. J. Camonis for the yeast two-hybrid expression vectors and for technical assistance. We thank Prof. J. Mazieres for critical reading of the manuscript, Dr. B. Canguilhem for preparing figures, and Remi Gence for technical assistance.

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