

CLIPR-59, a new trans-Golgi/TGN cytoplasmic linker protein belonging to the CLIP-170 family

Franck Perez,¹ Karin Pernet-Gallay,¹ Clément Nizak,¹ Holly V. Goodson,² Thomas E. Kreis,³ and Bruno Goud¹

¹Institut Curie, CNRS UMR144, 75248 Paris, France

²University of Notre Dame, Department of Chemistry and Biochemistry, Notre Dame, IN 46556

³University of Geneva, Department of Cell Biology, 1211 Geneva 4, Switzerland

The microtubule cytoskeleton plays a fundamental role in cell organization and membrane traffic in higher eukaryotes. It is well established that molecular motors are involved in membrane–microtubule interactions, but it has also been proposed that nonmotor microtubule-binding (MTB) proteins known as CLIPs (cytoplasmic linker proteins) have basic roles in these processes. We report here the characterization of CLIPR-59, a CLIP-170–related protein localized to the trans-most part of the Golgi apparatus. CLIPR-59 contains an acidic region followed by three ankyrin-like repeats and two CLIP-170–related MTB motifs. We show that the 60–amino acid–long carboxy-terminal domain of CLIPR-59 is necessary and sufficient to achieve

Golgi targeting, which represents the first identification of a membrane targeting domain in a CLIP-170–related protein. The MTB domain of CLIPR-59 is functional because it localizes to microtubules when expressed as a fragment in HeLa cells. However, our results suggest that this domain is normally inhibited by the presence of adjacent domains, because neither full-length CLIPR-59 nor a CLIPR-59 mutant missing its membrane-targeting region localize to microtubules. Consistent with this observation, overexpression of CLIPR-59 does not affect the microtubule network. However, CLIPR-59 overexpression strongly perturbs early/recycling endosome–TGN dynamics, implicating CLIPR-59 in the regulation of this pathway.

Introduction

The cytoskeleton plays a fundamental role in cell organization. The microtubule network is particularly important in higher eukaryotes, determining organelle localization and regulating the exchange between membranous compartments (Cole and Lippincott-Schwartz, 1995; Schroer, 2000). In particular, it has long been known that microtubules are required for the acquisition and maintenance of Golgi complex localization and structure, which in fibroblastic cells normally consists of long stacks of cisternae juxtaposed to the microtubule organizing center (Thyberg et al., 1980; Ho et al., 1989; Cole et al., 1996; Tian et al., 1996; Kreis et al., 1997). The intrinsic asymmetry of microtubules is used by the cells to generate cytosolic polarity. The rapidly polymerizing microtubule plus ends generally point toward the cell periphery, whereas the more slowly polymerizing minus ends are associated with the microtubule organizing center. Steady-state organelle localizations are thought to result

mainly from a “balance of power” between the activity of plus end–directed kinesin family motors and minus end–directed dynein-related motors (Goodson et al., 1997), and this “equilibrium model” has been particularly well studied in the case of Golgi complex localization (Burkhardt, 1998).

Although these data might suggest that the steady-state localization of organelles like the Golgi complex relies only on the function of these microtubule-based motors, it has been suggested that membranous organelles can also interact with microtubules via nonmotor microtubule-binding (MTB)* proteins known as cytoplasmic linker proteins (CLIPs). One hypothesis suggests that these CLIPs promote the initial interaction of membranous organelles with microtubules and, once docking is achieved, may participate in the regulation of motor activity (Rickard and Kreis, 1996). CLIP-170 was the first characterized CLIP. *In vitro* experiments have shown that CLIP-170 is essential for efficient

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Address correspondence to Franck Perez, Institut Curie, CNRS UMR144, 26 rue d’Ulm, 75248 Paris Cedex 05, France. Tel.: 33-14-234-6440. Fax: 33-14-234-6382. E-mail: franck.perez@curie.fr

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*Abbreviations used in this paper: CLIP, cytoplasmic linker protein; CLIPR, CLIP-related protein; E/P, glutamic acid and proline; GalT, β 1,4-galactosyltransferase; GFP, green fluorescent protein; GoLD, Golgi localization domain; LBPA, lyso-bis-phosphatidic acid; MTB, microtubule binding; Rh-Tf, rhodamine-labeled transferrin; STxB, Shiga toxin B subunit; TfR, transferrin receptor.

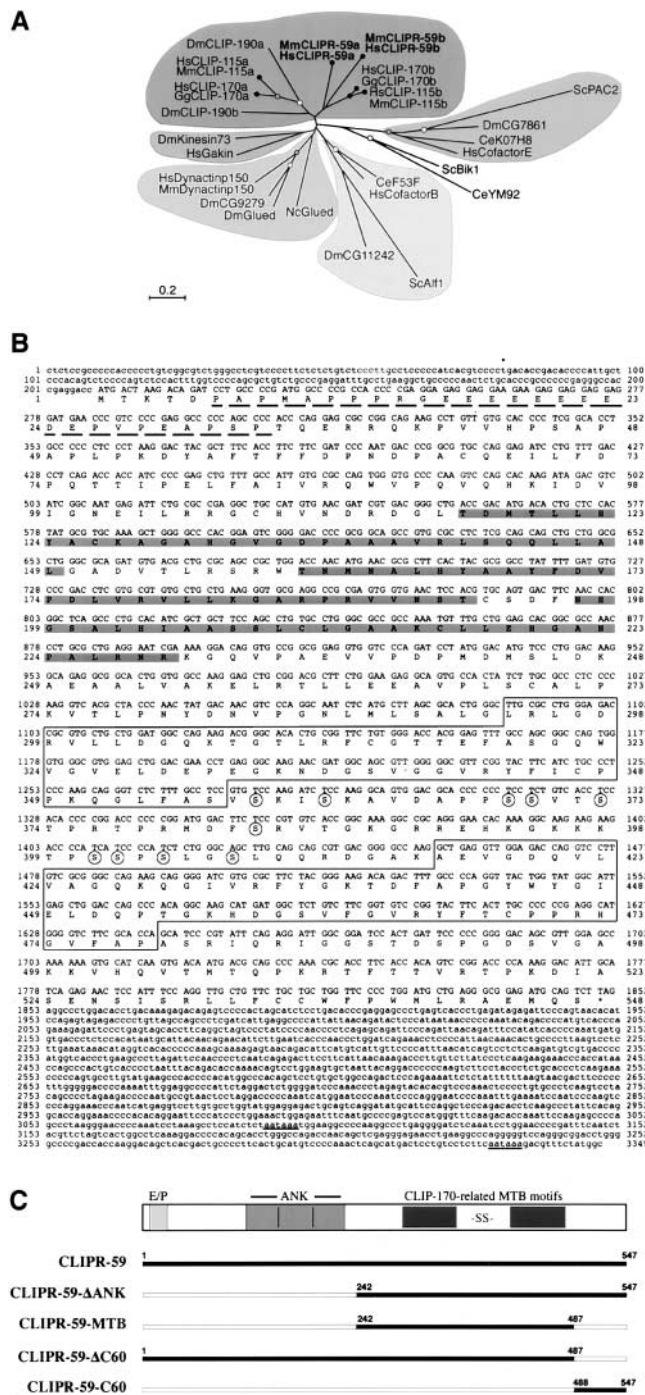


Figure 1. Identification of CLIPR-59, a new CLIP-170 family member. (A) Phylogenetic tree of the MTB motifs of CLIP-170-related proteins. A phylogenetic tree was generated by the neighbor joining method (as described in Online supplemental material) after using ClustalX to align the MTB motifs of CLIP-170-related proteins. Confidence in the sequence groupings was estimated by bootstrap analysis (black circle, groupings found in >90% of trials; gray circle, 75%; white circle, 50%). This bootstrap analysis showed that the new protein identified (CLIPR-59, containing two MTB motifs “a” and “b”) did not strongly cluster with any previously characterized subfamilies, i.e., the CLIP-170, glued, cofactor B, or cofactor E subfamilies. (B) Cloning of CLIPR-59. CLIPR-59 cDNA contains an open reading frame coding for a protein of 547 amino acids (59.5 kD). Inspection of this sequence identifies an in-frame stop (asterisk) upstream from the first ATG and two polyadenylation signals at the

binding of endocytic carrier vesicles to microtubules (Rickard and Kreis, 1990; Pierre et al., 1992), whereas experiments *in vivo* have suggested that CLIP-170 interacts, directly or indirectly, with the dynein regulator dynactin complex (Valetti et al., 1999; Vaughan et al., 1999).

CLIP-170 is an elongated homodimeric protein bearing an amino-terminal MTB domain. The MTB domain allows CLIP-170 to interact in a particular way with microtubules because it is specifically and dynamically localized at the plus extremity of growing microtubules (Pierre et al., 1992; Perez et al., 1999), probably through a rapid association with polymerizing tubulin subunits (Diamantopoulos et al., 1999). The MTB domain of CLIP-170 contains two repeats of an MTB motif (referred to as the CAP-GLY motif in the PROSITE database) found in other tubulin-interacting proteins (Pierre et al., 1992). The family of proteins containing this motif includes other proteins that may be functionally considered to be CLIPs. Notably, one of the subunits of the dynactin complex, p150^{glued}, contains a CLIP-170-related MTB domain (Swaroop et al., 1987; Holzbaur et al., 1991). A close relative of CLIP-170, CLIP-115, has been localized in neurons on dendritic lamellar bodies (De Zeeuw et al., 1997), and a CLIP-170 homologue in *Drosophila*, CLIP-190, has been localized on Golgi membranes (Sisson et al., 2000). Another yet unidentified CLIP-170-related protein is involved in the interaction of peroxysomes with microtubules *in vitro* (Thiemann et al., 2000).

Not all CLIPs are related to CLIP-170, as exemplified by CLIMP63 (Klopfenstein et al., 1998), an integral membrane protein that links ER membranes to microtubules. A number of other proteins, including the Golgi microtubule-associated protein GMAP-210 (Infante et al., 1999), have also been implicated in membrane-microtubule interactions. However, it is attractive to speculate that the CLIP-170 family contains additional CLIPs. We thus systematically analyzed sequence databases to identify new members of the CLIP family (HVG, FP, and TK in preparation). We report here the cloning and characterization of a 59.5-kD member of this family, CLIPR-59 (CLIP-170-related protein of 59 kD). Like CLIP-170, CLIPR-59 contains two MTB motifs separated by a serine-rich region. Three ankyrin-like repeats and an acidic domain are present amino-terminal to the CLIP domain, whereas the carboxy-terminal region is not strongly related to any protein in the databases. We show that in HeLa cells, CLIPR-59 is localized to Golgi-like structures, most likely related to the TGN, and the carboxy-terminal 60 amino acids are necessary

end of the 3' untranslated region. A region rich in glutamic acid and proline (underlined) is present in the amino-terminal part of CLIPR-59, followed by three ankyrin-like repeats (grayed). The two CLIP domains (boxed) are present in the second half of the protein, separated by a serine-rich region (the 10 serines are circled). (C) Summary and nomenclature of the CLIPR-59 deletion mutants used in this study. The structure of CLIPR-59 is schematized at the top, and the black lines underneath represent the portion of CLIPR-59 retained in the respective deletion mutants. Numbers indicate the position of the first and last amino acid in the deletion construct relative to full-length CLIPR-59. All the constructs were tagged amino terminally with either an HA or a GFP tag. The sequence of human CLIPR-59 cDNA is available from Genbank/EMBL/DDJB under accession no. AJ427922.

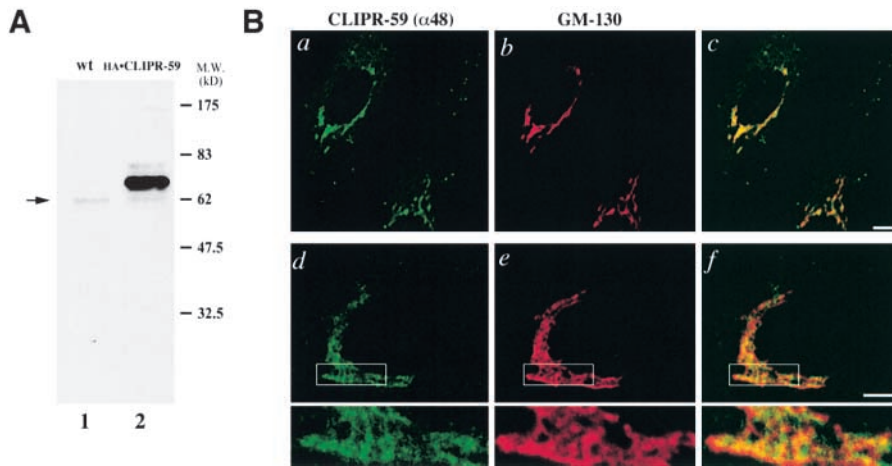


Figure 2. CLIPR-59 is localized on Golgi-like structures. An antibody directed against recombinant CLIPR-59 was raised in rabbit ($\alpha 48$) and affinity purified. (A) Western blot analysis showed that $\alpha 48$ detects a faint band at 60 kD (indicated by the arrow) in wild-type HeLa cells (lane 1). In HeLa cells transfected with a plasmid coding for HA-tagged CLIPR-59, $\alpha 48$ detects a strong additional band at a higher molecular weight (lane 2). (B) HeLa cells were fixed in paraformaldehyde and processed for immunofluorescence using the $\alpha 48$ antibody (green; a and d) together with a monoclonal anti-GM-130 antibody (red; b and e) to stain the Golgi complex. Two examples of images

acquired by confocal microscopy are shown (a–c and d–f), indicating that the $\alpha 48$ antibody stains the same reticular structure as the anti-GM-130 antibody, hence it is likely to be the Golgi complex. It should be noted that although the colocalization between CLIPR-59 and GM-130 is extensive, it is not perfect, as is particularly visible on the boxed area shown enlarged underneath c–f. Bars, 10 μ m.

and sufficient for this localization. We further show that the activity of the MTB motifs of CLIPR-59 is regulated in vivo by the adjacent domains, preventing cytoskeletal rearrangement upon overexpression. In contrast, major reorganization and perturbation of TGN/endosomal compartments, together with alteration of retrograde transport, can be observed in these overexpression conditions. Altogether, these data suggest that CLIPR-59 is a TGN CLIP involved in early endosome–TGN dynamics.

Results

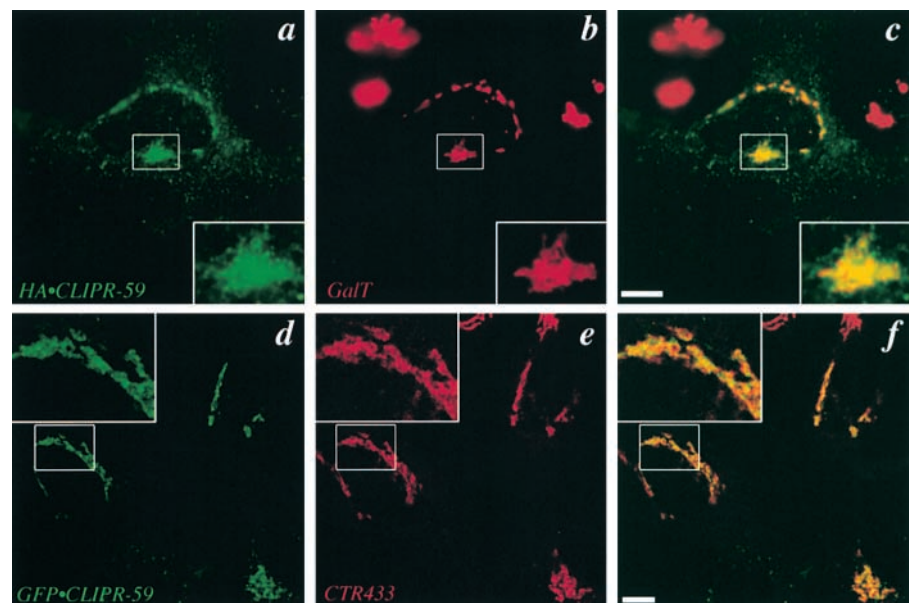
CLIPR-59 is a new CLIP-170-related protein localized to Golgi structures

We used the CLIP-170 MTB motifs to search the sequence databases for the family of proteins related to CLIP-170. Two sequences (ESTH2 and ESTH4) grouped with each other at a moderate confidence level, suggesting that they might encode related proteins, but did not group with any other known

CLIP-170-related protein (Fig. 1 A). The ESTH4 sequence was chosen for further analysis. Corresponding cDNA clones were obtained from the IMAGE consortium and, with the help of RT-PCR, we reconstructed a full-length clone of the 3.4-kb cDNA (Fig. 1 B). The corresponding gene, localized on chromosome 19q13, has been sequenced by the human sequencing project and found to be spliced in 14 exons spanning 17 kb. The open reading frame codes for a 59.5-kD protein that we call CLIPR-59. Because the nomenclature is very confusing in the CLIP field, we propose that the name “CLIPR” may only be given to proteins possessing at least one CLIP-170-related MTB motif. CLIPR-59 is expressed in many different tissues as indicated by EST database analysis (see Unigene Cluster hs.7357). Northern blot analysis confirmed the presence of CLIPR-59 mRNA in various tissues, although we observed that expression in the brain is particularly high (unpublished data).

Schematically, CLIPR-59 possesses a four-domain structure (Fig. 1, B and C). The amino terminus contains an

Figure 3. Transfected HA- and GFP-tagged CLIPR-59 localize to Golgi-like structures. (A) HeLa cells were transiently transfected for 36 h with a plasmid coding for HA•CLIPR-59 (a–c), or stably transfected with GFP•CLIPR-59 (d–f), and fixed in paraformaldehyde. Immunofluorescence was then performed using a mixture of monoclonal anti-HA antibody (green; a and c) together with a polyclonal anti-GaT (red; b and c), or using the natural green fluorescence of the GFP tag (green; d and f) and the CTR433 anti-Golgi antibody (red; e and f). Both HA- and GFP-tagged CLIPR-59 are targeted to Golgi-like structures. Careful examination of the overlaid images (c and f) shows that the colocalization is only partial with the different Golgi markers used. Bars, 10 μ m.



acidic region rich in glutamic acid and proline (E/P region) followed by three ankyrin-like repeats (ANK domain). The second half of the protein contains the two CLIP-170-related MTB motifs separated, as is the case in CLIP-170, by a serine-rich region. Finally, the carboxy-terminal region following the two MTB motifs shows no significant similarity to any protein in the databases.

We raised anti-CLIPR-59 antibodies in rabbits, and Western blot analysis using affinity-purified anti-48 antibody (Fig. 2 A) showed that this antibody detects a faint band at around 60 kD in HeLa cell extracts. An additional stronger band is detected in the extract of cells transfected with HA•CLIPR-59 (also detected by an anti-HA antibody; unpublished data). Immunofluorescence experiments showed that anti-48 stains a Golgi-like structure (Fig. 2 B), although careful analysis suggested that it only partially colocalizes with the *cis*/medial marker GM-130. This staining was obtained in nontransfected HeLa cells only upon long antibody incubation time and was much stronger after transfection of tagged or untagged CLIPR-59 (unpublished data), further suggesting that CLIPR-59 is only weakly expressed in HeLa cells. Immunofluorescence analysis of HeLa cells transiently expressing HA•CLIPR-59 (Fig. 3, a–c) or stably expressing green fluorescent protein (GFP)•CLIPR-59 (Fig. 3, d–f) showed that the recombinant proteins localized to Golgi-like structures very similar to the structures stained by the anti-48 antibodies in untransfected HeLa cells. Similarly, the colocalization was only partial with the different Golgi markers tested so far (CTR433, GM-130, mannosidase II, galactosyltransferase, TGN46; Figs. 3 and 5, and unpublished data).

We further analyzed CLIPR-59 localization by immunogold labeling of cryosections of HeLa cells transiently or stably expressing GFP•CLIPR-59 (Fig. 4). In agreement with our immunofluorescence data, GFP•CLIPR-59 was detected on Golgi stacks as well as on tubulovesicular elements juxtaposed to Golgi cisternae. Colabeling experiments indicated that GFP•CLIPR-59 was localized on the same side of Golgi stacks as galactosyltransferase. This suggests that CLIPR-59 is localized to membranes of the trans-Golgi/TGN.

Golgi and microtubule localization domains of CLIPR-59

Thus far, no membrane-targeting domains have been characterized in proteins of the CLIP-170 family. Therefore, we were particularly interested in studying the regions of CLIPR-59 responsible for membrane targeting. Deletion of the ankyrin repeat-containing amino terminus (CLIPR-59- Δ ANK) had no effect on the subcellular localization of the expressed protein (Fig. 5 b). In contrast, the deletion of the last 60 amino acids of CLIPR-59 was sufficient to completely abolish Golgi targeting (Fig. 5 c). This 60-amino acid domain is not only necessary but sufficient for Golgi targeting because the HA-tagged (and GFP-tagged, unpublished data) carboxy-terminal domain of CLIPR-59 efficiently localized to the Golgi (Fig. 5 d). This indicates that the carboxy terminus of CLIPR-59 is a Golgi localization domain (GoLD), but its sequence is different from previously described GoLDs (see Discussion).

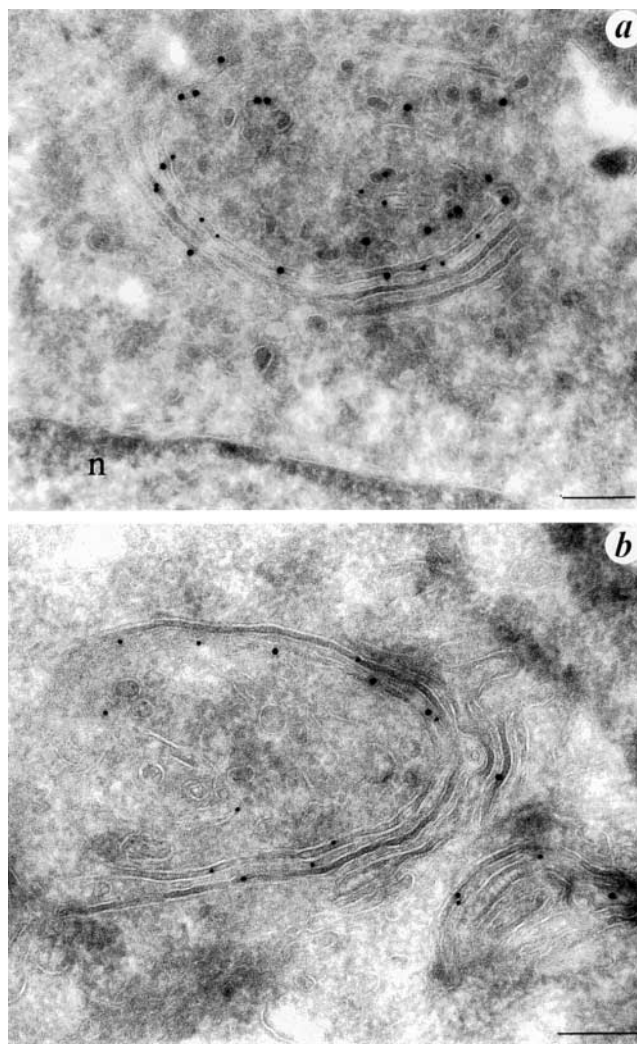


Figure 4. GFP-tagged CLIPR-59 localizes to the trans-Golgi/TGN. Cells transiently (a) or stably (b) transfected with GFP•CLIPR-59 were fixed by glutaraldehyde and processed for cryoelectron microscopy. Cryosections were labeled using an anti-GFP antibody and an anti-GalT antibody revealed with protein-A coupled to 15- or 10-nm gold particles, respectively. Electron microscopic observation confirmed that GFP•CLIPR-59 is targeted to membranous structures colocalized or juxtaposed to GalT-positive Golgi cisternae, indicating that CLIPR-59 is predominantly localized to the trans-most part of the Golgi complex. Bars, 200 nm.

Although CLIPR-59 possesses two MTB motifs highly related to those of CLIP-170, neither endogenous nor recombinant CLIPR-59 was observed to localize to microtubules, and overexpression of CLIPR-59 had no discernible effect on the microtubule cytoskeleton. This was unexpected because constructs containing the MTB motifs of CLIP-170 localize to microtubules and provoke microtubule bundling upon overexpression (Pierre et al., 1994). A possible explanation was that the GoLD signal of CLIPR-59 was dominant to the MTB domain, precluding quantitative microtubule localization of CLIPR-59. We thus tried to reveal microtubule binding of the cytosolic mutant CLIPR-59- Δ C60 by preextracting the cells with Triton X-100 before fixation (Fig. 6). Under these conditions, full-length GFP-

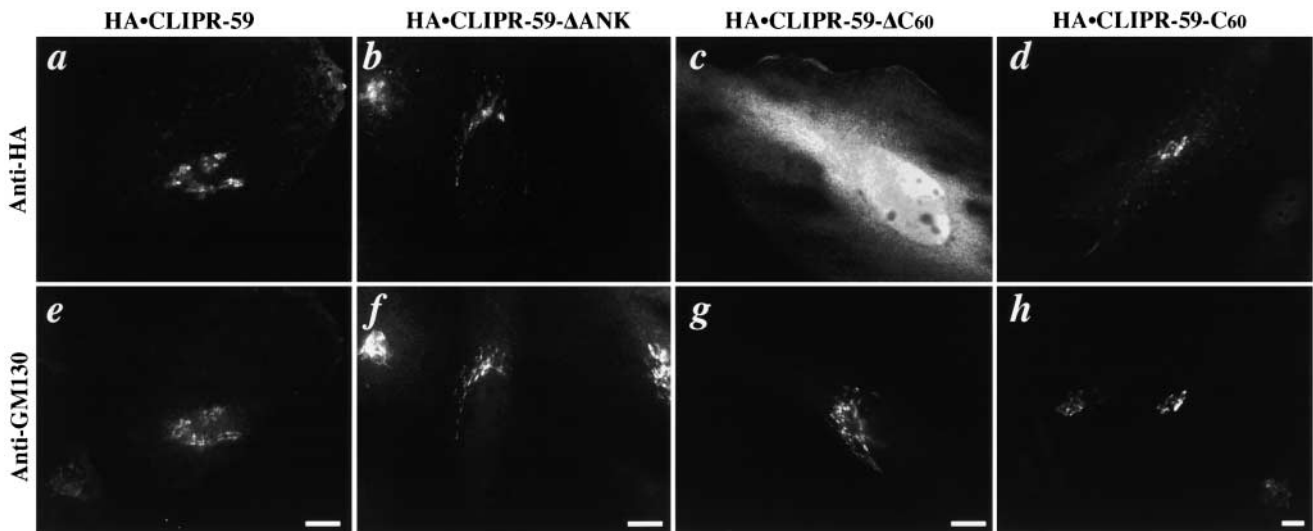


Figure 5. The carboxy-terminal 60 amino acids of CLIPR-59 are necessary and sufficient for Golgi targeting. HeLa cells were transfected with plasmids coding for HA-tagged full-length CLIPR-59 (a and e), or one of the following deletion mutants: HA-tagged CLIPR-59 missing its amino-terminal, ankyrin repeat-containing domain (b and f) or CLIPR-59 missing its carboxy-terminal 60 amino acids (c and g). Alternatively, a construct encompassing only these 60 amino acids fused to the HA tag was transfected (d and h). Cells were fixed 36 h later in paraformaldehyde and processed for immunofluorescence using a monoclonal anti-HA antibody (a–d) together with a polyclonal anti-GM-130 antibody (e–h). These experiments showed that the amino-terminal half of CLIPR-59 is dispensable for proper Golgi targeting, whereas the carboxy-terminal 60 amino acids are required. This carboxy-terminal domain is not only necessary but sufficient to achieve Golgi targeting, and thus represents a novel GoLD. Bars, 10 μ m.

tagged CLIPR-59 stained central structures, and no significant microtubule localization could be observed (Fig. 6, a–c). As expected, GFP•CLIPR-59– Δ C60 was dispersed in the cytosol but still did not show extensive microtubule localization (Fig. 6, d–f). However, upon closer examination, it was

clear that the GFP•CLIPR-59– Δ C60 faintly stained some filamentous structures that were colocalized with microtubules (Fig. 6, arrowheads). This observation suggested that CLIPR-59 devoid of GoLD can bind microtubules in vivo. However, the apparent weakness of this association sug-

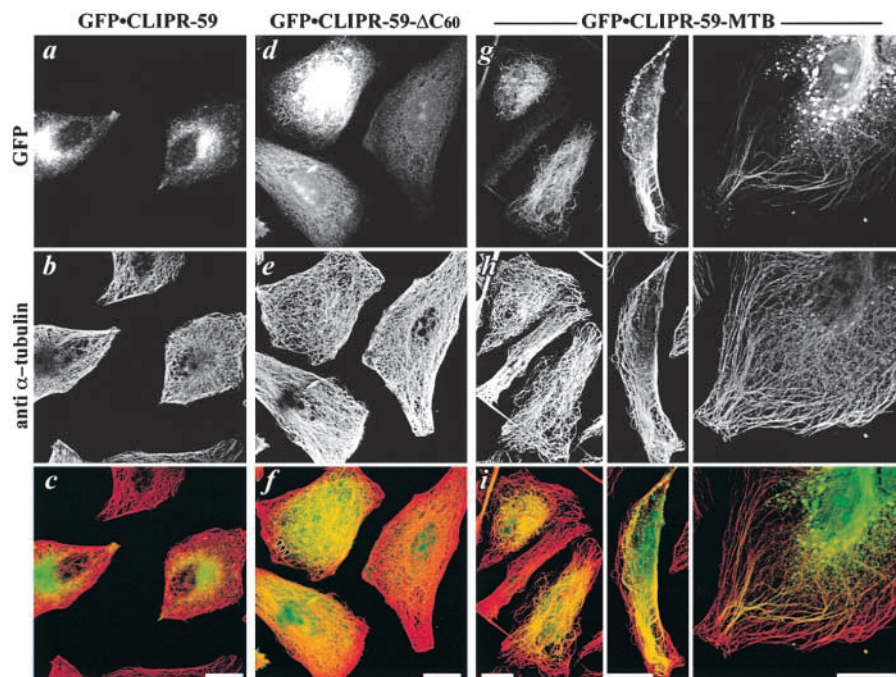


Figure 6. Microtubule association of the CLIPR-59 CLIP domain is inhibited by the ankyrin repeat-containing region together with the GoLD. HeLa cells were transfected with plasmids coding for GFP•CLIPR-59 (a–c), GFP•CLIPR-59– Δ C60 (d–f), or the GFP-tagged CLIP domain (GFP•CLIPR-59–MTB; g–i). 24 h later, cells were preextracted in 0.5% Triton X-100 and fixed in methanol before being processed for immunofluorescence. Transfected proteins were detected using the natural fluorescence of GFP (a, d, and g), whereas microtubules were stained using a monoclonal anti- α -tubulin antibody followed by Texas red anti-mouse secondary antibody (b, e, and h). Images were acquired by confocal microscopy and the overlay of the green and red channels is shown in the bottom pictures (c, f, and i). We observed no clear microtubule labeling for full-length CLIPR-59 and only a very faint microtubule staining can be observed for CLIPR-59– Δ C60 (arrowheads). In contrast, clear microtubule labeling could be observed when the

CLIP domain was expressed fused to GFP (three different fields are shown in g–i). Bundling of microtubules can even be observed upon CLIPR-59 MTB overexpression, in a dose-dependent manner. It is also worth pointing out that only a subfraction of microtubules seems to be recognized by the CLIPR-59 MTB domain. Note that the bright fluorescent spots observed upon GFP•CLIPR-59–MTB expression were not seen using the HA-tagged version of this protein and are likely due to nonspecific precipitation. Bars, 20 μ m.

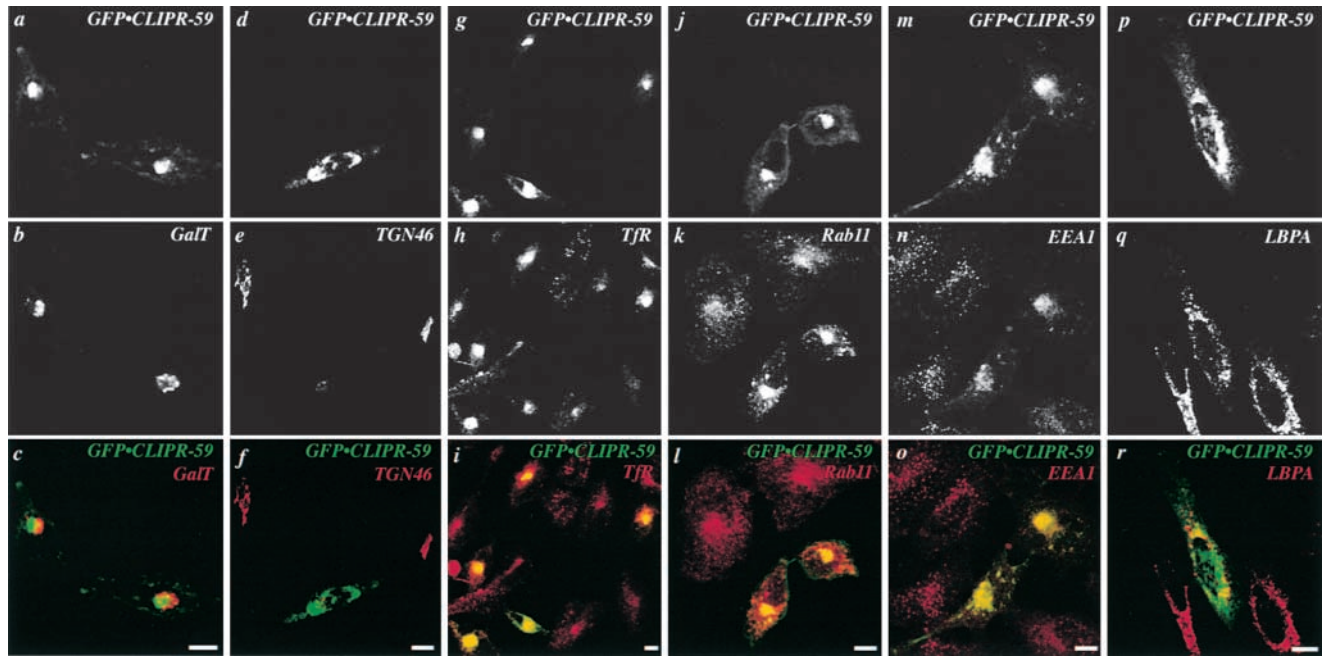


Figure 7. Overexpressed CLIPR-59 does not localize to the Golgi complex and perturbs early endosome/TGN compartments. (A) HeLa cells were transfected with GFP•CLIPR-59 (green; a, d, g, j, and m), fixed in paraformaldehyde 36 h later, and processed for immunofluorescence. Cells were stained with antibodies directed against GalT (a–c), TGN46 (d–f), TfR (g–i), Rab11 (j–l), EEA1 (m–o), or LBPA (p–r) followed by Texas red–labeled secondary antibodies, and images were acquired by confocal microscopy. The bottom panels show the overlaid images. Strong overexpression of CLIPR-59 leads to a segregation between CLIPR-59 and Golgi staining. In these conditions, the labeling for TGN46 is strongly reduced and TfR- and Rab11-positive membranes coaccumulate with CLIPR-59 at the center of the cells. EEA1-positive early endosomes also tend to coaccumulate with CLIPR-59, although EEA1 staining seems more diffuse. In contrast, no significant effect is observed on late endosomes/lysosomes. Bars, 10 μ m.

gested that the amino terminus containing the E/P domain and the ankyrin repeats might also be inhibitory. To test this hypothesis, we expressed in HeLa cells the GFP-tagged CLIPR-59 MTB domain and observed under the same conditions as above. GFP•CLIPR-59–MTB (or HA•CLIPR-59–MTB, unpublished data) showed comparatively strong microtubule binding (Fig. 6, g–i). Indeed, this construct was even able to induce microtubule bundling upon overexpression, in a dose-dependent manner. Careful examination revealed that the colocalization between GFP•CLIPR-59–MTB and microtubules was only partial. This suggests that only a subset of microtubules are recognized by CLIPR-59. Similar behavior had already been described for CLIP-170 (Pierre et al., 1992; Diamantopoulos et al., 1999; Perez et al., 1999).

Overexpression of CLIPR-59 affects membrane dynamics of early endosome and TGN membranes

We noticed that high overexpression of full-length CLIPR-59 often resulted in loss of colocalization between the CLIPR-59 proteins and Golgi markers. In as many as 35% of transfected cells (depending on length and efficiency of transfection), overexpressed CLIPR-59 accumulated in one or more discrete locations, usually juxtaposed to Golgi membranes (Fig. 7, a–c). Because immunoelectron microscopy indicated that CLIPR-59 localizes to the trans/TGN part of the Golgi, we tested the localization of the TGN marker TGN46 under these conditions. We observed that CLIPR-59 overexpression leads to reduced TGN46 stain-

ing in the Golgi region (Fig. 7, d–f; see also Fig. 10 A). The steady-state localization of TGN46 results from active recycling between the plasma membrane, early/recycling endosomes, and the TGN. It was thus interesting to check the localization of other markers in this pathway. We found that the localization of both transferrin receptor (TfR, a marker of early/recycling endosomes) and Rab11 (a marker of the recycling endosome) was strongly perturbed. Moreover, we observed extensive colocalization of TfR- and Rab11-positive membranes with juxtannuclear CLIPR-59 aggregates (Fig. 7, g–l). Similarly, we found that the punctiform early endosomes positive for EEA1 were depleted from the cell periphery and coaccumulated with CLIPR-59, although EEA1 staining seemed more diffuse than that of Rab11 or TfR (Fig. 7, m–o). In the same conditions, we observed no significant effect on the localization of lyso-bis-phosphatidic acid (LBPA)–containing late endosomes or lysosomes (Fig. 7, p–r), or on the localization of cation-independent mannose-6-phosphate receptor (unpublished data). In agreement with our previous experiments (Fig. 6), no effect on microtubule organization could be observed under these conditions. It is also worth noting that CLIPR-59 lacking the GoLD does not affect TGN/endosome localization (unpublished data).

Immunoelectron microscopy confirmed the presence of accumulated, CLIPR-59–positive, vesicular membranes in a juxta-Golgi location (Fig. 8 a). These tubulo-vesicular membranes were densely packed and not homogenous in size (Fig. 8, b and c). It is worth noting that some of the vesicu-

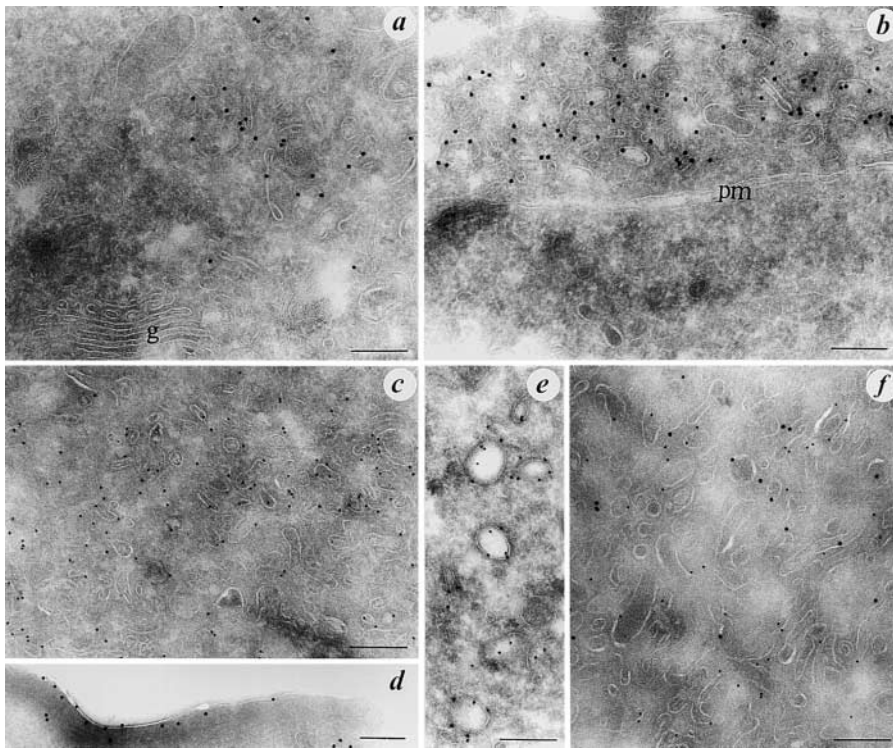


Figure 8. Overexpression of CLIPR-59 leads to endosome-like membrane aggregation in a peri-Golgi region, as observed by immunoelectron microscopy.

Cells were transfected and processed for immunocryoelectron microscopy using anti-GFP to stain for overexpressed GFP•CLIPR-59. As observed by immunofluorescence, electron microscopic observation shows that GFP•CLIPR-59 overexpression leads to accumulation of endosome-like membranes in a peri-Golgi region (a). Under these conditions, CLIPR-59 does not stain Golgi cisternae but is found on the aggregated membranes. These CLIPR-59-positive membranes have many sizes and shapes, and are reminiscent of early endosomes present in nontransfected control cells (b and c). Plasma membrane staining is sometimes visible (d), and some coated structures can also be seen in these vesicular clusters (e). In agreement with immunofluorescence data, TfR-positive membranes (15-nm gold) coaccumulate with CLIPR-59-positive membranes (10-nm gold) in overexpressing cells (f). g, Golgi complex; pm, plasma membrane. Bars, 200 nm.

lar structures present in these membrane clusters appeared to be coated (Fig. 8 e), although more work is necessary to address the nature of this coat and the frequency of such a coating. We noticed that overexpression sometimes led to plasma membrane staining (Fig. 8 d), which was also observed by immunofluorescence (unpublished data). Finally, in agreement with our immunofluorescence data, immunoelectron microscopic analysis showed that TfR-positive vesicular membranes were present in these aggregated membranes (Fig. 8 f).

Because CLIPR-59 overexpression altered the localization of early and recycling endosomes, we tested whether these aggregated endosomes were still functional in internalizing rhodamine-labeled transferrin (Rh-Tf). We found that transferrin could be actively endocytosed by GFP•CLIPR-59-transfected cells. As observed before for its receptor, the localization of endocytosed transferrin was altered in overexpressing cells. Under the same conditions, endocytosed α 2-macroglobulin was still transported to late endosomes/lysosomes and did not reach the CLIPR-59-positive aggregated membranes (unpublished data). Internalized transferrin was transported to the center of the cells where it extensively, although not perfectly, colocalized with GFP•CLIPR-59 (Fig. 9 A). In agreement with the observed depletion of the punctiform EEA1-positive structures from the cell periphery, fewer Rh-Tf-positive peripheral endosomes were present. In comparison, no effect of GFP•CLIPR-59- Δ C60 on internalized transferrin localization could be detected (Fig. 9 A).

We then quantified the effect of CLIPR-59 overexpression on transferrin uptake and recycling. Because only cells strongly overexpressing CLIPR-59 showed the aggregation phenotype, we had to devise a way to measure the kinetics of transferrin endocytosis in the population of moderate and

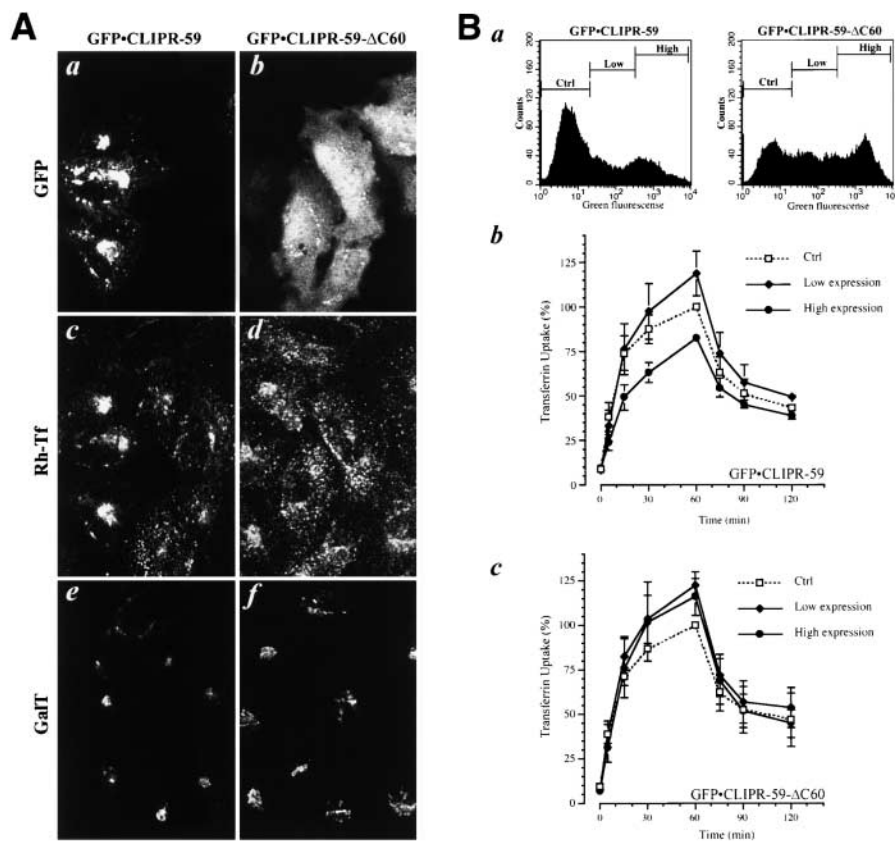
strong overexpressing cells, respectively. We used FACS[®] analysis to this end, measuring in parallel the green fluorescence of transfected GFP-tagged protein and the red fluorescence of internalized transferrin. Three windows were defined, corresponding to control, low, or high levels of green fluorescence (Fig. 9 B, a), and the kinetics of transferrin endocytosis and recycling were measured (Fig. 9 B, b and c). In agreement with immunofluorescence data, we observed that cells strongly overexpressing CLIPR-59 were still able to internalize transferrin. However, we also observed, in these high overexpressers, a reproducible reduction of transferrin uptake (80% of control) whereas the kinetics of transferrin release were not affected. This could indicate a reduction in the motility of early endosomes or in the pool of endosomes participating in transferrin endocytosis. No such reduction was observed for low level GFP•CLIPR-59 expression. Indeed, both low levels of GFP•CLIPR-59 expression and any level of GFP•CLIPR-59- Δ C60 expression led to a slight increase in transferrin internalization. This observation may indicate that the normal function of CLIPR-59 is to accelerate the rate of internalization, and the CLIPR-59- Δ C60 mutant titrates out a negative regulator of endogenous CLIPR-59.

Immunofluorescence data (Fig. 6) suggested that not only early endosomes, but also the TGN was affected by CLIPR-59 overexpression. Triple staining immunofluorescence analysis of CLIPR-59-overexpressing cells (Fig. 10 A) indicated that cells with accumulated TfR-positive membranes also had reduced TGN46 labeling. In comparison, β 1,4-galactosyltransferase (GalT) staining was much less affected. We thus conducted uptake experiments to test whether the TGN46 pathway, from plasma membrane to early endosomes and the TGN, still occurs in cells overexpressing CLIPR-59. We used a well-known and easy to follow marker of this pathway, the B

Figure 9. Endocytosed transferrin is transported to aggregated early endosomes in cells overexpressing GFP•CLIPR-59 with slightly slower kinetics.

(A) HeLa cells were transfected with plasmids encoding GFP•CLIPR-59 (A and B, left panels) or, as a control, CLIPR-59- Δ C60 (right panels). 36 h after transfection, cells were incubated either with 25 μ g/ml Rh-Tf (A) for 90 min at 37°C. Cells were then washed with medium and fixed with paraformaldehyde before being processed for immunofluorescence using a polyclonal anti-GaIT antibody followed by a Cy5-labeled anti-rabbit antibody. Note that aggregated early/recycling endosomes obtained upon overexpression of CLIPR-59 are still accessible to internalized transferrin even though fewer peripheral early endosomes are visible than in control conditions. (B) Cells were transfected as in A, scraped, pelleted, and resuspended in Alexa633-Tf-containing medium. Internalization and recycling of transferrin was then measured by FACS[®] as described in the Materials and methods. The kinetics of transferrin uptake and release were quantified in three different cell populations that were defined according to cell green fluorescence (Control [Ctrl], Low, and High). The mean Alexa633-Tf fluorescence was

then calculated for the three populations and expressed as a percentage of the fluorescence obtained in the control cell population after 60 min of internalization. The plots represent the means \pm SEM of four independent experiments. Cells strongly overexpressing CLIPR-59 are still able to internalize and recycle transferrin but with slightly reduced kinetics (b, High). In contrast, no such reduction was observed in cells moderately expressing CLIPR-59 (b, Low) or expressing CLIPR-59- Δ C60 at low and high levels (c). In these conditions, we instead observed increased transferrin endocytosis.



subunit of the Shiga toxin (STxB). STxB is transported from the plasma membrane to the TGN via early/recycling endosomes (Wilcke et al., 2000). GFP•CLIPR-59-transfected cells were incubated with fluorescently labeled STxB for 90 min at 37°C, and the localizations of endocytosed STxB, GFP•CLIPR-59, and the Golgi complex were analyzed (Fig. 10 B). Cells transfected with GFP•CLIPR-59- Δ C60 transported STxB to the Golgi complex normally. In contrast, although mild GFP•CLIPR-59 overexpression did not perturb STxB transport, cells strongly overexpressing GFP•CLIPR-59 showed a reduced ability to transport the endocytosed STxB to the Golgi complex. Interestingly, the colocalization of STxB with CLIPR-59 was clear at the cell periphery but far less at the cell center, suggesting that STxB might be blocked in the prerecycling endosomal compartment.

Because only strong overexpression led to perturbation of STxB transport, we were unable to directly quantify this effect. To further document the interaction between the CLIPR-59-sensitive pathway and the pathways followed by STxB and transferrin, we followed GFP•CLIPR-59 and fluorescent endocytosis markers in living cells (unpublished data). These experiments revealed that although GFP•CLIPR-59 does not colocalize with either endocytosed transferrin or STxB, it is “dynamically juxtaposed” with these markers, moving beside them in the cytoplasm without apparent mixing. This observation suggests that the two

target compartments somehow interact during transportation and/or sorting steps.

Discussion

CLIPR-59 is a new Golgi CLIP from the CLIP-170 family

We have identified a new member of the CLIP-170 family that behaves as a cytoplasmic linker protein involved in the TGN-endosome dynamics. The structure of CLIPR-59 differs notably from that of the other CLIPs from the CLIP-170 family because it has its MTB motifs near the carboxy terminus, has no identifiable coiled-coil region, and possesses three ankyrin-like repeats. The function of the CLIPR-59 ankyrin repeats is unknown, but it has generally been observed that ankyrin repeats form exposed domains involved in protein-protein interactions (Michaely and Bennett, 1992; Gorina and Pavletich, 1996). It is thus attractive to propose that the CLIPR-59 ankyrin domain mediates interaction with other proteins, although it could also be a regulatory domain (see below).

The localization of CLIPR-59 is also unusual for the CLIP-170 family. Both endogenous and recombinant CLIPR-59 are localized on Golgi membranes *in vivo*, and not on microtubules. In contrast, although association of CLIP-170, CLIP-115, or p150^{glued} (as part of the dynactin complex) has been documented on various cellular organelles (Pierre et al., 1992; De Zeeuw et al., 1997; Haber-

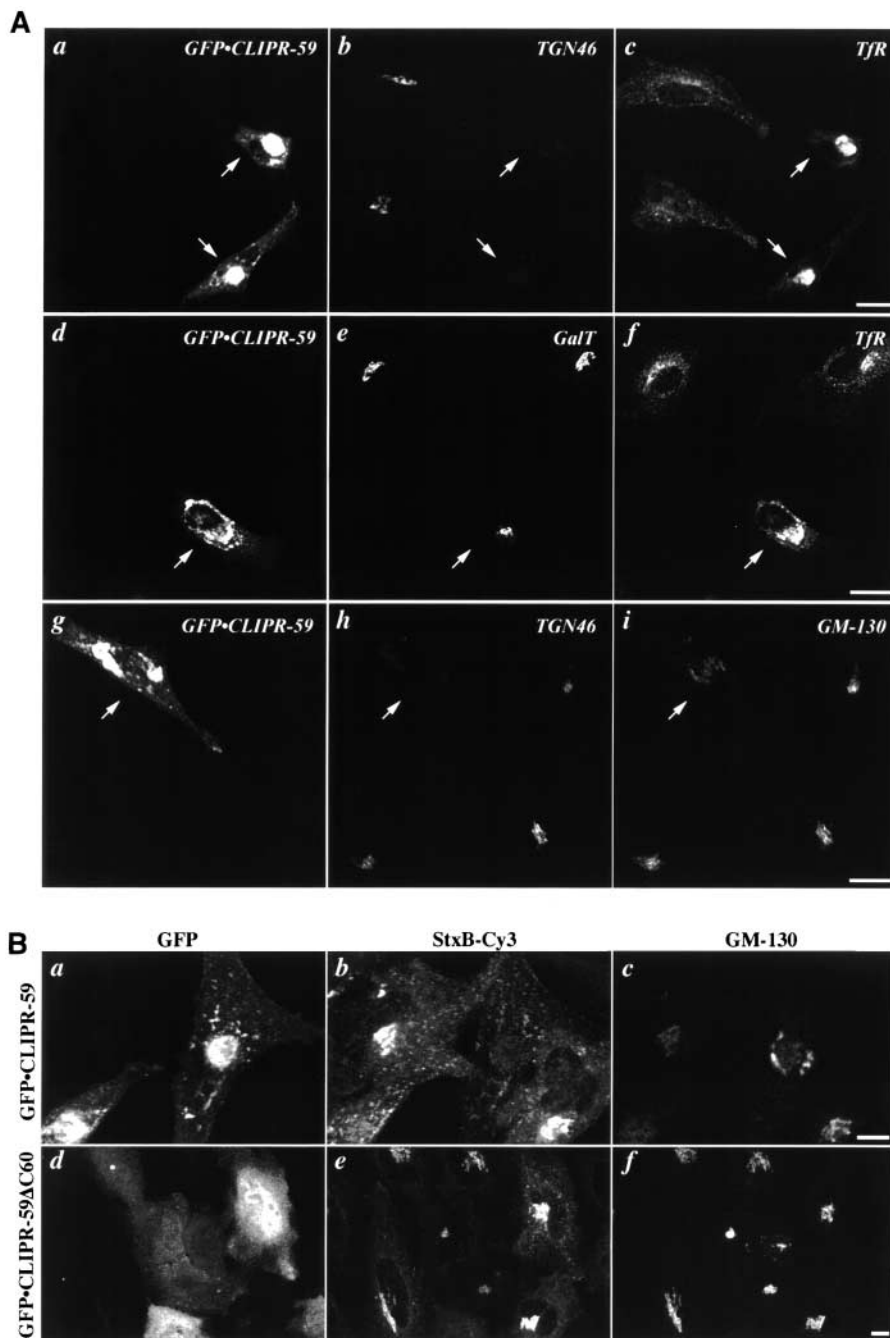


Figure 10. Overexpression of CLIPR-59 perturbs the TGN46-STxB pathway. HeLa cells were transfected as in Fig. 8 and either fixed and processed for immunofluorescence (A) or incubated with 10 μ g/ml STxB-Cy3 (B) for 90 min at 37°C. (A) Triple staining of HeLa cells overexpressing GFP•CLIPR-59 shows that in conditions where CLIPR-59 coaccumulates with TfR-positive early endomes, the TGN46 staining is strongly reduced (a–c), whereas the Golgi complex, detected by the GalT marker, seems unaffected (d–f). Indeed, some cells lose nearly all TGN46 staining while retaining essentially normal GM-130 staining (g–i). Bars, 20 μ m. (B) Strong overexpression of CLIPR-59 perturbs STxB-Cy3 transport to the Golgi complex. Both nontransfected cells and cells overexpressing CLIPR-59- Δ C60 transport STxB-Cy3 to the Golgi complex efficiently, however, cells strongly expressing GFP•CLIPR-59 retain STxB-Cy3 in the cell periphery. Note that STxB-Cy3 is not accumulated in the central CLIPR-59 aggregate, although it colocalizes to some extent with peripheral CLIPR-59 aggregates. Bars, 10 μ m.

mann et al., 2001), exogenous expression of these proteins systematically leads to microtubule targeting and not to membrane association. CLIPR-59 is the first member of this linker family for which a membrane localization domain has been identified.

CLIPR-59 possesses a new GoLD and a down-regulated CLIP domain

Whereas other ankyrin repeat-containing proteins are targeted to intracellular membranes (for review see Michaely and Bennett, 1993; De Matteis and Morrow, 2000), the CLIPR-59 ankyrin repeats are not involved in targeting to the Golgi complex. We directly demonstrate that the carboxy-terminal 60 amino acids of CLIPR-59 encode the GoLD of the protein. This domain, which is both necessary and sufficient for

addressing a cytosolic protein to the Golgi complex (most likely to the trans-Golgi/TGN), does not show any strong conservation with already described GoLDs. In particular, no similarity could be detected to the GRIP domains present in certain golgins (Barr, 1999; Kjer-Nielsen et al., 1999; Munro and Nichols, 1999). The CLIPR-59 GoLD thus represents a new GoLD, and we will now mutagenize it to dissect the molecular basis of its targeting activity.

Also particular to CLIPR-59 is its apparent lack of interaction with microtubules. Neither endogenous nor transfected CLIPR-59 localized to microtubules in tissue culture cells. Moreover, overexpression of CLIPR-59 failed to obviously alter the microtubule network. This failure is in marked contrast to CLIP-170 and many other MTB proteins, which induce microtubule bundling upon overexpression.

It should be noted that we did observe cosedimentation of in vitro–translated CLIPR-59 with taxol-stabilized microtubules in vitro (unpublished data). However, this type of experiment is prone to artifacts, and sedimentation was still observed when the CLIP domain was removed.

Finally, deletion experiments demonstrated that both the amino-terminal region and the membrane-targeting domain of CLIPR-59 inhibit microtubule association. The isolated CLIP domain did indeed behave as a MTB domain, eventually bundling microtubules upon overexpression. Careful analysis of immunofluorescence data suggested that the MTB domain of CLIPR-59 differentially recognized a subset of microtubules, but more experiments are necessary to establish both the nature of this subset as well as the function of this discrimination. It would not be unexpected that the MTB domain of CLIPR-59 could recognize a subset of microtubules, because the CLIP domain seems to confer conformation- (or structure-) sensitive tubulin binding to at least some members of the family. For example, CLIP-170 interacts with growing microtubule plus ends probably through a copolymerization mechanism (Diamantopoulos et al., 1999; Perez et al., 1999), a property that may be shared by p150^{glued}. Another group of CLIPRs also seems to be sensitive to tubulin conformation, as these proteins bind tubulin in a prefolded form (Lewis et al., 1997).

CLIPR-59 plays a role in TGN–endosome membrane dynamics

The function of CLIPR-59 is still uncertain. It was proposed by Rickard and Kreis (1996) that specific CLIPs, phylogenetically related to CLIP-170 or not, play a role at the interface between membranous organelles and microtubules. Some described CLIPs are indeed members of the CLIP-170 family: CLIP-170 for endocytic carrier vesicles (Pierre et al., 1992); CLIP-115 for dendritic lamellar bodies (De Zeeuw et al., 1997); and a yet unidentified CLIPR for peroxisomes (Thiemann et al., 2000). But so far, p150^{glued}, as part of the dynactin complex, is the only CLIP-170–related protein for which clear involvement in membrane dynamics has been established (Burkhardt et al., 1997; Presley et al., 1997).

Although early observations indicated the existence of Golgi CLIPs, the nature of the proteins encoding this activity has largely remained unknown (Karecla and Kreis, 1992; Rickard and Kreis, 1996). GMAP-210 has recently been characterized as a CLIP, mediating interactions between Golgi membranes and stable microtubules (Infante et al., 1999). Hook3 appears to be a CLIP located in the cis-Golgi (Walenta et al., 2001). It is also worth mentioning that the *Drosophila* CLIP-170 homologue, CLIP-190, colocalizes with Golgi markers during cellularization of the embryo, although no direct involvement of CLIP-190 in Golgi localization has yet been obtained. In this context, CLIPR-59 may be one of the elusive Golgi CLIPs, more particularly involved in trans-Golgi/TGN interaction with microtubules.

CLIPR-59 overexpression strongly perturbs membrane dynamics in the early endosome–TGN pathway, leading to the accumulation of membranes positive for both Rab11 and TfR, hence likely to be recycling endosomes (Ullrich et al., 1996). EEA1-positive structures coaccumulate with overexpressed CLIPR-59, indicating that early endosomes

are also affected. Immunofluorescence experiments together with quantification of transferrin uptake and recycling by FACS[®] analysis showed that transferrin can be internalized by cells overexpressing CLIPR-59, although less efficiently. It will be interesting to test whether these defects are due to a general inhibition of endosome motility or a failure of aggregated endosomes to participate in transferrin recycling.

Finally, a reduction in TGN46 staining was observed in CLIPR-59–overexpressing cells. CLIPR-59 overexpression also correlated with perturbation of STxB retrograde transport, which also transits through the recycling endosome to the TGN pathway (Mallard et al., 1998; Wilcke et al., 2000). Interestingly, although most of the TfR accumulated at the center of CLIPR-59–overexpressing cells, the STxB appeared to be blocked in peripheral structures partially positive for overexpressed CLIPR-59. However, it is difficult to use overexpression experiments to precisely pinpoint the site of CLIPR-59 action. In addition, we were not able to quantify this effect because cells overexpressing CLIPR-59 represent only a minority of transfected cells. We will now try to reconstitute this effect in semi-intact cells to gather more quantitative data and identify the perturbed stage.

The central accumulation of TfR-positive early/recycling endosomes observed upon CLIPR-59 overexpression may result from either direct or indirect effects on endosome function. Indirect effects could be due to the titration of essential components necessary for function of early endosome–TGN pathways, for example components involved in sorting (receptors), budding (coats), or delivery (fusion machinery and motors). One such component could be the clathrin adaptor PACS-1 (Wan et al., 1998), because the CLIPR-59 E/P domain closely resembles the PACS-1 interaction domain present in furin and HIV-Nef (Wan et al., 1998; Pigué et al., 2000). Moreover, electron microscopy suggested that CLIPR-59 overexpression induced some accumulation of coated membranes. However immunofluorescence analysis showed no major effect on clathrin-coated membranes, or on AP-1, -2, and -3–positive membranes (unpublished data).

What model for CLIPR-59 function?

According to the proposed model for CLIP function (Rickard and Kreis, 1996; Schroer, 2000), CLIPR-59 may stably link its target membranes to microtubules until they are mature enough to be translocated by molecular motors. It could also help the mature membrane to select a particular subset of microtubules for movement. Because we observed a slight acceleration of endocytosis upon moderate expression of CLIPR-59, this step may represent a necessary checkpoint along the TGN46–STxB pathway. It will be important to determine whether specific kinesins are involved in this pathway, as was shown for the M6PR pathway (Nakagawa et al., 2000). When overexpressed, CLIPR-59 may attach membranes to microtubules too strongly, thus perturbing their motility. It is worth mentioning that CLIPR-59 mutants missing their amino-terminal domain can still efficiently perturb the endosome–TGN membrane dynamics, whereas the additional deletion of the MTB domain prevents this effect. Overexpressed CLIPR-59 would thus behave like the rotavirus protein NSP4 that stably attaches ER-derived membranes to microtubules

and inhibits secretory transport (Xu et al., 2000), thus behaving like a nonregulatable CLIP. It is, however, worth noting that the FACS[®] experiments also suggested that moderate expression of GFP•CLIPR-59, as well as expression of GFP•CLIPR-59-ΔC60, had a weak stimulatory effect on transferrin endocytosis. This may indicate that low levels of endogenous CLIPR-59 may be an activator of this pathway.

Our domain mapping experiments suggest a model where the membrane-targeting domain of CLIPR-59 is dominant over the MTB domain, and the ankyrin repeat-containing amino-terminal half of CLIPR-59 interferes with microtubule binding. We thus propose that newly synthesized CLIPR-59 is unable to bind to microtubules and first associates with membranes. This membrane association may then allow microtubule binding of CLIPR-59, possibly by displacing the ANK domain or after posttranslational modifications. According to this model, CLIPR-59 would be the first CLIP that binds microtubules only when already localized to its target membrane. In contrast to the other previously characterized CLIPs, its overexpression would thus only affect its target compartment without affecting the microtubule network. This could be of some importance because we observed that CLIPR-59 is strongly expressed in some neurons during development (Bloch-Gallego, E., and C. Sotelo, personal communication; unpublished data), and may thus be used during neuronal maturation to regulate the function of the TGN and recycling endosomes.

In conclusion, we have identified a new CLIP-170-related cytoplasmic linker protein that is involved in the early/recycling endosome-TGN transport pathway. Its unusual characteristics, including its membrane interactions and fine-tuned microtubule interactions, suggest that it plays an important role in membrane-microtubule interactions.

Materials and methods

Antibodies and reagents

Antibodies against CLIPR-59 were raised in rabbits using a GST fusion protein produced in bacteria. Extensive carboxy-terminal degradation of CLIPR-59 in bacteria resulted in the production of antibodies primarily directed against the amino-terminal domain. Sera were depleted of anti-GST antibodies before being affinity purified on GST-CLIPR-59 resin. Other polyclonal antibodies used in this study were: anti-GFP (Molecular Probes), anti-EEA1 (Santa Cruz Biotechnology, Inc.), anti-galactosyltransferase (provided by E.G. Berger, Institute of Physiology, University of Zurich, Zurich, Switzerland), and anti-Rab11 (Wilcke et al., 2000). Monoclonal antibodies used were anti-GM-130 (Transduction Laboratories), anti-α-tubulin (Sigma-Aldrich), anti-Golgi CTR433 (provided by M. Bornens, Institut Curie, Paris, France), anti-HA, anti-LBPA (6C4; provided by J. Gruenberg, University of Geneva, Geneva, Switzerland), and anti-TfR OKT9, or H68.4 (for immunoelectron microscopy; Zymed Laboratories). Fluorescent secondary antibodies were from Jackson ImmunoResearch Laboratories. Rhodamine- and Alexa633-labeled transferrin were from Molecular Probes.

Culture medium, sodium pyruvate, and glutamine were from GIBCO BRL, restriction enzymes and T4 DNA ligase were from New England Biolabs, Inc., and oligonucleotides were obtained from Sigma-Genosys. DNA was purified using Jetstar columns (Genomed).

Cloning of CLIPR-59, phylogenetic analysis, and plasmid construction

Phylogenetic analysis as well as cloning and tagging of CLIPR-59 are described in the online supplemental material (available at <http://www.jcb.org/cgi/content/full/200111003/DC1>).

Cell culture, transfection, and immunofluorescence analysis

HeLa cells were grown as previously described (Mallard et al., 1998) and transfected using the calcium phosphate precipitate method. 24 or 36 h after transfection, cells were fixed with 3% paraformaldehyde and permeabi-

lized with 0.05% saponin (ICN Biomedicals). Alternatively, when indicated, cells were prepermeabilized with 0.5% Triton X-100 as described by Kreis (1987) and fixed in methanol (4 min, -20°C). Fixed cells were incubated with antibodies for 30 min (except anti-48, which was incubated overnight). Images were then acquired using a Leica Microsystem confocal microscope (TCS4D or SP2) or, in the case of Fig. 3 (a-c), with a cooled CCD camera (CH250; Photometrics) installed on an Axiovert TV135 microscope (ZEISS). Figures were prepared using Adobe Photoshop[®] 6.0 running on a Power Macintosh (Apple Computer, Inc.).

Immunoelectron microscopy

HeLa cells were plated on tissue culture dishes 24 h before the experiment to obtain 80% confluency at the time of infection. For transient expression, cells were then infected with the vT7 recombinant vaccinia virus (Fuerst et al., 1986) and cotransfected using DOTAP (Roche) with GFP-CLIPR-59 (subcloned in pSP72 under the T7 promoter). Cells were fixed 4 h after the transfection with 2% paraformaldehyde and 0.125% glutaraldehyde and processed for cryosectioning. The cryosections were made at -120°C using a cryo-ultramicrotome (Leica-Reichert) and retrieved with a 1:1 solution of 2.3 M sucrose and 2% methyl cellulose. Cryosections were then incubated with primary antibodies and revealed with protein A gold (purchased from J.W. Slot, Utrecht Medical School, Utrecht, Netherlands). Labeled cryosections were analyzed with a CM120 electron microscope (Philips Electronic Instrument).

Uptake of transferrin and STxB

Rh-Tf was obtained from Molecular Probes and Cy3-labeled STxB (STxB-Cy3) was provided by L. Johannes (Institut Curie, Paris, France; Mallard et al., 1998). Transfected cells were incubated with 25 μg/ml of Rh-Tf or 10 μg/ml of STxB-Cy3 in DME for 90 min at 37°C to achieve steady-state labeling of their respective target compartment. Cells were then washed in medium and fixed in 3% paraformaldehyde before being processed for immunofluorescence.

Quantification of transferrin uptake

Cells transfected by GFP•CLIPR-59 or GFP•CLIPR-59-ΔC60 for 24 h were detached in PBS-EDTA, pelleted, resuspended in endocytosis medium (DME, 10 mM Hepes, pH 7.4, 0.1% BSA, 5 μg/ml Alexa633-labeled transferrin), and incubated at 37°C. After 60 min of internalization, cells were diluted in cold PBS, pelleted, resuspended in recycling medium (DME, 10% FCS, 10 mM Hepes, pH 7.4, 100 μg/ml unlabeled transferrin), and incubated for another 60 min at 37°C. At the indicated time during the endocytosis and recycling periods, aliquots of incubated cells were taken, diluted five times in PBS and left for 10 min at 4°C in the presence of 100 μg/ml unlabeled transferrin (Sigma-Aldrich). Cells were then pelleted, resuspended in PBS-EDTA, and fixed in 1% paraformaldehyde. FACS[®] analysis was then performed using a FACScalibur[®] (Becton Dickinson), measuring GFP fluorescence in FL1 and Alexa633 in FL4. The mean Alexa633 fluorescence was then calculated in three separate windows chosen according to relative green fluorescence (control, low, and high). In separate experiments, we checked that cells from the lowest fluorescence window behaved as mock-transfected cells, and could thus be taken as an internal control. At least 7.5×10^2 and up to 10^4 cells were counted in the high window.

Online supplemental material

Additional Materials and methods concerning the cloning and phylogenetic analysis of CLIPR-59, as well as additional references related to the monoclonal antibodies used in this study are available online (available at <http://www.jcb.org/cgi/content/full/200111003/DC1>).

This article is dedicated to the memory of Thomas Kreis.

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