CLIPR-59 is a lipid raft-associated protein containing a cytoskeleton-associated protein glycine-rich domain (CAP-Gly) that perturbs microtubule dynamics*

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We recently identified a new cytoplasmic linker protein (CLIP), CLIPR-59, which is involved in the regulation of early endosomes/trans-Golgi network dynamics. In contrast with CLIP-170, CLIPR-59 is not localized to microtubules at steady state but is associated with the trans-Golgi network and the plasma membrane. Here we show that the last 30 amino acids (C30) are sufficient for membrane targeting and that two cysteines in the C30 domain are palmitoylated. We demonstrate that CLIPR-59 is associated with lipid rafts via its C-terminal palmitoylated domain. In vitro experiments suggest that CLIPR-59 and its microtubule-binding domain alone have a better affinity for unpolymerized tubulin or small oligomers than for microtubules. In contrast with the CLIP-170 microtubule-binding domain, the CLIPR-59 microtubule-binding domain diminishes microtubule regrowth after nocodazole washout in vivo, showing that this domain can prevent microtubule polymerization. In contrast with the role of linker between membranes and microtubules that was proposed for CLIP function, CLIPR-59 thus may have an “anti-CLIP” function by preventing microtubule-raft interactions.

The microtubule network is required for intracellular organization and movement. The dynamic instability of microtubules allows a rapid exploration of the cytoplasmic space, and the intrinsic asymmetry of the tubulin polymer is used by molecular motors to establish intracellular polarity. Cytoskeleton linker proteins (CLIPs),1 which are non-motoric microtubule-binding proteins, were also proposed to be involved in intracellular organization and organelle movement. Rickard and Kreis (1) proposed that CLIPs would transiently link membranes to microtubules, regulating molecular motor function and organelle movement. This model was proposed following the identification of CLIP-170, which was shown to be necessary for the interaction of endosomes with the microtubules (2). This model was further strengthened by the characterization of the Golgi-associated microtubule-binding protein GMAP-210 (3) and the cytoskeleton-linking membrane protein CLIMP-63 (4).

The microtubule-binding domain of CLIP-170 contains a repetition of a microtubule binding motif, the CAP-Gly motif, found in other tubulin-interacting proteins. Interestingly, most of CLIP-170-related proteins are active at the organelle-microtubule interface as proposed for p150Gluad (5, 6), CLIP-115 (7), Drosophila CLIP-190 (8), or a yet unidentified peroxisomal CLIP (9). Another key aspect of CAP-Gly-containing proteins is that they usually interact with tubulin in a tightly regulated way. Certain proteins containing CAP-Gly are tubulin-folding cofactors that interact with α-tubulin in a preactivated state (10), whereas others, like CLIP-170, CLIP-115, or p150Gluad, interact more strongly with polymerizing microtubule plus ends than with the rest of the polymer (11–13). This interaction of CLIP-170 with microtubule plus ends may be because of an interaction of CLIP-170 with soluble, non-polymerized tubulin and subsequent co-polymerization during tubulin assembly (11), and this may be important to promote microtubule rescue (14).

We recently identified a new CLIP-170-related protein of 59 kDa (CLIPR-59) (15). Like CLIP-170, the CLIPR-59 MTB (C59MTB) consists of two CAP-Gly motifs separated by a serine-rich region. Unlike CLIP-170, which is essentially partitioned between the microtubules and the cytosol, the full-length protein does not label microtubules quantitatively in vivo. Indeed, C59MTB was found to bind microtubules in vivo; however, no restricted plus end binding was observed, and only a subset of microtubules is labeled along their entire span. At steady state, CLIPR-59 is targeted to the plasma membrane and to trans-Golgi network (TGN) membranes by its 60-amino acid-long C-terminal membrane-targeting sequence (15). We proposed that CLIPR-59 was a TGN-associated CLIP that, according to the CLIP model, would establish a static interaction between TGN membranes and microtubules, hence regulating membrane dynamics. CLIPR-59 is indeed involved in intracellular trafficking as its overexpression induces endosome relocation and perturbs transferrin recycling as well as Shiga toxin B-fragment retrograde transport to the Golgi apparatus (15).

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1 The abbreviations used are: CLIP, cytoplasmic linker protein; CLIPR-59, CLIP-170-related protein of 59 kDa; CAP-Gly, cytoskeleton-associated protein glycine-rich domain; MTB, microtubule-binding domain; C59MTB, the MTB of CLIPR-59; TGN, trans-Golgi network; MEF, mouse embryonic fibroblast; GFP, green fluorescent protein; GPP-C59, GFP-labeled CLIPR-59; GPP-ΔC60, GFP-labeled CLIPR-59 with the last 60 amino acids deleted; GPP-C90, enhanced GFP CDN fused with the sequence encoding the last 30 amino acids of CLIPR-59; HA, hemagglutinin; MBP, maltose-binding protein; TTR, transferrin receptor; MβCD, methyl-β-cyclodextrin; PIPES, 1,4-piperazinediethanesulfonic acid; PNS, postnuclear supernatant; N2, nocodazole; DRM, detergent-resistant membrane(s); GPl, glycolylphosphatidyl inositol; PIC, protease inhibitor mixture.
The mechanism by which CLIPR-59 is targeted to the TGN is still unknown. In integral membrane proteins, the length of the transmembrane domain is thought to be involved in this process, and CLIPR-59 could bind to such an integral membrane receptor on Golgi membranes. Alternatively, CLIPR-59 may recognize the TGN directly through its membrane-targeting domain. Cellular membranes contain lateral heterogeneities, thus defining membrane subdomains, and CLIPR-59 could be targeted to specific membrane subdomains. Rafts are 10–300-nm microdomains enriched in cholesterol and sphingolipids that exist as aggregated-phase bilayers (lipid rafts) within more fluid membranes (16). Depending on the cholesterol concentration, rafts are thought to exist from the TGN to the plasma membrane (17, 18). The recruitment of proteins to the ordered membrane seems to play a role in diverse functions such as signal transduction or intracellular trafficking. Here, we demonstrate that CLIPR-59 is associated with lipid rafts. We found that the last 30 amino acids of CLIPR-59 are necessary and sufficient to target it to the TGN and to the plasma membrane and that a double palmitoylation on tandem cysteines within this domain is responsible for the raft targeting. In addition, to test whether CLIPR-59 could function as a CLIP, we studied the activity of C59MTB. We show that in contrast with the CLIP-170 MTB, the CLIPR-59 MTB reduces microtubule elongation and displays a specific behavior in a microtubule co-sedimentation assay. This first raft-associated CLIP could play an atypical role at the raft-microtubule junction.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Mouse embryonic fibroblasts (MEFs) stably transfected with a pCDNA6/TO plasmid from Invitrogen were given by H. de The (St. Louis Hospital, Paris, France) and cultured as described previously (19). HeLa cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Transient and stable transfections were performed with the calcium phosphate precipitate method. Stable GFP-CLIPR-59 (GFP-C59) and GFP-ΔC60-overexpressing MEFs were obtained by transfecting the GFP-C59 or ΔC60 pCDNA6/TO constructs (Invitrogen) into MEF-TR cells and were selected for 3 weeks with 50 μg/ml Zeocin. Dilutions were performed, and different clones were selected for CLIPR-59 expression after induction with 2 μg/ml doxycycline (Clontech Laboratories) for 24–48 h.

Plasmid Constructions—GFP- and HA-C59, ΔC60, and C59MTB cDNAs cloned into the pCB6 plasmid were described previously (15). HA- or GFP-C59 cDNA, AA, C534A, C535A, and W536A point mutants were obtained by replacing the wild type sequence with a mutated sequence (MWG, Biotech France SA). pCDNA6/TO vector from Invitrogen was used to clone GFP-C59 and ΔC60 cDNA for stable cell lines. The MBP-C59MTB fusion was obtained by cloning the C59MTB sequence in the pMalC3 plasmid, and the fusion His-C170H1 was described previously (20).

Antibodies—Monoclonal antibody against GFP was obtained from Roche Applied Science, and antibodies against p150^GLued, golgin-84, flotillin-2, and GM-130 were from BD Transduction Laboratories. Purified polyclonal anti-Rab6 antibody was obtained as described previously (21). Monoclonal anti-HA and polyclonal antitubulin (T13) antibody were described previously (15). Monoclonal H88.4 was used for mouse TR in Western blotting on MEF extracts; horseradish peroxidase-tagged cholera toxin (Sigma) was used for detection of GM1 lipids on dot blot. Cy3-aerolysin was kindly provided by G. van der Goot (Centre Medico-Universitaire, Geneva, Switzerland). Alexa488-labeled secondary antibodies were obtained from Molecular Probes, and Cy3-labeled and fluorescein labeled (1:1000) were from Invitrogen.

Cell Treatments, Immunofluorescence, and Confocal Microscopy—HeLa or MEF cells were cultured on glass coverslips. 24 h after transfection or doxycycline induction, cells were fixed for 10 min in 3% paraformaldehyde and permeabilized for 5 min (in phosphate-buffered saline, 0.1% bovine serum albumin, 0.05% saponin) or fixed for 4 min in methanol at –20 °C. For pre-extraction, coverslips were incubated in 0.5% Triton X-100, PEM buffer (100 mM PIPES, 1 mM EGTA, 1 mM MgCl2) for a few seconds five times and then fixed in methanol. To deplete cholesterol from membranes, transfected HeLa cells grown on coverslips were treated with 5 mg/ml methyl-β-cyclodextrin (MβCD) for 10 min at 37 °C and then fixed in 3% PFA. 200 μM bromoacetamide was used to inhibit palmitoylation on doxycycline-induced MEF cells grown on coverslips prior to fixation in PFA. Wide field images were acquired on a Leica microscope DMRA controlled by the Metamorph software (Universal Imaging) running on a Dell personal computer. Confocal images were acquired on a Leica SP2 microscope. Figures were then assembled using Adobe Photoshop 7.0 running on a Macintosh G4.

Membrane Extraction and Raft Purification—10^6 HeLa cells transfected with GFP-C59 or GFP-ΔC60-overexpressing MEF cells were resuspended in 3 mM imidazole, 0.25 M sucrose buffer with PIC on ice for 15 min, then broken with a 22-gauge needle or with a ball-bearing cell cracker, and centrifuged to remove nuclei and large cell debris. Postnuclear supernatant (PNS) was incubated with ice-cold 1% Triton X-100, 1 mM NaCl, or 0.1 mM Na2CO3, pH 11.5, and centrifuged at 60,000 × g for 30 min at 4 °C in a TL100 centrifuge. Supernatants and pellets were analyzed by Western blot.

To purify lipid rafts, 3 × 10^6 MEF cells were resuspended in 300 ml of TE buffer (25 mM Tris, pH 7.4, 2.5 mM EDTA) with PIC and lysed with a cell cracker. An equal volume of 2% Triton X-100 or Lubrol WX in TE, 300 mM NaCl was then added. PNS was obtained by centrifugation (1000 × g for 5 min) and kept on ice for 30 min. Optiprep gradient was performed as follows. Optiprep (800 μl) was added to the PNS (400 μl) to obtain a 40% final concentration and loaded at the bottom of the centrifugation tube. 2.3 ml of 30% Optiprep, 150 mM NaCl, TE and 0.8 ml of 5% Optiprep, 150 mM NaCl, and TE were carefully added without perturbing the interfaces. The gradient was centrifuged for 4 h at 4 °C at 28,000 rpm (rotor SW41). Fractions were collected from the top to the bottom, and fraction 2, corresponding to the 0.530% interface, contained lipid rafts.

In Vitro Vino Microtubule Polymerization and Co-sedimentation Assay—Extraction co-sedimentation assays were performed in the presence of 10 μM nocodazole (N2)-treated cells lysed and incubated at 4 °C. Supernatants and pellets were analyzed by Western blot. Microtubule polymerization was performed as above with or without purified MBP-C59MTB or His-C170MTB (40 μg/ml) in the presence of an increased concentration of purified tubulin (0.5–2.5 mg/ml) instead of cell extract. This compares well with the concentration of tubulin in the cytosol (20 μM; Ref. 22).
For the microtubule regrowth assay, the microtubules of GFP-C59MTB- or GFP-C170H1-overexpressing HeLa cells growing on coverslips were depolymerized by incubating the cells at 4 °C for 20 min before adding 10 μM NZ at 37 °C for 2 h. Microtubule regrowth was allowed by washing out NZ and incubating at 20 °C; the coverslips were prepermeabilized with 0.5% Triton X-100, PEM buffer to eliminate soluble tubulin and GFP-C59MTB proteins and then were fixed in methanol (4 min at −20 °C) at different periods of time. Cells were then processed for immunofluorescence to stain microtubules and imaged as described above, using the same exposure time for each condition. Quantification of microtubule regrowth was done using the Metamorph software to quantify the mean fluorescence (in the tubulin channel) of control cells or cells overexpressing GFP-C59MTB or GFP-C170H1.

RESULTS

The Last 30 Amino Acids of CLIPR-59 Are a Membrane-targeting Domain—We have shown previously that CLIPR-59 is targeted to the TGN and the plasma membrane via the C-terminal part of the protein; the deletion of the last 60 amino acids (GFP-Δ60) prevents CLIPR-59 from membrane targeting, inducing a cytosolic distribution (Figs. 1A and 2) (15). Within this region, colored in gray in Fig. 1B, a short C-terminal sequence of 10 amino acids is hydrophobic. Although its size would not be enough to cross membranes, we wanted to determine whether this hydrophobic sequence is enough to target CLIPR-59 to membranes. We fused the enhanced GFP cDNA to the sequence coding for the last 30 amino acids of CLIPR-59 (GFP-C30). Immunofluorescence experiments performed on GFP-C30 transiently transfected HeLa cells showed that the GFP-C30 protein was addressed to membranes and co-localized with the cis-Golgi marker GM-130 (Fig. 1A). This distribution is similar to the TGN localization of the GFP-CLIPR-59 full-length protein (GFP-C59; Fig. 1A, arrows). In cells expressing a higher amount of GFP-C30 or GFP-C59 proteins, the plasma membrane was also labeled indicating that CLIPR-59 distribution was not restricted to but concentrated on Golgi membranes (Fig. 1A, arrowhead). The C30 domain of CLIPR-59 is thus sufficient for membrane targeting.
The Membrane Targeting of CLIPR-59 Requires a Tandem of Palmitoylated Cysteines—The C30 domain of CLIPR-59 contains two adjacent cysteines, which are amino acids commonly used to target cytoplasmic proteins to membranes after post-translational modifications (farnesylation, geranylgeranylation, or palmitoylation). To investigate whether such posttranslational modifications are involved in the membrane targeting of CLIPR-59, we generated point mutants by replacing both of these cysteines or each of them separately with alanines. We also replaced with alanines the amino acids preceding and following the tandem cysteines, a phenylalanine and a tryptophan, respectively, to control the selective effect of the mutations. This was particularly interesting because a phenylalanine preceding tandem cysteines was found in other proteins like SCG10 (23), which localized like CLIPR-59 from the TGN to the plasma membrane (see “Discussion”). Each mutant was HA-tagged and transfected in HeLa cells, and its subcellular localization was analyzed by double immunofluorescence with anti-HA and anti-GM-130 antibodies. Like the mutant deleted from the C60 domain, the HA-C59-CC/AA double mutant and HA-C59-F533A phenylalanine mutant were completely cytosolic (Fig. 2). On the other hand, the HA-C59-W536A mutant co-localized with GM-130 and localized to the plasma membrane. The HA-C59-C534A and the HA-C59-C535A single mutants were partly cytosolic and partly associated with membranes, but HA-C59-C535A was more widely distributed in the cytosol than HA-C59-C534A (Fig. 2). The same subcellular localizations were observed with GFP-tagged mutants (not shown). This indicates that both cysteines, as well as the upstream phenylalanine, are necessary for membrane targeting of CLIPR-59.

We then tested whether these cysteines were palmitoylated. HeLa cells overexpressing the GFP-C59 protein or the GFP-ΔC60 cytosolic mutant were labeled with [3H]palmitate, and palmitoylation of the recombinant proteins was analyzed after immunoprecipitation. As shown in Fig. 3A, CLIPR-59 incorporated [3H]palmitate, whereas the ΔC60 deletion mutant did not, indicating that the C-terminal part of the protein was modified by palmitate or a palmitate derivative. We repeated [3H]palmitate labeling and immunoprecipitation with GFP-tagged point mutants (n = 2). The C59-CC/AA mutant was not palmitoylated (Fig. 3A). The C594A, the C535A, and the F533A single mutations reduced but did not completely prevent CLIPR-59 palmitoylation. In addition, a higher amount of radioactivity seems to be incorporated in the C59-C534A mutant than in the C59-C535A mutant, suggesting that the Cys135 is the major site for palmitoylation. The downstream tryptophan is not involved in CLIPR-59 palmitoylation because the C59-W536A mutant was normally modified. Thus, both cysteines are palmitoylated, and this modification requires the conservation of the adjacent phenylalanine. The membrane localization of CLIPR-59 wild type and mutants is thus the exact reflection of their respective palmitoylation modification, further indicating that tandem palmitoylation is required for correct CLIPR-59 localization. We then...
used bromopalmitate to decrease CLIPR-59 palmitoylation (24). GFP-C59 labeling was restricted to TGN membranes in treated cells showing that efficient CLIPR-59 palmitoylation is necessary for its localization (Fig. 3B). Note that by Western blot, wild type and mutant CLIPR-59 migrate as a double band (Figs. 3A, 4A, and 5). Why these doublets were observed is unclear; they are not due to palmitoylation because they were still observed with the mutants.

The C-terminal Domain of CLIPR-59 Is Necessary for Triton X-100-resistant Interaction with Membranes—To further characterize the membrane association of CLIPR-59, we generated stable cell lines that overexpressed either the full-length GFP-C59 protein or the GFP-ΔC60 soluble mutant. The tetracycline-inducible system was used in the MEF cell line to overcome the toxicity of stable CLIPR-59 overexpression. Clones with a low expression level were selected to conserve the TGN and plasma membrane localization of CLIPR-59 and to prevent side effects observed upon overexpression (Figs. 3B and 4B). PNSs of these overexpressing cells were treated or not with ice-cold 1% Triton X-100 buffer, and membranes were subsequently purified with high speed centrifugation. CLIPR-59 distribution was then analyzed by Western blotting with anti-GFP antibody; TfR (Fig. 4).

**Fig. 4.** Interaction of CLIPR-59 with detergent-resistant membranes. A. MEF cells stably overexpressing GFP-CLIPR-59 (GFP-C59) or GFP-CLIPR-59ΔC60 (GFP-ΔC60) were treated or not with 1% Triton X-100 (TX-100), 1 M NaCl, or 0.1 M Na₂CO₃, pH 11.5, and fractionated to separate high speed pellet (P) and supernatant (S). The analysis of protein partition was done by Western blotting using anti-GFP and anti-TfR antibodies. A representative experiment of eight independent experiments is shown here. B, confocal analysis of immunofluorescence staining performed on GFP-C59-overexpressing cells pre-extracted with 0.5% Triton X-100 or treated with 5 mg/ml MβCD. Cy3-tagged aerolysin (Aerolysin-Cy3) staining of GPI-anchored proteins was used as a control for DRM-associated protein. The “glow” look-up table was used to visualize variations in fluorescence intensity (increasing fluorescence is represented from red to yellow, and blue corresponds to saturation). Bar, 10 μm.
4A) and GD1β (not shown) were used as controls for transmembrane and cytosolic proteins, respectively. As expected, in untreated cells, GFP-C59 was found in the membrane fraction (pellet), whereas GFP-ΔC60 was mainly in the cytosol (supernatant) (Fig. 4A). Interestingly, this interaction was partly resistant to Triton X-100 extraction; only part of CLIPR-59 was solubilized in 1% Triton X-100, whereas the integral membrane TIR protein was completely removed from the pellet by such a treatment. Although this suggests that CLIPR-59 is inserted into detergent-resistant membrane (DRM), it is possible that the Triton X-100-resistant CLIPR-59 sedimentation is a result of an interaction with a raft-associated receptor, with cytoskeletal elements, or with insoluble protein complexes. We thus repeated the same experiment using 1 M NaCl or sodium carbonate (pH 11.5) extractions, which prevent most protein interactions and remove most peripheral membrane-associated proteins. The fraction of the GFP-ΔC60 mutant that sedimented at high speed was not sensitive to Triton X-100 but was solubilized by high salt and basic pH. Thus, GFP-ΔC60 sedimentation was probably because of an indirect interaction with membranes, a cold-resistant cytoskeleton, or simply because of aggregation. In contrast, GFP-C59 was completely insoluble in high salt or basic pH, suggesting that CLIPR-59 interacts directly with DRM (Fig. 4A, n = 8).

To investigate the DRM association of CLIPR-59 in vivo, we compared the distribution of CLIPR-59 to that of glycosyolphosphatidylinositol (GPI)-anchored proteins, which are mostly lipid raft-associated luminal proteins targeted to the membrane by a C-terminal GPI anchor, using fluorescent aerolysin (25, 26). Confocal analysis of the localization of GFP-C59 and GPI-anchored proteins showed that these proteins co-localized on Golgi and plasma membranes (Fig. 4B, arrows). The plasma membrane localization of CLIPR-59 was not homogenous, and its co-localization with GPI-anchored proteins was particularly clear at membrane ruffles. GFP-C59-overexpressing HeLa cells were then subjected to 0.5% Triton X-100 extraction or MβCD treatment, which can be used to deplete cholesterol from membranes. Confocal analysis was performed with the same settings as those used for untreated cells to visualize variations of fluorescence intensity. Triton X-100 reduced GFP-C59 fluorescence on membranes, but most of the proteins remained associated with TGN membranes and to a lesser extent with the plasma membrane (Fig. 4B and data not shown). The difference in Triton X-100 sensitivity between plasma and Golgi membranes may be because of differences in the accessibility of this drug. Nevertheless, the membrane association of CLIPR-59 is resistant to Triton X-100 similarly to GPI-anchored proteins. In most of the MβCD-treated cells, plasma membrane and intracellular punctuate labeling greatly decreased (Fig. 4B), although in some cells Golgi labeling was still bright (not shown). Thus, GFP-C59 proteins are associated with DRM containing GPI-anchored proteins in a cholesterol-dependent way.

**CLIPR-59 Is Associated with Lipid Raft**—We next tested whether CLIPR-59 interaction with DRMs is a result of lipid raft association. The ordered organization of cholesterol-rich raft microdomains confers cold non-ionic detergent resistance to these microdomains and allows their isolation by flotation in sucrose density gradient. Thus, we tested the ability of CLIPR-59 proteins to co-fractionate with these rafts. GFP-C59- or GFP-ΔC60-overexpressing MEF cells were extracted with cold 1% Triton X-100, and their PNSs were analyzed using flotation equilibrium Optiprep density gradient (Fig. 5A, n = 4). Western blot analysis of the collected fractions showed that a significant amount of GFP-C59 was found in fraction 2, corresponding to 5.37% interface where the raft-associated protein flotillin-2 was found almost exclusively (Fig. 5A, lane F2). In contrast, GFP-ΔC60 did not float to this low density fraction but stayed in denser fractions with the Rab6 protein. These results show that CLIPR-59 association to low density Triton X-100-resistant membranes is dependent on the palmitoylated C-terminal domain. Depending on their lipid composition, distinct rafts could be defined and characterized based on their different non-ionic detergent insolubility (27, 28). We used Lubrol-WX, another non-ionic detergent, to better characterize CLIPR-59-interacting rafts. As in Triton X-100 assay, GFP-C59 but not GFP-ΔC60 floated to low density fraction 2 (Fig. 5B, n = 3). GM1, the lipid raft-associated receptor of the cholera toxin, was used as a control. We then confirmed that cholesterol was required for CLIPR-59 flotation by extracting membranes with 0.5% Triton X-100 and 0.5% saponin (Sap) (29). As shown in Fig. 5C (n = 2), saponin prevented GFP-C59 to float after Triton X-100 extraction, indicating that CLIPR-59-associated DRMs are dependent on cholesterol to form rafts. Thus, CLIPR-59 is targeted to lipid rafts, both resistant to Triton X-100 and Lubrol-WX, through its palmitoylated C-terminal domain.

**CLIPR-59 Co-sedimentation with Microtubules Is Inhibited by Increased Concentration of Tubulin**—CLIP-170 contains two CAP-Gly motifs and was proposed to link microtubule plus ends to endosomes. Because CLIPR-59 is associated with lipid rafts, we wondered whether it could play the role of cytoplasmic linker between the lipid raft microdomains and microtubules. CAP-Gly sequences are known to interact with microtubules in other CLIPs, and the CLIPR-59 MTB co-localized with microtubules when expressed in cells (Fig. 7A) (15). We thus inves-
tigated the ability of CLIPR-59 to interact with microtubules using co-sedimentation experiments. Microtubules were polymerized at 37 °C from the endogenous tubulin of the PNS of GFP-C59-overexpressing cells in the presence of 1 mM GTP. Taxol was added at the end of the reaction to stabilize polymerized microtubules, and centrifugation at 20,000 × g was performed to separate pelleted microtubules from unpolymerized tubulin. Western blot analysis showed that although most of the GFP-C59 proteins were found in the supernatant, part of GFP-C59 co-sedimented with microtubules, like the component of the dynein-dynactin complex p150Glued used as an internal control (not shown and Fig. 6A). Although membranes are usually not quantitatively pelleted under these conditions, we could not exclude that a fraction was present in the 20,000 × g pellet that could contribute to GFP-C59 sedimentation. To rule out this possibility, we tested GFP-C59 sedimentation in conditions that perturb microtubule assembly or protein-protein interactions. NZ or cold treatments were used to depolymerize microtubules, and 1 mM NaCl was used to disrupt protein interactions. Note that as shown above (Fig. 4A), high salt treatment does not disrupt CLIPR-59 interaction with membranes. GFP-C59 sedimentation, like p150Glued sedimentation, was reduced by cold treatment and inhibited by NaCl or NZ and very closely parallels the amount of polymerized microtubules (Fig. 6A). These results indicate that CLIPR-59 sedimentation is a result of its interaction with microtubules, but not with membrane, and thus suggest that CLIPR-59 behaves like a microtubule-associated protein.

This polymerization/sedimentation assay using the PNS of CLIPR-59-overexpressing cells was repeated, adding increased concentration of brain purified tubulin to the reaction. In these conditions, microtubules were polymerized depending on tubulin concentration, and p150Glued was proportionally co-sedimented with microtubules (Fig. 6B, n = 6). Unexpectedly, the amount of pelleted GFP-C59 was inversely proportional to tubulin concentration. The more tubulin was added, the less CLIPR-59 sedimented. Upon purified tubulin addition, the amount of tubulin recovered in the pellet was increased, corresponding to enhancement of microtubule polymerization, but the amount of unpolymerized tubulin in the supernatant was also greatly increased (supernatants not shown). The displacement of CLIPR-59 toward the supernatant fractions suggests that CLIPR-59 may have a better affinity for unpolymerized tubulin or for small unsedimented oligomers than for microtubules. The reasons for such a preference are unclear at the moment; the CLIPR-59 MTB may directly bind to tubulin dimers as observed before for CLIP-170 (11) or to unfolded tubulin as described for other CAP-Gly-containing proteins (10). Alternatively, CLIPR-59 could prefer slow polymerizing microtubules, the proportion of which is decreased in the presence of high tubulin concentrations. Because cell extracts contain many other factors that could indirectly modulate CLIPR-59 co-sedimentation, we wanted to test directly the behavior of purified CLIPR-59 MTB in similar experiments. Co-sedimentation assay was thus repeated with purified tubulin and with purified CLIPR-59 MTB fused to the maltose-binding protein (MBP-C59MTB). Like GFP-C59 in cell extract, the sedimentation of purified MBP-C59MTB with microtubules decreased when the amount of tubulin increased from 0.5 to 2.5 mg/ml (Fig. 6, C and D, n = 5). Note that the amount of MBP-C59MTB in the supernatant increased from 0.5 to 2.5 mg/ml (Fig. 6, C and D, n = 5). Note that the amount of MBP-C59MTB in the supernatant increased from 0.5 to 2.5 mg/ml (Fig. 6, C and D, n = 5). Note that the amount of MBP-C59MTB in the supernatant decreased when the amount of tubulin increased from 0.5 to 2.5 mg/ml (Fig. 6, C and D, n = 5). Note that the amount of MBP-C59MTB in the supernatant was correlated to that of tubulin (Fig. 6D, Supernatant). In contrast, the sedimentation of the His-tagged head domain of CLIP-170 (His-C170H1), which contains the CLIP-170 MTB, was not prevented by increased concentration of tubulin but was proportional to microtubule sedimentation (Fig. 6D, 1 and 2 mg/ml tubulin). Thus, although the decreased sedimentation of CLIPR-59 observed in increasing tubulin concentrations directly depends on the CLIPR-59 MTB, this behavior is not displayed by all CAP-Gly-containing proteins.

**CLIPR-59 MTB Perturbs Microtubule Polymerization in Vivo**—We then asked whether, in vivo, C59MTB overexpression perturbs microtubule dynamics. We have shown previously that the CLIPR-59 MTB is partly localized on microtubules and could be associated with microtubule bundles in transfected cells (Fig. 7A). We compared the effects of the CLIPR-59 MTB on microtubule polymerization in vivo with those of the CLIP-170 MTB. HeLa cells transfected with the GFP-tagged MTB of CLIPR-59 (GFP-C59MTB) or with GFP-C170H1 were incubated at 4 °C and then treated with NZ to induce complete
Fig. 7. **GFP-C59MTB inhibits microtubule regrowth after NZ washout.** Immunofluorescence was performed with antitubulin antibody on Triton X-100-pre-extracted GFP-C59MTB-transfected HeLa cells (A). The cells were treated with NZ for 2 h (B) and then washed out for 15 and 30 min (C and D, NZ + WO 15 min and 30 min). Immunofluorescence was performed with antitubulin antibody on GFP-C170H1-transfected HeLa cells after 10 and 20 min of NZ washout (E and F, NZ + WO 10 min and 20 min). Left panels, GFP fluorescence; middle panels, tubulin immunostaining; right panels, merge images. Bar, 10 μm.
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FIG. 8. Quantification of microtubule regrowth upon NZ washout. Cells treated as described in Fig. 7 were imaged by fluorescent microscopy, and the mean fluorescence/cell in the tubulin channel was quantified using the Metamorph software. In control cells, as well as in cells transfected by GFP-C59MTB (C59-MTB) or by GFP-C170H1 (H1), the mean fluorescence increased as a function of time. However, a clear reduction of total microtubule polymerization was detected after GFP-C59MTB overexpression. In contrast, GFP-C170H1 seems to slightly enhance microtubule polymerization in vivo. The number of cells analyzed was, respectively, 47, 45, 30, 53, 49, and 47. The results are shown as mean ± S.D.

microtubule depolymerization. We followed microtubule regrowth at 20 °C during NZ washout by performing immunofluorescence on cells pre-extracted with Triton X-100 and fixed at increasing periods of time. Pre-extraction was necessary to remove unpolymerized tubulin and soluble CLIPR-59 MTB that masked the microtubule-associated fraction. Upon NZ treatment, GFP-C59MTB did not localize on the remaining stable microtubules but localized in dots dispersed in the cytoplasm (Fig. 7B). During NZ washout, GFP-C170H1 rather enhanced microtubule regrowth in a classical centric microtubule network, confirming previous results on CLIP-170 microtubule stabilization effects (Fig. 7, E and F) (14, 30). In contrast, the size of asters was reduced in the GFP-C59MTB-overexpressing cells compared with adjacent untransfected cells after both 15 and 30 min of recovery from NZ treatment (Fig. 7, C and D). GFP-C59MTB was not detected on centric polymerizing microtubules but was distributed in foci through the cytoplasm. Quantification of microtubule regrowth, done by measuring the mean fluorescent intensity of control and transfected cells (Fig. 8), confirmed the opposite effect of CLIP-170 and CLIPR-59 MTBs. A similar microtubule regrowth inhibition was observed in HA-C59MTB-transfected cells (not shown). Thus, although the MTBs of both CLIPR-59 and CLIP-170 proteins contain CAP-Gly motifs, the CLIPR-59 MTB inhibits microtubule re-polymerization, whereas the CLIP-170 MTB enhances it.

DISCUSSION

CLIPR-59 Is Targeted to Lipid Raft by Tandem Palmitoylation—We have characterized the mechanism of CLIPR-59 membrane targeting and demonstrated that CLIPR-59 is a raft-associated CLIP endowed with an unusual inhibition of microtubule regrowth activity. We have shown that a short C-terminal sequence constituted by the last 30 amino acids of the protein is sufficient for TGN and plasma membrane targeting. Point mutation analysis together with metabolic labeling have shown that the palmitoylation of two tandem cysteines within this C30 domain is responsible for membrane localization. Palmitoylation of CLIPR-59 targets it to lipid rafts as shown by flotation in density gradient after detergent extraction and co-localization with GPI anchor proteins. High salt or basic pH does not perturb the membrane association of CLIPR-59, suggesting that it does not interact with a raft-associated receptor but interacts directly with lipid raft microdomains. Many proteins are targeted to lipid rafts via dual myristoylation and palmitoylation on N-terminal glycine and cysteine, like p59(fyn), or by C-terminal farnesylation and palmitoylation, which are both required for H-Ras insertion in caveolin microdomains (31–33). In these cases, palmitoylation is dependent on prior myristoyl or farnesyl modification. Because only tandem palmitoylation at the C terminus of CLIPR-59 appears to be required, the mechanism of CLIPR-59 raft association is different from these dual modifications. Interestingly, the membrane-targeting signal of CLIPR-59 is similar to that of SCG10. SCG10 is a neuron-specific stathmin-like protein harboring a 34-amino acid-long N-terminal sequence (not conserved in stathmin), which allows its interaction with membranes and is required for Golgi localization (23, 34). Like the CLIPR-59 C30 domain, the membrane-targeting sequence of SCG10 presents two palmitoylated cysteines and an adjacent phenylalanine. Other stathmin-like proteins, SCLIP, RB3, and the RB3’ and RB3” variants, contain this targeting sequence; they are all neuron-specific and distributed from the Golgi to plasma membranes at nerve ruffles (35). Additionally, CLASP26, a CLIP-170- and CLIP-115-interacting protein expressed in neurons, presents similar dual cysteines required for its Golgi membrane targeting (36). GAP-43 and SNAP-25 are two other nerve-terminal proteins, which present two and four tandem palmitoylated cysteines, respectively, mediating membrane association (37–40). Although conflicting, SNAP-25 was proposed to be enriched in lipid rafts in PC12 cells (38, 41), and GAP-43 and SCG10 were shown to be associated with brain lipid rafts (37, 42). CLIPR-59 is also strongly enriched in the brain, and it is interesting to note that all of these multiple palmitoylated proteins are neuron-specific, suggesting that these domains play particularly important roles in neurons. Moreover, the growing number of multipalmitoylated proteins shown to be raft-associated suggests that palmitoylation on tandem cysteines is an alternative lipid raft microdomain-associating signal. Like myristoylation and farnesylation, other signals, allowing a first interaction with membrane, could be required for tandem cysteine palmitoylation. A short hydrophobic sequence in the SCG10 raft-targeting sequence seems to be required for membrane targeting. In SNAP-25, five amino acids required for palmitoylation were proposed to interact with a membrane receptor that could facilitate interaction with a palmitoyltransferase (40). As shown by hydrophytalysis analysis, the dual palmitoylated cysteines of CLIPR-59 are contained within a short hydrophobic sequence that could similarly promote palmitoylation (Fig. 1B).

The diversity of raft microdomains, which present different lipid compositions, can be characterized by their insolubility in different non-ionic detergents (27). We have shown that the CLIPR-59-positive lipid rafts float in density gradient after both Triton X-100 and Lubrol-WX extractions. Palmitoylation on tandem cysteines could confer an affinity for microdomains with a specific cholesterol and sphingolipid composition, insoluble in these two non-ionic detergents. It is worth noting that SNAP-25, which contains four cysteines, is targeted to the plasma membrane at steady state, whereas GAP-43, SCG10,
and CLIPR-59, which present two tandem cysteines, are targeted from Golgi to plasma membranes. Lipid raft composition is not only different laterally within the bilayer but also from the TGN to the plasma membrane. Thus, the number of palmitoylated cysteines could be responsible for the specificity of the interacting rafts.

**CLIPR-59 Is an Atypical CLIP That Could Prevent Microtubules from Associating to Rafts**—Only a few proteins have been shown to be both associated with lipid rafts and with tubulin, like the stathmin-like proteins or the molecular motor KIFC3 (42, 43), although it has been shown that the movement of raft depends on microtubules (44). Furthermore, CLIPR-59 is the first CAP-Gly protein shown to be associated with membranes at the steady state and in particular to lipid raft microdomains. According to CLIP function, the model we proposed before was that CLIPR-59 could stably link membrane subdomains to microtubules until they could be transported by molecular motors. However, in vitro experiments indicated that only part of CLIPR-59 co-sediment with microtubules from PNS and that surprisingly this sedimentation was decreased by the addition of purified tubulin. The same result was obtained with purified C59MTB and increased concentration of purified tubulin, showing that CLIPR-59 behavior is directly a result of its MTB. This is in contrast with the sedimentation properties of p150Glued, the CLIP-170 MTB, or other CAP-Gly-containing proteins. Under these experimental conditions, not only microtubule polymerization increased, enhancing microtubule sedimentation, but also a large excess of unpolymerized tubulin was present in the supernatant. Thus, CLIPR-59, like CLIP-170 (11), may have a better affinity for monomeric tubulin, or at least short non-sedimenting polymers, than for microtubules. Alternatively, the CLIPR-59 MTB may prefer slow polymerizing microtubules. This could explain why it binds only to a subset of cellular microtubules. Finally, the CLIPR-59 MTB, like tubulin-folding cofactors (10), may have a stronger affinity for some misfolded tubulin that may be present in our experiments.

NZ wash-out experiments showed that C59MTB inhibits microtubule regrowth, in contrast with C170MTB, which enhances polymerization (Fig. 7, B and C). C59MTB could either directly interact with slow polymerizing microtubules or microtubule plus ends or sequestrate unpolymerized tubulin or tubulin oligomers, preventing microtubule polymerization. Under this last hypothesis, C59MTB would function like stathmin, which forms a ternary complex with α- and β-tubulin and induces both inhibition of microtubule polymerization and catastrophes (45). Alternatively, this effect on microtubule polymerization could be because of a dominant negative effect of the C59MTB mutant, which could prevent CLIPR-59 and the other CLIPs, like CLIP-170, CLIP-115, or p150Glued, from binding microtubules by direct competition between CAP-Gly sequences. Indeed, CLIP-170 was shown to promote microtubule rescue (14), and p150Glued was shown to be required for microtubule anchoring at the centrosome (46). However, C59MTB does not localize at microtubule plus ends or at the centrosome, neither at the steady state nor during NZ wash-out experiments. Moreover, no depletion of p150Glued from centrosomes or plus ends was detected in C59MTB-overexpressing cells (not shown). Thus, soluble C59MTB may reveal the function of CLIPR-59, normally restricted at the microtubule-raft interface. Regardless of the molecular mechanism underlying this effect, full-length CLIPR-59 could inhibit microtubule polymerization near the lipid raft microdomain, preventing raft-microtubule linkage. Such a function would be difficult to visualize and could explain why full-length CLIPR-59 does not prevent microtubule regrowth during recovery from NZ treatment (not shown). A similar effect was proposed recently in a study using SCG10-based chimeric proteins, where the authors suggested that only local microtubule perturbation could be achieved by membrane-targeted microtubule destabilizing proteins (47). Although CLIPR-59 cannot sequester a large pool of the very abundant tubulin in vivo, it may be locally concentrated enough to slow down microtubule growth around particular raft domains. Alternatively or additionally, the CLIPR-59 MTB may directly act on microtubule plus ends in the raft periphery to reduce their polymerization. Our in vitro experiments suggested that CLIPR-59 may bind slowly polymerizing microtubules better. As the overall structure of microtubule plus ends was shown to be sensitive to growth rate (48), it will be interesting to test whether the CLIPR-59 MTB binds directly to a subset of microtubule plus ends.

In addition, we have observed previously that the MTB of CLIPR-59 is inhibited by the N-terminal part of the protein (in the ΔC60 mutant), and thus cytoplasmic CLIPR-59 may not be active (15). The following model thus may be proposed for CLIPR-59 function. In the cytosol, newly synthesized CLIPR-59 proteins present an inactive MTB but are then targeted to lipid microdomains by tandem palmitoylation. The MTB could be activated, either by conformational changes or by an unknown membranous activator (counteracting the N-terminal inhibition effect), on membranes, where it could locally inhibit microtubule polymerization. A signal may then inactivate CLIPR-59 function and allow microtubules to grow in the raft vicinity. Like CLIP-170, the two CAP-Gly of CLIPR-59 are separated by a serine-rich region whose phosphorylation is implicated in the regulation of CLIP-170 activity. The negative regulation of CLIP function by phosphorylation was shown recently to be more complicated because CLIP-170 is phosphorylated on two sites with opposite effects on microtubule binding (49). Stathmin proteins are also negatively regulated by serine phosphorylation to prevent microtubule destabilization in mitosis (35). We thus will have to test whether CLIPR-59 is phosphorylated on the serine-rich region and whether this phosphorylation could regulate CLIPR-59 function. The doublet revealed by Western blotting could correspond to such a modification of CLIPR-59.

We have shown previously that strong CLIPR-59 overexpression perturbs Shiga toxin B-fragment retrograde transport, leading to its retention in the cell periphery. This suggested that CLIPR-59 was controlling the interaction between Shiga toxin B-fragment-containing transport intermediates and microtubules (15). This is consistent with our model where CLIPR-59 would prevent raft-microtubule interaction. Shiga toxin B-fragment receptor Gal3 is indeed associated with lipid raft microdomains (50), and by preventing raft-microtubule association, CLIPR-59 could inhibit both efficient Gal3 delivery to the plasma membrane and Gal3/Shiga toxin B-fragment-associated raft retrograde transport.

**CAP-Gly, a Signature for Regulated Tubulin Binding Behavior?**—In contrast with the CLIP model that was proposed based on CLIP-170 function (1), CLIPR-59 does not seem to stably attach membrane domains to microtubules but rather could be used for microtubule destabilization near raft microdomains. A common theme seems to exist, however, between the different CAP-Gly proteins from the CLIP-170 family. CLIP-170 associates preferably with polymerizing microtubule plus ends and seems to recognize tubulin dimers or short oligomers in a non-polymerized form (11). The same plus end association is observed for CLIP-115 and p150Glued, suggesting that the same preference for unpolymerized tubulin may be at work. Similarly, cofactor B and its yeast ortholog Alf1p specifically interact with α-tubulin by their CAP-Gly with a strong preference.
for monomer versus polymer, facilitating the formation of an α/β-tubulin dimer (51). Alfip overexpression actually induces defects in microtubule function by α-tubulin sequestration. We have thus to reconsider the CAP-Gly motif not as a signature for proteins "CLIPping" membranes to microtubules but instead for proteins interacting with tubulin in a conformation- or structure-dependent way, preferably with non-polymerized tubulin. This may allow them to regulate positively or negatively microtubule dynamics and consequently, depending on their subcellular localization, linkage to membranes. It will be interesting to test the effect of the CLIP-115/-170-interacting protein CLASP2 on microtubule polymerization. Because CLASP2 and CLIPR-59 share a similar C-terminal membrane-targeting sequence, it may also be targeted to lipid raft domains to observe its microtubule control in a manner opposite of CLIPR-59. The role of CLASP proteins in the microtubule network could vary from an ant catastrophe/prorescue effect by protecting plus ends (+Tip) to destabilization (CLIP-59) or dual function (cofactor B). It will thus be interesting to analyze systematically whether all of the different CAP-Gly-containing proteins interact with tubulin in such a regulated way and to reveal their specific influences on microtubule dynamics.

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