A Cycling *cis*-Golgi Protein Mediates Endosome-to-Golgi Traffic

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Toxins can invade cells by using a direct endosome-to-Golgi endocytic pathway that bypasses late endosomes/prelysosomes. This is also a route used by endogenous proteins, including GPP130, which is an integral membrane protein retrieved via the bypass pathway from endosomes to its steady-state location in the *cis*-Golgi. An RNA interference-based test revealed that GPP130 was required for efficient exit of Shiga toxin B-fragment from endosomes en route to the Golgi apparatus. Furthermore, two proteins whose Golgi targeting depends on endosome-to-Golgi retrieval in the bypass pathway accumulated in early/recycling endosomes in the absence of GPP130. GPP130 activity seemed specific to bypass pathway trafficking because the targeting of other tested proteins, including those retrieved to the Golgi via the more conventional late endosome route, was unaltered. Thus, a distally cycling Golgi protein mediates exit from endosomes and thereby underlies Shiga toxin invasion and retrieval-based targeting of other cycling Golgi proteins.

INTRODUCTION

Plant and bacterial toxins with enzymatic activity toward intracellular targets enter cells by endocytosis and then, in some cases, traffic to the Golgi apparatus and the endoplasmic reticulum (ER) before translocating into the cytosol where they exert their toxic effect. As such, studies of toxin trafficking reveal novel aspects of membrane transport, offer possibilities for treatment of infectious diseases where toxins are involved, and offer new modes for drug delivery to the cytosolic compartment (Sandvig and van Deurs, 2000). Bacterial Shiga-like toxins have a monomeric A subunit bound to a homopentameric B-subunit (Fraser *et al.*, 1994). Whereas the A-subunit contains the enzymatic activity that attacks the 28S RNA of the 60S ribosomal subunit (Endo *et al.*, 1988), the B-subunit interacts with the cellular receptor for the toxin, the glycolipid globotriaosylceramide, and mediates Shiga toxin trafficking (St Hilaire *et al.*, 1994; Johannes *et al.*, 1997; Hagnerelle *et al.*, 2002). Interestingly, studies of Shiga toxin revealed, for the first time, retrograde trafficking from the plasma membrane to the ER (Sandvig *et al.*, 1994), and in addition, exposed a novel endocytic pathway that bypasses late endosomes/prelysosomes en route from the cell surface to the Golgi (Johannes *et al.*, 1997; Mallard *et al.*, 1998). Presumably, the bypass pathway is advantageous to toxins in that it allows trafficking to the Golgi apparatus along a route that prevents contact with degradative activities present in late endosomes/prelysosomes.

The bypass pathway is not restricted to toxins. Rather, it seems to be an endosome-to-Golgi route used by a growing list of endogenous proteins that cycle distally out of, and back to, the Golgi apparatus (Ghosh *et al.*, 1998; Mallard *et al.*, 1998; Puri *et al.*, 2002; Medigeschi and Schu, 2003; Umeda *et al.*, 2003; Lin *et al.*, 2004). A defining example is the

trafficking itinerary of TGN38/46, which can be contrasted to that of the more classic late endosomal itinerary of the endoprotease furin. Each protein continuously leaves its steady-state location in the *trans*-Golgi network (TGN), moves to the plasma membrane, and undergoes endocytosis to early endosomes (Reaves *et al.*, 1993; Molloy *et al.*, 1994). Furin then moves, together with the bulk of the endocytosed fluid, from early endosomes to late endosomes. In late endosomes, furin is finally sorted away from the degradative route and moves into vesicles that will fuse with the TGN (Bosschart *et al.*, 1994; Wan *et al.*, 1998; Mallet and Maxfield, 1999; Crump *et al.*, 2001). In contrast, TGN38/46 is sorted from the degradative route immediately in early endosomes (Mallet and Maxfield, 1999). From there, it reaches the TGN either directly or indirectly via recycling endosomes (Ghosh *et al.*, 1998; Mallet and Maxfield, 1999). Thus, the bypass pathway can be considered a Golgi-directed branch out of the pathway that mediates plasma membrane recycling of endocytosed receptors. As with toxin trafficking, early sorting of endogenous cycling proteins away from the degradative route could reduce the amount of degradation that otherwise might occur in late endosomes/prelysosomes.

Somewhat surprisingly, two proteins localized to the *cis*-Golgi at steady state are among the proteins that seem to cycle in the bypass pathway (Puri *et al.*, 2002). GPP130 and GP73 are single-pass transmembrane proteins of unknown function (Linstedt *et al.*, 1997; Kladney *et al.*, 2000). In the absence of acidified luminal compartments GPP130 and GP73 redistribute from the Golgi to endosomes (Linstedt *et al.*, 1997; Puri *et al.*, 2002). On restoration of normal pH, the proteins traffic back to the Golgi via the bypass pathway, suggesting that targeting of these proteins involves pH-sensitive cycling in the bypass pathway (Puri *et al.*, 2002). Indeed, each protein contains luminal targeting determinants that mediate endosome-to-Golgi retrieval; and in the case of GPP130, a separable endosomal targeting determinant has been shown to be required for its pH-sensitive Golgi targeting (Bachert *et al.*, 2001). Although the steady-state level of either protein in endosomes seems low, surface biotinylation readily detects a surface pool that undergoes

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efficient endocytosis (Puri *et al.*, 2002). Furthermore, endosomal localization is clearly detected after relatively slight increases in expression level (Linstedt *et al.*, 1997; Bachert *et al.*, 2001; Puthenveedu *et al.*, 2003).

Dynamic cycling itineraries allow proteins to function efficiently at various intracellular locales. Thus, GPP130 and GP73 cycling in the bypass pathway raises the possibility that these proteins mediate functions distal to the Golgi apparatus. As a test of their role in bypass pathway trafficking, the expression of GPP130 and GP73 was inhibited using RNA interference. GP73 inhibition was not associated with an obvious defect. In contrast, in the absence of GPP130, sorting of Shiga toxin from early endosomes was inhibited as was retrieval-based targeting of Golgi proteins that cycle in the bypass pathway. These defects seemed specific to the bypass pathway, suggesting that GPP130 cycles from the *cis*-Golgi to carry out a direct role in the sorting of both invasive toxins and cellular proteins into an endocytic route of emerging importance.

MATERIALS AND METHODS

Cell Culture and Transfection

HeLa cells or HeLa cells stably expressing *N*-acetylglucosamine transferase T2 tagged with green fluorescent protein (kindly provided by J. White, Cancer Research Center, Massachusetts General Hospital, Charlestown, MA) were grown and transfected as described previously (Elbashir *et al.*, 2001). Transfections were carried out in the absence of antibiotics in 60-mm plates, 35-mm plates, and 24-well dishes with culture volumes of 4, 2, and 0.5 ml, respectively. The day after plating, the cells were placed in fresh media. Per milliliter of culture volume, 26 μ l of OptiMEM (Invitrogen, Carlsbad, CA) and 6 μ l of Oligofectamine (Invitrogen) were mixed in a microcentrifuge tube. After 10 min at room temperature, a mixture of 80 μ l of OptiMEM and 6 μ l of small interfering RNA (siRNA) were added to the microcentrifuge tube. After an additional 25 min at room temperature, the transfection mixture was added to the cells. The final siRNA concentration was 0.6 nM. The following day, an additional 0.5 ml (per milliliter of culture volume) of fresh media was added. After 65 h, cells were processed for immunostaining, transport assays, or immunoblotting. The siRNAs were either purchased (QIAGEN, Valencia, CA) or made using the Silencer siRNA kit (Ambion, Austin, TX). siRNAs targeted GPP130 sequences AAAAGCCAACACGAGGAGCTA (#377) and AATAGCAGATACAGTGCCTG (#335) and GP73 sequence AACAGCTGTACCAGGACGAA. Except where indicated, all experiments used #377. Control siRNAs included one against GAPDH (Ambion) and the following: AACACGAGGAGCTAAAGAAAC (#384). To examine furin localization in siRNA-transfected cells, the cells in a 35-mm dish were transfected with 8 μ g of vector DNA (pECE-FLAG-furin) in 12 μ l of FuGENE 6 (Roche Diagnostics, Indianapolis, IN) 24 h before siRNA transfection.

Assays

Immunoblotting was as described previously (Jesch and Linstedt, 1998). Primary antibodies were used at 1:1000 (anti-GPP130, anti-giantin, anti- β -tubulin; Santa Cruz Biotechnology, Santa Cruz, CA) or 1:3000 (anti-GP73). Immunostaining was as described previously (Puri and Linstedt, 2003). Antibodies against GPP130, giantin, p115, and GM130 were used at 1:500 (Linstedt *et al.*, 1997; Puthenveedu and Linstedt, 2001; Puri *et al.*, 2002). Anti-GRASP65 and anti-GRASP55, used at 1:500, were kind gifts of V. Malhotra (University of California at San Diego, La Jolla, CA). Other antibody dilutions were anti-GP73 at 1:400 (Kladney *et al.*, 2000), anti-TGN46 at 1:200 (Serotec, Raleigh, NC), anti-M6PR at 1:500 (a kind gift from S. Kornfeld, Washington University School of Medicine, St. Louis, MO), anti-lysosomal associated membrane protein 2 (LAMP2) at 1:50 (Zymed Laboratories, South San Francisco, CA), anti-FLAG epitope at 1:300 (Sigma-Aldrich, St. Louis, MO), and anti-golgin-97 at 1:200 (Molecular Probes, Eugene, OR). Fluorescein-labeled goat anti-mouse, rhodamine-labeled goat anti-rabbit, Cy5-labeled goat anti-mouse (Zymed Laboratories), and Texas Red donkey anti-sheep (Rockland, Gilbertsville, PA) were used at 1:200. Unlabeled rabbit anti-goat antibody (diluted to 1:300) was added during TGN46 staining to block unwanted cross-reactivity. To label internalized transferrin receptor, Alexa Fluor 647 conjugated-transferrin (Molecular Probes) was added to cells at 1 μ g/ml for 5 min followed by chase incubations in growth media before fixation. For antibody uptake, cells were incubated at 37°C with rabbit anti-GP73 (1:50) or sheep anti-human TGN46 (1:50) in culture media for 60 min. The cells were then transferred to 0.2 M glycine, 0.5 M NaCl (pH 2.4) for 20 min on ice before fixation. Shiga toxin transport was assayed as described previously (Johannes *et al.*, 1997). Mock and siRNA-transfected HeLa cells were incubated with 70 μ g/ml Shiga toxin B-fragment, purified as described previously (Su *et al.*,

1992), on ice for 30 min. Unbound Shiga toxin was removed by washing with ice-cold growth medium, and the cells were either directly transferred to 37°C for various times, or before the 37°C incubations, they were shifted to 19.5°C for 60 min to allow accumulation in endosomes. The cells were then fixed and costained with mouse anti-StxB 13C4 at 1:200 (kindly provided by L. Johannes, Institut Curie, Paris, France) and rabbit anti-GPP130 or rabbit anti-GP73 antibodies. To quantify the assay, the percentage of cells exhibiting predominantly Golgi-localized toxin was determined, i.e., those cells that exhibited a strong Golgi ribbon pattern and weak, or no, punctate Shiga staining. In GPP130 siRNA-transfected cells, analysis was restricted to cells lacking detectable GPP130 staining. To measure fluid phase uptake, cells were incubated with 2 μ g/ml fluorescein-labeled dextran (Molecular Probes) for 30 min or 2 h before fixation and staining with anti-LAMP2. Colocalization was determined as described below.

Image Analysis

Microscopy was performed using a conventional fluorescence microscope (Linstedt *et al.*, 1997), or where indicated, a spinning disk confocal scan head equipped with three-line laser excitation and independent excitation and emission filter wheels (PerkinElmer, Fremont, CA) mounted on an Axiovert 200 microscope with a 100 $\times$, 1.4 numerical aperture apochromat objective (Carl Zeiss, Thornwood, NY). To determine the level of Golgi area fluorescence, pixels in each full field image exhibiting juxta-nuclear fluorescence were selected, and the average pixel intensity was determined for the region by using Adobe Photoshop (Adobe Systems, Mountain View, CA). Average background pixel intensity, taken from an adjacent cellular area, was then subtracted. To determine the level of marker coincidence, six optical sections were projected (max value) to generate single multicolor images. Objects in one channel were considered to be positive for a marker in another channel only if pixels in each object exhibited extensive overlap. Fluorescence intensity in objects was determined using mean pixel intensity for each selected object. Where indicated, objects were identified after application of a fixed threshold value. Total object fluorescence was mean pixel intensity multiplied by object size. Total cellular fluorescence in objects was the sum of total object fluorescence for each cell. Unless noted otherwise, all deviations are reported as SE of the mean.

RESULTS

Expression of GPP130 was inhibited by transfection of HeLa cells with an siRNA specific to the GPP130 mRNA sequence. Immunoblotting analysis indicated that by day 3 posttransfection, GPP130 levels were >80% reduced compared with mock-transfected cells (Figure 1, A and B). The loading control, β -tubulin, was unchanged. Analysis of the cells by immunofluorescence yielded control or mock-transfected cells with strong staining for GPP130 in a typical Golgi ribbon pattern (Figure 1C), whereas most cells transfected with the GPP130 siRNA exhibited undetectable GPP130 staining (Figure 1D). As determined by measuring Golgi-associated GPP130 staining intensity across the population of transfected cells, an average of $91 \pm 2\%$ of cells had no detectable GPP130 staining, $7 \pm 2\%$ showed faint GPP130 staining, and $4 \pm 1\%$ had GPP130 staining comparable with control cells ($n = 5$, >70 cells analyzed per experiment).

Golgi targeting of the protein GP73 seems to depend on endosome-to-Golgi retrieval via the bypass pathway (Puri *et al.*, 2002). If so, inhibition of GP73 retrieval to the Golgi should cause loss of GP73 from the Golgi and its accumulation in endosomes. Therefore, to test whether GPP130 mediates bypass pathway trafficking, we determined GP73 localization in cells lacking detectable GPP130. As an internal specificity control, the Golgi localization of a stably expressed protein, *N*-acetylglucosamine transferase T2 tagged with green fluorescent protein (NAGT2), whose targeting does not depend on the bypass pathway, was tested in the same cells. In mock or control siRNA-transfected cells, GPP130, GP73, and NAGT2 yielded localizations that were restricted to a Golgi ribbon pattern (Figure 2, A–D). In contrast, in cells lacking GPP130, there was a significant accumulation of GP73 in both peripheral and juxta-nuclear punctate structures, whereas NAGT2 in the same cells was clearly present in a normal Golgi ribbon pattern (Figure 2,

Figure 1. Inhibition of GPP130 expression by RNA interference. (A) Mock and GPP130 siRNA-transfected HeLa cell lysates corresponding to the indicated number of days posttransfection were analyzed by immunoblotting (10 μ g/lane) by using antibodies against GPP130 ("130") and β -tubulin ("tub."). (B) Immunoblotting was also used to detect GPP130 ("130") and GP73 ("73") in control, mock (–) and GPP130 siRNA (+) lysates (micrograms per lane is indicated). Note that the lanes were overloaded to allow detection of the residual GPP130. This caused overexposure of the highest signals yet the titration shows that the signals remained proportional to the protein loaded. Thus, GPP130 knockdown may have caused, at most, a slight change in GP73 levels. Mock (C) and GPP130 siRNA (D)-transfected HeLa cells were stained 65 h posttransfection by using antibodies against GPP130. As determined by measuring Golgi-associated GPP130 staining intensity across the population of transfected cells in five experiments, an average of $91 \pm 2\%$ of cells exhibited undetectable GPP130 staining. Bar, 10 μ m.

E–H). Quantitation indicated that average GP73 staining intensity in the Golgi was reduced from 85 ± 20 in mock-transfected cells to 16 ± 8 in siRNA-transfected cells ($n = 4$). GP73 loss from the Golgi reflected redistribution rather than degradation because total GP73 levels, as determined by immunoblotting, were apparently unchanged (Figure 1B).

The specificity of the GPP130 knockdown and its associated phenotype was indicated by several observations. First,

immunoblotting showed that transfected cells with inhibited GPP130 expression contained normal levels of β -tubulin, giantin, and GP73 (Figure 1; our unpublished data). Second, a comparison of staining intensities after immunofluorescence analysis of control and siRNA-transfected cells yielded indistinguishable levels for a variety of organelle marker proteins (see below). Third, siRNAs without effect on GPP130 expression did not effect GP73 localization (Figure 2; our unpublished observations). Finally, and most important, a second siRNA against a nonoverlapping GPP130 sequence also knocked down GPP130 expression and was associated with the same phenotype. That is, GP73 was redistributed, whereas GFP-NAGT2 was normal (Figure 2, I–L). That GPP130 knockdown causes displacement of GP73 with no apparent effect on GFP-NAGT2 localization or Golgi structure indicates that GPP130 is required for GP73 targeting to the Golgi apparatus.

Because TGN46 also depends on bypass pathway retrieval for its Golgi targeting, we next determined the localization of TGN46 in cells lacking detectable GPP130. Cells transfected with GPP130 siRNA exhibited a clear shift of TGN46 from its Golgi localization to peripheral punctate structures (Figure 3, A and B). Average TGN46 staining intensity in the Golgi was reduced from 130 ± 50 arbitrary units in mock-transfected cells to 20 ± 10 in siRNA-transfected cells. We were unable to detect TGN46 by immunoblot. Thus, it remains unclear whether, in common with GP73, the redistributed TGN46 escaped degradation. However, costaining revealed that GP73 and TGN46 accumulated in the same peripheral punctate structures and quantitation indicated that they did so to the same extent (our unpublished observations).

If GPP130 mediates bypass pathway trafficking then the structures containing redistributed GP73 and TGN46 should be endosomes. Therefore, it was tested whether the peripheral punctate structures became labeled by externally added antibodies against luminal epitopes in GP73 and TGN46. Under the conditions of the assay, mock-transfected cells exhibited weak uptake of externally added anti-GP73 antibodies, whereas antibody uptake was dramatically increased in cells lacking GPP130 (Figure 3, C and D). A

Figure 2. GP73 is mistargeted in the absence of GPP130. HeLa cells were either transfected with control siRNA #384 (A–D) or transfected with either of two nonoverlapping GPP130 siRNAs, as indicated (E–H and I–L), and then triple stained 65 h posttransfection by using anti-GPP130, anti-GP73, and GFP-NAGT2. Cells lacking GPP130 showed a redistribution of GP73 from the Golgi to peripheral and juxta-nuclear punctate structures. An average of $71 \pm 3\%$ of transfected cells yielded redistribution of the GP73 staining pattern ($n = 3$, >70 cells per experiment). Bar, 10 μ m.

Figure 3. Displacement of TGN46 and GP73 to endosomes. (A and B) Mock (A) and GPP130 siRNA (B)-transfected HeLa cells were costained 65 h posttransfection with anti-GPP130 and anti-TGN46. All GPP130 siRNA-transfected cells shown lacked detectable GPP130 staining. In the absence of GPP130, TGN46 Golgi staining was appreciably reduced and it accumulated in peripheral punctate structures. The inset shows a peripheral region at a higher exposure to demonstrate TGN46-positive punctate structures. (C–F) Mock (C and E) and GPP130 siRNA (D and F)-transfected HeLa cells were allowed to internalize anti-GP73 (C and D) or anti-TGN46 (E and F) antibodies for 1 h and noninternalized antibody was removed with an acid wash. Cells lacking GPP130 exhibited a dramatic increase in specific antibody uptake. Bar, 10 μ m.

similar increase was noted for anti-TGN46 antibody uptake in cells lacking GPP130 (Figure 3, E and F). As determined by comparison of object associated fluorescence of identically thresholded images the level of increase for each antibody was at least 10-fold. In the case of GP73, the average number of anti-GP73-labeled endosomes increased from 8 ± 1 in cells mock-transfected cells to 101 ± 6 in cells lacking GPP130 ($n = 2$, >25 cells per experiment). Control antibodies added at the same concentration yield no detectable internalization signal in this assay, indicating that fluid phase antibody uptake does not contribute to the signal (Puri *et al.*, 2002). Thus, uptake of anti-GP73 and anti-TGN46 antibodies in transfected cells depends on surface cycling of the redistributed antigens and is dramatically increased by GPP130 inhibition. Costaining was used to test whether the redistributed Golgi proteins accumulated in the same endocytic structures labeled by antibody uptake. Indeed, the TGN46-positive punctate structures induced by GPP130 knockdown were clearly labeled by internalized anti-GP73 antibodies (Figure 4, A–C). Also, simultaneous internaliza-

Figure 4. Redistributed GP73 and TGN46 accumulate in early endosomes. (A–C) At 65 h posttransfection with the GPP130 siRNA, anti-GP73 antibody uptake incubations were carried out for 1 h and noninternalized antibody was removed with an acid wash. Staining of internalized anti-GP73 (A) is compared with the redistributed TGN46 pattern (B) by using a merged image with GP73 in green and TGN46 in red (C). (D–I) At 65 h posttransfection with the GPP130 siRNA, transferrin uptake incubations were carried out for 5 min followed by 0 or 45 min of chase, as indicated. The redistributed GP73 pattern (GP73) is compared with internalized transferrin (Tf) by using a merged image with GP73 in green and transferrin in red (merge). As an example, many of the costained objects are indicated by arrowheads. Projected images are shown after confocal acquisition.

tion of both antibodies yielded striking costaining. Thus, the two Golgi proteins redistributed to the same structures and these were endosomes.

Endocytic pathway markers were then used to determine the identity of the endosomes containing the redistributed Golgi proteins. Strikingly, many GP73 endosomes seemed to be early endosomes based on the presence of transferrin after internalization for 5 min. Under these conditions, 54% (± 11 , $n = 3$) of the endosomes containing GP73 were positive for transferrin (Figure 4, D–F). When the internalized transferrin was then allowed to move to recycling endosomes during a 45-min chase incubation, the level of coincidence dropped to 12% (Figure 4, G–I). This suggests that redistributed GP73 was primarily accumulating in early endosomes. Consistent with this, <25% of the GP73 positive structures contained the recycling endosome marker Rme-1 (our unpublished observations). Also, LAMP2 was rarely present in GP73-positive structures, indicating that few GP73-positive structures were late endosomes/lysosomes. Thus, absence of GPP130 causes a shift in the distribution of GP73 and TGN46 from the Golgi to early endosomes, indicating that GPP130 mediates exit of GP73 and TGN46 from early endosomes during their Golgi retrieval.

To test whether GPP130 function is specific to the targeting of proteins that cycle in the late endosome bypass pathway, we also determined the localization of a number of marker proteins that do not significantly cycle this pathway. Similar to the results for GFP-NAGT2 presented above, Golgi markers giantin, GM130, and GRASP65 each yielded a normal Golgi ribbon staining pattern in cells lacking GPP130

Figure 5. Selective displacement of GP73 and TGN46. (A–F) Mock (A, C, and E) and GPP130 siRNA (B, D, and F)-transfected HeLa cells were costained 65 h posttransfection with anti-GPP130 and either anti-giantin (A and B), anti-golgin-97 (C and D), or anti-FLAG to detect FLAG-tagged furin (E and F). All GPP130 siRNA-transfected cells shown lacked detectable GPP130 staining. Bar, 10 μ m. (G) Golgi fluorescence for marker proteins was determined in mock and GPP130 siRNA-transfected cells. Values represent average pixel intensity in the Golgi region per cell plotted as percentage of mock-transfected controls ($n = 3$, >30 cells per experiment).

(Figure 5, A and B; our unpublished data). Importantly, an unperturbed localization pattern was also observed for golgin-97, a TGN marker (Luke *et al.*, 2003), indicating that the TGN itself was intact in cells that contained redistributed TGN46 and GP73 (Figure 5, C and D). Note that costaining was used to confirm not only GPP130 knockdown but also

GP73 redistribution in the same cells that contained normal golgin-97 patterns. The localization of two ER markers, calreticulin and p63, and the ER-Golgi intermediate compartment marker ERGIC53, were also compared in control cells and cells lacking GPP130. Indistinguishable localization patterns were observed (our unpublished observations). Also unaffected were the localization patterns of cation-independent mannose-6-phosphate receptor, lysosomal associated membrane protein 1, Rme-1, and internalized transferrin receptor (our unpublished observations). Perhaps most striking, the localization of furin, which cycles in the classic late endosome-to-Golgi pathway, was also unaffected by the absence of GPP130 (Figure 5, E and F). Quantitative determination of Golgi staining in mock and GPP130 siRNA-transfected cells confirmed the selective displacement of the two bypass pathway markers GP73 and TGN46, compared with the nonbypass pathway Golgi markers (Figure 5G). Therefore, the role of GPP130 in targeting is highly specific to proteins that depend on retrieval in the bypass pathway for their localization. Further, even though GPP130 knockdown causes redistribution of GP73 and TGN46, GPP130 is apparently not required for the integrity of the ER, ERGIC, Golgi, TGN, recycling endosomes, late endosomes, or lysosomes.

Based on these results, it seemed possible that GPP130 might act generally to facilitate sorting into the bypass pathway. Therefore, we tested whether GPP130 mediates endosome-to-Golgi trafficking of Shiga toxin using the nontoxic B-subunit of Shiga toxin as a marker (Mallard *et al.*, 1998). Control and GPP130 knockdown cells internalized Shiga toxin B-subunit with equal efficiency, indicating that absence of GPP130 does not affect endocytosis of the toxin into the cell. To synchronize transport, the toxin was allowed to accumulate in early and/or recycling endosomes by using incubation at 19.5°C (Mallard *et al.*, 1998). As expected, a return of control cells to 37°C was accompanied by rapid transport of the toxin from peripheral punctate structures to the Golgi apparatus (Figure 6, “mock”). In contrast, in cells lacking GPP130, transport of Shiga toxin was significantly delayed (Figure 6, “GPP130 siRNA”). Rather than transfer to the Golgi, there was an extended period of Shiga toxin localization to peripheral punctate structures. Consistent with a kinetic block during exit from early endosomes, Shiga toxin-positive structures at the 30-min time point seemed to be identical to the endosomes containing redistributed GP73 and TGN46. That is, $93 \pm 4\%$ ($n = 3$) of GP73-positive punctate structures in these cells were also positive for Shiga toxin.

Kinetics of Shiga toxin transport were quantified by determining the percentage of cells at each time point yielding a predominantly Golgi-localized Shiga toxin pattern (Figure 7A). By 30 min, Shiga toxin was already mostly Golgi-localized in mock-transfected controls. In contrast, even at 90 min in cells lacking GPP130, there was little Shiga toxin evident in the Golgi. As indicated in the figure, the inhibition was apparent for each of the two nonoverlapping siRNAs effective in causing GPP130 knockdown. Interestingly, at the 60- and 90-min time points in cells lacking GPP130, the toxin subunit was frequently associated with larger juxta-nuclear punctate structures (e.g., Figure 6O). Given that Golgi markers yield a normal ribbon pattern in the absence of GPP130 (see above), the punctate pattern may reflect Shiga trafficking by an alternate route. Possibly, misrouting in early endosomes allows access of the toxin subunit to the late endosome route. Cells with surface-bound toxin subunit were also shifted directly to 37°C to avoid any possibility that the 19.5°C incubation altered the normal trafficking of

Figure 6. GPP130 mediates endosome-to-Golgi transport of Shiga toxin. Shiga toxin B-fragment staining is shown after the indicated times of 37°C incubation (0, 10, 20, 30, and 60 min) in cells that had been mock transfected (column 1), GP73 siRNA transfected (column 2) or GPP130 siRNA transfected (column 3). Before the 37°C incubations, the B-fragment was added to cells on ice and then allowed to accumulate in endosomes by using a 19.5°C incubation (see *Materials and Methods*). Bar, 10 μm.

the toxin subunit. Similar to the case for endosome-to-Golgi trafficking, surface-to-Golgi trafficking of the toxin subunit was significantly inhibited in the absence of GPP130 and the toxin subunit persisted in endosomal structures (Figure 7B). In sum, although GPP130 is not absolutely required, its inhibition caused a potent kinetic block in endosome-to-Golgi trafficking of Shiga toxin B-fragment.

Three additional specificity controls were carried out. First, the Shiga toxin trafficking assay was performed in cells in which GP73 expression was knocked down by siRNA transfection. Despite the finding that GP73 levels were reduced to levels below detection, this was not associated with

any effect on Golgi targeting of GPP130 or other Golgi markers (our unpublished observations). There was also no effect on trafficking of Shiga toxin, which moved to the Golgi with kinetics indistinguishable from mock-transfected controls (Figure 6, "GP73 siRNA"). Second, endocytic transport of a fluid phase tracer from the surface to lysosomes was also tested. In an internalization time course spanning 2 h, fluorescein isothiocyanate (FITC)-dextran moved to lysosomes as indicated by costaining with LAMP2. Neither the rate nor the extent of FITC-dextran appearance in lysosomes was significantly different in mock versus GPP130 siRNA-transfected cells (Figure 8A). Third, the kinetics of Golgi

Figure 7. Quantification of Shiga toxin transport in cells lacking detectable GPP130. (A) Cell-bound Shiga B-fragment was allowed to accumulate in endosomes during a 19.5°C incubation. After transfer to 37°C for the indicated times, the percentage of cells showing predominantly Golgi localized B-fragment was determined for mock and the two nonoverlapping GPP130 siRNAs (#377 and #335). Values shown are averages ($n = 3$, >40 cells per time point in each). (B) The percentage of cells showing predominantly Golgi localized B-fragment was also determined for mock and siRNA-transfected cells (#377 and #335) in which the Shiga transport assay was carried out in the absence of the 19.5°C accumulation. After addition of the B-fragment to cells on ice, the cells were transferred directly to 37°C for the times indicated (>30 cells per time point).

apparatus reassembly was determined after brefeldin A washout. Analysis of multiple time points by using immunofluorescence yielded no indication that GPP130 mediates any of the anterograde and retrograde trafficking reactions that underlie Golgi biogenesis (Figure 8B).

DISCUSSION

GPP130 is a ubiquitous homodimeric type II integral membrane protein with a short, 12-amino acid, cytoplasmic tail (Lindstedt *et al.*, 1997). The 664-amino acid luminal domain has two distinct regions. The membrane proximal region is strongly predicted to form a coiled-coil stem structure and contains distinct Golgi and endosomal targeting determinants that mediate GPP130 bypass pathway trafficking and targeting (Bachert *et al.*, 2001; Puri *et al.*, 2002). The outermost luminal region is rich in acidic amino acids. Inhibition of GPP130 expression was used to test its role in bypass

Figure 8. Traffic to lysosomes and Golgi reassembly are normal after GPP130 knockdown. (A) The fluorescence intensity of lysosome-localized FITC-dextran was determined for each time point. Values shown are averages ($n = 3$, >40 cells per experiment). Only FITC-dextran fluorescence that was coincident with LAMP2 staining in the doubly stained cells was included. (B) Levels of giantin fluorescence after thresholding were determined at various time points of Golgi assembly during brefeldin A washout. For all samples, staining was carried out in parallel, images were acquired at a constant exposure, and a constant threshold (value = 80) was applied. The threshold value was chosen to exclude fluorescence contributed by ER-localized giantin, thereby allowing measurement of Golgi emergence and assembly.

pathway trafficking. In sum, the defects observed in cells lacking GPP130 were remarkably specific to this Golgi-directed branch off the endocytic recycling pathway. Endogenous proteins that cycle in this pathway became displaced from the Golgi and accumulated in early endosomes. Trans-

port of a toxin subunit in this pathway was impaired, causing its accumulation in early endosomes. The localization of proteins that do not cycle in the bypass pathway was unperturbed as were transport in the endocytic degradative pathway and reassembly of the Golgi apparatus. These observations indicate that a *cis*-Golgi protein that cycles via the bypass pathway plays a functionally important role in traffic from endosomes to the Golgi.

Most likely, GPP130 facilitates the crucial sorting step in which certain molecules are directed out of the recycling pathway into membranous carriers that will fuse with the TGN. Similar to luminal pH disruption, GPP130 absence alters the steady-state distribution of GP73 and TGN46 such that a significant pool is now present cycling between the cell surface and endosomes. This implies increased trafficking through the surface, which, given the controls, is strongly supported by the observed increased antibody uptake and transferrin colocalization. That is, without efficient sorting into the bypass pathway there is increased trafficking via the alternative routes. This would not only include the recycling route back to the cell surface, but also the late endosome route. In the latter case, bypass pathway markers might inefficiently reach the Golgi in the absence of GPP130. As stated above, the presence of Shiga toxin in relatively large juxta-nuclear endosomes in cells lacking GPP130 may reflect Shiga trafficking via this alternate route.

The molecular requirements in the bypass pathway are being identified. Unlike the more classical late endosome pathway, the bypass pathway requires neither intact microtubules nor phosphoinositide 3-kinase activity (Kundra and Kornfeld, 1998; Mallet and Maxfield, 1999). Rab11, which is present on recycling endosomes, plays a role not only in recycling to the plasma membrane but also in mediating efficient trafficking of TGN38 to the Golgi (Wilcke *et al.*, 2000). Export from early and/or recycling endosomes toward the Golgi also involves the EH-domain protein Rme-1, EpsinR, and clathrin (Mallard *et al.*, 1998; Lin *et al.*, 2001; Saint-Pol *et al.*, 2004). Rab6a' is required for Shiga toxin transport to the Golgi, leading to the suggestion that it controls docking of bypass pathway vesicles at the TGN (Mallard *et al.*, 2002). Finally, fusion at the TGN of membranes that are sorted away from the recycling pathway depends on one of two v-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), vesicle-associated membrane protein (VAMP)3 or VAMP4, interacting with a Syn16-based t-SNARE complex that contains Syn6 and Vti1a (Mallard *et al.*, 2002).

Where might GPP130 fit into the emerging mechanisms of bypass pathway trafficking? One critical aspect that remains unclear is how a linkage is made from sorting factors acting on the cytoplasmic face of the membrane, such as epsinR and clathrin, to luminal cargo molecules such as Shiga toxin. Formation of sorting complexes for targeting to the bypass pathway could involve lipid rafts (Brown and London, 1998; Falguieres *et al.*, 2001) and/or integral membrane components. One possibility is that the GPP130 luminal domain binds to, and sorts, molecules trafficking in the bypass pathway. If so, its interactions with TGN46 and GP73 are likely to be weak or indirect as neither affinity chromatography nor yeast two-hybrid tests have revealed any interaction (Puri *et al.*, 2002). Another possibility is that GPP130 facilitates a lipid-based sorting mechanism. GPP130 is present in a detergent-insoluble complex (our unpublished observations), and it contains a consensus sequence for *N*-myristoylation. Detergent insolubility is a feature exhibited by Shiga toxin and evidence suggests that this is important in its trafficking (Falguieres *et al.*, 2001). Thus, the presence of

GPP130 may exert an effect on lipid composition that serves to increase the local concentration of cargo molecules and thereby their detergent insolubility during exit from early endosomes.

Together, the membrane topology of GPP130, its presence in early endosomes, and its knockdown phenotype suggest that GPP130 could link membrane domains rich in cargo proteins to vesicle coats, presumably clathrin based (Mallard *et al.*, 1998), forming on the cytoplasmic side of endosomal membranes. The identification of a role for GPP130 in endosome-to-Golgi trafficking sets the stage for a structure/function analysis to dissect its mechanism of action. The ultimate characterization of the mechanisms underlying trafficking in the bypass pathway will not only reveal reactions critical to the targeting and function of distally cycling Golgi proteins but also reactions exploited by pathogenic toxins to invade cells.

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