AGAP2 regulates retrograde transport between early endosomes and the TGN

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Summary
The retrograde transport route links early endosomes and the TGN. Several endogenous and exogenous cargo proteins use this pathway, one of which is the well-explored bacterial Shiga toxin. ADP-ribosylation factors (Arfs) are ~20 kDa GTP-binding proteins that are required for protein traffic at the level of the Golgi complex and early endosomes. In this study, we expressed mutants and protein fragments that bind to Arf-GTP to show that Arf1, but not Arf6 is required for transport of Shiga toxin from early endosomes to the TGN. We depleted six Arf1-specific ARF-GTPase-activating proteins and identified AGAP2 as a crucial regulator of retrograde transport for Shiga toxin, cholera toxin and the endogenous proteins TGN46 and mannose 6-phosphate receptor. In AGAP2-depleted cells, Shiga toxin accumulates in transferrin-receptor-positive early endosomes, suggesting that AGAP2 functions in the very early steps of retrograde sorting. A number of other intracellular trafficking pathways are not affected under these conditions. These results establish that Arf1 and AGAP2 have key trafficking functions at the interface between early endosomes and the TGN.

Key words: Shiga toxin, Arf, ArfGAP, ARAP, Mannose 6-phosphate receptor, TGN46, Cholera toxin, Clathrin, Retromer, VSVG, Transferrin

Introduction
Proteins and lipids traffic via the retrograde route from endosomes to the Golgi complex (Bonifacino and Rojas, 2006; Johannes and Popoff, 2008). In mammalian cells, this pathway enables a functional cycle for lysosomal enzyme delivery by mannose-6-phosphate receptors (MPRs) (Duncan and Kornfeld, 1988; Snider and Rogers, 1985), and trafficking of TGN38/46 between TGN and plasma membrane (Reaves et al., 1993). Retrograde trafficking is crucial for the maintenance of Golgi morphology (Ghosh et al., 2003; Naslavsky et al., 2009; Yoshino et al., 2005), and a range of other cellular and pathological functions depend on retrograde transport, including the cellular entry of pathogens and pathogenic factors (for reviews, see Bonifacino and Rojas, 2006; Johannes and Popoff, 2008). A well-studied example is the bacterial Shiga toxin (Johannes and Römer, 2010). After binding to its cellular receptor, the glycosphingolipid Gb3, the toxin follows the retromer (Bonifacino and Hurley, 2008), Shiga toxin is segregated from TIRs in early endosome-linked tubular structures that have been termed retrograde tubules (Popoff et al., 2007). With the recent demonstration of a function for Rab7 in retromer recruitment (Rojas et al., 2008; Seaman et al., 2009), the combined evidence suggests that the clathrin requirement in retrograde sorting on early or maturing endosomes precedes that for retromer.

The ADP-ribosylation factor (Arf) GTPases are ~20 kDa GTP-binding proteins that regulate membrane traffic through the recruitment of clathrin or COPI coats, the modulation of lipid-modifying enzyme activity, or by controlling actin dynamics at membrane surfaces (D’Souza-Schorey and Chavrier, 2006; Donaldson and Lippincott-Schwartz, 2000). Mammals express six Arf isoforms, Arf1-Arf6, which are grouped into three classes based on primary sequence and gene organization (Kahn et al., 2006). The best-characterized Arf proteins are Arf1 and Arf6. Arf6 regulates endosomal trafficking and plasma membrane organization, whereas Arf1 is thought to be localized specifically to the Golgi complex (Peters et al., 1995). Recent studies have shown that Arf1 can also be recruited to endosomal membranes (Gu and Gruenberg, 2000) and the plasma membrane (Kumari and Mayor, 2008), and that pairs of Arf1 with Arf3, Arf4 or Arf5 regulate transferrin recycling (Volpicelli-Daley et al., 2005). Arf proteins cycle between active GTP-bound and inactive GDP-bound conformations. Hydrolysis of GTP is mediated by GTPase-activating proteins (ArfGAPs), whereas the exchange of GDP for triphosphate nucleotide is mediated by guanine nucleotide-exchange factors (ArfGEFs).

The function of Arf proteins and their GAPs and GEFs in retrograde transport from early endosomes to TGN remains largely
unknown. When cells were treated with brefeldin A (BFA), a fungal metabolite that inhibits ArfGEFs, exit of Shiga toxin B-subunit (STxB) from Tf-positive tubular membranes was prevented (Mallard et al., 1998). In addition, GBF1, a BFA-sensitive ArfGEF was suggested to function in retrograde transport of STxB (Saenz et al., 2009). This conclusion was based on the use of a small-molecule compound, golgicide A, as an inhibitor of GBF1. Whether GBF1 functions directly in endosome-to-TGN transport, or indirectly through its effects on maintaining Golgi structure, is currently unknown (Saenz et al., 2009).

ArfGAPs were originally considered as simple regulators of ARFs, and it has only recently been shown that ArfGAPs themselves can be effectors that transduce signals in cells (Inoue and Randazzo, 2007). In Golgi-to-ER trafficking, ArfGAP1 is necessary for COPI vesicle budding (Lanoix et al., 1999; Nickel et al., 1998; Pepperkok et al., 2000; Yang et al., 2002). Very few studies have addressed ArfGAP function in post-Golgi membrane traffic. SMAP2 is an ArfGAP that binds to clathrin heavy chain and clathrin assembly protein CALM. SMAP2 colocalizes on endosomes with the clathrin adaptor epsinR, and the overexpression of a SMAP2 clathrin-binding mutant inhibits retrograde transport of murine TGN38 in COS-7 cells (Natsume et al., 2006). These findings suggest that ArfGAPs are effectors of Arf in retrograde transport.

Here, we have found that ARF1, but not ARF6 is required for early-endosomes-to-TGN transport of Shiga toxin. We inhibited the expression of six ArfGAP family members that have GAP activity to Arf1, and found that AGAP2 is required for retrograde transport of STxB. In AGAP2-depleted cells, Shiga toxin localizes in endosomes that are positive for the transferrin receptor and Rab4, and to a lesser extent with the retromer protein Vps26. These results establish that ARF1 and AGAP2 participate in very early steps of retrograde sorting on early endosomes.

Results
ARF involvement in STxB transport from early endosomes to the TGN

As a first approach to testing the function of Arf proteins in retrograde transport to the TGN in HeLa cells, we expressed GFP-tagged protein fragments that bind to Arf1-GTP and Arf6-GTP. Expression of GFP alone had no effect on retrograde transport, and STxB accumulated efficiently in perinuclear Golgi membranes labeled by the medial-Golgi marker CTR433 (Fig. 1A). By contrast, cells that expressed the GFP-tagged Arf-GTP binding domain (ARFBD) of ARHGAP10 (Dubois et al., 2005) showed increased peripheral STxB labeling (Fig. 1B), and in a small fraction of cells, STxB did not reach Golgi membranes at all (Fig. 1H). Similar results were observed in cells expressing the HA-tagged VHS-GAT domain of GGA1 that also binds to ARF-GTP (data not shown).

A permeabilized-cells approach (Amessou et al., 2006) was used to further test the effect of ARFBD on STxB trafficking between early endosomes and the TGN (Fig. 1C). In this approach, a STxB variant with a tandem sulfation signal, termed STxB-Sulf2, is used to measure arrival in the TGN. STxB-Sulf2 was accumulated in early endosomes by incubation with cells at 19.5°C. After plasma-membrane permeabilization with SLO and removal of endogenous cytosol, the permeabilized cells were incubated for 20 minutes at 37°C in the presence of GST or GST-ARFBD and radioactive sulfate. Sulfation signal in the presence of exogenous cytosol was set to a 100% for maximal TGN arrival, and the

Fig. 1. ARF involvement in retrograde transport of STxB. (A,B,D-G) HeLa cells were transfected with the indicated constructs, and then incubated with Cy3-labeled STxB for 45 minutes at 37°C. The cells were fixed and stained with anti-CTR433 antibody. In ARFBD- and ARF1QL-transfected cells, strongly increased STxB localization to peripheral structures is found. Scale bars: 10 μm. (C) STxB-Sulf2 transport to the TGN was assayed by sulfation analysis on permeabilized cells in the presence of the indicated concentrations of recombinant GST or ARFBD. Sulfation signals are expressed as percentages of signal observed under control conditions (+cytosol and 1 μM GST). The means ± s.e.m. of three independent experiments are shown. (H) Among the 50 cells that were transfected and had internalized STxB, the percentage of cells showing colocalization of STxB and CTR433 was determined. Independent transfections were repeated three times, and the colocalization means ± s.e.m. were calculated.
background signal was determined in the absence of exogenous cytosol (Fig. 1C). The addition of GST-ARFBD, but not of GST alone, led to a dose-dependent inhibition of retrograde transport (Fig. 1C), thus confirming the results obtained on intact cells (Fig. 1B).

These results strongly suggest the involvement of Arf proteins in membrane trafficking at the interface between early endosomes and the TGN. To identify the Arf isoform that is involved in this trafficking step, we expressed in intact HeLa cells constitutively active mutants of Arf1 and Arf6, i.e. Arf1Q71L or Arf6Q67L, or mutants defective in nucleotide binding, Arf1N126I or Arf6N122I. In Arf1Q71L-expressing cells, STxB remained blocked in peripheral endosomes (Fig. 1D), and in many cells the protein failed to reach the Golgi altogether (Fig. 1H). In cells expressing Arf1N126I, a similar albeit weaker effect was observed (Fig. 1E,H). It should be noted that Golgi integrity was slightly affected in these cells (Fig. 1D-E), and high expression of Arf1N126I often led to the disruption of the Golgi (data not shown). The dispersed Golgi fragments were not reached by STxB, however (Fig. 1D-E).

In cells expressing the different Arf6 mutants, endosomal STxB accumulation was as low, as in control cells (Fig. 1F-G), and STxB transport to the Golgi was not visibly altered (Fig. 1H). These results demonstrate that Arf1, but not Arf6, is involved in STxB transport from early endosomes to the TGN.

Identification of ArfGAP proteins that function in retrograde transport

Based on the finding that Arf1, but not Arf6 was required for retrograde transport to the TGN, we performed sulfation analysis on intact cells that were transfected with validated siRNAs pools (except SMAP2) against six ArfGAPs with preferential GAP activity on Arf1 (Miura et al., 2002; Natsume et al., 2006; Nie et al., 2005; Vitale et al., 2000; Yoon et al., 2004). The depletion of the individual proteins was not assayed, and negative results can therefore not be interpreted. As shown in Fig. 2, the strongest inhibition of STxB sulfation was observed in cells transfected with a smart pool of four siRNAs against ARAP1, and a weaker inhibition in cells transfected with siRNA against AGAP2. For AGAP1 and SMAP2-1, inhibition was not significant and immunofluorescence analysis revealed that STxB efficiently accumulated in Golgi membranes (data not shown). In the case of cells transfected with GIT2 and SMAP2-2 siRNA, sulfation levels were increased. The significance of these findings is not clear at this stage. For further analysis, we focused on ARAP1 and AGAP2.

ARAP1 is not required for retrograde transport to the TGN

To study ARAP1 function in retrograde transport, the sulfation assay was repeated using the four siRNAs of the smart pool against ARAP1 individually. All four siRNAs efficiently depleted ARAP1 protein (Fig. 3A). Sulfation levels on STxB were decreased in all cases, most strongly with sequences 3 and 4 (Fig. 3B). Inspection of STxB labeling by fluorescence microscopy showed that many cells that were transfected with these siRNAs had reduced signals of cell-associated STxB (Fig. 3C, arrows). This finding suggested that in ARAP1-depleted cells, plasma membrane Gb3 levels were reduced, or that Gb3 molecules were organized in a way such that STxB could not be bound efficiently. In cells in which STxB binding could still be detected (Fig. 3C, arrowheads), retrograde transport to the TGN was apparently not affected. Quantification confirmed that 68% or 70% of cells failed to bind STxB in cells transfected with ARAP1 siRNA sequences 3 and 4, respectively, whereas this percentage was much smaller in cells transfected with control siRNA (7%). Dosage of Gb3 after lipid extraction and overlay (Falguières et al., 2001) revealed that total cellular Gb3 levels were not altered in cells transfected with ARAP1 siRNA (data not shown). ARAP1 is probably required for Gb3 transport from the Golgi to the plasma membrane, but other interpretations cannot be excluded at this stage.

AGAP2 functions at the interface between early endosomes and the TGN

To study AGAP2, we generated a peptide antibody that detected the protein by western blotting only upon overexpression (not shown), and by immunofluorescence only when cells were fixed in methanol. Under these fixation conditions, endogenous AGAP2 was found in the perinuclear region in good colocalization with TGN46 (Fig. 4A), to a lesser extent with the Golgi marker giantin (supplementary material Fig. S1A), and not with the late endosomal or lysosomal marker Lamp-1 (supplementary material Fig. S1B). TIR only weakly overlapped with AGAP2 (supplementary material Fig. S1C), which for peripheral sites might be due to poor preservation under conditions of methanol fixation. GFP-tagged AGAP2 partially colocalized with STxB after short times of internalization (5 minutes; Fig. 4B). These findings and other published results (Nie et al., 2005) show that AGAP2 is localized at the TGN and on endosomes.

As above for ARAP1, the function of AGAP2 was addressed in sulfation experiments by depleting AGAP2 expression individually with each of the four siRNA sequences of the smart pool. Since our antibody did not work for western blotting, we relied on RTPCR (supplementary material Fig. S2A) and immunofluorescence (see below) to confirm the efficacy of the AGAP2 siRNAs. The STxB sulfation signal was strongly reduced with each of the four siRNAs that were used to deplete AGAP2 (Fig. 4C). Upon prolonged incubation (120 minutes), sulfation still remained much lower in the depletion condition (supplementary material Fig. S2B), suggesting that STxB failed to reach TGN membranes altogether. Since STxB degradation was not detected in AGAP2-depleted cells upon incubation for at least 4 hours (supplementary material Fig. S2C), it appears likely that STxB remained in the early endosomal membrane system (see below), as we described before.
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in cells in which retrograde transport was abolished upon BFA treatment (Mallard et al., 1998).

The perinuclear AGAP2 labeling that was seen with the methanol-fixation protocol in control cells (Fig. 4A and supplementary material Fig. S3, top panel) was strongly diminished in cells that were transfected with AGAP2 siRNAs 1 to 4 (supplementary material Fig. S3). This loss of perinuclear AGAP2 labeling was not observed in cells transfected with ARAP1 siRNA (data not shown), confirming the specificity of the labeling. In cells transfected with control siRNA (supplementary material Fig. S3, top panel), perinuclear STxB labeling at the Golgi was well preserved in the methanol-fixation protocol. In siRNA-transfected cells, this perinuclear STxB labeling was lost (supplementary material Fig. S3, lower panels for siRNA sequences 1 to 4; see right column for Golgi labeling with giantin). As opposed to ARAP1, the apparent loss of global STxB signal was in this case not due to loss of STxB binding. Indeed, when cells that were transfected with AGAP2 siRNA sequence 3 (Fig. 4D, lower panel) were fixed using paraformaldehyde, STxB (red) was largely absent from perinuclear Golgi membranes (giantin, blue), as in the methanol-fixation condition. However, STxB could now be detected in peripheral structures.

We noticed that Golgi morphology was somewhat affected in AGAP2 siRNA-transfected cells (Fig. 4D and supplementary material Fig. S3). We therefore used the permeabilized cell assay to validate the function of AGAP2 in an experimental set-up that allows interference with protein function without going through prolonged depletion conditions. Recombinant glutathione-S-transferase (GST) and tagged wild-type AGAP2 were used as purified proteins. As shown in Fig. 4E, GST had no effect on retrograde transport of STxB. By contrast, wild-type AGAP2 potently inhibited, confirming a function for AGAP2 in retrograde transport.

**STxB accumulates in early endosomes of AGAP2-depleted cells**

The analysis of sites of STxB accumulation in AGAP2-depleted cells was performed in paraformaldehyde-fixed cells. Transfection of siRNA sequence 3 induced an efficient inhibition of retrograde transport with minimal effects on Golgi morphology, and this siRNA sequence was chosen for all experiments described below.

In AGAP2-depleted cells, STxB colocalized with TIR on perinuclear and peripheral endosomes (Fig. 5A). The GFP-tagged early endosomal marker Rab4 also decorated STxB-positive enlarged structures under these conditions (Fig. 5B). Importantly, GFP-Rab4 expression by itself did not affect STxB trafficking to the TGN (data not shown). Clathrin is a crucial component for retrograde sorting. In clathrin-depleted cells, STxB fails to reach Golgi membranes, and remains blocked in TIR-positive early endosomes (Popoff et al., 2007; Saint-Pol et al., 2004), similarly to the situation described here for AGAP2-depleted cells. We also found that clathrin was often juxtaposed to sites of STxB accumulation (Fig. 5C). The retromer complex has been suggested to be involved in the processing of retrograde tubules in which STxB is taken out of TIR-positive early endosomes (Popoff et al., 2007), and therefore appears to function consecutively to clathrin (Johannes and Popoff, 2008). Little or no colocalization was observed here between the retromer component Vps26 and STxB (Fig. 5D). Taken together, the colocalization of STxB with TIR in AGAP2-depleted cells, the proximity to clathrin and the lack of overlap with Vps26 suggest that AGAP2 functions in very early steps of retrograde sorting. In agreement with this hypothesis, the recycling endosomal marker Rab11 (Fig. 5E) and the late endosomal or lysosomal marker Lamp-1 (Fig. 5F) were not colocalized with STxB in AGAP2-depleted cells.

Live-cell imaging was used to confirm the AGAP2 function in very early steps of retrograde sorting. We previously showed that depletion of the retromer protein Vps26 leads to the prolonged appearance of STxB in retrograde tubules that although connected to early endosomes, were devoid of Tf (Popoff et al., 2007). By contrast, here we found that in AGAP2-depleted cells, STxB and Tf were colocalized in early endosomal tubules (Fig. 6A and supplementary material Movie 1), similarly to the colocalization
on static images that we observed in clathrin-depleted cells (Saint-Pol et al., 2004). The live-cell imaging also confirmed that after 45 minutes of incubation with AGAP2-depleted HeLa cells, STxB and Tf were still dynamically associated, notably in the perinuclear area (Fig. 6B and supplementary material Movie 2, right), whereas in control cells, STxB has efficiently reached the Golgi at this time point, and no overlap with Tf was seen (Fig. 6C and supplementary material Movie 2, left).

AGAP2 regulates retrograde transport of several exogenous and endogenous cargos

The GM1-binding B-subunit of cholera toxin (CTxB) shares with STxB some of the trafficking requirements that have been analyzed to date (Amessou et al., 2007; Lu et al., 2004). Here, we found that retrograde transport of CTxB to the Golgi was also inhibited in AGAP2-depleted cells (Fig. 7A-B). Trafficking of the endogenous retrograde cargo protein TGN46 was followed using a dynamic antibody-uptake protocol. In control cells, the antibody efficiently accumulated in the perinuclear region, in colocalization with the TGN marker Golgin-97 (Fig. 7C). By contrast, the anti-TGN46 antibody remained in peripheral structures that did not colocalize with Golgin-97 in AGAP2-depleted cells (Fig. 7D). Similarly, STxB also failed to reach Golgin-97-positive membranes in AGAP2-depleted cells (supplementary material Fig. S4). In these experiments, we noticed a slight effect of AGAP2 depletion on the distribution of Golgin-97, which appeared less compact than in control cells, suggesting that TGN morphology was affected.

In a further experiment, the steady-state localization of cation-independent MPR (CI-MPR) was analyzed. In our HeLa cell clone, most CI-MPR was localized in perinuclear membranes in colocalization with the Golgi matrix protein GM130 (Fig. 7E). In AGAP2-depleted cells, more CI-MPR labeling was visible in peripheral structures (Fig. 7F). Taken together, these findings strongly suggest that AGAP2 functions in retrograde transport of several exogenous and endogenous retrograde cargo proteins.

Effects of AGAP2 depletion on other intracellular pathways

A number of intracellular-trafficking routes were analyzed to address the specificity of AGAP2 function at the early-endosome-TGN interface. Since AGAP2 depletion has a slight effect on TGN morphology (see above), we analyzed anterograde transport along the biosynthetic or secretory pathway in control and depletion conditions. A temperature-sensitive version of the glycoprotein from vesicular stomatitis virus (VSVG) was blocked in the ER at the restrictive temperature. Upon shift to the permissive temperature for 2 hours, the protein was transported in a brefeldin A (BFA)-sensitive manner to the cell surface. We found here that VSVG transport was...
40% inhibited in AGAP2-depleted cells, when compared with siRNA-transfected control cells (Fig. 8A). Since VSVG might also traffic via endosomes to the cell surface (Cancino et al., 2007; Chen et al., 1998), we believe that this effect is consistent with a post-Golgi function of AGAP2, as described above for the retrograde toxin cargos and the endogenous proteins TGN46 and CI-MPR. A function of AGAP2 in anterograde transport at the TGN cannot be excluded either. However, since STxB and anti-TGN46 antibody fail to reach the extended TGN in AGAP2-depleted cells (see above), a direct role of AGAP2 in post-Golgi retrograde transport remains the most likely possibility. In quantitative biochemical assays we could finally show that the endocytosis of STxB (Fig. 8B) and Tf (Fig. 8C) and the recycling of Tf (Fig. 8D) were not affected by the depletion of AGAP2.

Discussion
In this study, we demonstrate the involvement of Arf1 in retrograde transport between early endosomes and the TGN, using quantitative biochemical tools and morphological approaches. Furthermore, we identify the ARF1 GAP AGAP2 as a crucial factor for retrograde sorting on early endosomes.

Arf proteins and Arf1-sensitive coatomer have previously been involved in trafficking to the late endocytic pathway (Aniento et al., 1996; Daro et al., 1997; Gu and Gruenberg, 2000; Whitney et al., 1995). Our finding that Arf1 is also required for retrograde sorting adds further complexity to the endosomal coat network. Indeed, clathrin, the clathrin adaptors epsinR, AP-1 and OCRL, and the clathrin uncoating ATPase Hsc70 and its early endosomal adaptor RME-8 are also required for efficient retrograde sorting of endogenous and exogenous cargo proteins (Choudhury et al., 2005; Folsch et al., 2001; Lauvrak et al., 2004; Popoff et al., 2009; Saint-Pol et al., 2004; Shi et al., 2009). How exactly Arf1 links into this sorting machinery still remains to be determined. Our unpublished evidence suggests that coatomer I proteins (COPI) are not required for retrograde transport of Shiga toxin to the TGN, and it therefore appears likely that Arf1 rather interacts with the clathrin machinery, as it has been found at the TGN (Puertollano et al., 2001; Traub et al., 1993).

A likely link between Arf1 and the clathrin machinery might operate via AGAP2. A previous study had shown that AGAP2 is localized on endosomes and interacts with the clathrin adaptor AP-1 (Nie et al., 2005). We found here that depletion of AGAP2 leads to an inhibition of retrograde transport of Shiga toxin in a way such that

Fig. 5. Detailed analysis of STxB localization in AGAP2-depleted cells. HeLa cells were transfected for 3 days with AGAP2 siRNA sequence 3 and after binding was incubated with Cy3-STxB for 45 minutes at 37°C. The cells were then labeled with antibodies against Tfr (A), clathrin heavy chain (CHC) (C), Vps26 (D) and Lamp-1 (F). Alternatively, siRNA-treated cells were transfected for 24 hours with GFP-Rab4 (B) or GFP-Rab11 (E). Note that in GFP-Rab4-expressing cells, enlarged endosomes were observed. Scale bars: 10 μm.
Fig. 6. Live-cell-imaging analysis. HeLa cells were transfected with AGAP2 siRNA (A, B) or control siRNA (C) and incubated with Alexa-Fluor 488-STxB and Alexa Fluor 568-Tf on ice for 30 minutes. After washing, cells were shifted to 19.5°C for 60 minutes, then subjected to imaging at 37°C. After 5 minutes, STxB colocalized with Tf in the same tubules (A, arrows) and this colocalization persisted up to 60 minutes in the perinuclear region (B). In control cells, the majority of STxB is localized at the Golgi by 60 minutes and only a remnant of Tf is observed in recycling endosomes (C, arrows). Time in minutes is shown on bottom left of images.
STxB accumulates in colocalization with TfR in Rab-positive endosomes that are close to clathrin patches. This phenotype is similar to the one observed upon clathrin depletion (Saint-Pol et al., 2004), and different from the retromer protein Vps26 depletion condition, in which STxB moves away from TfR-positive membranes (Popoff et al., 2007). Our body of data therefore suggest a function for Arf1/AGAP2 in clathrin-dependent retrograde tubule formation on early endosomes. Since our previous studies had shown that AP-1 was not required for retrograde transport of STxB (Saint-Pol et al., 2004), one must assume that AGAP2 can also interact with the clathrin machinery via other molecules.

The inhibition of retrograde transport by AGAP2 depletion might at first sight appear surprising, since one might expect Arf1 to be preferentially in the active GTP-bound conformation under these conditions and thereby be stimulating the trafficking step. However, in the COPI-dependent vesicle-formation model, it is now well established that cargo sorting is inhibited when the

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**Fig. 7.** AGAP2 is required for retrograde transport of various exogenous and endogenous cargos. HeLa cells were transfected with control or AGAP2 siRNA sequence 3 for 3 days. (A,B) Fluorescently labeled cholera toxin B-subunit (CTxB, red) was incubated with HeLa cells for 45 minutes. Note that CTxB fails to reach Golgi membranes (giantin, green) in AGAP2-depleted cells. Arrows indicate CTxB-positive structures that are positive or negative for giantin (A or B, respectively). (C,D) Anti-TGN46 antibody (red) was incubated with HeLa cells for 6 hours. In AGAP2-depleted cells, the antibody fails to reach the distended TGN, labeled with Golgin-97 (green). (E,F) Cells labeled with anti-CIMPR (red) and anti-GM130 (green, arrow) antibodies. The presence of CIMPR at peripheral sites is increased in AGAP2-depleted cells.

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**Fig. 8.** The effects of AGAP2 depletion on other trafficking pathways. (A) VSVG-tsO45-GFP was transfected into HeLa cells that were previously treated with AGAP2 siRNA sequence 3. VSVG levels at the plasma membrane were measured by FACS after a trafficking pulse of 0 or 2 hours. BFA was used as a positive control. Means ± s.e.m. of three determinations. (B,C) Endocytosis assays. After binding, STxB-S-S-biotin (B) or Tf-S-S-biotin (C) was incubated for the indicated times with cells transfected with control siRNA or AGAP2 siRNA. The percentages of cell-surface-inaccessible (internalized) STxB or Tf were determined. Means ± s.e.m. of three determinations. (D) Tf recycling assay. Tf-S-S-biotin was incubated with HeLa cells for 40 minutes at 37°C. After washing, cells were incubated for the indicated times in the presence of an excess of non-biotinylated Tf. Residual cell associated Tf was determined at each time point. A representative of two determinations is shown.
reaction is performed in the presence of the non-hydrolysable GTP analogue GTPγS (Lanoix et al., 1999; Nickel et al., 1998; Peperkock et al., 2000), suggesting that inhibition of GAP activity would also lead to a blockage.

AGAP2 has been localized to focal adhesions, interacts with focal adhesion kinase, and by interfering with AGAP2 function impacts the integrity of focal adhesions (Zhu et al., 2009). Put in perspective with our current study on the involvement of AGAP2 in retrograde sorting, the possibility arises of a molecular link between retrograde trafficking and focal-adhesion dynamics. Such a link could be achieved through trafficking of focal-adhesion proteins via the retrograde route, which has, however, not yet been described. Alternatively, a link might exist between retrograde sorting and recycling machineries. Focal-adhesion dynamics clearly depends on recycling from Rab4-positive early endosomes (Roberts et al., 2001), and we found here that in AGAP2-depleted cells, STxB is trapped in the Rab4 compartment. Cholera toxin, which similarly to Shiga toxin, follows the retrograde route, has been found to alternate between retrograde sorting and recycling, depending on cell adhesion (Balasubramanian et al., 2007). These data imply that the machinery of retrograde transport could have an additional role in the recycling pathway under certain conditions. The mechanisms by which recycling and retrograde pathways are linked should be the subject of future studies.

Materials and Methods

Recombinant proteins, antibodies, siRNAs and other reagents

STxB-Sulf2 was purified as described (Mallard and Johannes, 2003). Briefly, periplasmic extracts were loaded on a QHP column (GE Healthcare) and eluted in a linear NaCl gradient (25 mM Bis-Tris-HCl, pH 6). STxB-Sulf2, eluted from the column at about 500 mM. Wild-type STxB was purified as above, with a NaCl gradient at pH 8. STxB eluted around 150-250 mM, and was dialyzed against coupling buffer (20 mM HEPES-KOH, pH 7.4, 150 mM NaCl), and subjected to coupling with Cy3 mono-reactive Dye Pack (GE Healthcare). The Cy3-coupled STxB was purified by PD-10 column (GE Healthcare), quickly frozen and stored at –80°C. Streptolysin-O (SLO) was purchased from Sucharit Bhakdi, Institute of Medical Microbiology and Hygiene, Mainz, Germany. The monoclonal anti-STxB antibody 13C4 was purified with Protein-A-Sepharose from culture medium of the corresponding mouse hybridoma cells (ATCC). Glutathione S-transferase (GST) and GST-ARFBD were purified on glutathione Sepharose beads, according to the manufacturer’s instructions (GE Healthcare). The polyclonal antibody against AGAP2 was raised in rabbits against the following two peptides: UNNLRLKLAERVDDP and TPSNTATPSRRPR. The antisera recognizes overexpressed AGAP2 by western blotting, and endogenous AGAP2 by immunofluorescence. The specificity of the antibody was verified on siRNA-transfected cells. The monoclonal antibodies CTR433 and anti-VSVG were generous gifts from Michel Bornens and Franck Perez (Institut Curie, Paris, France). The rabbit polyclonal antibody against ARAP1 was the generous gift from Paul Randazzo (NIH, Bethesda, MD). The monoclonal antibodies against gianin (Abcam), Golgin-97 (Invitrogen) and Lamp-1 (BD Bioscience) were purchased from the indicated suppliers. The cDNA constructs for all ARF proteins, GFP-Rab4, GFP-Rab5, GFP-Rab11, GFP-ARFBD, GFP-RHOGAP and GST-ARFBD were generous gifts from Kazuhisa Nakayama (Kyoto University, Kyoto, Japan), Jean Salamero, Bruno Goud and Philippe Chavrier (Institut Curie). The pool of four siRNAs against ARF-GAPs that target different sequences from the same mRNAs were purchased from Pharmacia. The endocytosis assay was performed as described previously (Saint-Pol et al., 2004). Briefly, STxB and human dfferic transferrin were biotinylated using NHS-SS-biotin (Pierce). HeLa cells were serum starved for 1 hour and detached with 2 mM EDTA in PBS. Detached cells were incubated on ice for 30 minutes with 2.5 μg/ml biotinylated STxB and 20 μg/ml biotinylated transferrin. After washing with 5 mM glucose, 0.2% BSA in PBS+, the cells were divided into 2×10^5 cells per data point and incubated at 30°C for indicated times. Endocytosis was terminated by placing cells on ice. Biotin on cell-surface-exposed STxB or transferrin was cleaved by incubation on ice for 30 minutes with 200 μM MesNa in TNB buffer (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.02% BSA). The reaction was quenched with 5 mM EDTA. Supernatant was precipitated using TCA. The beads were washed with buffer IV (50 mM Tris-HCl, pH 8), dried with Exmire microsyringe (ITO corporation), boiled in sample buffer, and eluates were loaded on Tris-Tricine gels. The gels were fixed and dried, and analyzed by autoradiography using PhosphorImager (Molecular Dynamics). The radioactive bands were quantified by ImageQuant (Molecular Dynamics). For total sulfation analysis, the TCA precipitated proteins from the supernatant were glass fiber filtered (Whatman), and radioactivity was quantified in a scintillation counter. Variations of total sulfation counts were within 10% of control conditions.

Transfection and immunofluorescence

HeLa cells were transfected with siRNAs at 200 nM for 72 hours using oligofectamine (Invitrogen), or for 6-24 hours with CDNs using FuGene (Roche). The cells were incubated with DMEM containing Cy3-STxB for 3 minutes at 37°C, washed, chased in DMEM for 45 minutes at 37°C, fixed with 4% paraformaldehyde, quenched with 50 mM NH4Cl, and permeabilized with saponin buffer (0.2% saponin, 2% BSA in PBS). Primary and secondary antibodies were diluted with saponin buffer. After treatment with secondary antibody, the cells were washed in water and mounted with Mowiol. Images were acquired on a Leica SP2 confocal microscope.

VSVG transport

0° HeLa cells were transfected with GFP-VSVG-f6045 plasmid using calcium phosphate (Invitrogen). After 4 hours, the cells were incubated overnight at 4°C, trypsinized for 30 minutes at 4°C, washed with 12 mg/ml of HeLa cell cytosol (Mallard et al., 2002) and preincubated with 12 μg/ml of 13C4 and 40 μl of 13C4-Sepharose beads (GE Healthcare). After end-over-end rotation for 90 minutes at 4°C, the beads were collected and the supernatant was precipitated using TCA. The beads were washed with buffer IV (50 mM Tris-HCl, pH 8), dried with Exmire microsyringe (ITO corporation), boiled in sample buffer, and eluates were loaded on Tris-Tricine gels. The gels were fixed and dried, and analyzed by autoradiography using PhosphorImager (Molecular Dynamics). The radioactive bands were quantified by ImageQuant (Molecular Dynamics). For total sulfation analysis, the TCA precipitated proteins from the supernatant were glass fiber filtered (Whatman), and radioactivity was quantified in a scintillation counter. Variations of total sulfation counts were within 10% of control conditions.

Endocytosis and recycling assay

The endocytosis assay was performed as described previously (Saint-Pol et al., 2004). Briefly, STxB and human dfferic transferrin were biotinylated using NHS-SS-biotin (Pierce). HeLa cells were serum starved for 1 hour and detached with 2 mM EDTA in PBS. Detached cells were incubated on ice for 30 minutes with 2.5 μg/ml biotinylated STxB and 20 μg/ml biotinylated transferrin. After washing with 5 mM glucose, 0.2% BSA in PBS+, the cells were divided into 2×10^5 cells per data point and incubated at 30°C for indicated times. Endocytosis was terminated by placing cells on ice. Biotin on cell-surface-exposed STxB or transferrin was cleaved by incubation on ice for 30 minutes with 200 μM MesNa in TNB buffer (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.02% BSA). The reaction was quenched with 5 mM EDTA. Supernatant was precipitated using TCA. The beads were washed with buffer IV (50 mM Tris-HCl, pH 8, 7.4, 8.37 mM CaCl2, 78 mM KCl, 4 mM MgCl2, 10 mM EGTA, 1 mM DTT), incubated for 10 minutes on ice with 2 μg/ml SLO in ICD/TDT, washed, and incubated for plasma membrane permeabilization for 10 minutes at 37°C with ICD/TDT containing the indicated molecular tools. The permeabilized cells were then incubated for 30 minutes at 37°C with 12 mg/ml of HeLa cell cytosol (Mallard et al., 2002) containing 960 μCi/ml [35S] sulfate in the continued presence of the molecular tools. The cells were lysed with 900 μl of RIPA buffer (1% NP-40, 0.5% deoxycholate, 0.5% SDS in PBS), and STxB-Sulf2 was immunoprecipitated with 12 μg/ml of 13C4 and 40 μl of protein-G-Sepharose beads (GE Healthcare). After end-over-end rotation for 90 minutes at 4°C, the beads were collected and the supernatant was precipitated using TCA. The beads were washed with buffer IV (50 mM Tris-HCl, pH 8), dried with Exmire microsyringe (ITO corporation), boiled in sample buffer, and eluates were loaded on Tris-Tricine gels. The gels were fixed and dried, and analyzed by autoradiography using PhosphorImager (Molecular Dynamics). The radioactive bands were quantified by ImageQuant (Molecular Dynamics). For total sulfation analysis, the TCA precipitated proteins from the supernatant were glass fiber filtered (Whatman), and radioactivity was quantified in a scintillation counter. Variations of total sulfation counts were within 10% of control conditions.

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