

The retromer complex and clathrin define an early endosomal retrograde exit site

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Summary

Previous studies have indicated a role for clathrin, the clathrin adaptors AP1 and epsinR, and the retromer complex in retrograde sorting from early/recycling endosomes to the trans Golgi network (TGN). However, it has remained unclear whether these protein machineries function on the same or parallel pathways. We show here that clathrin and the retromer subunit Vps26 colocalize at the ultrastructural level on early/recycling endosomes containing Shiga toxin B-subunit, a well-studied retrograde transport cargo. As previously described for clathrin, we find that interfering with Vps26 expression inhibits retrograde transport of the Shiga toxin B-subunit to the TGN. Under these conditions, endosomal tubules that take the Shiga toxin B-subunit out of transferrin-containing early/recycling endosomes appear to be stabilized. This situation differs from that previously described for low-temperature incubation and clathrin-depletion conditions

under which Shiga toxin B-subunit labeling was found to overlap with that of the transferrin receptor. In addition, we find that the Shiga toxin B-subunit and the transferrin receptor accumulate close to multivesicular endosomes in clathrin-depleted cells, suggesting that clathrin initiates retrograde sorting on vacuolar early endosomes, and that retromer is then required to process retrograde tubules. Our findings thus establish a role for the retromer complex in retrograde transport of the B-subunit of Shiga toxin, and strongly suggest that clathrin and retromer function in consecutive retrograde sorting steps on early endosomes.

Supplementary material available online at

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Key words: Retrograde transport, Retromer, Shiga toxin, Endosome, Golgi, Clathrin

Introduction

Shiga toxin is a bacterial protein toxin that is produced by *Shigella dysenteriae* (O'Brien et al., 1992) and is highly related to verotoxins (or Shiga-like toxins) from enterohemorrhagic strains of *Escherichia coli*. These toxins all have an AB₅ subunit structure. The A-subunit has N-glycanase activity that catalyzes the depurination of ribosomal 28S RNA, causing inhibition of protein biosynthesis. To reach its intracellular target, the A-subunit must be transported to the cytosol. This function is carried out by the B-subunit (i.e. STxB in the case of Shiga toxin), which the A-subunit binds non-covalently (Johannes and Decaudin, 2005; Sandvig and van Deurs, 2005). STxB assembles as a homopentamer of B-fragments that are arranged around a central pore, giving the complex a doughnut-like structure. STxB binds with high affinity to the cellular toxin receptor, glycosphingolipid globotriaosyl ceramide (Gb3 or CD 77). Following receptor binding, Shiga toxin is internalized to early/recycling endosomes (EE/RE), and then directly transported to the trans Golgi network (TGN), bypassing the late endocytic pathway (Mallard et al., 1998). After reaching the endoplasmic reticulum (Sandvig et al., 1992), the A-subunit is translocated to the cytosol where it inactivates ribosomes (Lord et al., 2005).

Some elements of the molecular machinery involved in retrograde transport of Shiga toxin have already been

identified. At all levels of the retrograde route, Shiga toxin is associated with detergent-resistant membranes (Falguieres et al., 2001). Passage from EE/RE to the TGN is blocked by a dominant-negative mutant of dynamin (Lauvrak et al., 2004), and requires clathrin (Lauvrak et al., 2004; Saint-Pol et al., 2004) and the clathrin adaptor epsinR (Saint-Pol et al., 2004) that binds phosphatidylinositol (4)-phosphate (Mills et al., 2003). The clathrin adaptor AP1 is localized on Shiga-toxin-containing EE/RE (Mallard et al., 1998), but is not required for toxin transport to the TGN (Saint-Pol et al., 2004), at variance with its documented function in retrograde trafficking of some transmembrane proteins (Crump et al., 2001; Folsch et al., 2001; Meyer et al., 2000; Valdivia et al., 2002). Two soluble N-ethylmaleimide-sensitive factor attachment protein receptor complexes formed around syntaxin 16 and syntaxin 5 (Amessou et al., 2007; Mallard et al., 2002; Tai et al., 2004), GTPases of the Rab family (del Nery et al., 2006; Mallard et al., 2002; Wilcke et al., 2000), and tethering factors (Lu et al., 2004; Yoshino et al., 2005) also play crucial roles in retrograde transport of Shiga toxin. How these machineries function together is not yet fully understood.

Another factor involved in retrograde transport between endosomes and the TGN is the retromer complex, which in the yeast *Saccharomyces cerevisiae* is composed of the five subunits Vps5p, Vps17p, Vps26p, Vps29p and Vps35p

(Seaman et al., 1998). The orthologs of these subunits in mammals are known as sorting nexin-1 (SNX1), SNX2, Vps26, Vps29 and Vps35 (reviewed in Bonifacino and Rojas, 2006; Carlton et al., 2005; Seaman, 2005). The mammalian retromer complex is localized to early endosomes and mediates endosome-to-TGN transport of the mannose 6-phosphate receptors (MPRs) (Arighi et al., 2004; Carlton et al., 2004; Seaman, 2004). The retromer complex is organized into two subcomplexes (Arighi et al., 2004; Rojas et al., 2007). SNX1 and SNX2 are sorting nexins that mediate localization to highly curved EE/RE membranes. Both proteins contain phagocyte oxidase (phox) homology domains that bind phosphatidylinositol (3)-phosphate and phosphatidylinositol (3,5)-bisphosphate, and a Bin-amphiphysin-Rvs (BAR) domain that acts as a membrane curvature sensor (Gallop and McMahon, 2005). The subcomplex composed of Vps26, Vps29 and Vps35 is important for cargo selection. In association with Vps29, Vps35 binds particular cargos, such as the cation-independent MPR (Arighi et al., 2004). Vps35 also interacts with SNX1, thus linking the two retromer subcomplexes. By interacting with both SNX1 and Vps35, Vps26 improves the stability of the retromer complex (Haft et al., 2000).

In this study, we have analyzed the function of retromer in endosome-to-TGN transport of Shiga toxin. We find that the retromer subunits Vps26 and Vps29 localize to STxB-containing membrane tubules that emanate from transferrin receptor (TfR)-positive EE/RE. In Vps26 small interfering RNA (siRNA)-treated cells, retrograde endosome-to-TGN transport of STxB is impaired. Immunofluorescence and immunoelectron analysis revealed that in these cells, STxB accumulates in structures that are juxtaposed, but not superposed with TfR containing EE/RE. Live cell fluorescence imaging shows that these structures have tubular morphologies. This phenotype differs from the one previously described for clathrin RNA interference (RNAi) conditions (Saint-Pol et al., 2004), in which case STxB accumulates in close superposition with TfR on endosomal membranes. Our data suggest that retromer and clathrin define a TGN-bound exit site emanating from early endosomes.

Results

Retromer and clathrin partially colocalize on STxB-positive endosomes

A previously described adaptation of the whole-mount transmission electron microscopy technique pioneered by Stoorvogel and colleagues (Stoorvogel et al., 1996) allows visualization of the Shiga toxin containing endosome on non-sectioned cells (Saint-Pol et al., 2004). For this, a variant of Shiga toxin B-subunit (STxB) that was specifically constructed for site-directed chemical coupling, termed STxB/Cys (Amessou et al., 2006), is covalently linked to horseradish peroxidase (HRP). Immunofluorescence and immunoelectron microscopy showed that the trafficking of STxB is not altered by this procedure (Saint-Pol et al., 2004). STxB-HRP is internalized into HeLa cells at 19.5°C, a temperature that inhibits retrograde transport to the TGN, and causes accumulation of STxB in EE/RE (Mallard et al., 1998). A horseradish peroxidase (HRP)-mediated reaction can then be used to stabilize STxB-HRP-containing membranes on non-fixed cells, which appear electron-dense due to the deposition

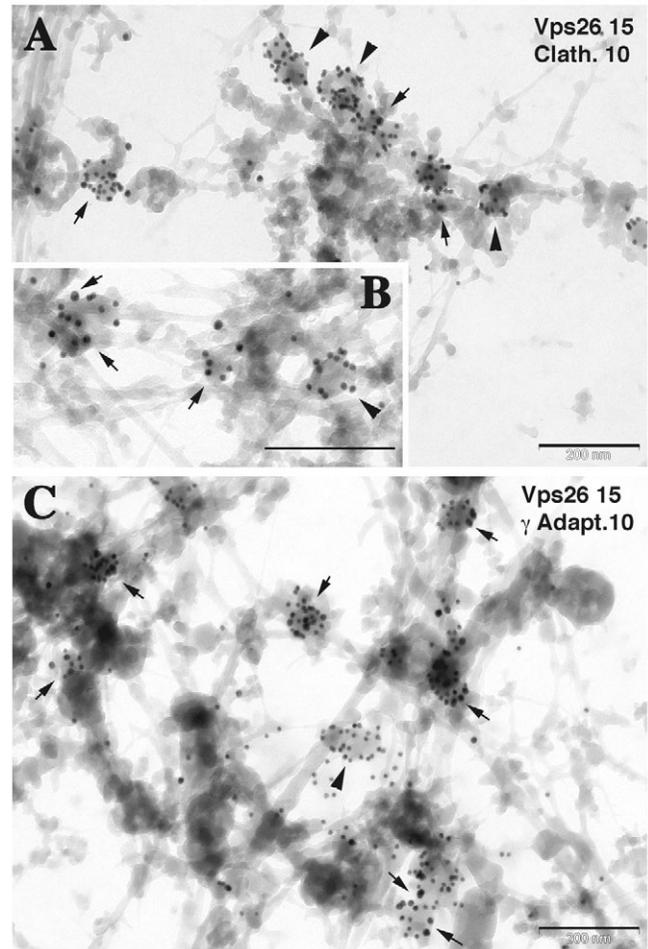


Fig. 1. (A-C) Clathrin, AP1 and retromer localization on STxB-containing EE/RE. STxB-HRP was bound to HeLa cells kept on ice that were then incubated for 45 minutes at 19.5°C. Cells were prepared for whole-mount analysis, followed by immunogold-labeling with antibodies against (A,B) clathrin or (C) the AP1 subunit γ -adaptin (10-nm gold particles), and in all cases to Vps26 (15-nm gold particles). STxB-HRP-containing membranes appeared electron-dense owing to the polymerization product. Note that some structures are double-labeled for clathrin-AP1 and Vps26 (arrows), whereas others are stained for clathrin or γ -adaptin only (arrowheads). Bars, 200 nm.

of a polymerization product. After cytosol wash-out, cells are fixed and immunolabeled.

As previously reported, the whole-mount analysis revealed the presence of STxB on tubular and vacuolar endosomal structures (Saint-Pol et al., 2004). These STxB-containing endosomes were also labeled by antibodies against the retromer subunit Vps26 (Fig. 1A-C). In most cases, Vps26-positive structures are also labeled with antibodies against clathrin (Fig. 1A,B) or the endosomal clathrin adaptor γ -adaptin (Fig. 1C); however, endosomal elements that were only decorated by one of the markers were also observed. These observations thus demonstrated the presence of the retromer subunit Vps26 on the Shiga-toxin-containing early endosome, which led to the hypothesis that retromer plays a role in the

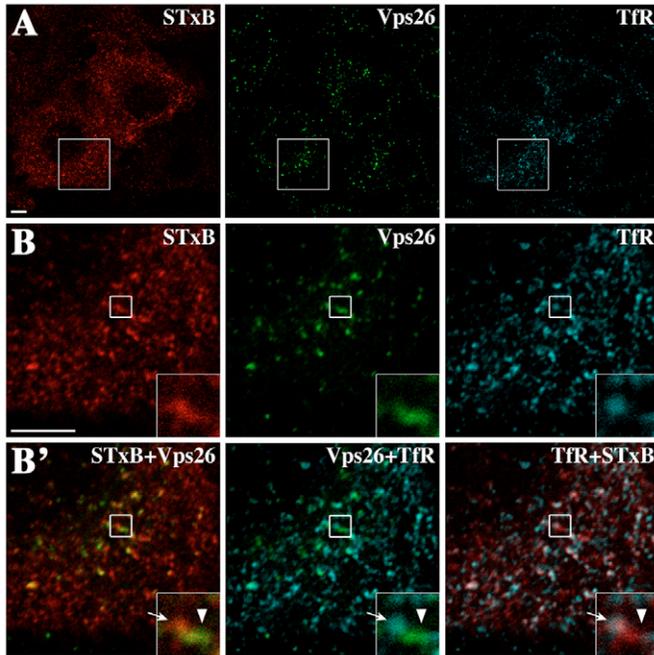


Fig. 2. Retromer protein Vps26 on STxB-containing endosomes. (A) Cy3-STxB was bound to HeLa cells kept on ice that were then transferred to 37°C for 10 minutes, fixed and labeled for the indicated proteins. Bar, 10 μ m. (B) Magnification of boxed areas in A. Insets show detailed view of an STxB-containing tubular structure. (B') Pair-wise overlays of images shown in B. Note that TfR overlaps with vacuolar parts of STxB structures (arrows), whereas Vps26 overlaps with tubular parts (arrowheads). (B,B') Bar, 5 μ m.

retrograde transport of Shiga toxin from early endosomes to the TGN and that it cooperates with clathrin in this process. This hypothesis is addressed in the following sections.

STxB exits TfR-containing EE/RE in tubular transport intermediates

The presence of Vps26 on STxB-containing endosomal structures was confirmed by immunofluorescence (Fig. 2A). After 10 minutes of uptake into cells, many STxB-positive structures were found to overlap with Vps26- and TfR-containing endosomes. Detailed analysis revealed that the vacuolar parts of the STxB-positive structures co-stained with TfR, whereas the tubular parts co-stained with Vps26.

This differential distribution was analyzed in further detail by live cell imaging. In one series of experiments, fluorescently labeled STxB and transferrin (Tf) were accumulated in EE/RE by incubation of cells for 60 minutes at 19.5°C. The cells were shifted to 37°C and immediately imaged. STxB and Tf were found to colocalize to a large population of vesicles (Fig. 3A). Tubules emanating from the bigger vesicles contained only STxB (Fig. 3A and supplementary material Movies 1 and 2), or only Tf (Fig. S1 and supplementary material Movies 1 and 3). In another set of experiments we observed that fluorescently labeled STxB also colocalized with Vps29 on a large population of vesicles (Fig. 3B). In addition, STxB was present in highly dynamic tubules that were also positive for Vps29 (Fig. 3B and supplementary material Movies 4 and 5). From

these experiments we concluded that STxB segregates from Tf upon exit from early endosomes on retromer-containing tubules.

Retrograde transport of STxB is impaired in Vps26 siRNA-treated cells

To analyze whether Vps26 is involved in mediating endosome-to-TGN transport of STxB, we used sulfation analysis under control and Vps26-depletion conditions. This assay is based upon the use of an STxB variant carrying a tandem sulfation site termed STxB-Sulf₂ (Amessou et al., 2006; Mallard et al., 1998; Mallard and Johannes, 2003). Upon arrival at the TGN, sulfotransferase catalyzes the transfer of radiolabeled inorganic sulfate onto the recognition sequence, thus allowing the quantification of endosome-to-TGN transport of STxB-Sulf₂ by autoradiography.

We designed three different siRNAs against Vps26 that all reduced Vps26 levels, albeit with different efficacies (Fig. 4A). Under all conditions, we observed an inhibition of retrograde transport that correlated with the decrease of Vps26 levels (Fig. 4B). To further confirm the specificity of the observed effect, we performed dose-response experiments with siRNA number 3 (see Materials and Methods), which was the most efficient siRNA used at depleting Vps26. siRNA concentrations between 2 and 200 nM were used, and Vps26 expression levels were quantified by western blotting with tubulin as a loading control (Fig. 4A). In Fig. 4C, the efficiency of retrograde transport is represented as a function of Vps26 depletion. At Vps26 expression levels below 25%, cells started to detach from the dish; these conditions were not considered. Linear regression analysis of the results revealed a correlation coefficient of 0.715, i.e. the greater the decrease of Vps26 levels, the less efficient retrograde transport of STxB. Statistical analysis showed that the two variables (Vps26 depletion and retrograde STxB transport to the TGN) are correlated with a probability higher than 99%. These biochemical data indicated that depletion of Vps26 inhibits retrograde transport of STxB to the TGN.

Early endosomal retrograde exit sites can be visualized in Vps26-depleted cells

Immunofluorescence and immunoelectron microscopy was performed to determine in which compartment STxB accumulated in Vps26-depleted cells. STxB was bound to control and Vps26-depleted cells on ice. After washing, cells were incubated for 45 minutes at 37°C. In control cells, STxB accumulated in a perinuclear compartment, in colocalization with the medial Golgi marker CTR433 (Fig. 5A), as described previously (Johannes et al., 1997; Mallard et al., 1998). RNAi cells were recognized by reduced Vps26 staining (Fig. 5B). In these cells, STxB was no longer detectably transported to the Golgi complex, but appeared in peripheral structures (Fig. 5).

To further characterize the compartment in which STxB accumulated in Vps26-depleted cells, these were double-labeled for endosome-specific markers. It has previously been shown that upon downregulation of clathrin expression, STxB perfectly overlapped at the photonic level of resolution with TfR-positive EE/RE structures (Saint-Pol et al., 2004). Strikingly, we found here that in Vps26-depleted cells, labeling for STxB and TfR did not overlap, but were found in closely juxtaposed structures (Fig. 6A,A'). A shift pattern similar to

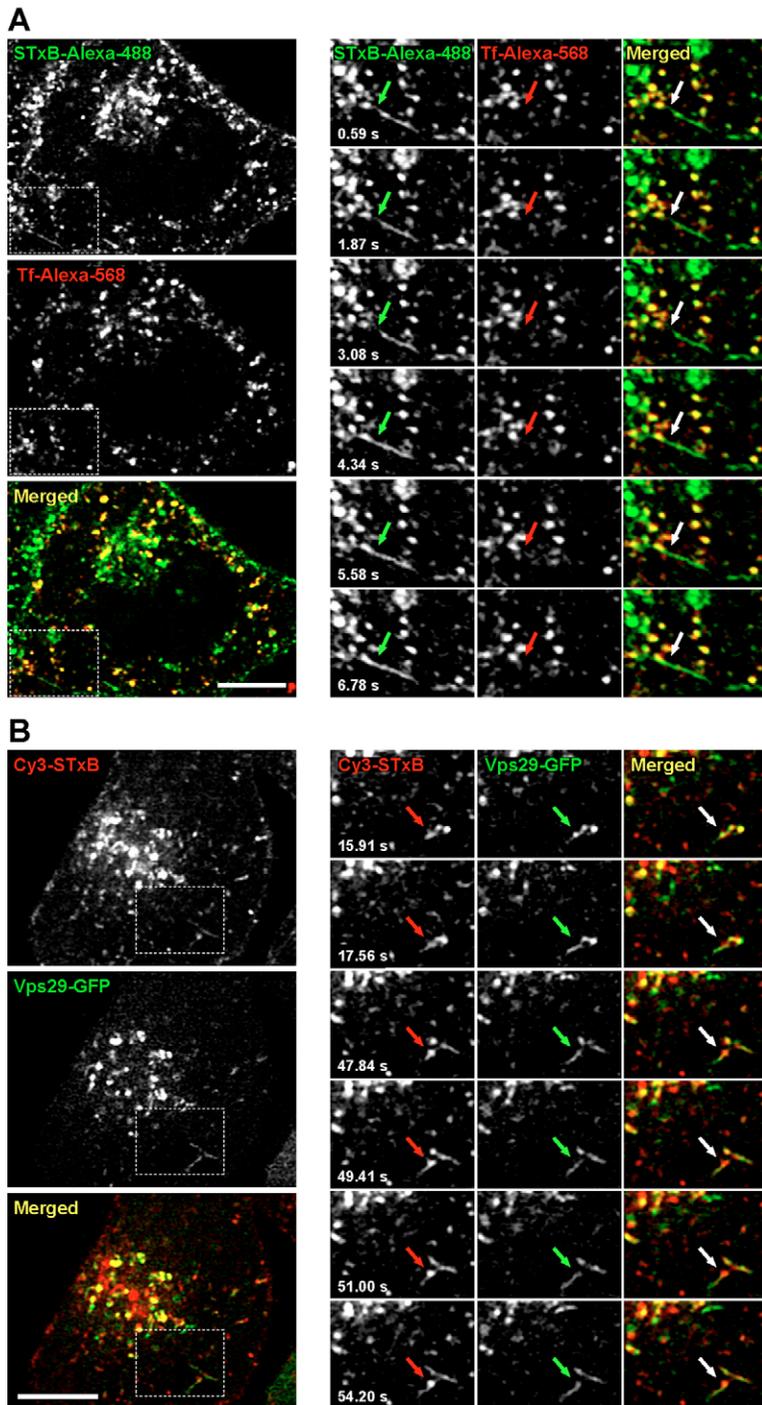


Fig. 3. Live cell imaging of retrograde STxB sorting on Tf-containing EE/RE. (A) HeLa cells were incubated with Alexa Fluor-488-labeled STxB (green) and Alexa Fluor-568-labeled human Tf (red) for 60 minutes at 19.5°C. After transferring cells to 37°C, they were immediately imaged by time-lapse microscopy. Shown is one cell with STxB and Tf accumulated in early endosomes. Merging images indicate overlapping localization (yellow). Images of the right panels are magnifications of the boxed areas on the left. The series show an STxB-containing tubule moving away from an early endosome (green arrows), while Tf remains associated only with the endosome (red arrows). (B) HeLa cells transiently expressing Vps29-GFP (green) were incubated with Cy3-labeled STxB (red) for 60 minutes at 19.5°C. After transferring cells to 37°C, they were imaged by time-lapse microscopy. Images of the right panels are magnifications of the boxed areas on the left. The series show an STxB-containing tubule moving away from an early endosome (red arrows) and Vps29-GFP associated to the same tubules (green arrows). Time after the start of imaging is shown in seconds. Bars, 10 μ m.

block protocol (see Fig. 1). We could previously demonstrate that, at the photonic level of resolution, STxB- and TfR-specific labelings superpose perfectly in EE/RE membranes under low-temperature incubation conditions (Mallard et al., 1998). We found here that a similar overlap between STxB and TfR could be detected in Vps26-depleted cells at 19.5°C (Fig. 6C,C',C''). These data strongly suggest that STxB and TfR are in the same endosomal compartment, and that Vps26 function is required for the physical separation of subdomains with which both proteins are associated.

Immunoelectron microscopy lent further support to this hypothesis. After a 45-minute incubation of HeLa cells at 37°C, internalized STxB strongly accumulated in TGN/Golgi membranes (Fig. 7A), as described previously (Mallard et al., 1998). By contrast, in Vps26 siRNA-treated cells, Golgi cisternae were almost devoid of STxB, and STxB was found in peripheral structures (Fig. 7B). Double labeling for STxB and TfR showed that both proteins accumulated in juxtaposed areas, with very little overlap (Fig. 7C).

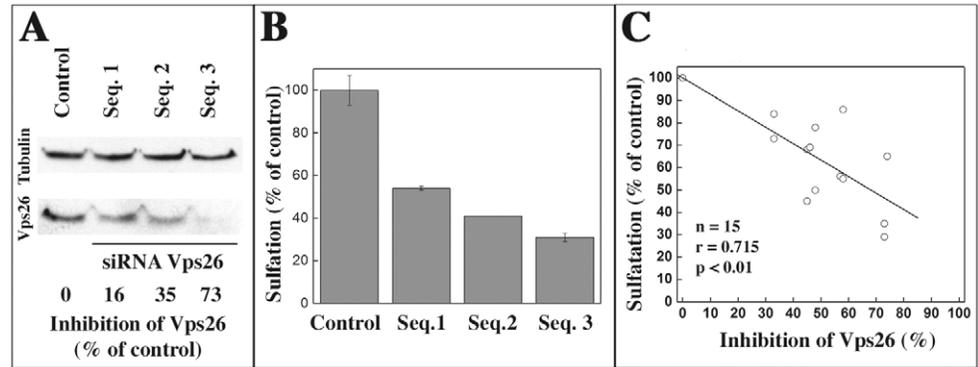
The situation was different in clathrin-depleted cells (Fig. 7D,E). Here, STxB and TfR localized in the same structures. In some cases, they were associated with tubular extensions from vacuolar endosomes mostly devoid of internal vesicles, which probably represented early endosomes (Fig. 7D). In many cases, both labelings were found close to multivesicular endosomes and were preferentially associated with the limiting membrane (Fig. 7E), in contrast to previous studies that failed to detect STxB on these structures in control cells (Mallard et al., 1998).

We conclude that STxB depends on clathrin (Saint-Pol et al., 2004) and retromer (this study) for efficient retrograde sorting. The presence of STxB on multivesicular endosomes in clathrin-depleted cells suggests that, when an early step of retrograde sorting on vacuolar endosomes is inhibited, STxB gets mislocalized to late endocytic compartments. Upon

that of TfR was observed upon double labeling Vps26-depleted cells that had internalized STxB with antibody to EEA1 (Fig. 6B,B'), with the difference that, in this case, fewer structures exhibited the shift-phenotype (Fig. 6B'').

The juxtaposition of STxB-positive and TfR- (or EEA1)-positive membranes in Vps26 siRNA-treated cells could mean that both proteins are localized to two early endosomal subcompartments that have failed to fuse or that they are in two subdomains of the same compartment that have failed to separate. To address this question, we used the temperature-

Fig. 4. Sulfation analysis of retrograde transport in Vps26 siRNA-treated cells. (A,B) Western blot (A) and sulfation analysis (B) performed on HeLa cells transfected with siRNAs targeting Vps26. Three different siRNA sequences (Seq.1, Seq.2, Seq.3) have been used, which all induced the downregulation of Vps26 expression. Loss of Vps26 expression correlated with inhibition of Shiga toxin transport to TGN/Golgi membranes. Results are given as the mean \pm s.e.m. of three determinations. (C) Dose-response experiment with siRNA number 3. A correlation was observed between the expression levels of Vps26 (as determined by western blotting) and retrograde Shiga toxin trafficking (as determined by sulfation analysis). Linear-regression analysis shows that both variables are linked with a probability higher than 99%.



inactivation of retromer function, STxB partly segregates from TfR-positive membranes, suggesting that retromer is involved in the processing of post-early endosomal retrograde trafficking structures.

Post-early endosomal retrograde tubules are stabilized under Vps26 RNAi conditions

Live cell imaging was again used to analyze in further detail the morphology of STxB-containing structures that accumulate in Vps26-depleted cells. STxB was incubated alone or together with Tf for 45 minutes with control, mock-transfected or Vps26-depleted cells. In control or mock-transfected cells, STxB strongly accumulated in perinuclear Golgi structures (see supplementary material Movie 6). By contrast, in Vps26-depleted cells the perinuclear accumulation was almost non-existent, and STxB often appeared in tubular structures (Fig. 8A and supplementary material Movie 6).

Life cell analysis in Vps26-depleted cells showed that the peripherally accumulated STxB was present in endosomal domains in which it only partially colocalized with Tf (Fig. 8B,B' and supplementary material Movies 7 and 8). Tubules emanating from these domains were exclusively labeled for STxB (Fig. 8B'' and supplementary material Movies 7 and 8). In conclusion, it appeared that the STxB-containing tubules

that emanate from TfR-containing early endosomes under control conditions cannot be processed under Vps26-depletion conditions.

Discussion

Early and recycling endosomes play a crucial role in the sorting of proteins and lipids to different destinations within cells. Retrograde sorting to TGN/Golgi membranes has only recently been discovered (Ghosh et al., 1998; Mallard et al., 1998) and remains a poorly explored cellular process. We here show that retromer is required for efficient retrograde transport of STxB. Our data indicates that low-temperature incubation or interfering with clathrin function (Saint-Pol et al., 2004) blocks retrograde STxB trafficking upstream of the site of function of retromer, suggesting that clathrin and retromer act in a sequential manner.

The retromer complex has previously been shown to bind to cytosolic MPR tails and to be required for efficient retrograde MPR transport to the TGN (Arighi et al., 2004; Carlton et al., 2004; Seaman, 2004). The cytosolic tails of MPRs have further interaction motifs that mediate binding to the clathrin adaptor AP1, and evidence was provided for a role of AP1 in retrograde transport of MPRs (Meyer et al., 2000) and of other transmembrane proteins (Crump et al., 2001; Folsch et al.,

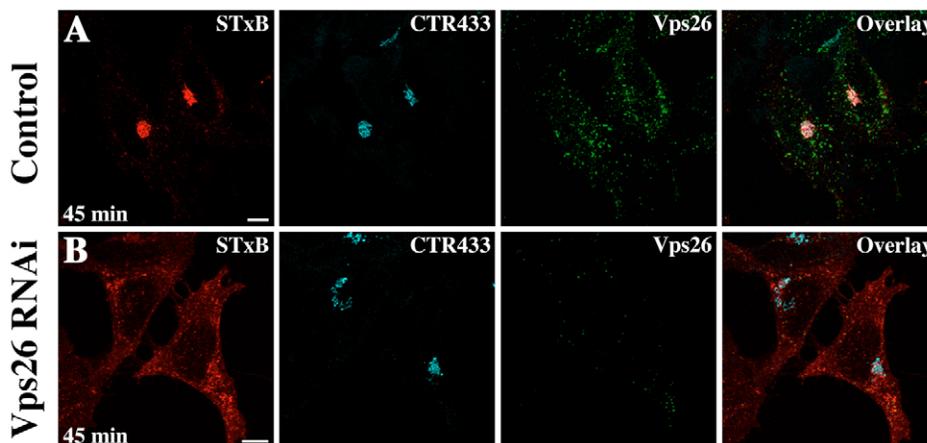


Fig. 5. Immunofluorescence analysis of STxB transport in Vps26 siRNA-treated cells. (A,B) Cy3-labeled STxB (red) was incubated for 45 minutes at 37°C with (A) mock-transfected or (B) Vps26 siRNA-transfected cells. After fixation, cells were stained for Vps26 (green) and CTR 433 (blue), a marker of the Golgi apparatus. In control cells, STxB accumulated in Golgi membranes, whereas it failed to do so in Vps26 siRNA-treated cells. There, it accumulated in peripheral structures. Bars, 10 μ m.

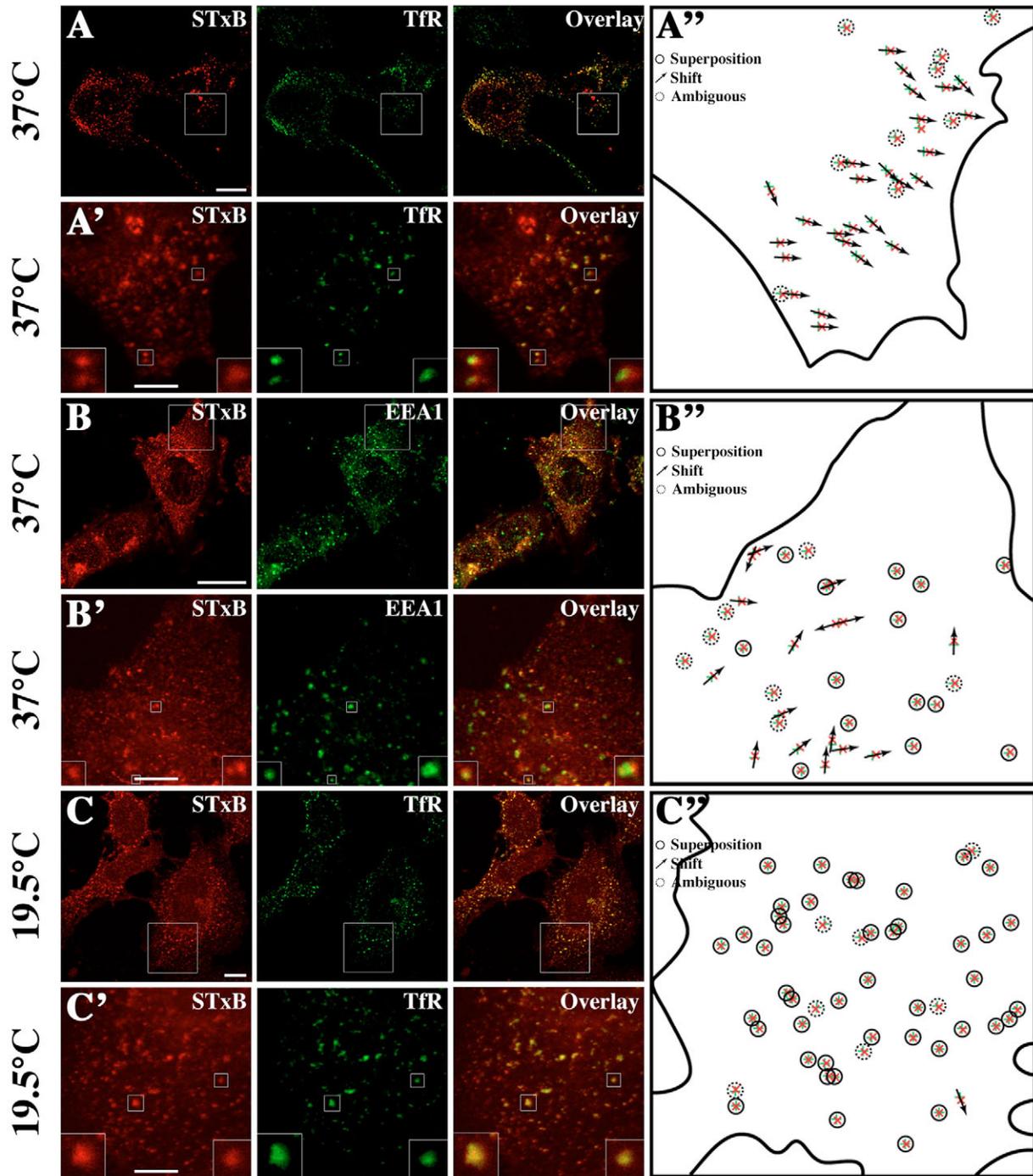


Fig. 6. (A-C) Endosomal STxB accumulation in Vps26 siRNA-treated cells. Cy3-STxB (red) was incubated with Vps26-depleted cells for 45 minutes at (A,B) 37°C or (C) 19.5°C. After fixation, cells were labeled for Vps26 (not shown) and Tfr or EEA1 (green), as indicated. (A'-C') Magnified views of boxed areas of A-C. Additional magnification views are shown as insets in A', B', C'. (A''-C'') The orientation of the shifts between STxB and Tfr at 37°C (A''), STxB and EEA1 at 37°C (B''), and STxB and Tfr at 19.5°C (C'') are shown for the boxed areas in A-C, respectively. Centers of STxB-positive structures are marked 'x' (red), centers of Tfr or EEA1 positive structures are marked '+' (green). Colocalized structures are encircled, structures with shifts are marked by arrows. Dotted circles indicate ambiguous situations. Note that at 37°C, STxB-positive structures are neighboring to Tfr- or EEA1-positive structures, whereas at 19.5°C, exact superposition is observed for STxB and Tfr. Bars, 10 μ m (A-C) and 5 μ m (A'-C').

2001; Valdivia et al., 2002). In another study, interfering with the putative clathrin adaptor epsinR was also found to affect MPR transport from endosomes to the TGN (Saint-Pol et al.,

2004). For STxB, efficient retrograde sorting depends on clathrin (Lauvrak et al., 2004; Saint-Pol et al., 2004) and epsinR, but not on AP1 (Saint-Pol et al., 2004). Of note, these

studies addressed trafficking between EE/RE and the TGN, different from the late-endosome-to-TGN pathway described previously (Krise et al., 2000). The question arises of how the activities of clathrin and retromer are articulated at the endosome-TGN interface. That this might actually be the case is suggested by recent proteomics data in which the loss of clathrin-coated vesicles from a highly enriched membrane fraction was paralleled by a loss of retromer from the very same preparation (Borner et al., 2006).

In principle, these protein complexes could function in parallel or sequential transport steps. However, we consider the parallel-pathway hypothesis less likely. First, in cells whose Vps26 or clathrin expression is highly reduced by RNAi, retrograde transport of STxB is almost completely inhibited (Saint-Pol et al., 2004) (and this study). If parallel pathways existed, we would expect to be confronted with partial inhibition under such conditions. Second, the colocalization of Vps26 and clathrin (and the clathrin adaptor AP1), as shown in this study, also argues against the parallel-pathway hypothesis.

A number of observations favor the sequential-transport-step hypothesis. Under low-temperature-incubation conditions, STxB- and TfR-specific labeling overlap perfectly at the light-microscopy resolution limit, independently of Vps26 (this study) or clathrin (Saint-Pol et al., 2004) expression. A temperature shift to 37°C has different effects in each of these conditions. In the case of clathrin depletion, STxB and TfR still remain in perfect overlap (Saint-Pol et al., 2004) (and this study). By contrast, in Vps26-depleted cells, the specific staining patterns for both markers shift, which suggests an accumulation in distinct subdomains of the same compartment that fail to separate (this study). Indeed, ultrastructural analysis shows STxB and TfR in separate domains, and our live cell imaging analysis reveals that STxB labels tubular structures that emanate from the TfR-positive EE/RE and appear to be stabilized in Vps26-depleted cells.

It has remained unclear on which part exactly of the early endosomal pathway retrograde sorting occurs. Indeed, the presence of retrograde transport cargoes in recycling endosomes has been taken as an indication for their function in this process (Ghosh et al., 1998; Wilcke et al., 2000). However, it was also reported that STxB accumulated in early-endosomal antigen-1 (EEA1)-positive membranes when retrograde transport was inhibited by overexpression of epsinR (Saint-Pol et al., 2004), suggesting that the early endosome is also competent in retrograde sorting. Our current observation that, STxB – instead of being transported to TGN/Golgi membranes – accumulates in juxtaposition to EEA1-positive membranes upon inhibition of Vps26 function, further confirms this contention. Another argument in favor of retrograde sorting from the early endosome stems from our finding that STxB gets mislocalized to multivesicular endosomes in clathrin-depleted cells, suggesting a retrieval defect from the maturing early endosome. It has also been described that retromer can be found on tubular profiles that emanate from vacuolar early endosomes, which often exhibit flat coats that resemble bilayered clathrin lattices (Arighi et al., 2004); these data also put retromer in the vicinity of the early endosome.

Based on our findings and those described in the literature, we suggest the following model (Fig. 9): Clathrin is required on the maturing early-endosome to induce curvature changes

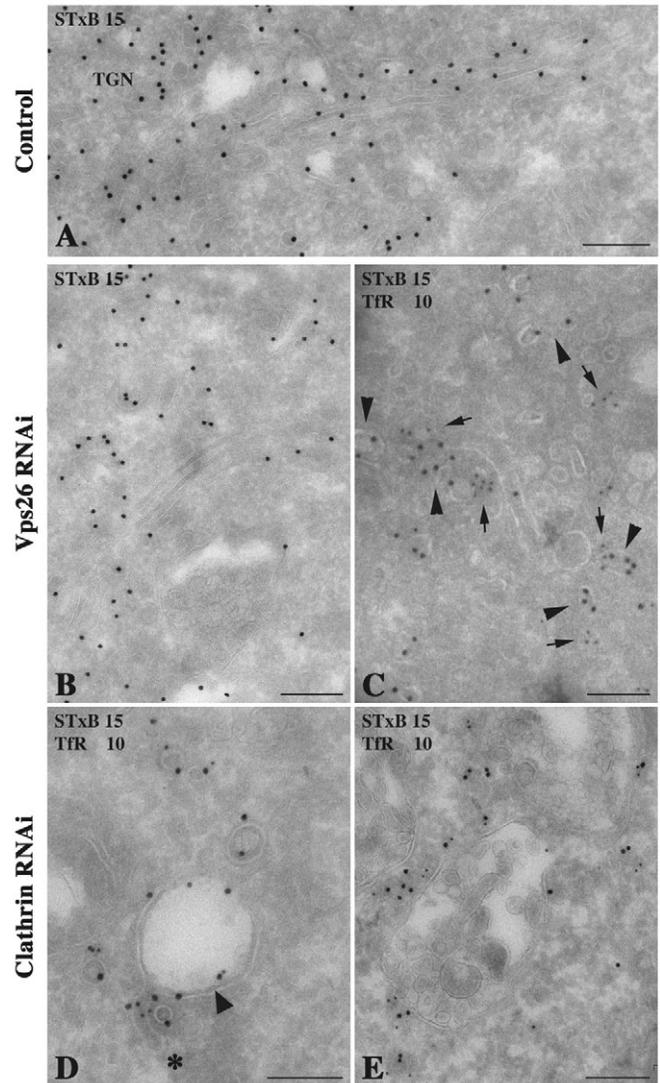


Fig. 7. Ultrastructural localization of STxB in Vps26 and clathrin RNAi conditions. (A–E) STxB was incubated for 45 minutes at 37°C with (A) mock transfected cells, (B,C) Vps26-depleted and (D,E) clathrin-depleted cells. After fixation and cryosectioning, preparations were labeled with anti-STxB antibody (A–E, 15-nm gold particles) and with anti-TfR antibody (C–E, 10-nm gold particles). In C, arrows indicate clusters of TfR-specific labeling, arrowheads indicate clusters of STxB-specific labeling. In D, the star indicates a tubule in which both TfR and STxB can be detected, arrowhead indicates the vacuolar endosome from which the tubule emanates. Note that in Vps26-depleted cells, STxB is found throughout the cytoplasm, whereas it is often localized close to multivesicular endosomes in clathrin-depleted cells. Bars, 200 nm.

that lead to retrograde tubule formation (step 1). The retromer complex would then function in a processing event leading to further trafficking to TGN/Golgi membranes (step 2). In clathrin-depleted cells (Fig. 9B), the curvature-inducing step 1 cannot function and STxB remains associated with TfR-positive membranes on tubular recycling structures, and also on maturing multivesicular bodies. By contrast, under Vps26-depletion conditions (Fig. 9C), retrograde tubules still form but

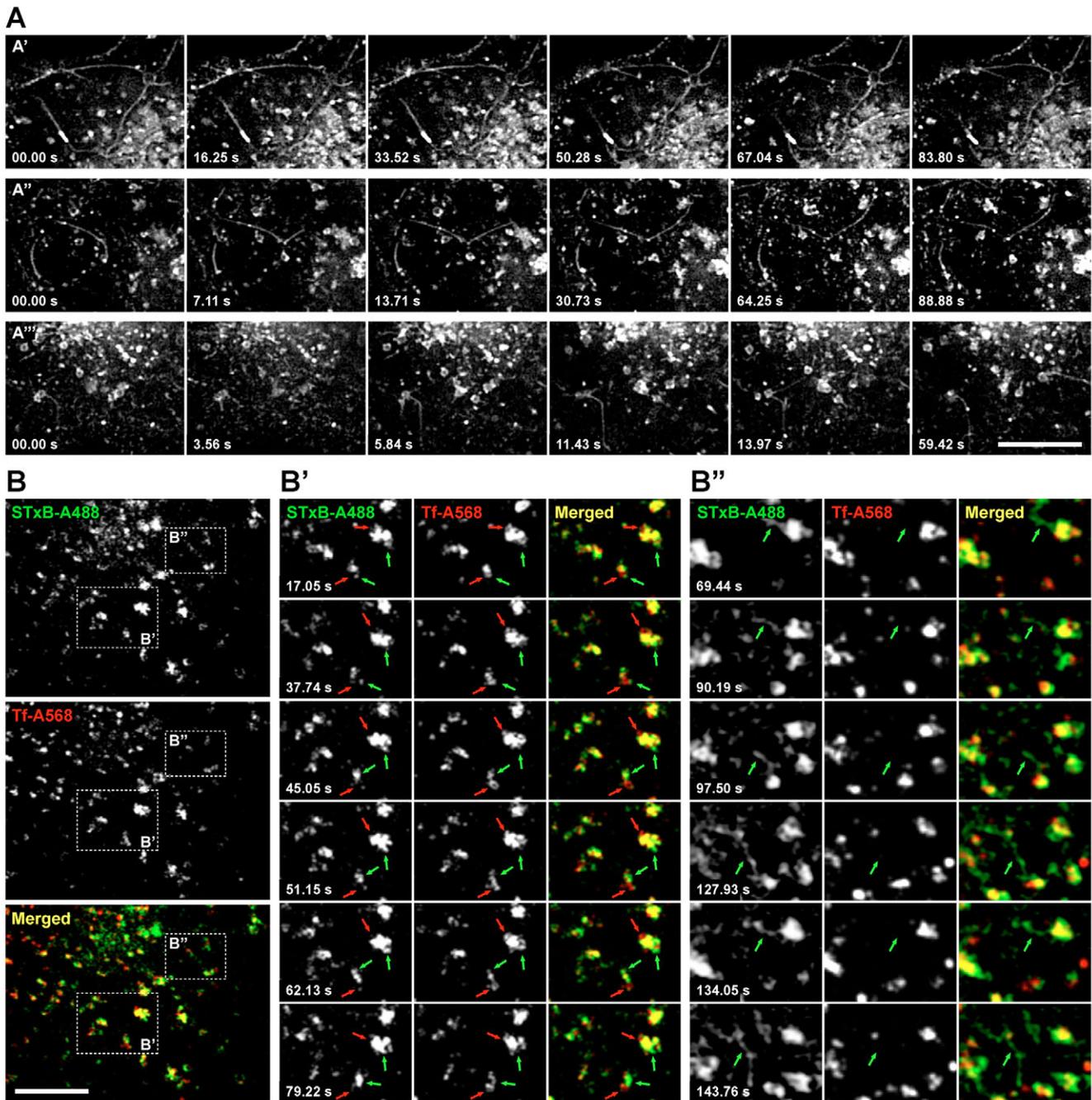


Fig. 8. STxB-containing endosomal tubules appear to persist longer in Vps26 siRNA-treated cells. (A,B) Vps26-depleted HeLa cells were incubated for 45 minutes at 37°C with (A) Alexa Fluor-488-labeled STxB alone (green), or (B) Alexa Fluor-488-labeled STxB (green) together with Alexa Fluor-568-labeled human Tf (red) and then imaged by time-lapse microscopy. In B, merged image indicates overlapping localization (yellow). (B',B'') magnified images of the boxed areas in B. In addition to colocalization, the series in B' show regions in the endosomes that contain either only STxB (green arrows) or only Tf (red arrows). The series in B'' show endosomal tubules that contain only STxB. Time after the start of imaging is shown in seconds. Bars, 10 μm .

cannot be processed correctly for retrograde trafficking to TGN/Golgi membranes. However, STxB can nonetheless still be retrieved from TfR-positive membranes.

The precise function of the retromer complex remains to be determined. Another report by Bujny and colleagues in this issue of *Journal of Cell Science* (Bujny et al., 2007) shows that SNX1 is also required for efficient retrograde trafficking of

Shiga toxin. We describe here that, under SNX1-depletion conditions, a shift similar to that observed in Vps26-depleted cells can be detected between STxB and TfR (supplementary material Fig. S2). Considering the SNX1 protein structure with phosphatidylinositol-binding phox domains and membrane-curvature-sensing BAR domains, it is tempting to ascribe to SNX1 a function in curvature-sensing and stabilization. Such

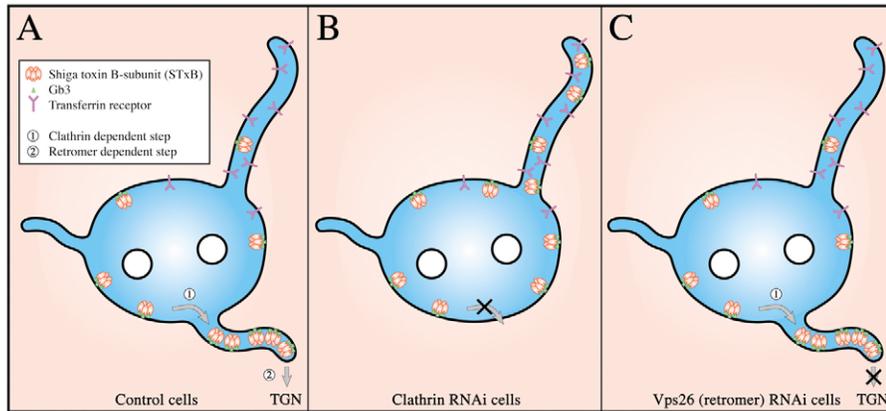


Fig. 9. Working model for retrograde sorting on the early endosome. (A) In control cells, clathrin organizes microdomains with which Shiga-toxin associates (step 1). We hypothesize that microdomain construction induces initial membrane-curvature changes leading to tube formation. A retromer-dependent process then allows stabilizing and processing of membrane curvatures, leading to the formation of retrograde transport intermediates (step 2). (B) In clathrin-depleted cells, the early endosomal retrograde sorting domain cannot form any more. Under these conditions, retrograde transport to TGN/Golgi membranes is inhibited and Shiga toxin distributes throughout the early endosomal pathway, perfectly overlapping with TfR. (C) In Vps26-depleted cells, retrograde transport intermediates cannot form and STxB remains blocked in retrograde tubules that are devoid of TfR. For further details see text.

hypothesis is supported by the previous finding on membrane tubulation in the presence of SNX1 (Carlton et al., 2004). Stabilizing and processing of clathrin-induced curvature-changes by retromer and its possible interacting partners represent a tempting model to integrate clathrin and retromer functions in retrograde sorting on the early endosome

Materials and Methods

Cells

HeLa cells were grown at 5% CO₂ in DMEM (GIBCO BRL) medium containing 4.5 g/l glucose supplemented with 10% FCS, 0.01% penicillin-streptomycin, 4 mM glutamine and 5 mM pyruvate.

Recombinant proteins and antibodies

Cy3-STxB, STxB-Sulf2, monoclonal anti-STxB antibody (13C4) (Johannes et al., 1997; Mallard et al., 1998), and polyclonal antibody anti-Vps26 antibody (Arighi et al., 2004) were obtained as described previously. The monoclonal anti-CTR433 antibody was a gift from M. Bornens (Institut Curie, France). Monoclonal anti-TfR (anti-CD71, Santa Cruz Biotechnology), polyclonal anti-EEA1 (BD Bioscience), and FITC- or Cy5-coupled secondary antibodies (Jackson ImmunoResearch) were purchased from the indicated suppliers.

Recombinant DNA constructs

The Vps29-GFP construct was generated by PCR amplification of human Vps29 (residues 1-182; the stop codon TAA at position 183 was mutated to TAT, resulting in a tyrosine residue) and cloned in-frame into the *EcoRI* and *BamHI* sites of the pEGFP-N1 vector (BD Biosciences Clontech, Palo Alto, CA).

RNA interference

Small interfering RNAs (siRNAs) to downregulate hVps26 (no. 1 5'-CTCT-ATTAAGATGGAAGTGdTdT-3', no. 2 5'-AGAGGCTAGAACCAAGGdTdT-3', no. 3 5'-AACTCCTGTAAACCTTGAGdTdT-3') were obtained from Prologico. HeLa cells were transfected in six-well dishes with Oligofectamine (Invitrogen) according the manufacturer's protocols. Experiments were performed after 3 days of transfection. Clathrin RNAi was performed as described previously (Saint-Pol et al., 2004) by electroporation of a pSUPER plasmide containing the human clathrin target sequence (5'-AAGACCAAUUUCAGCAGACAG-3').

Biochemical analysis and imaging

STxB/Cys was conjugated to horseradish peroxidase (HRP) by chemical coupling with the heterobifunctional crosslinker sulfo-m-maleimidobenzoyl-N-

hydroxysuccinimide ester (Sulfo-MBS, Pierce), as described (Amessou et al., 2006; Saint-Pol et al., 2004). Whole-mount (Saint-Pol et al., 2004), sulfation (20-minute sulfation step) (Mallard and Johannes, 2003) and immunofluorescence analyses (Mallard et al., 1998) were performed as published.

Immunoelectron microscopy

Cryosectioning and immunoelectron microscopy were carried out as described previously (Johannes et al., 1997).

Time-lapse microscopy

HeLa cells grown to 40-50% confluency on 35-mm-glass-bottom culture dishes (MatTek Corporation, Ashland, MA) were transfected with 4 μg of Vps29-GFP using Lipofectamine-2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Cells were imaged 36 hours post transfection. For siRNA-mediated suppression of endogenous Vps26, HeLa cells grown to 30% confluency were transfected twice at 24-hour intervals with 80 pmol siRNA oligonucleotides [SMART pool[®] reagents, M-013195-00 (Vps26), Dharmacon, Chicago, IL] using Oligofectamine (Invitrogen) following the manufacturer's protocol. Cells were imaged 48 hours after the second round of transfection. For imaging, HeLa cells plated on 35-mm-glass-bottom culture dishes (MatTek Corporation, Ashland, MA), were placed on ice, and incubated for 30 minutes with 1 μg/ml Alexa Fluor-488-labeled STxB or 2 μg/ml Cy3-STxB and 10 μg/ml of either Alexa Fluor-568-labeled or Alexa Fluor-488-labeled human Tf (Invitrogen) in Hepes-buffered culture medium (Invitrogen). Cells were then washed three times with ice-cold culture medium and incubated with regular medium at 19.5°C for 60 minutes in a CO₂ incubator. After incubation, the medium was replaced with Phenol-Red-free Hepes-buffered medium (Invitrogen). On the microscope stage, cells were kept at 37°C by using an ASI 400 Air Stream Stage Incubator (Nevtek, Burnsville, VA). Time-lapse fluorescence images were acquired with an Ultraview Confocal Scanner (Perkin Elmer, Norwalk, CT) in a Nikon Eclipse TE2000-S inverted microscope (Nikon, Melville, NY) equipped with a PlanApo 100× oil immersion objective (NA 1.40; Nikon), and a 12-bit charge-couple device (CCD) camera, ORCA (Hamamatsu, Bridgewater, NJ). Image capture and data acquisition were performed using Ultraview LCI software (Perkin Elmer). Images were acquired in binning 2×2 modes to increase the signal-to-noise ratio at 0.25-second to 1.6-second intervals. Sequence images were exported as single TIFF files and processed with ImageJ 1.36b (Wayne Rasband, NIH). To prepare figures, single frames were processed with Adobe Photoshop 7 (Adobe Systems, Mountain View, CA). Quicktime movies were produced using ImageJ 1.36b.

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