Over-Expression of Rififylin, a New RING Finger and FYVE-like Domain-containing Protein, Inhibits Recycling from the Endocytic Recycling Compartment

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Endocytosed membrane components are recycled to the cell surface either directly from early/sorting endosomes or after going through the endocytic recycling compartment (ERC). Studying recycling mechanisms is difficult, in part due to the fact that specific tools to inhibit this process are scarce. In this study, we have characterized a novel widely expressed protein, named Rififylin (Rffl) for RING Finger and FYVE-like domain-containing protein, that, when overexpressed in HeLa cells, induced the condensation of transferrin receptor-, Rab5-, and Rab11-positive recycling tubulovesicular membranes in the perinuclear region. Internalized transferrin was able to access these condensed endosomes but its exit from this compartment was delayed. Using deletion mutants, we show that the carboxy-terminal RING finger of Rffl is dispensable for its action. In contrast, the amino-terminal domain of Rffl, which shows similarities with the phosphatidylinositol-3-phosphate–binding FYVE finger, is critical for the recruitment of Rffl to recycling endocytic membranes and for the inhibition of recycling, albeit in a manner that is independent of PtdIns(3)-kinase activity. Rffl overexpression represents a novel means to inhibit recycling that will help to understand the mechanisms involved in recycling from the ERC to the plasma membrane.

INTRODUCTION

Endocytosis is a fundamental cellular process, whereby extracellular material as well as plasma membrane proteins and lipids are internalized and transported to various intracellular compartments (Mukherjee et al., 1997). Initially, internalized molecules are delivered to early/sorting endosomes, which constitute the first sorting station of the pathway. Ligands may dissociate from their receptor at the mildly acidic pH of the early/sorting endosomes and are then targeted to the late endosome degradation compartment, whereas receptors may be transported back to the plasma membrane through recycling endosomes. Underlying the complexity and dynamic nature of the endocytic compartment, a large array of cellular proteins act together to ensure complex sorting decisions and precise targeting of internalized molecules.

About 95% of endocytosed membrane is recycled to the plasma membrane. This recycling is important for the maintenance of membrane composition and for receptors involved in nutrient uptake as well as for other several cellular processes. To study the recycling pathway, the transferrin receptor (TfR) and its ligand transferrin (Tf) have been used extensively (reviewed in Mellman, 1996; Mukherjee et al., 1997; Maxfield and McGraw, 2004). Within early endosomes, Fe³⁺ dissociates from Tf and the TfR-Tf complexes, either return directly to the plasma membrane or reach a network of tubular membranes, called the endocytic recycling compartment (ERC) before returning to the plasma membrane. These two pathways are believed to rely on distinct molecular machineries and the functional significance of these two recycling pathways is poorly understood. Some evidence indicates that ERC has sorting abilities. Indeed, ERC has been shown to be instrumental in basolateral/apical sorting in epithelial cells (Apodaca et al., 1994; Knight et al., 1995) as well as to be involved in the delivery of membrane proteins to the trans-Golgi network (TGN; Ghosh et al., 1998). ERC may therefore represent, in addition to early/sorting endosomes, another sorting station along the recycling pathway.

Only a few molecules involved in the function of the ERC have been identified so far. Among the large Rab family (Zerial and McBride, 2001), Rab11 has been shown to local-
ize to and function in endocytic recycling from the ERC to the plasma membrane (Ullrich et al., 1996) and the TGN (Wicke et al., 2000). Several Rab11-interacting proteins have been isolated: Rab11BP/Rabphilin11 (Zeng et al., 1999), myosi


and religation with annealed oligonucleotides 5’D0 (CCTCCCACATGGTGG). RfI/H9004 Cter was obtained after digestion of RfI/H11032-CGAGATCTTCATGTGGGCATCCTGCTG-


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was achieved by subcloning the full-length coding region of RfI from the C-terminus (RfI deletion of GFP in the RfI-CAACTGGT) and 3 residues 3/98 from the N-terminus (RfI fl-finger. It is instrumental for RfI recruitment to recycling endosomes, where it plays a role in membrane fusion events during recycling of plasma membrane proteins (Prekeris et al., 1998). Rme-1, a protein identified in a genetic screen for endocytosis mutants in Caenorhabditis elegans, has been reported to be involved in the exit of membrane proteins from the ERC to the TGN and the plasma membrane (Lin et al., 2001). Altogether, very little is known about the regulation of membrane trafficking through the ERC (Maxfield and Gragg, 2004). A major difficulty stems from the paucity of specific tools to inhibit recycling and from the existence of several recycling pathways.

We have identified a novel gene coding for a protein, Rifilin (Rfl), containing both a FYVE-like domain and a RING finger in the amino- and carboxy-terminal region, respectively. Using deletion mutant analysis, we found that the amino-terminal domain is a key component of Rfl. This domain presents sequence similarities with the well-characterized phosphatidyl-inositol-3-phosphate (PtdIns(3)P)-binding FYVE finger. It is instrumental for Rfl recruitment to recycling endosome, but in contrast to FYVE fingers, is not dependent on PtdIns(3)-kinase activity. We show that overexpression of Rfl alters both the morphology and the function of recycling endosomes, as illustrated by the massive accumulation of TIR and Rab11 in the perinuclear region and the inhibition of Tf recycling.

MATERIALS AND METHODS

Plasmid Constructs
cDNA sequences encoding the full-length 363 amino acid coding region of Rfl were PCR-amplified from mouse liver mRNA using the primer 5’T5’-GTTCCTGACCCCATCTGGGATCCCTGCACTG (CACTG) that contains an XhoI site and a Kozak translational start consensus, and the primer 3’T3’-ACCCTGATGGACCCGAAGACGTCATGC (GTCATG), which includes a BamHI site. The resulting sequence was cloned into the XhoI and BamHI sites of pEGFP-N1 (Clontech, Palo Alto, CA) to give rise to a mouse-Rfl-GFP expression vector. We also derived an expression vector for the Rfl protein fused in its amino-terminal region with GFP (GFP-Rfl). This was achieved by subcloning the full-length coding region of Rfl-RFP into the BglII and BamHI sites of pEGFP-C1 (Clontech). The myc-Rfl expressing vector was obtained by PCR amplification on mouse liver cDNA (by using the primers 5’myc-5’-CGAGAATCTTCTGACCCGAAGACGTCATGC (CACTG) and 3’myc-3’-CGAGAATCTTCCACCATCTGGGATCCCTGCACTG (GTCATG) and cloned into the BamHI and HindIII sites of pCMV-TagLk (Stratagene, La Jolla, CA). Untagged Rfl-expressing vector was obtained after deletion of GFP in the Rfl-GFP construct. Two mutants that lack either residues 9/98 from the N-terminus (Rfl-GFP-DNter) or residues 336/363 from the C-terminus (Rfl-GFP-DCter) were generated by restriction enzymes digestion. Rfl-GFP-DNter was obtained after digestion of Rfl-GFP construct with Xhol/Sacl and religation with annealed oligonucleotides 5’T5’T (TCGACCCGATCCCATCTGGGATCCCTGCACTG) and 3’D3’ (CTCCACCATCTGGGATCCCTGCACTG). Rfl-GFP-DCter was obtained after digestion of Rfl-GFP construct with Xhol/XbaI and religation with annealed oligonucleotides 5’T5’T (CTCCACCATCTGGGATCCCTGCACTG) and 3’D3’ (CTCCACCATCTGGGATCCCTGCACTG). The single-FYVE (SFL) and double-FYVE (DFL) constructs consist of one and two copies of residues 4–95 fused to pEGFP-N1 (Clontech). Junctions and PCR-amplified regions were sequenced to ensure that no errors were introduced.

Cell Culture and Transient Transfections

Hela cells were grown at 37°C in the presence of 8% CO2 in DMEM supplemented with 10% FCS (complete medium) and transiently transfected by electroporation as follows: cells were plated in 10/200 ml medium, 100/200 ml, respectively, placed in 0.45-cm gap cuvette along with 10–30 μg of plasmid DNA and electroporated (200 V and 975 μF) with a Bio-Rad GenePulser II (Richmond, CA). Under these conditions, we observed that 70–90% of transfected cells expressed Rfl-GFP. Cells were then plated on 12-mm gelatin-coated glass coverslips and processed for immunofluorescence analysis 4 or 16 h after electroporation. Treatment with wortmannin (Sigma, St. Louis, MO) was carried out at 100 nM for 40 min at 37°C. Concentration of the drug and duration of the treatment were increased up to 400 nM and 3 h, respectively. Treatment with LY294002 (Calbiochem, San Diego, CA) was carried out at 100 μM for 40 min at 37°C.

Immunofluorescence Microscopy

Transfected cells were washed in PBS, fixed for 20 min in 4% PFA, and incubated twice for 8 min in PBS-glycine, 0.1 M, before immunostaining. The cells were then blocked in PBST buffer (PBS supplemented with 10% FCS, 0.2% BSA, and 0.05% saponin) for 20 min at room temperature. Primary and secondary antibody incubations were performed in PBST buffer for 1 h at room temperature. Monoclonal antibodies against EEA1, GM130, and Rab5 were from Transduction Laboratories (Lexington, KY). Monoclonal anti-TIR and anti-myc antibodies were from Sigma. Monoclonal anti-Lamp2 was from Pharmingen (San Diego, CA). Rabbit polyclonal anti-TGN38 was obtained from George Banting, Alexei and Aleska™-conjugated secondary antibodies and Alexa Fluor Probes (Eugene, OR). Coverslips were mounted in mowiol containing 100 mg/ml DABCO (Sigma) and examined using a Zeiss LSM 510 confocal microscope (Thornwood, NY). Z-series of optical sections were acquired at 0.3-μm increments. Images were acquired with a setting allowing the maximum detection below saturation limits. For double staining, sequential acquisitions per optical section were performed, in order to prevent fluorescence passage between the two channels. Three-dimensional reconstructions using 36 projections around the Y axis were performed using LSM-510 software. Images were processed using Adobe Photoshop v5.0 software (San Jose, CA). Other images were obtained using a Zeiss Axiosplan 2 fluorescence microscope equipped with a Zeiss AxioCam camera.

Immunoblotting

Recombinant fusion protein of the FYVE-like domain of Rifilin (1–100 amino acids) was produced in Escherichia coli as a GST fusion protein from pGEX-4T1 expression vector (Pharmacia Biotechnology, Piscataway, NJ) and purified using a gluthathione-conjugated affinity column (Pharmacia Biotech). Purified protein (37 kDa) was then used as an immunogen for the production of rabbit antisera (Agro-Bio, La Ferriere Saint-Aubin, France). Serum batches showing higher titers were subjected to antigen-affinity chromatography. Although the antigen-affinity purified anti-Rfl antibody allowed the detection of overexpressed Rfl, they never detected endogenous Rfl. In addition, it cross-reacted with many unidentified proteins (see Figure 5B, right panel). For immunoblotting, total cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to standard methods. The blots were then probed with affinity-purified anti-Rfl (1/1000 dilution) or anti-GFP (Clontech, 1/7500 dilution) and peroxidase-conjugated anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, 1/50 000 dilution) followed by ECL detection (Pharmacia Biotechnology).

Transferrin Recycling Assays

Tf recycling was first monitored using a fluorescence microscope. Sixteen hours after transfection, Hela cells were incubated for 90 min in a serum-free medium supplemented with 0.1% BSA. Then, cells were allowed to internalize AlexaFluor™-Ti (Molecular Probes) for 90 min at 37°C and washed three times in ice-cold PBS. Recycling was induced by warming the cells to 37°C in medium containing 0.1% BSA, and 100 μM DABCO (Sigma). After 30 min, cells were fixed, stained with DAPI, and observed under a fluorescence microscope. Identification of Tf recycling was performed by flow cytometry. Cells were transfected with plasmids encoding Rfl-GFP or GFP. Sixteen hours later, transfected cells were incubated for 30 min in a serum-free medium at 37°C, and AlexaFluor™-Ti (Molecular Probes) was added for 30 min at 37°C. The cells were then washed in serum-containing medium at 4°C, and membrane-bound Tf was removed by acid wash (DMEM medium, 20 mM sodium acetate, pH 3.0, for 3 min at 4°C), followed by neutralization with an excess of DMEM medium, 30 mM HEPES buffer, pH 7.4, as described in Duprez et al. (1998). After efficiency of acid wash was confirmed by cell surface Ti, recycling was then induced by rapidly warming the cells to 37°C, in DMEM medium containing a 100-fold excess of unlabelled Tf. When indicated, 100 μM LY294002 was added to loading and recycling medium. At various times, recycling was stopped by adding ice-cold DMEM medium
supplemented with 10% fetal calf serum. Fluorescence intensity due to GFP and to Alexa488/432 was then measured by flow cytometry (FACS-LSR, BD Biosciences, San Diego, CA). The mean fluorescence intensity of 10,000 cells expressing Riffl-GFP or GFP was obtained for each time point.

Electron Microscopy
Transfected HeLa cells were fixed with a mixture of 2% PFA and 0.2% glutaraldehyde in 0.2 M phosphate buffer, pH 7.4, for 2 h at room temperature. Cells were embedded in 10% gelatin, infused in 2.3 M sucrose, and frozen in liquid nitrogen as described previously (Raposo et al., 1997). Ultra-thin cryosections were prepared with a Leica UC6 ultracryotome (Leica, Deichert, Wetzlar, Germany). Cells were then incubated with a mixture of 2% w:v gelatin, 2.3 M sucrose (vol/vol; Liou et al., 1996). Sections were single immunogold labeled with a rabbit polyclonal anti-GFP (Molecular Probes) or double immunogold labeled using, in a first step, a mouse monoclonal anti-FLAG (clone H68.4, Zymed Laboratories, South San Francisco, CA) or a mouse monoclonal anti-CD63 (clone CBL/γ, Caltag Laboratories, Burlingame, CA) followed by a rabbit anti-mouse IgG (Dako, Carpinteria, CA) and, in a second step the anti-GFP antibody. Antibodies were detected with protein A gold conjugates 10 or 15 nm (PAG 10; PAG 15) that were purchased from Proteins (Molecular Probes) or double immunogold labeled using, in a first step, a mouse monoclonal anti-FLAG (clone H68.4, Zymed Laboratories, South San Francisco, CA) or a mouse monoclonal anti-CD63 (clone CBL/γ, Caltag Laboratories, Burlingame, CA) followed by a rabbit anti-mouse IgG (Dako, Carpinteria, CA) and, in a second step the anti-GFP antibody. Antibodies were detected with protein A gold conjugates 10 or 15 nm (PAG 10; PAG 15) that were purchased from Department of Cell Biology, Utrecht University, The Netherlands. Double labelings were performed according to Slot et al. (1991). A 1% glutaraldehyde fixation step was included between the primary and the secondary antibody/protein A gold conjugates immunogold labeling steps in order to avoid the first antibody to be recognized by the second protein A gold conjugate. Controls were performed without the primary or secondary antibody reagents (Raposo et al., 1997).

Northern Blot Analysis
A mouse multiple tissue Northern Blot from Clontech was hybridized in HybriDexpress buffer (Clontech) with a 32P-labeled probe corresponding to the ORF of Riffl. Hybridization and washes were performed at 68 and 50°C, respectively. Autoradiography was performed using X-Omat film (Eastman Kodak, Rochester NY).

RESULTS
Cloning of a Novel Protein that Contains Two Zinc Finger Domains
During our global effort of gene identification and characterization in the Ovum mutant locus region on mouse chromosome 11 (Babinet et al., 1990; Cohen-Tannoudji et al., 1996; 2000; Le Bras et al., 2002), we have identified several ESTs that mapped in the nonrecombinant Omm region. One of these ESTs (accession no. AA174545) from CD-1 mouse testis library was obtained from the IMAGE consortium and fully sequenced. To complete this analysis, we performed a 5'RACE RT-PCR reaction on BALB/c testis cDNAs that were submitted to the public databases while this work was in progress (accession no. AK007189, NM026097, and AK006757). Upon sequencing, we determined that the ORF of a protein which we named Riffl encoded a single 1 kbp transcript, as do adult mouse liver and kidney cells majorly expressed Riffl transcripts contained the additional sequence (Figure 1E). These data suggest that Riffl codes for two ubiquitously expressed proteins of 336 and 364 aa, respectively, and that their relative level of expression varies between tissues. Hela cells express almost exclusively the 363 aa isoform, as do adult mouse liver and kidney cells.

Overexpression of Riffl Affects the Morphology of Recycling Endosomes
We first examined the intracellular distribution of Riffl in HeLa cells. We constructed a vector expressing the full-length (363 aa) protein fused, at its C-terminus, to GFP (Riffl-GFP). HeLa cells were electroporated with this vector and the distribution of the fusion protein was examined 16 h later. In most cells, we observed that Riffl-GFP localized to a limited number of brightly stained globular structures at the perinuclear region (Figure 2, left panels). This situation was not restricted to HeLa cells because similar globular structures were observed upon expression of Riffl-GFP in various cell types (COS7, mouse embryonic fibroblasts, Neuro2A, A203P (MOC, ILCF, our unpublished results)). Moreover, this phenotype was due to Riffl overexpression per se because similar observations were made in cells expressing either untagged Riffl (detected using anti-Riffl antibody), the Riffl protein tagged with GFP in its N-terminus (GFP-Riffl), or with a c-myc epitope at the N-terminus (myc-Riffl; our unpublished results).

We analyzed the structures labeled by Riffl-GFP in transfected cells, with markers from subcellular compartments. In cells overexpressing Riffl, the pattern of some endosomal markers was perturbed. Thus, TR, a marker of peripheral early endosomes and perinuclear recycling endosomes, was severely redistributed in Riffl-GFP expressing cells as most TR immunostaining was detected in aggregated perinuclear structures in these cells. Although we observed that TR and
Figure 1. Rififylin expression and protein structure. (A) Nucleotide and predicted amino acid sequence of mouse Rif cDNA. Sequences 5’ of nt 492 (arrowhead) have been derived from a testis 5’ RACE RT-PCR product. The FYVE-like and RING domains are underlined. The alternative coding sequence is shadowed and not numbered. Sequence data for the testis cDNA and the alternative coding sequence are available from GenBank/EMBL/DDBJ under accession nos. AY163902 and AY163903, respectively. (B) Schematic representation of the two Rif protein isoforms. (C) ClustalW alignments of Rif FYVE-like domain with previously characterized FYVE domains. The amino-acids that defined the FYVE finger signature (Stenmark et al., 2002) are indicated in bold. Arrowheads point to critical residues of the core R(R/K)HHCR motif that are not conserved in the FYVE-like domain. (D) Northern blot (mouse MTN, Clontech) and (E) RT-PCR analysis of Rif expression in adult mouse and embryonic tissues: heart (H), brain (B), spleen (S), lung (Lu), liver (Li), skeletal muscle (SM), kidney (K), testis (T), thymus (Ty), embryo 14.5 dpc (E), placenta 14.5 dpc (P), and in ES cells (ES), mouse embryonic fibroblasts (EF), and human HeLa cells (He). Region from nt 662-1115 was amplified by RT-PCR. PCR products of 537 and 453 base pairs in length correspond to transcripts with and without the alternative coding sequence, respectively.
Figure 2. Effects of Rifflin overexpression on the morphology of endocytic compartments. HeLa cells were transfected with Riffl-GFP expressing vector and analyzed 16 h post-transfection. Transfected cells were fixed, immunostained with antibodies against TfR (A), Rab11 (B), Rab5 (C), EEA1 (D), Lamp2 (E), and TGN38 (F), and analyzed by confocal microscopy. Images show a medial orientation of a three-dimensional reconstruction. Bars, 10 μm. In each case, the right panel shows a higher magnification of the area delimited by the insets showing the Riffl-positive compartment (magnification, ×4, left) and the corresponding area of a nontransfected cell (magnification, ×4, right). Bars, 5 μm.
Rfl-GFP colocalized to some extent, the two markers often appeared tightly intermingled but not fully colocalized (Figure 2A). Similarly, immunostaining of Rab11, a marker of the ERC and TGN, or Rab5, a marker of early endosomes, were shifted to the Rfl-positive structures (Figure 2, B and C). In contrast, the staining of EE1, a marker of early sorting endosomes, was unaffected by Rfl overexpression and appeared as a punctate vesicular pattern indistinguishable from that observed in untransfected HeLa cells (Figure 2D). Finally, we found that Rfl-positive structures were distinct from Lamp2-positive late endosomes/lysosomes (Figure 2E) and from the trans-Golgi network, labeled with an anti-TGN38 antibody (Figure 2F). Moreover, other Golgi markers, such as GM130 and Rab6, were unaffected by Rfl overexpression (our unpublished results). It is intriguing that Rab5 and EE1 localization were differentially affected by Rfl overexpression. Indeed, it was reported that Rab5 physically interacts with EE1 and colocalizes with it in early endosomes, where it regulates the homotypic fusion between early endosomes as well as the transport of clathrin-coated vesicles from the plasma membrane to early endosomes (Gorvel et al., 1991; Bucci et al., 1992; Stenmark et al., 1994). Altogether, these data suggest that Rfl-positive structures belong to the endocytic recycling compartment. It should also be noted that the condensation of endosomal structures upon Rfl overexpression did not appear to be due to the disorganization of cytoskeleton networks because similar distributions of actin and tubulin were observed in Rfl-transfected and nontransfected cells (our unpublished results).

To gain further insight as to the nature of the endosomal membranes in which Rfl-GFP accumulates, we analyzed the subcellular distribution of Rfl on ultrathin cryosections of transfected HeLa cells. We observed that the Rfl-positive compartment consisted of massive accumulation of tubulovesicular membrane profiles decorated with Rfl at high density (Figure 3A). In addition, we observed some Rfl staining on the outer membranes of vacuolar structures containing a few internal membranes (star on Figure 3A). In agreement with our immunofluorescence data, double immunogold labeling revealed an intermingled distribution of Rfl (10-nm gold particles) and TfR (15-nm gold particles) in the tubulovesicular membrane profiles (Figure 3B). In contrast, double immunogold labeling of Rfl and CD63, a protein present in late endosomes and lysosomes of HeLa cells (Stumptner-Cuvelette et al., 2003), indicated that the two proteins localized to distinct membranes subdomains, CD63 being mostly restricted to the internal membranes of endosomal compartments (star on Figure 3C). In conclusion, morphological analysis revealed that aggregation of perinuclear TfR positive tubules is the main phenotype induced by Rfl overexpression.

Overexpression of Rifilysin Slows Tf Recycling from the ERC

The morphological alteration of the endosomal compartment induced by Rfl expression prompted us to investigate whether Rfl overexpression could alter protein traffic in this compartment. To follow the recycling of Tf from the ERC to the cell surface, transfected cells were first allowed to internalize Alexa594-Tf at 37°C for 90 min and then were subjected to a chase with an excess of unlabeled Tf for various times. Initially, Alexa594-Tf was found in vesicular structures in the perinuclear area of the cytoplasm as well as in the cell periphery of the nontransfected cells (Figure 4A). In Rfl-overexpressing cells, Alexa594-Tf was found accumulated in the perinuclear Rfl-positive structures (Figure 4A). Some Tf-containing vesicles were also present in the peripheral cytoplasm but to a lesser extent than in nontransfected cells (Figure 4A). After 30 min, most labeled Tf was chased from nontransfected cells (Figure 4B). In contrast, in Rfl-expressing cells, Alexa594-Tf had disappeared from peripheral vesicles, but a significant amount was retained in the Rfl-positive compartment up to a 240-min chase (Figure 4, B–D).

Quantification of the effect of Rfl-GFP expression on the kinetics of Tf recycling was performed by flow cytometry. Rfl-GFP– and GFP-transfected cells were loaded for 30 min at 37°C with Alexa633-Tf. Then, plasma membrane-associated Alexa633-Tf was removed by an acid wash at 0°C, and the cells were incubated at 37°C to allow recycling of internalized Tf. Fluorescence intensity due to Alexa633-Tf in cells transfected with Rfl-GFP was then compared with that of control cells transfected with GFP. The rate of Alexa633-Tf recycling was significantly lower in Rfl-expressing cells than in control cells (Figure 4E). Consistently, the surface levels of TfR were reduced by twofold in Rfl-GFP–positive cells as compared with controls (our unpublished results). Altogether, these data show that overexpression of Rfl significantly slows recycling from the ERC to the plasma membrane.

Inhibition of Recycling by Rifilysin

The Amino-terminal FYVE-like Domain Is Essential for the Effect of Rifilysin on Recycling Endosomes

To study the role of Rfl zinc finger domains, we constructed expression vectors coding for deletion mutants of Rfl lacking either the amino-terminal FYVE-like domain or the carboxy-terminal RING finger motif (Figure 5A). GFP-tagged full-length and truncated Rfl proteins were expressed in HeLa cells (Figure 5B). Rfl–ΔNter harbors a truncated and therefore nonfunctional RING finger. Its distribution was indistinguishable from that of full-length Rfl (Figure 5, D and C, respectively). In contrast, Rfl–ΔNter was uniformly distributed in the cytoplasm and the nucleus (Figure 5E). This pattern was indistinguishable from that of GFP alone (our unpublished results). We next analyzed the effect of overexpression of the deleted proteins on Tf recycling. The kinetics of Tf recycling was not affected by the expression of Rfl–ΔNter, whereas Rfl–ΔNter induced an inhibition of Tf recycling similar to that of full-length Rfl (Figure 5F).

Because the intracellular localization of Rfl required the presence of the FYVE-like domain, we constructed expression vectors coding for one (SFL), or two tandemly arranged (DFL), Rfl FYVE-like domain fused to GFP. When expressed in HeLa cells, SFL-GFP was mainly cytosolic, whereas DFL-GFP localized on vesicular structures (Figure 6, A and B). Immunofluorescence analysis showed that DFL-GFP and TfR partially colocalized (Figure 6C). Morphological alteration of the endocytic compartment was less pronounced upon expression of DFL-GFP than Rfl-GFP. Thus, vesicular structures containing DFL-GFP were smaller and more dispersed in the cytoplasm than those containing full-length Rfl-GFP (compare Figures 2 and 6C). Yet, inhibition of Tf recycling was similar in DFL-GFP– and Rfl-GFP–expressing cells (Figure 6D). Altogether, these results indicate that the amino-terminal FYVE-like domain is necessary and sufficient for targeting Rfl to the ERC and to inhibit Tf recycling. In contrast, the carboxy-terminal zinc finger domain is dispensable for the effect mediated by Rfl overexpression on recycling endosomes.
The effect of Rif-1 on PtdIns(3)-kinase Activity

The amino-terminal zing finger domain of Rif-1 resembles the FYVE domain that has been shown to bind specifically to PtdIns(3)P (Burd and Emr, 1998; Gaullier et al., 1998; Patki et al., 1998). However, it exhibits important sequence differences (see above), suggesting that Rif-1 might not bind to PtdIns(3)P. To investigate this point, HeLa cells expressing
Rfi-GFP were depleted of PtdIns(3)P by treatment with LY294002, an inhibitor of PtdIns(3)-kinases. In treated cells, we observed a much less intense vesicular EEA1 immunostaining, indicating that most EEA1 had been displaced by the treatment (Figure 7, A and B). In contrast, Rfi distribution was not modified by the treatment (Figure 7, A and B). Likewise, we observed that DFL-GFP distribution was not affected upon inhibitor treatment (Figure 7, C and D). Similar observations were made using wortmannin, a structurally unrelated inhibitor of PtdIns(3)-kinase activity. Increasing the concentration and/or the duration of wortmannin treatment had no effect on Rfi cellular distribution (our unpublished results). We next compared the effect of PtdIns(3)-kinases inhibitors and Rfi-GFP expression on Tf recycling (Figure 7E). Consistent with previous studies (Martys et al., 1996; van Dam et al., 2002), an inhibitory effect of LY294002 treatment on Tf recycling was observed. The inhibitory effect of LY294002 on Tf recycling was comparable to that induced by Rfi overexpression. Interestingly, a cumulative effect was observed when cells overexpressing Rfi were treated with LY294002 (Figure 7E). These data suggest that Rfi and PtdIns(3)-kinase inhibitors affect different recycling pathways.

**DISCUSSION**

The term ERC refers to a set of endocytic membranes, essentially tubules, that usually concentrate in the perinuclear region of the cell and in which membrane components following the endocytic recycling pathway travel. Few proteins were shown to localize to the ERC (Prekeris et al., 1996; van Dam et al., 2002; Lindsay et al., 2002; Lindsay and McCaffrey, 2002; Meyers and Prekeris, 2002; Wallace et al., 2002a, 2002b). However, how these different proteins intervene in ERC function is largely unknown (Maxfield and McGraw, 2004).

In the present study, we have identified a novel protein, Rfi, that, when overexpressed in HeLa cells, localized to and induced the condensation of endosomal structures displaying TfR, Rab5, and Rab11 markers into several perinuclear globular structures. In contrast, the localization of EEA1, another marker of the early endocytic endosomes was not altered by Rfi overexpression. Finally, lysosomal markers,
like Lamp2, or markers of secretory organelles, including the
TGN, were not affected by Rfl overexpression. Consistently
with confocal microscopy data, ultrastructural analysis
showed that Rfl was mainly located on densely packed
tubulovesicular membrane profiles. Altogether, these data
suggest that the compartment affected by Rfl overexpres-
sion is the ERC.

Furthermore, recycling of TfR was inhibited by Rfl over-
expression. Indeed, the aggregated recycling endosomes
were accessible to endocytosed TfR-Tf complexes. However,
recycling from the Rfl-positive compartment was delayed,
Tf being still detected in the perinuclear region of trans-
fected cells after several hours of chase. Recycling of mem-
brane proteins can occur through at least two different
routes that appear to depend on different molecular machin-
erys (Hao and Maxfield, 2000; Sheff et al., 2002). Rfl is
involved in the recycling of only part of endocytosed TfR,
suggesting that it affects the dynamics of a subdomain of
recycling endosomes. The cumulative inhibitory effects of
Rfl overexpression and of LY294002 treatment on Tf recy-
cling suggests that Rfl acts mainly on a PtdIns(3)-kinase
independent recycling pathway. This is consistent with our
observations that Rfl affects the ERC and the proposal that,
in HeLa cells, PtdIns(3)-kinase activity is required for recy-
cling directly from early endosomes, a recycling route that
bypasses the ERC (van Dam et al., 2002).

We observed a major redistribution of Rab5 immu
nostaining upon Rfl overexpression. In normal cells, Rab5 overlaps
extensively with EEA1 (Simonsen et al., 1998; Lawe et al.,
2000), whereas it shows little colocalization with Rab11 (Son-
nichsen et al., 2000). In transfected cells, both Rab5 and
Rab11 but not EEA1 concentrate in the aggregated recycling

Figure 5. Analysis of deletion mutants lacking either the FYVE-like domain or the RING finger. (A) Schematic representation of Rfl deletions constructs. (B) Western blot detection of the various Rfl fusion proteins. Total cell lysates were prepared from either nontransfected (NT) or transfected HeLa cells. Full length Rfl-GFP (68 kDa), Rfl-ΔNter-GFP (58 kDa), Rfl-ΔCter-GFP (65 kDa), and GFP (27 kDa) were visualized with anti-GFP antibody (B, left panel). SFL-GFP (40 kDa) and DFL-GFP (49 kDa) were visualized with anti-Rfl antibodies raised against the FYVE-like domain (B, right panel). The arrowheads indicate the bands corresponding to the various fusion proteins. Nonspecific bands cross-reacting with both antibodies are also observed. (C–E) HeLa cells were transfected with Rfl-GFP (C), or Rfl-GFP deleted for either the carboxy-terminal domain (Rfl-ΔCter) (D), or the amino-terminal domain (Rfl-ΔNter) (E). Cells were fixed, stained with DAPI, and observed under a fluorescence microscope. Bars, 5 μm. (F) Quantification of the effect of the different constructs on Tf recycling was carried out by flow cytometry as in Figure 4. The graph shows the average ± SD of three independent experiments.
endosomes. This is a striking observation because EEA1 is a Rab5 effector (Simonsen et al., 1998) and perturbations of the endocytic pathway that affect Rab5 without affecting EEA1 have not been reported so far. The way in which Rab5-positive endocytic membranes are disturbed by Rifl overexpression is unknown. However, it is unlikely to rely on direct physical interactions between Rab5 and Rifl, because the two proteins failed to interact in a yeast two-hybrid assay (F.Coumailleau and M.Cohen-Tannoudji, unpublished observations).

Concerning late endosomes, we noticed that some CD63-positive vesicles showed a tendency to be distributed around the aggregated Rifl-GFP–positive recycling endosomes. These surrounding endosomes appeared at the electron microscopy level as vacuoles with a limited amount of internal CD63-containing membranes and may correspond to early multivesicular bodies (MVB). We observed some Rifl staining on the limiting membrane of MVB-like endosomes, suggesting that Rifl overexpression might also perturb this compartment but to a much lesser extent than ERC.

Rifl is a previously uncharacterized protein that contains a zinc finger domain at both ends. The amino-terminal domain is similar to the well-characterized PtdIns(3)P-binding FYVE finger but presents noticeable departures from the consensus sequence in particular at the level of the core R(R/K)HHCR sequence, which is the principal site of interaction with PtdIns(3)P (Dumas et al., 2001; Stenmark et al., 2002). For this reason, we named it FYVE-like domain. Besides sequence similarities, the FYVE-like domain of Rifl shares other similarities with bona fide FYVE domains. Indeed, we have shown that it was necessary for the recruitment of Rifl to endocytic membranes. In addition, it is sufficient, if duplicated, to target GFP to endosomes. This is reminiscent of what has been described for the FYVE domains of EEA1 and Hrs (Stenmark et al., 1996; Gillooly et al., 2000; Lawe et al., 2000). In these cases, the fact that isolated

Figure 6. Analysis of the FYVE-like domain of Rififlin. (A and B) HeLa cells were transfected with SFL-GFP (A) or DFL-GFP (B) constructs, fixed, stained with DAPI, and observed under a fluorescence microscope. Sixteen hours after transfection, cells were fixed and processed for immunostaining with anti-TfR antibody and analyzed by confocal microscopy (C). Images show a medial orientation of a three-dimensional reconstruction. Bars, 10 μm. The right panel shows a higher magnification of the area delimited by the square inset showing the Rifl-positive compartment. Bars, 5 μm. (D) Quantification of the effect of the different constructs on Tf recycling was carried out by flow cytometry as in Figure 4. The graph shows the average ± SD of three independent experiments.
FYVE domains were not targeted to endosomes has been interpreted as due to a weak affinity for PtdIns(3)P that could be compensated by duplication. Similarly, the FYVE-like domain of Rfl may mediate weak interactions with components of recycling endocytic membranes, the nature and the identity of which remain to be identified. Importantly, major differences exist that distinguish the Rfl FYVE-like domain from bona fide FYVE domain. First, Rfl was

**Figure 7.** Effect of Rififlin-GFP is not dependent on PtdIns(3)-kinase activity. Sixteen hours after transfection with Rififlin-GFP (A and B) or DFL-GFP (C and D), HeLa cells were incubated in medium alone (A and C) or containing 100 µM LY294002 (B and D) for 40 min at 37°C. The cells were then fixed and stained with an anti-EEA1 antibody and analyzed by confocal microscopy. Images show a medial orientation of a three-dimensional reconstruction. Bars, 10 µm. In each case, the right panel shows a higher magnification of the area delimited by the square inset showing the Rfl-positive compartment. Bars, 5 µm. (E) Recycling of Tf in Rfl-GFP and GFP-expressing cells in the absence or in the presence of 100 µM LY294002 was measured by flow cytometry as in Figure 4. The graph shows the average ± SD of three independent experiments. Ly: LY294002.
found on membranes with characteristic of recycling endosomes and did not overlap with EE1-positive early endosomes membranes. On the contrary, bona fide FYVE domain-containing proteins localize to PtdIns(3)P-containing membranes, i.e., early endosomes and internal vesicles of the MVB (Gillooly et al., 2000). These proteins play important roles in endocytic membrane trafficking such as early endosome fusion (EE1; Simonsen et al., 1998), traffic from early endosome to recycling endosomes (Rabip4 and Rabenosyn-5; Nielsen et al., 2000; Cormont et al., 2001; de Renzis et al., 2002), or from early endosome to late endosomes (Hrs and PtkFYVE; Komada and Soriano, 1999; Ikonomonov et al., 2001; Raiborg et al., 2002) but are not involved in traffic from ERC to plasma membrane. A second important difference is that endosomal localization of full-length Rif or DFL-GFP is independent of PtdIns(3)-kinase activity, whereas that of bona fide FYVE domain-containing proteins is not. This indicates that, as already suggested by the amino-acid sequence, Rif FYVE-like domain does not bind to PtdIns(3)P. It is therefore not surprising that Rif does not localize to PtdIns(3)P-enriched membranes. Of special interest will be the characterization of the natural ligand of the FYVE-like domain.

The second zinc finger domain of Rif is a carboxy-terminal RING finger motif. Within the past few years, evidence has accumulated arguing in favor of a general role in ubiquitination for RING finger-containing proteins (Freemont, 2000; Weissman, 2001). Recently, it has been shown that SAKURA, the rat ortholog of Rif, exhibited E3 ubiquitin ligase activity in vitro and ex vivo (Araki et al., 2003). However, the RING domain of Rif does not appear to participate in the recycling inhibition mediated by Rif overexpression because similar perturbations of recycling endosomes were obtained upon transfection of full-length and RING finger-deleted version of Rif (Rif ΔCter).

Exactly how Rif could affect the morphology and the function of the ERC is unclear. However, it should be noted that inhibition of Tf recycling was obtained by overexpressing the full-length protein. This is in contrast with other proposed ERC regulatory proteins for which recycling was affected upon overexpression of constitutively active or inactive forms (Rab4, Rab11) or dominant negative or truncated proteins (RCP, Rmel, Rab11BP/Rabhillin11), whereas the overexpression of full-length proteins had little or no effect on the Tf recycling pathway (van der Sluijs et al., 1992; Ulrich et al., 1996; Zeng et al., 1999; Wickle et al., 2000; Lin et al., 2001; McCaffrey et al., 2001; Lindsay et al., 2002). Therefore, the phenotype observed in cells overexpressing Rif may be due to enhancement of the normal functions of Rif, resulting in excess Rif activity. Underlying this hypothesis is the assumption that in un transfected cells Rif is a limiting component. When the FYVE-like domain of Rif was removed, the protein remained cytosolic and inhibition of Tf recycling was no longer observed. This suggests that association of Rif with ERC membranes is necessary for its interference with recycling. Interestingly, we found that DFL-GFP has an inhibitory effect on recycling. This could be the consequence of the occupancy of FYVE-like domain binding sites on ERC membranes by DFL-GFP and the consecutive displacement of endogenous Rif or other proteins targeted to ERC membranes through similar mechanism.

In conclusion, we have characterized a new protein that, when overexpressed, alters both the morphology and the function of the ERC. Further studies on how Rif overexpression inhibits recycling from ERC to the plasma membrane will certainly help to understand the molecular mechanisms underlying membranes proteins trafficking along this poorly characterized compartment.

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