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Clathrin Adaptor epsinR Is Required for Retrograde Sorting on Early Endosomal Membranes

Agnès Saint-Pol,[1,6] Belén Yélamos,[1,6] Mohamed Amessou,[1] Ian G. Mills,[4,7] Marc Dugast,[3] Danièle Tenza,[2] Peter Schu,[5] Claude Antony,[4] Harvey T. McMahon,[4] Christophe Lamaze,[1] and Ludger Johannes[1,*]

[1]Laboratoire Trafic et Signalisation
[2]Unité de Microscopie Electronique
UMR144 Curie/CNRS
[3]INSERM U520
Institut Curie
26 rue d'Ulm
75248 Paris Cedex 05
France
[4]MRC Laboratory of Molecular Biology
Neurobiology Division
Hills Road
Cambridge CB2 2QH
United Kingdom
[5]Georg-August-Universität Göttingen
Zentrum für Biochimie und Molekulare Zellbiologie
Heinrich-Düker-Weg 12
37073 Göttingen
Germany

Summary

Retrograde transport links early/recycling endosomes to the trans-Golgi network (TGN), thereby connecting the endocytic and the biosynthetic/secretory pathways. To determine how internalized molecules are targeted to the retrograde route, we have interfered with the function of clathrin and that of two proteins that interact with it, AP1 and epsinR. We found that the glycosphingolipid binding bacterial Shiga toxin entered cells efficiently when clathrin expression was inhibited. However, retrograde transport of Shiga toxin to the TGN was strongly inhibited. This allowed us to show that for Shiga toxin, retrograde sorting on early/recycling endosomes depends on clathrin and epsinR, but not AP1. EpsinR was also involved in retrograde transport of two endogenous proteins, TGN38/46 and mannose 6-phosphate receptor. In conclusion, our work reveals the existence of clathrin-independent and -dependent transport steps in the retrograde route, and establishes a function for clathrin and epsinR at the endosome-TGN interface.

Introduction

Clathrin plays a central role in endocytosis at the plasma membrane. However, alternative internalization pathways also exist. Cellular uptake via these alternative routes is commonly termed clathrin-independent endocytosis (Nichols and Lippincott-Schwartz, 2001; Johannes and Lamaze, 2002; Conner and Schmid, 2003). The molecular mechanisms of clathrin-independent endocytosis remain to be established. Recent data have shown that multiple pathways exist. Some of the clathrin-independent uptake pathways, including IL2 receptor endocytosis (Lamaze et al., 2001) and SV40 endocytosis (Pelkmans and Helenius, 2002), are controlled by the GTPase dynamin. Other uptake pathways, including those used by some GPI-anchored proteins, are independent of the commonly used dynamin isoforms (Skretting et al., 1999; Sabharanjak et al., 2002).

Many endocytosed molecules reach the conventional transferrin receptor (TfR)-positive early endosome (EE), which serves as a sorting station (Gruenberg and Maxfield, 1995; Mellman, 1996). Some receptors are recycled to the plasma membrane to be recharged with ligands, whereas others enter the late endocytic pathway for degradation. The retrograde route bypasses the recycling and degradation pathways, allowing specific proteins and lipids to reach other intracellular compartments such as the trans-Golgi network (TGN), the Golgi cisternae, the endoplasmic reticulum and, in some instances, the cytosol (Mallard et al., 1998; for a review, see Johannes and Goud, 1998). This pathway has been studied in detail to explain the cellular entry of some protein toxins, such as cholera toxin, ricin, and Shiga toxin (Lord and Roberts, 1998; Sandvig and van Deurs, 2000). Other studies have shown that the cellular proteins TGN38/46 (Mallard et al., 1998, 2002; Ghosh et al., 1998), GPP130, and GP73 (Puri et al., 2002), all of unknown function, use the retrograde route.

In some cases, clathrin-independent endocytosis has been described to be linked to endosomes that are devoid of classical markers of the early endocytic pathway (Pelkmans et al., 2001; Nichols et al., 2001). The relationship between theses structures and the conventional EE has remained debatable (see Nichols, 2002, versus Sabharanjak et al., 2002).

Although the conventional EE is known to be a sorting station, the molecular mechanisms underlying the differential distribution of internalized molecules have not been studied in detail. COP-type coats are localized on the EE and implicated in sorting into the late endocytic pathway (Whitney et al., 1995; Aniento et al., 1996). The role of clathrin in the early endocytic pathway is more uncertain, even though ultrastructural imaging has clearly shown that it is present on this compartment (Stoorvogel et al., 1996; Mallard et al., 1998; Sachse et al., 2002). Clathrin may play a role in transferrin (Tf) recycling (Bennett et al., 2001; Wettey et al., 2002), possibly via perinuclear recycling endosomal (RE) tubules (van Dam and Stoorvogel, 2002). Several studies have suggested that clathrin coats containing the adaptor protein 1 (AP1) are involved in retrograde transport (Mallard et al., 1998; Meyer et al., 2000; Folsch et al., 2001; Crump et al., 2001; Valdivia et al., 2002).

EpsinR, one of the proteins that interacts with AP1, can also directly associate with clathrin (Kalthoff et al.,

*Correspondence: johannes@curie.fr
[6]These authors contributed equally to this work.
[7]Present address: Department of Oncology, University of Cambridge, c/o Hutchinson/MRC Cancer Research Centre, Hills Road, Cambridge CB2 2XZ, United Kingdom.

2002; Wasiak et al., 2002; Mills et al., 2003; Hirst et al., 2003). In addition, epsinR can bind to phosphatidyl inositol (PtdIns) lipids via its ENTH domain. Like AP1, epsinR has been localized to Golgi membranes and peripheral structures that are most likely endosomes (Mills et al., 2003; Hirst et al., 2003). However, this localization of epsinR does not depend on AP1 expression (Hirst et al., 2003). Unlike epsins involved in coated pit formation at the plasma membrane, epsinR is strongly enriched in clathrin-coated vesicles and stably associates with them (Mills et al., 2003).

In this study, we showed that the bacterial Shiga toxin can enter cells efficiently even when clathrin expression was inhibited. However, the retrograde transport of the Shiga toxin from EE/RE to the TGN was strongly reduced in these conditions. EpsinR was localized to endosomal membranes, and involved in the retrograde transport of exogenous Shiga toxin and endogenous TGN38/46 and mannose 6-phosphate receptor of 300 kDa (MPR300, also termed cation-independent MPR). Thus, our study establishes a role for epsinR/clathrin in retrograde sorting at the EE/RE-TGN interface.

Results

A Specific Clathrin Machinery on Endosomal Membranes of the Retrograde Route

The whole-mount technique developed by Stoorvogel and colleagues allows the in-depth inspection of cytoplasmic membranes by using an ultrastructural method (Stoorvogel et al., 1996). For the analysis of the retrograde route, horseradish peroxidase (HRP) was covalently linked to a variant of the Shiga toxin B-subunit (STxB) that was specifically constructed for site-directed chemical coupling (Haicheur et al., 2003). STxB is a nontoxic protein used as a bona fide retrograde transport marker (see Johannes, 2002, for a review). Immunofluorescence (Figure 1A) and immunoelectron microscopy (Figures 1B–1C) showed that HRP-coupled STxB was targeted normally to the TGN and Golgi cisternae. This confirms that the trafficking of STxB was not altered by chemical crosslinking.

We then used the whole-mount technique (Stoorvogel et al., 1996) to compare the intracellular distribution of STxB/HRP to that of Tf/HRP, which has previously been used for these studies. Both Tf/HRP and STxB/HRP were internalized by HeLa cells at 19.5°C, i.e., in conditions in which retrograde transport of STxB to the TGN/Golgi is inhibited (Mallard et al., 1998). Cells were prepared for whole-mount analysis and stained with anti-clathrin antibody. Membranes containing HRP-coupled ligands appeared dark due to the deposit of a polymerization product. Some Tf/HRP was detected in early endosomal vacuoles while most was found in tubules and in 60 nm clathrin-coated buds (Figure 1D, arrows), as described before (Stoorvogel et al., 1996). In contrast, large amounts of STxB/HRP were found in clathrin-containing early endosomal vacuoles (Figure 1E, arrowheads), endosomal tubules, and clathrin-coated buds of different size (arrows). Thus, although STxB and Tf colocalize extensively when examined by immunofluorescence microscopy (Mallard et al., 1998), both proteins appeared to be differentially enriched in subdomains of the early/recycling endosomal membrane system.

To address the function of clathrin in STxB trafficking, we designed an RNA interference (RNAi) tool that allowed us to inhibit clathrin heavy chain (CHC) expression to 20% of control levels (Figure 1F). We carried out whole-mount analysis of clathrin RNAi cells and mock-transfected cells after 15 min internalization of STxB/HRP at 37°C. This confirmed that clathrin was present on STxB-containing early/recycling endosomal buds of different sizes both at low temperatures (Figure 1E) and at 37°C (Figure 1G, left). Interestingly, even in CHC RNAi-treated cells, in which clathrin immunolabeling was almost lost, STxB/HRP still entered the endosomal system (Figure 1G, left). STxB-containing endosomal membranes were strongly labeled for the γ-adaptin subunit of the clathrin adaptor AP1, even in CHC RNAi-treated cells that were recognized via thick tubular elements in their cytoplasm (Figure 1G, middle). Similar observations were made for the clathrin, AP1, and PtdIns lipid binding protein epsinR (Kalthoff et al., 2002; Wasiak et al., 2002; Mills et al., 2003; Hirst et al., 2003), another candidate for recruiting clathrin to internal membranes (Figure 1G, right). These data clearly show that a specific clathrin machinery is present on STxB-containing early/recycling endosomal membranes.

Functional Analysis of Clathrin in the Retrograde Route

We first studied the effect of CHC RNAi treatment on the retrograde transport of STxB by using immunofluorescence. In control conditions, Tf was efficiently internalized and STxB was transported to the TGN (Figure 2A). In CHC RNAi-treated cells, the uptake of Tf was strongly inhibited (Figure 2B), as expected. In these conditions, STxB appeared in tubular and vesicular structures, instead of being concentrated in perinuclear Golgi membranes (Figure 2B). The tubular structures contained TfR (Figure 2C), suggesting that they were of early/recycling endosomal origin. The TfR cell surface signal was 8-fold stronger in these conditions than in control conditions, indicating that a significant fraction of the receptor was blocked at the plasma membrane (data not shown).

To characterize further the TfR-positive compartment in which STxB accumulated in CHC RNAi cells, we used three known early and recycling endosomal markers. The STxB-containing tubules were strongly labeled with anti-Rab11 antibody and with GFP-tagged Rab4 (Figures 2D–2E), clearly indicating that they belong to the recycling branch of the early endocytic pathway (Sonnichsen et al., 2000). Consistently, they were only lightly labeled by EEA1 (Figure 2F), a PtdIns(3)P binding protein that interacts with Rab5 on the EE (Simonsen et al., 1998).

Morphological analysis suggested that the transport of STxB to the TGN/Golgi, but not endocytosis into EE/RE, was inhibited in CHC RNAi-treated cells. We used quantitative biochemical techniques to test this hypothesis. When measuring endocytosis with biotinylated ligands (i.e., Tf or STxB), we observed that in CHC RNAi-treated cells, Tf uptake was inhibited (Figure 3A) to a similar extent to cells in which the clathrin gene was genetically inactivated (Wettey et al., 2002). In contrast to that of Tf, the endocytosis of STxB was only slightly affected in these conditions (Figure 3B). The

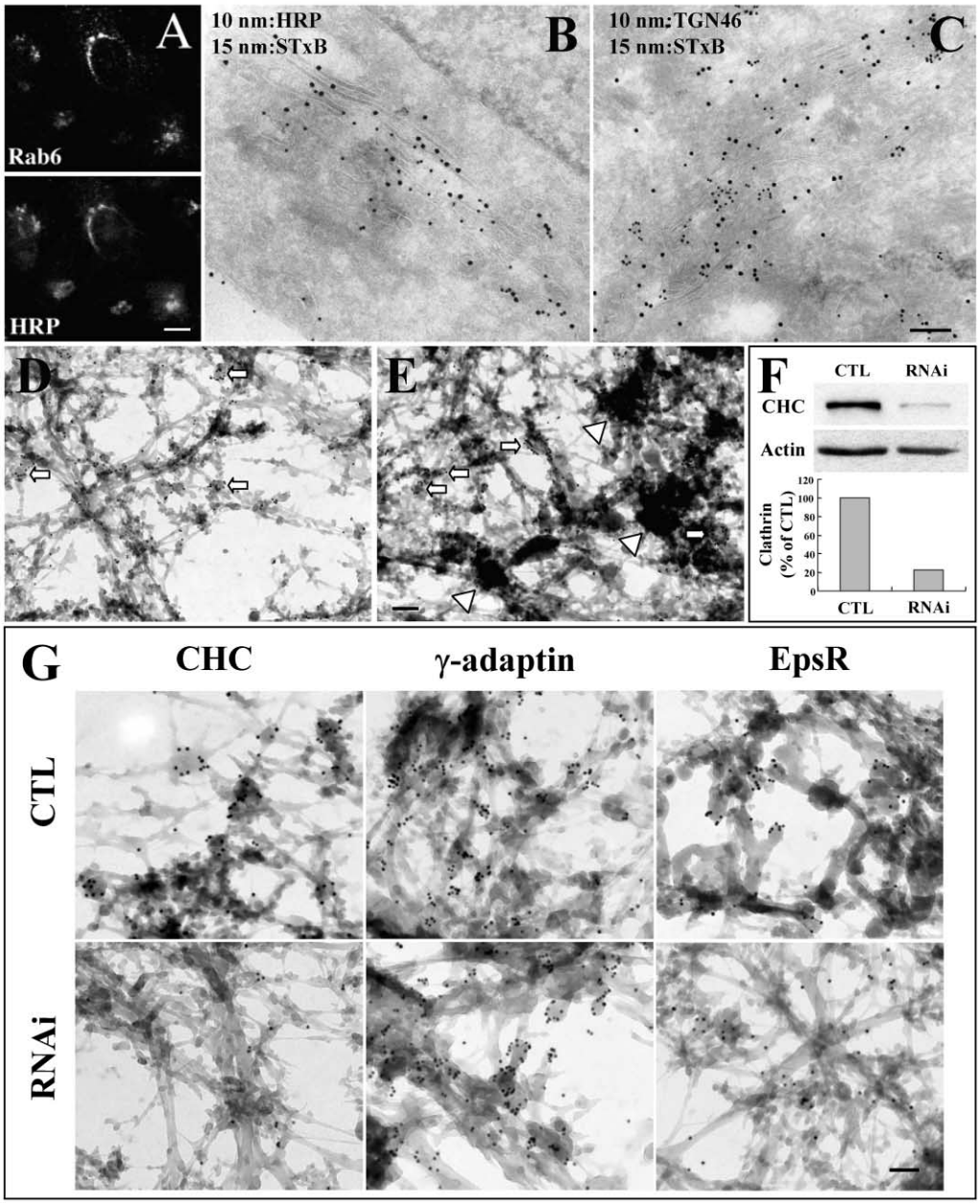

Figure 1. Analysis of the Clathrin Machinery on the STxB-Containing Early/Recycling Endosome

(A) HRP was chemically coupled to a STxB variant. The coupling product was bound to HeLa cells on ice and incubated for 45 min at 37°C before fixation and immunolabeling with antibodies against HRP and the Golgi/TGN marker Rab6. Note that when coupled to STxB, HRP efficiently accumulated in perinuclear Golgi membranes. Bar = 10 μm.

(B and C) STxB/HRP was internalized into HeLa cells for 45 min at 37°C. After cryosectioning, cells were fixed and immunolabeled. Note that HRP and STxB were readily detected in the TGN and in the cisternae of the Golgi apparatus. Bar = 100 nm.

(D and E) Whole-mount preparations with (D) Tf/HRP and (E) STxB/HRP internalized at 19.5°C. STxB-HRP- or Tf/HRP-containing membranes appear dark due to the formation of the DAB precipitate. Clathrin: 10 nm gold particles. Bar = 200 nm.

(F) RNAi efficiently down-modulated CHC expression in HeLa cells as detected by Western blot analysis. The histogram shows the mean of two determinations.

(G) Whole-mount analysis of clathrin (CHC), γ-adaptin, and epsinR (EpsR) localization on EE/RE of HeLa cells. STxB/HRP was internalized for 15 min at 37°C into mock-transfected (CTL) or CHC RNAi-transfected cells before sample preparation. Note that γ-adaptin and epsinR labeling on membranes could be detected independently of clathrin expression. Bar = 100 nm.

initial rate of STxB uptake and the plateau reached after about 20 min were largely unaltered compared to control cells transfected with the empty vector. These data confirm that STxB can enter cells even when clathrin-dependent endocytosis is severely impaired (Falguières et al., 2001; Nichols et al., 2001), meaning that it is possible to study its intracellular fate in these conditions.

Sulfation analysis was chosen to quantify retrograde transport of sulfation site-carrying STxB to the TGN (Johannes et al., 1997; Mallard et al., 1998, 2002). In CHC

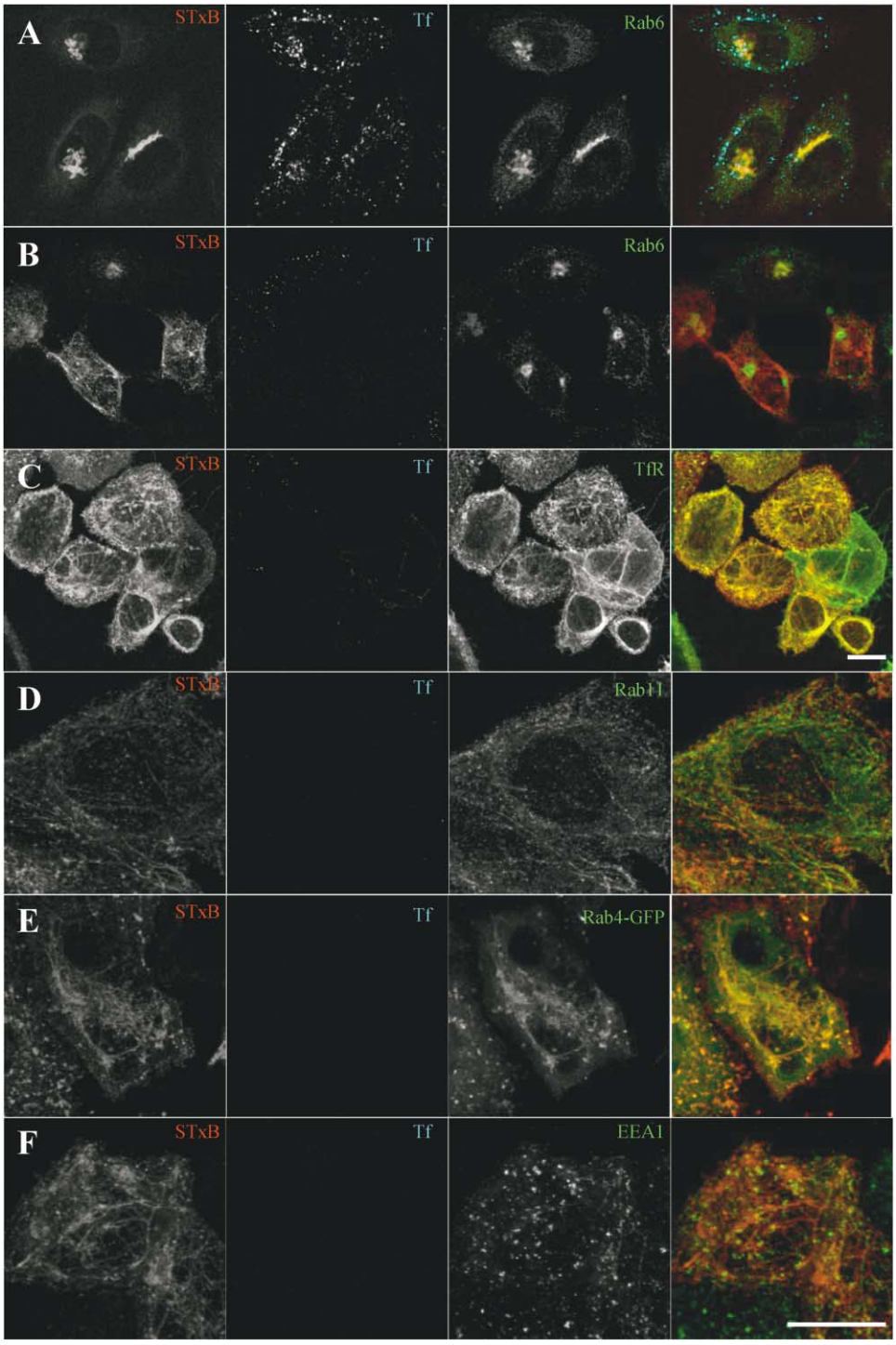

Figure 2. Immunofluorescence Analysis of Retrograde Transport of STxB in CHC RNAi HeLa Cells

(A–F) STxB was internalized for 45 min at 37°C by mock-transfected (A) or CHC RNAi-transfected (B–F) HeLa cells. During the last 15 min, Tf was added continuously and accumulated in EE/RE. In (E), the cells were also transfected with GFP-Rab4. The cells were then stained for the indicated markers. Bars = 10 μm.

RNAi cells, only a small fraction of sulfation site-carrying STxB was transported to the TGN (9% of that observed in control cells) (Figure 3C). A correlation was observed between the level of CHC expression and retrograde transport efficiency (data not shown). To confirm the effect of the CHC RNAi tool, we showed that the retrograde transport of STxB to the TGN was also strongly inhibited in cells expressing the CHC Hub fragment (Bennett et al., 2001) (Figure 3C), whereas STxB endocytosis again was unaffected in these conditions (data not shown).

To determine whether the observed effects of CHC depletion on the retrograde transport of STxB were direct or indirect, we used an assay that measures

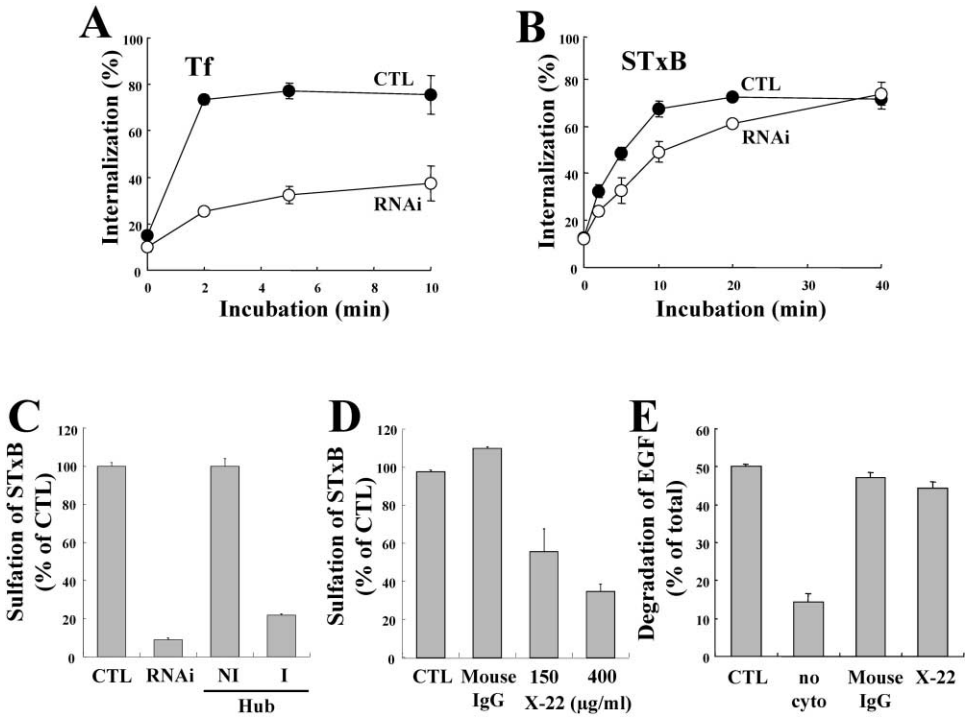

Figure 3. Biochemical Analysis of Endocytosis and Retrograde Transport under Clathrin Dysfunction Conditions

(A and B) HeLa cells were transfected with control plasmid (CTL) or RNAi plasmid against CHC (RNAi). The following experiments were done in the indicated conditions: (A) endocytosis of Tf; (B) endocytosis of STxB. Means of three independent experiments are shown. Note that in CHC RNAi-treated cells, Tf uptake was strongly inhibited, as opposed to STxB uptake.

(C) HeLa cells were transfected with empty vector (CTL) or CHC RNAi (RNAi). Alternatively, HeLa Hub cells were induced (I) or not (NI). Retrograde transport to the TGN (20 min at 37°C) was measured by sulfation analysis on intact cells.

(D) Permeabilized cell approach. STxB-$Sulf_2$ was internalized by HeLa cells at low temperatures. The cells were permeabilized and sulfation analysis was done in the presence of the indicated concentrations of X22 or 0.4 mg/ml control mouse IgGs.

(E) In the same experimental conditions as shown in (D), cytosol-dependent degradation of EGF was not inhibited by 0.4 mg/ml X22, demonstrating the specificity of the observed effect on retrograde transport. (C–E) Means of three to seven independent experiments.

EE/RE-to-TGN transport on permeabilized cells (Mallard et al., 2002). In this assay, STxB is internalized into the EE/RE of HeLa cells at low temperatures. The cells are then permeabilized and retrograde transport to the TGN measured in the presence or absence of exogenous molecules. The interfering anti-clathrin antibody X22 (Doxsey et al., 1987) had a strong dose-dependent inhibitory effect in this assay, whereas even the highest concentration of the control antibody had no effect (Figure 3D). STxB was not relocalized to the late endocytic pathway in the presence of X22, as indicated by the fact that STxB was not degraded over time (data not shown). As a further control, we showed that the degradation of epidermal growth factor (EGF) was not affected by the X22 antibody (Figure 3E) in identical experimental conditions to those used in Figure 3D. These observations demonstrate that clathrin directly and specifically controls retrograde transport at the EE/RE-TGN interface.

AP1 Is Not Required for the Retrograde Transport of STxB

Our previous data (Mallard et al., 1998) and the whole-mount analysis presented in this study (Figure 1G, middle) revealed the presence of the clathrin adaptor AP1 on STxB-containing early/recycling endosomal membranes. To test whether AP1 is required for the retrograde transport of STxB, we inhibited the expression of the AP1 subunit γ-adaptin by using RNAi. No effect was detected on STxB transport to the TGN in cells that expressed as little as 20% of endogenous γ-adaptin (Figure 4A), as shown by quantitative sulfation analysis (Figure 4B). To exclude the possibility that RNAi-mediated γ-adaptin downmodulation was not efficient enough to produce an effect on retrograde transport, we used fibroblasts from mice in which the μ1A subunit of AP1 had been genetically inactivated (Meyer et al., 2000). Again, no effect was detected on the retrograde transport of STxB to the TGN, as observed by immunofluorescence (Figure 4C). Transport in μ1A-positive control fibroblasts was identical (data not shown). In the permeabilized cell assay described above, concentrations of up to 3 mg/ml of the anti-γ-adaptin antibody 100/3 had no effect on retrograde transport to the TGN (data not shown). We concluded that AP1 could not be the only adaptor involved in clathrin-dependent transport intermediate formation on EE/RE.

The Clathrin Binding Protein epsinR Is Involved in Retrograde Transport to the TGN

The clathrin binding protein epsinR is localized on the STxB-containing endosome (Figure 1G, right). To address its function in retrograde transport, we overexpressed myc-tagged wild-type epsinR and analyzed

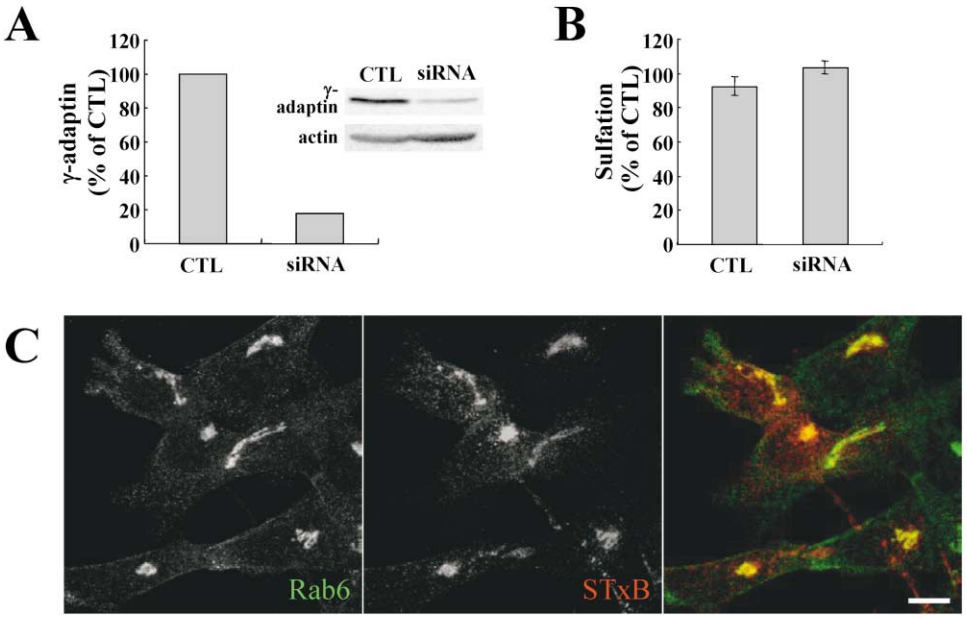

Figure 4. Analysis of the Role of AP1 in Retrograde Transport of STxB

(A) Expression of the AP1 subunit γ-adaptin was efficiently down-modulated using RNAi, as determined by Western blotting (see inset).

(B) In conditions of RNAi-mediated inhibition of γ-adaptin expression, retrograde transport was not inhibited, as assessed quantitatively using sulfation analysis on intact cells (20 min at 37°C).

(C) In fibroblasts from μ1A knockout mice, STxB was efficiently transported to the Golgi apparatus, labeled with Rab6. Bar = 10 μm.

STxB transport to the Golgi apparatus by immunofluorescence. This approach is based on the previous observation that overexpression of epsinR impairs procathepsin D trafficking (Mills et al., 2003). In cells that strongly expressed myc-epsinR, STxB could not be detected in perinuclear Golgi membranes labeled with the Golgi marker CTR433 (Figure 5A) or the TGN marker Rab6 (data not shown). STxB accumulated in membrane structures that contained the TfR (Figure 5B) or cointernalized Tf (see below), showing that they were of endosomal origin. Sulfation analysis of these cells confirmed that retrograde transport was indeed inhibited (data not shown).

The capacity of epsinR to bind the PtdIns lipids PtdIns(4)P and PtdIns(5)P via its ENTH domain (Kalthoff et al., 2002; Mills et al., 2003; Hirst et al., 2003) positions the molecule as a potential adaptor between membranes and clathrin. To understand the contribution of the different interactions to the observed overexpression phenotype, we used epsinR mutants with selectively affected binding to specific partners, as described previously (Mills et al., 2003). The D34G/R67L double mutation in the ENTH domain drastically reduces binding to PtdIns lipids (Mills et al., 2003). When the double mutant was expressed in HeLa cells, STxB transport to the TGN was inhibited (Figure 5C). The mutant phenotype was somewhat less robust, when compared to wild-type transfected cells, and the peripheral STxB-positive structures contained the TfR but were not decorated by the epsinR mutant (Figure 5C). This suggests that the mutant inhibits transport through interaction(s) with cytosolic partners. By analogy, an epsin1 lipid binding mutant inhibits Tf uptake by mislocalizing adaptors from the membrane (Ford et al., 2002).

To address this issue, we used mutations that interfere with AP1 or clathrin interactions. The D422R mutant shows a reduced binding affinity for clathrin, whereas its interaction with adaptors is not affected (Mills et al., 2003). The expression of this mutant inhibited STxB transport to the TGN and led to the accumulation of STxB in TfR-positive membranes that were also decorated by the mutant (Figure 5D). Combining the D34G/R67L mutations with D422R yielded a triple mutant with strongly reduced inhibitory activity on retrograde transport (Figure 5E). Similar observations were made with the D349R mutant, which is selectively deficient in adaptor binding but can still interact with clathrin (data not shown). These data suggest that the ENTH domain is necessary for localizing epsinR to endosomal membranes and that concomitant inactivation of lipid and clathrin or lipid and adaptor binding is required to release the inhibitory impact of myc-epsinR overexpression on retrograde transport.

To characterize further the endosomal TfR-positive structures that accumulate in myc-epsinR-expressing cells, we double labeled cells for endosomal markers. The early endosomal antigen EEA1 was clearly detected on myc-epsinR membranes (Figure 6A). Only partial overlap was observed with Rab11 (Figure 6B), a marker that is enriched in recycling endosomal membranes (Sheff et al., 1999), and GFP-Rab4 (Figure 6C). Thus, myc-epsinR membranes appear to be mostly of early endosomal origin. Strikingly, they were also strongly decorated with clathrin and the AP1 subunit γ-adaptin (Figures 6D–6E), in agreement with a role for epsinR as a coat-recruiting protein.

The effect of myc-epsinR overexpression was not restricted to the retrograde transport of STxB. Indeed,

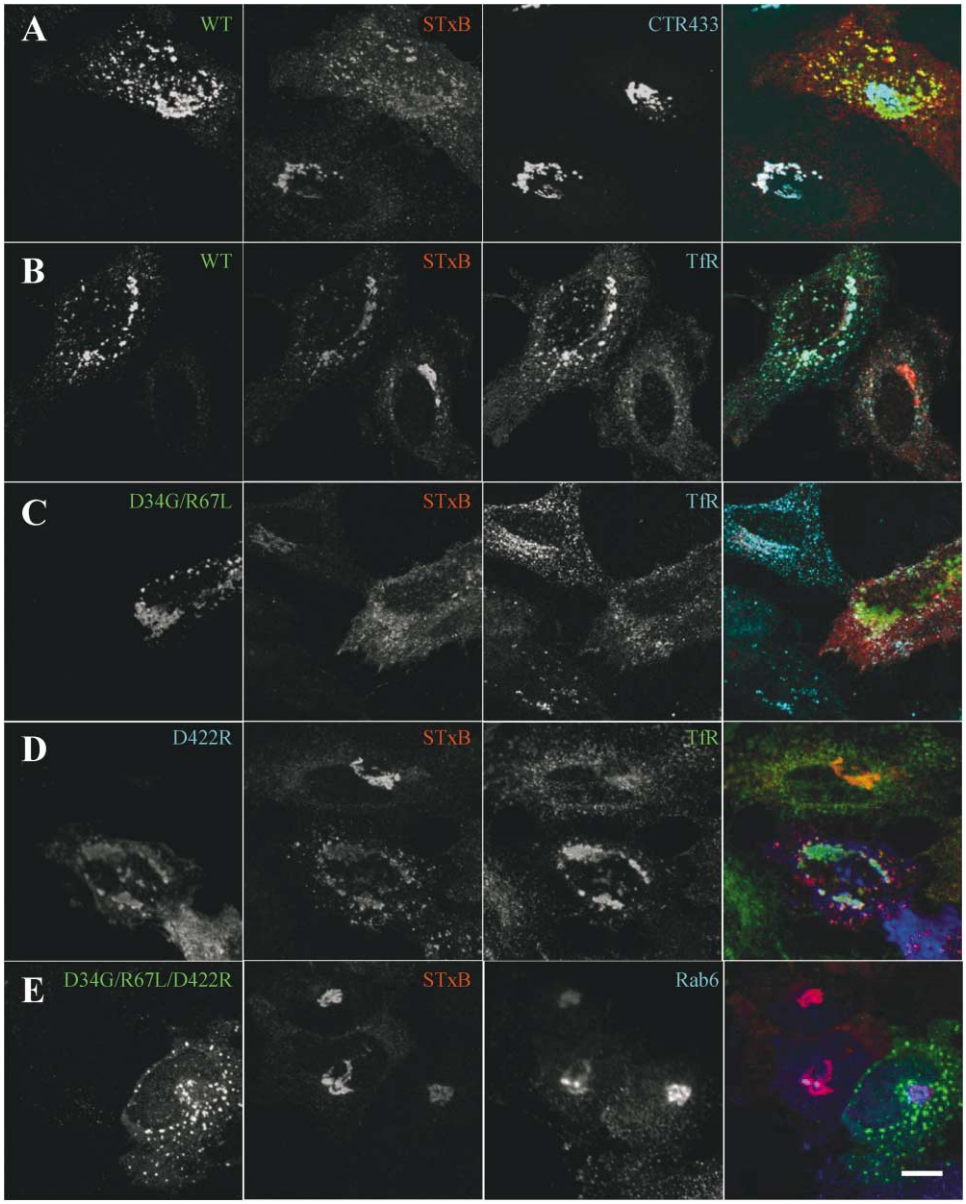

Figure 5. Morphological Analysis of epsinR Function in Retrograde Transport

In all conditions, HeLa cells were transfected for 1 day with the indicated myc-tagged epsinR mutants. STxB was internalized for 45 min at 37°C with these cells, before fixation and immunostaining, as indicated.

(A) In cells expressing myc-tagged epsinR, retrograde transport of STxB to the Golgi apparatus, labeled with CTR433, was inhibited.

(B) In these cells, STxB accumulated in membranes of early/recycling endosomal origin, labeled with TfR.

(C–E) Mutational analysis of epsinR interactions with membranes and coat components. (C) In cells expressing the ENTH domain double mutation, D34G/R67L, the transport of STxB to the Golgi apparatus was inhibited and STxB staining substantially overlapped with TfR staining. Unlike in cells overexpressing wild-type epsinR (see above), no mutant protein was detected on STxB-containing membranes. (D) In cells expressing the clathrin interaction-deficient D422R mutant, STxB did not reach the Golgi apparatus and accumulated on TfR-positive membranes that also were decorated by the D422R mutant. (E) When combined with the D34G/R67L double mutation, D422R was no longer inhibitory. Bar = 10 μm.

endogenous MPR300 and TGN46 were relocalized to myc-epsinR-containing endosomal membranes (Figures 6F and 6G). The labeling for endogenous TGN46 was lost in the most strongly myc-epsinR-expressing cells, similar to the apparent disappearance of the protein in CHC and γ-adaptin RNAi-treated cells (data not shown). Whether the protein is actually degraded in these conditions was not addressed directly. In antibody uptake experiments on rat TGN38-expressing HeLa cells, antibodies to TGN38 also accumulated in myc-epsinR membranes, whereas they were transported to the TGN in control conditions (data not shown).

Thus, proteins that depend on endosome-to-TGN transport for their intracellular localization, i.e., Shiga

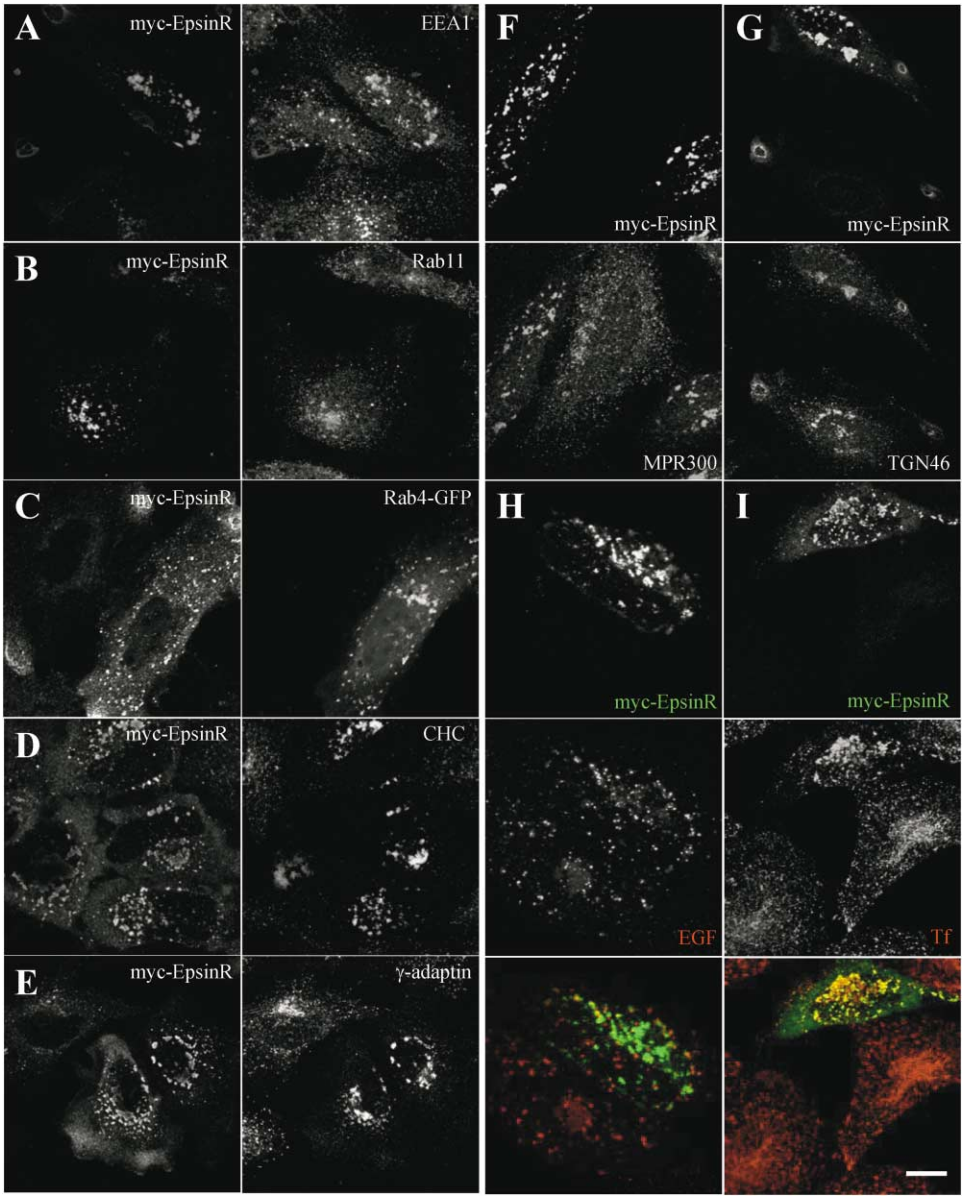

Figure 6. Characterization of Membranes Accumulating in Cells Overexpressing myc-epsinR

(A–E) HeLa cells were transfected with myc-epsinR for 24 hr and stained for the indicated markers. In (C), the cells were cotransfected with GFP-Rab4. In all conditions, myc-epsinR-positive membranes contained internalized STxB (data not shown). Note that myc-epsinR membranes are well recognized by anti-EEA1 antibody (A) and are strongly decorated with CHC (D) and the AP1 subunit γ-adaptin (E).

(F and G) Endogenous MPR300 and TGN46 relocalized from their steady-state localization at the TGN to myc-epsinR membranes.

(H and I) Fluorophore-labeled EGF (H) or Tf (I) were internalized by myc-epsinR-transfected HeLa cells for 40 min. The cells were then washed and fixed (H) or chased for 40 min (I) before fixation. Bar = 10 μm.

toxin, TGN38/46, and MPR300, accumulate in myc-epsinR membranes. Golgi markers that are not known to cycle, i.e., CTR433 and Rab6, were not relocalized. To explore the specificity of the transport block, fluorophore-labeled Tf and EGF were internalized by myc-epsinR-transfected and nontransfected cells. The distribution of EGF was similar in both conditions, and the fraction of the EGF labeling that was found in or close to myc-epsinR membranes was low (Figure 6H). Upon chase, EGF labeling disappeared in all conditions (data not shown). In agreement with these observations, we found that the endogenous EGF receptor was not relocalized to the myc-epsinR compartment (data not shown). When cells that had accumulated intracellular Tf were washed and then chased for 40 min, Tf labeling decreased in transfected and nontransfected cells, indicating that recycling was not inhibited. In the conditions that were used to detect the Tf remaining in these cells (Figure 6I), it became apparent that in nontransfected cells, Tf was located in short tubular membranes distributed

throughout the cell, characteristic of the RE in HeLa cells (Lin et al., 2002). In transfected cells, Tf was still in the myc-epsinR membranes and appeared to recycle from there. These morphological data suggest that neither access to the late endocytic pathway nor access to the recycling pathway is inhibited when epsinR function is perturbed. These conclusions were supported by quantitative biochemical techniques (see below).

The permeabilized cell assay showed that epsinR plays a direct role in transport from endosomal membranes to the TGN. A polyclonal antibody against full-length epsinR significantly inhibited retrograde transport, whereas the corresponding preimmune serum did not (Figure 7A). The recombinant clathrin/adaptor binding N3 fragment of epsinR (Mills et al., 2003) also displayed significant inhibitory activity in this assay when fused with glutathione-S-transferase or after cleavage, whereas GST had no effect (Figure 7A). The purified epsinR ENTH domain was not inhibitory at the concentrations used (up to 50 μM; data not shown).

To address whether epsinR is required for retrograde transport, we used siRNA to inhibit its expression. EpsinR expression was 60% lower in siRNA-treated cells than in control cells (Figure 7B, inset). Stronger reduction resulted in reduced growth (data not shown). Sulfation analysis (Figure 7B) showed that STxB transport to the TGN was inhibited in epsinR siRNA-treated cells (45% residual transport), strongly suggesting that epsinR is a necessary component of the clathrin machinery involved in retrograde sorting. Immunofluorescence studies showed that when compared to control cells in which STxB had efficiently accumulated in the Golgi (Figure 7C, left), STxB was still present in peripheral membranes in epsinR siRNA-treated cells where it colocalized with the TfR (Figure 7C, right).

We developed a method that allowed us to follow the retrograde transport of cellular proteins to the TGN by coupling specific antibodies to a sulfation site peptide. We found that the retrograde transport of antibodies to GFP-tagged MPR300 (Waguri et al., 2003) and to endogenous TGN46 was inhibited in epsinR siRNA-treated cells (Figures 7D–7E). This was expected for TGN38/46 as this protein uses the same molecular machinery as STxB to reach the TGN (Mallard et al., 2002). However, it was more surprising for MPR300 (see Discussion). Therefore, we showed that siRNAs to syntaxin 16, the heavy chain t-SNARE involved in EE/RE-to-TGN transport (Mallard et al., 2002), not only inhibited retrograde transport of STxB but also that of antibodies to MPR300 (Figure 7D). In contrast to the inhibition of retrograde trafficking of STxB, TGN46, and MPR300, degradation of EGF was barely affected in epsinR siRNA-treated cells (Figure 7F), and Tf recycling was not affected at all (Figure 7G). This suggests that epsinR plays a specific role in the clathrin-dependent retrograde transport of endogenous and exogenous proteins and lipids at the EE/RE-TGN interface.

Discussion

Using exogenous Shiga toxin as a marker for the retrograde route, we observed an unexpected sequence of clathrin-independent endocytosis followed by clathrin-dependent retrograde sorting at the level of the early/recycling endosome. Our data suggest that epsinR acts as a structural adaptor between lipids and clathrin on endosomal membranes, and that it is involved in the retrograde transport of Shiga toxin and the endogenous proteins TGN38/46 and MPR300 to the TGN.

Uncoupling of Clathrin-Independent and Clathrin-Dependent Transport Steps in the Retrograde Route

The molecular demonstration of the existence of clathrin-independent endocytosis raised the question of whether different internalization routes lead to the same EE and couple to the same intracellular pathways (Johannes and Lamaze, 2002). It appears possible that clathrin-independent endocytosis connects to intracellular routes, bypassing the conventional TfR-positive EE/RE reached by many markers of the endocytic clathrin pathway (Pelkmans et al., 2001; Nichols, 2002; Le and Nabi, 2003). Unlike the above-mentioned studies, our data show that in the retrograde route to the Golgi apparatus, the conventional EE/RE can be reached by clathrin-independent endocytosis. These observations are similar to those described by Pagano and colleagues on the cellular uptake of fluorophore-labeled sphingomyelin (Puri et al., 2001). Strikingly, the endosomal targeting of endogenous and exogenous markers to the retrograde route depends on clathrin function. Thus, Shiga toxin is recruited either into clathrin-coated or non-clathrin-coated structures depending on its molecular environment.

Earlier studies had suggested that clathrin located at the plasma membrane is involved in Shiga toxin endocytosis (Sandvig et al., 1989; but see also Falguières et al., 2001; Nichols et al., 2001). Our data do not contradict these findings. However, they show that when the clathrin pathway is blocked, Shiga toxin can use other endocytic routes, although the exact molecular nature of these routes remains to be established.

The role of clathrin/adaptors at the level of the EE/RE is a controversial matter (Stoorvogel et al., 1996; Mallard et al., 1998; Futter et al., 1998; Meyer et al., 2000; Bennett et al., 2001; Valdivia et al., 2002; Wettey et al., 2002; Deneka et al., 2003). Our study clearly shows that clathrin plays a functional role in retrograde transport. For endogenous cargo proteins of the retrograde route, such as TGN38/46 and VAMP4, coupling to the clathrin machinery may occur via AP1-interacting peptide signals in their cytosolic tails (Rapoport et al., 1998; Peden et al., 2001; Hinners et al., 2003; see below). Such direct interactions with elements of the cytosolic sorting machinery are not possible for Shiga toxin, which is associated with the exoplasmic leaflet. Shiga toxin may interact directly or indirectly via coclustering in membrane microdomains (Falguières et al., 2001) with trans-membrane domain proteins that are themselves targeted to the retrograde route. Alternatively, a specific lipid composition of the cytoplasmic leaflet of membrane microdomains that are implicated in Shiga toxin targeting to the retrograde route could allow the recruitment of clathrin, possibly via PtdIns lipid-binders such as epsinR.

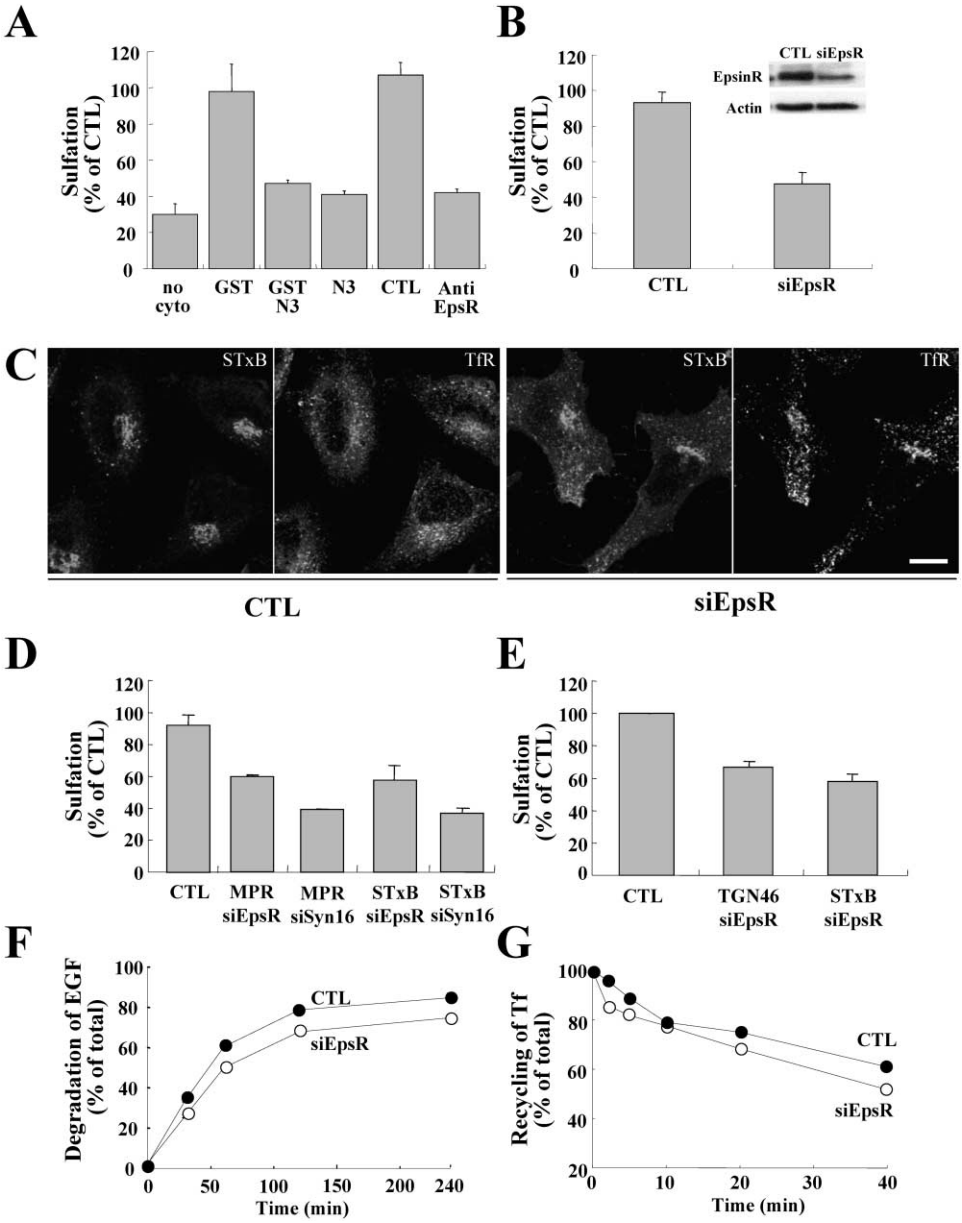

Figure 7. Biochemical Analysis of epsinR Function in Retrograde Transport

(A) Use of the permeabilized cell assay (see Figure 3D) with different epsinR-specific tools: GST-tagged N3 domain (residues 291–426; 1 mg/ml), GST (1 mg/ml), N3 domain (0.3 mg/ml), preimmune serum (CTL), and anti-epsinR serum Ra43 at 4-fold dilutions. As a control, the efficiency of retrograde transport in the absence of exogenous cytosol is shown. Means of three independent experiments.

(B) Use of siRNA to down-modulate epsinR expression. In siRNA-transfected cells, retrograde transport of STxB to the TGN (20 min at 37°C) was efficiently inhibited, as indicated by sulfation analysis. Means of five independent experiments. Inset: Western analysis of epsinR expression in mock-transfected (CTL) and epsinR siRNA-transfected cells (siEpsR).

(C) In epsinR siRNA-transfected cells (siEpsR, right), STxB was retained in peripheral TfR-positive membranes, whereas the protein was efficiently transported to the TGN/Golgi in control cells (CTL, left). Transfected cells were recognized using Ra43 anti-epsinR serum (data not shown). Bar = 10 μm.

(D) Retrograde transport to the TGN (60 min at 37°C) of sulfation site-tagged anti-GFP antibody (MPR) and of sulfation site-tagged STxB was inhibited in HeLa GFP-MPR300 cells in which epsinR (siEpsR) or syntaxin 16 (siSyn16) expression were down-modulated using specific siRNAs.

(E) Similar to (D), sulfation site-tagged anti-TGN46 antibody was used to show that retrograde transport (60 min at 37°C) of this marker was similarly inhibited to STxB in epsinR siRNA-transfected cells.

(F) Radiolabeled EGF was bound to mock-transfected (CTL) and epsinR siRNA-transfected (siEpsR) HeLa cells on ice. These were then shifted to 37°C for the indicated times and degradation of EGF was measured. Means of three independent experiments. Note that error bars are not visible under the symbols.

(G) Biotinylated Tf was internalized by HeLa cells for 40 min at 37°C. The cells were washed, and incubated at 37°C for the indicated times. Cell-associated Tf was determined in cells using ELISA. Means of two independent experiments.

A Specific Clathrin Machinery on Endosomal Membranes

We found that epsinR is a clathrin binding protein present on endosomal membranes where it functions in retrograde sorting of endogenous and exogenous proteins. We mutated the interactions with PtdIns lipids, clathrin, and clathrin adaptors to release the inhibition of retrograde transport that was observed upon overexpression of epsinR, suggesting that epsinR functions as a multivalent linker between membranes and coats.

Several ENTH domain proteins exist in yeast, including a potential epsinR ortholog (Duncan et al., 2003; Chidambaram et al., 2004). Deletion of these proteins results in distinct phenotypes at the TGN-endosome interface. Interestingly, a double deletion including the potential epsinR ortholog Ent3p and the ENTH protein Ent5p results in the secretion of precursor α factor (Duncan et al., 2003). This observation could be explained if the processing protease Kex2p failed to recycle from endosomes to the TGN to meet its substrate. However, other mutant combinations involving Ent3p result in altered vacuolar transport pathways (Duncan et al., 2003; Chidambaram et al., 2004), similar to the cathepsin D processing phenotype observed in mammalian cells that overexpress myc-epsinR (Mills et al., 2003).

The finding that retrograde sorting of MPR300 involves epsinR is intriguing in the light of recent data that showed that MPR300 was relocalized to peripheral cytoplasmic structures in cells in which PtdIns 4-kinase IIα was inactivated (Wang et al., 2003), suggesting that the endosomal retrieval of the receptor is deficient in these conditions. We also showed that retrograde trafficking of MPR300 depends on the TGN t-SNARE syntaxin 16, which we have previously shown to be involved in the retrograde route of Shiga toxin (Mallard et al., 2002). This surprising result is in agreement with other recent studies (Medigeshi and Schu, 2003; Umeda et al., 2003; Lin et al., 2003), and suggests that MPR300 uses two different pathways to cycle between endosomes and the TGN: the late endosome-to-TGN pathway described by Pfeffer and colleagues (Lombardi et al., 1993), and the EE/RE-to-TGN pathway that we and others have identified (Johannes, 2002).

Unlike epsinR, AP1 is not required for retrograde transport of Shiga toxin, despite its localization on the Shiga toxin-containing EE/RE (Mallard et al., 1998, and this study). This is in contrast to earlier studies on AP1 function in the retrograde transport of endogenous proteins (Meyer et al., 2000; Folsch et al., 2001; Crump et al., 2001; Valdivia et al., 2002). Notably, the resialylation of MPR46 (also termed cation-dependent MPR) is inhibited in cells in which the μ1A subunit of AP1 is genetically inactivated, strongly suggesting that the return of MPR46 from endosomes to the TGN is severely impaired in these cells (Meyer et al., 2000). One explanation for this apparent discrepancy is that AP1 might not be essential for the formation of retrograde transport intermediates, similarly to the recent observation that AP2 has cargo-specific functions at the plasma membrane (Motley et al., 2003). The role of these adaptors may be to recruit trans-membrane proteins to budding sites, similar to the role of arrestins in G protein-coupled receptor internalization (Goodman et al., 1996). Whether epsinR is another cargo adaptor, as suggested recently (Chidambaram et al., 2004), and whether epsinR has other functions in transport intermediate formation, remains to be established.

In summary, our study reveals the existence of an epsinR/clathrin coat on endosomes involved in retrograde sorting. Future work will focus on the functional interaction of epsinR with other endosomal clathrin adaptors and cargo proteins, and on the lipid organization that underlies epsinR/clathrin-dependent retrograde sorting.

Experimental Procedures

Cells

HeLa cells stably transfected with GFP-CI-MPR (B. Hoflack, Dresden, Germany) and HeLa-T7Hub cells (F. Brodsky, UC San Francisco) were grown in the presence of 0.5 mg/ml G418, or 0.2 mg/ml G418 and 0.4 mg/ml hygromycin (Life Technologies), respectively. The expression of the Hub domain was induced with 2 μg/ml of doxycycline (Sigma). μ1A knockout fibroblasts were grown as described previously (Meyer et al., 2000). μ1A knockout fibroblasts stably expressing Gb3 synthase were obtained after selection in the presence of G418 (2 mg/ml).

Recombinant Proteins and Antibodies

STxB-Cy3, Tf-Cy5, and STxB-Sulf$_2$, monoclonal (13C4) and polyclonal anti-STxB, and polyclonal anti-epsinR antibodies were obtained as described previously (Johannes et al., 1997; Mallard et al., 1998; Mills et al., 2003). Polyclonal anti-Tf antibodies, polyclonal anti-Rab11 antibodies, and monoclonal antibodies against clathrin (X-22) were kindly provided by E. Smythe (Sheffield, United Kingdom), Bruno Goud (Institut Curie, Paris), and F. Brodsky (UC San Francisco), respectively. The polyclonal anti-Rab6 antibody (Santa Cruz), the monoclonal antibodies against GFP (Roche), TfR (H68.4, Zymed), clathrin (BD Bioscience), and anti-CD71, Sigma, actin (AC-74, Sigma), TGN46 (Serotec), and FITC-, Cy3-, Cy5-, or AMCA-coupled secondary antibodies (Jackson Immunoresearch) were purchased from the indicated suppliers.

RNA Interference

Synthetic oligonucleotides (64-mers) containing the human clathrin (AAGACCAAUUUCAGCAGACAG) or γ-adaptin (AAACCGAAUUAA GAAAGUGGU) target sequences for cloning into pSUPER were synthesized (MWG-Biotech, Germany), annealed, and ligated into the pSUPER construct, as described previously (Brummelkamp et al., 2002). HeLa cells (8×10^6) were transfected with pSUPER vectors by electroporation using OptiMIX according to the manufacturer's instructions (Ozyme). Cells were split after 2 days and used 4 days after transfection. CHC siRNA-transfected cells were not apoptotic when used for experiments, as shown by intact nuclear morphology and the absence of annexin V labeling (data not shown). At later time points, cell survival was compromised, as described previously (Wettey et al., 2002). For epsinR, synthetic siRNA duplexes were purchased from Dharmacon (Lafayette, CO). The sequence was: AAGUGCCAGAGAACACAUUUA (Hirst et al., 2003). HeLa cells were transfected using oligofectamine (Invitrogen) according to the manufacturer's recommendations. Experiments were carried out 3 days after transfection.

Immunoelectron Microscopy and Whole-Mount Analysis

STxB-Cys (Haicheur et al., 2003) was conjugated to horseradish peroxidase by chemical coupling with the heterobifunctional crosslinker Sulfo-m-maleimidobenzoyl-N-hydroxysuccinimide ester (Sulfo-MBS, Pierce). Cryosectioning and immunoelectron microscopy were done as described previously (Johannes et al., 1997). In the case of mAbs, a rabbit anti-mouse linker antibody was used (DAKO-PATTS AB, Älvsjö, Sweden).

For whole-mount analysis, HeLa cells were cultured directly on electron microscopy grids (José Delville Technology), and STxB/HRP or Tf/HRP (Jackson Immunoresearch) was internalized after binding on ice, as described in the figure legends. The cells were then put on ice again and incubated in DAB-containing buffer to fix

HRP-containing compartments with DAB-polymer (Stoorvogel et al., 1996). In brief, cells were washed and incubated on ice in freshly prepared DAB buffer (1.5 mg/ml DAB, 70 mM NaCl, 50 mM ascorbic acid, 20 mM HEPES [pH 7.0], adjusted to 300 mosM with NaCl, and supplemented with 0.02% H_2O_2). After HRP-mediated crosslinking, soluble cytosolic proteins were removed by permeabilizing the cells on ice with 0.5 mg/ml saponin in PBS containing 1 mM EGTA, 0.5 mM $MgCl_2$. Cells were fixed for with 1% paraformaldehyde in PBS, and free reactive aldehyde groups were blocked by incubation in PBS, 20 mM glycine. The immunolabeling was done in blocking buffer (PBS, 0.5 mg/ml saponin, 20 mM glycine, 0.1% cold water fish gelatin, and 0.02% NaN_3). Labeling was performed using 10 nm Protein A colloidal gold particles. Monoclonal antibodies were detected using rabbit anti-mouse Ig as an intermediate step. After immunolabeling and fixation, the cells were extensively washed with H_2O, dehydrated in ethanol, and critical point dried. The grids were examined using a Philips CN120 transmission electron microscope.

Endocytosis and Recycling Assay

STxB and human diferric transferrin were biotinylated using NHS-SS-Biotin (Pierce). Endocytosis was measured as described previously (Mallard and Johannes, 2002) with the following modifications. Serum-starved cells were detached from plates with 2 mM EDTA in PBS and incubated in the presence of 1 μM biotin-STxB and 200 nM biotin-Tf for 30 min on ice. After washing, cells were incubated at 30°C for the indicated times (1.5×10^5 cells per data point). The biotin on cell surface-exposed STxB or transferrin was cleaved by subsequent treatment with 100 mM nonmembrane permeable reducing agent sodium 2-mercaptoethanesulfonic acid (MESNA) on ice for 20 min. After washing, excess MESNA was quenched with 150 mM iodoacetamide for 20 min. Cells were lysed in blocking buffer (10 mM Tris [pH 7.4], 1 mM EDTA, 50 mM NaCl, 0.2% BSA, 0.1% SDS, and 1% Triton X-100) before loading on ELISA plates coated either with anti-Tf or anti-STxB antibody (13C4). Biotinylated STxB or transferrin was detected using streptavidin-HRP (Roche). For the transferrin recycling experiments, biotinylated Tf was internalized into HeLa cells for 40 min at 37°C. After washing on ice, the cells were incubated at 37°C for the indicated times. The cells were directly lysed in blocking buffer, and Tf was quantified by ELISA.

Transfection and Immunofluoresence

HeLa cells were transfected with myc-tagged epsinR and mutants using FUGENE reagent according to the manufacturer's instructions (Roche). Immunofluorescence experiments were carried out 24 hr after transfection, as described previously (Johannes et al., 1997). In Figure 2, Tf was not stripped before fixation.

Retrograde Transport and EGF Degradation Assays on Intact Cells and in SLO-Permeabilized HeLa Cells

Anti-GFP (Roche) and anti-TGN46 (Serotec) antibodies were conjugated to a peptide carrying a tandem sulfation site. The experimental procedure will be described elsewhere. Sulfation analysis on intact or SLO-permeabilized cells was carried out as described previously (Mallard et al., 2002). Sulfation of endogenous proteins was used to normalize data obtained in various conditions. To measure degradation of EGF on intact cells, ^{125}I-EGF (Amersham) was bound to cells on ice. These were then shifted to 37°C for the indicated times. TCA-precipitable counts were determined in the culture medium and cell lysate. To measure EGF degradation on SLO permeabilized cells, ^{125}I-EGF was internalized at 19.5°C, as described in the permeabilized cell retrograde transport protocol (Mallard et al., 2002). After permeabilization and incubation at 37°C for 30 min, TCA-precipitable counts were determined in the conditions indicated in the figure legends.

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