A Functional Role for the GCC185 Golgin in Mannose 6-Phosphate Receptor Recycling


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Mannose 6-phosphate receptors (MPRs) deliver newly synthesized lysosomal enzymes to endosomes and then recycle to the Golgi. MPR recycling requires Rab9 GTPase; Rab9 recruits the cytosolic adaptor TIP47 and enhances its ability to bind to MPR cytoplasmic domains during transport vesicle formation. Rab9-bearing vesicles then fuse with the trans-Golgi network (TGN) in living cells, but nothing is known about how these vesicles identify and dock with their target. We show here that GCC185, a member of the Golgin family of putative tethering proteins, is a Rab9 effector that is required for MPR recycling from endosomes to the TGN in living cells, and in vitro. GCC185 does not rely on Rab9 for its TGN localization; depletion of GCC185 slightly alters the Golgi ribbon but does not interfere with Golgi function. Loss of GCC185 triggers enhanced degradation of mannose 6-phosphate receptors and enhanced secretion of hexosaminidase. These data assign a specific pathway to an interesting, TGN-localized protein and suggest that GCC185 may participate in the docking of late endosome-derived, Rab9-bearing transport vesicles at the TGN.

INTRODUCTION

Mannose 6-phosphate receptors (MPRs) bind newly synthesized lysosomal hydrolases in the Golgi complex and deliver them to prelyosomes (Ghosh et al., 2003). There, the enzymes are released from MPRs, which are then recycled to the trans-Golgi network (TGN) for another round of enzyme delivery. There are two MPRs in most cell types: the cation-independent (CI)-MPR of ~300 kDa and the cation-dependent (CD)-MPR, a dimer of ~45 kDa. Both MPRs seem to require similar cellular components for their transport between compartments within mammalian cells (Ghosh et al., 2003).

This laboratory has studied the process by which MPRs are transported from late endosomes to the Golgi. We have shown that MPR recycling requires Rab9 GTPase and its effector p40 (Lombardi et al., 1993; Riederer et al., 1994; Diaz et al., 1997), a cargo adaptor named TIP47 (for tail-interacting protein of 47 kDa; Diaz and Pfeffer, 1998; Burguete et al., 2005) and several other general transport factors (Itin et al., 1997). Using live cell video microscopy, we were able to track yellow fluorescent protein (YFP)-Rab9-containing transport vesicles and watch them fuse with a cyan fluorescent protein-labeled trans-Golgi compartment (Barbero et al., 2002). That study showed that Rab9 is present on transport vesicles en route to the Golgi complex, but it did not provide additional clues to the mechanisms by which such vesicles are targeted.

In an effort to understand the full scope of Rab9 function, we sought to identify additional partners for the active form of this GTPase. We report here the discovery that a Golgi-localized, putative tether of the Golgin protein family binds specifically to Rab9. Golgins contain long, predicted coiled-coil motifs and are important for Golgi organization, vesicle tethering, and secretory protein transport (for reviews, see Gillingham and Munro, 2003; Gleeson et al., 2004; Short et al., 2005). Several Golgins bind Rab GTPases, but the precise functional significance of Rab–Golgin interactions remains poorly defined. Rab1 binds to the well-characterized Golgin p115 (Allan et al., 2000) and to Golgin-84 (Diao et al., 2003); Rab6α recruits the coiled-coil, Bicaudal-D protein to the TGN (Matanis et al., 2002) and also binds to a Golgin named TATA element modulatory factor (Fridmann-Sirkis et al., 2004); Rab2 interacts with the medial Golgi matrix protein Golgin-45 (Short et al., 2001).

Four mammalian Golgins, named Golgin-97, Golgin-245, GCC88, and GCC185, contain so-called GRIP domains, which are ~50 amino acids in length and have each been shown to be sufficient to target green fluorescent protein (GFP) to the trans-Golgi (Munro and Nichols, 1999; Barr, 1999; Kjer-Nielsen et al., 1999a,b; Luke et al., 2003; Figure 1). Certain GRIP domains bind to the Arf-like GTPase Arl1 (Van Valkenburgh et al., 2001; Lu and Hong, 2003), which itself is Golgi localized via Arl3 (Setty et al., 2003; Panic et al., 2003b). Interaction with Arl1 at the TGN is the basis for the localization of several GRIP domain proteins.

We show here that the TGN localized, GCC185 protein binds to Rab9 with strong preference for its active, GTP-bound conformation. Importantly, the Rab9-dependent, retrograde trafficking of CD-MPRs to the TGN requires GCC185 but not the related Golgin-97. Unlike Golgin-245,
GCCC185 binds poorly (if at all) to Arl1 GTPase in vitro, distinguishing this Golgin from other GRIP domain-containing proteins.

MATERIALS AND METHODS

Yeast Two-Hybrid System

To construct plasmids pRB2510-Rab9Q66L acc and pRB2510-Rab9S21N acc, Rab9 reading frames were excised from pASl-CYH2-Rab9Q66L acc and pASl-CYH2-Rab9S21N acc, respectively (Diaz et al., 1997) with NcoI and BamH1 and cloned into plasmid pRSB2510 (Schwartz et al., 1997). A human placental cDNA library was amplified according to the manufacturer (Clontech, Mountain View, CA). Yeast strain Y190 (MATa and his3Δ1 leu2Δ0 trp1Δ0 ura3Δ1) was transformed sequentially with pRB2510-Rab9Q66L acc and pGBD-CYC1 (pGAL), or pRB2510-Rab9S21N acc and pGBD-CYC1 (pGAL) as a template (PGAL12-T7; BD Biosciences, San Jose, CA) to create plasmid pHis-GCC185. A glutathione S-transferase (GST)-tagged, 110 C-terminal amino acid fragment of GCC185 was generated by PCR using pHis-GCC185 as a template, followed by digestion with BamHI and EcoRI and ligation into BamHI-EcoRI–digested pGEX 4T-1 (GE Healthcare) equilibrated in binding buffer (5 mM MgCl2, 0.1 mg/ml BSA, 150 mM NaCl, and 20 mM HEPES/KOH, pH 7.4, for 30 min at 37°C in a total volume of 1 ml). Free nucleotide was removed on a PD-10 column (GE Healthcare) equilibrated in binding buffer (5 mM MgCl2, 0.1 mg/ml BSA, 150 mM NaCl, and 20 mM HEPES/KOH, pH 7.4) prior to 3 h at room temperature and processed as described above.

Small Interfering RNA (siRNA) Depletion of GCC185 and Rab9

Human Rab9 was targeted using the duplex: GUUGUAACACCCAGGUUCUC. Human GCC185 was targeted using two independent duplexes: GCC185/1: GAAACAAGCUGAUGUGCCUAA, GCC185/2: GAAUCAUAAACUAUGUGGCUU (Dharmacon, Lafayette, CO). HeLa or human embryonic kidney (HEK)-293 cell transfections were performed according to the manufacturer using Oligofectamine or Lipofectamine 2000, respectively (Invitrogen). Human Golgin-97 was targeted precisely as described previously (Lu et al., 2004).

Microscopy and Immunoblotts

Posttransfection (48–72 h), HeLa, or BS-C-1 cells were fixed and processed for immunofluorescence (Warren et al., 1984). For Figure 3C and Supplemental Figure 1, cells were imaged using an Axiovert 200 inverted microscope (Carl Zeiss Microimaging, Thornwood, NY) fitted with a 63×/1.4 Plan-Apochromat objective and a charge-coupled device (CCD) (CoolSNAP HQ, Photometrics, Tucson, AZ). For Figures 4, top, and 6C, cells were imaged using an Axioplan 2 fluorescence microscope (Carl Zeiss Microimaging) fitted with a Plan-Neofluar 63×/1.4 oil objective (Carl Zeiss Microimaging), and a CCD camera (AxioCam HRc; Carl Zeiss Microimaging) and controlled by Axiovision 4.2 software (Carl Zeiss Microimaging). Figures 5, 6A, and Supplemental Figure 2 were acquired using a deconvolution microscopy setup (Spectris; Applied Precision, Issaquah, WA), with an inverted epifluorescence microscope (IX70; Olympus America, Melville, NY), a PlanApo 60×, 1.40 numerical aperture oil immersion objective (Olympus America), a CCD camera (CoolSNAP HQ) and acquisition and deconvolution software (Delta Vision; Applied Precision). The quantitative analysis of fluorescence intensity in Figure 2B was performed using ImageJ (Wayne Rasband, National Institutes of Health, Bethesda, MD). All images were processed using ImageJ and Adobe Photoshop (Adobe Systems, Mountain View, CA).

When staining with anti-Rab9 or anti-furin, cells were washed once in ice-cold glutamate buffer (75 mM HEPES, pH 7.4, 25 mM KCl, 2.5 mM MgOac2, 5 mM EGTA, and 150 mM K glutamate). After removal of excess buffer, coverslips were frozen in liquid nitrogen, incubated for 1 min in glutamate buffer, washed twice on ice with ice-cold glutamate buffer, fixed, and then processed as described above but without detergent treatment. Mouse anti-MPR was labeled with the Zenon IgG labeling kit (Invitrogen) when containing required mouse anti-EEA1 antibody. For electron microscopy, GCC185 siRNA-transfected HeLa cells were grown on 12-mm glass coverslips for 48 h and processed as described previously (Canley et al., 2004). For immunoblots, 72 h after transfection with GCC185 siRNA, cells were washed three times with phosphate-buffered saline (PBS), followed by incubation with radiolabeled monosaccharide (RIPA) buffer for 15 min. Lysates were collected at 10,000 × g for 10 min, and equal amounts of protein were analyzed by immunoblot. Signals were detected by chemiluminescence (PerkinElmer Life and Analytical Sciences, Boston, MA).

Metabolic Labeling

HeLa cells seeded onto six-well plates were transfected with GCC185 siRNA. G3P is present in the vesicular stomatitis virus (VSV)-YFP transfectant across GCC185 siRNA-transfected HeLa cells 56 h later using FuGENE 6 (Roche Diagnostics). After a total of 72 h of transfection, cells were metabolically labeled as described previously (Burge et al., 2005). Cells were washed three times with ice-cold DMEM, followed by binding of cell surface VSVG-YFP with anti-GFP for 1 h at 4°C. Unbound antibody was washed away with PBS, and cells were lysed in RIPA buffer containing protease inhibitors for 15 min. Antibody–protein complexes were immunoprecipitated with protein A-agarose (Roche Diagnostics) and

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processed. MPR turnover was determined as described previously (Ganley et al., 2004). For CD-MPR maturation, HEK-293 HeLa cells stably expressing a modified CD-MPR (Itin et al., 1997) were transfected with siRNA for 48 h as described above. Cells were metabolically labeled for 30 min, washed, and chased (Burguete et al., 2005). At the indicated times, cells were transferred to ice, washed once, and lysed in RIPA buffer (Itin et al., 1997) containing protease inhibitors for 30 min. The cell lysate was centrifuged for 10 min at 100,000 × g at 4°C, and the supernatant was incubated with 40 μl of pre-washed Ni-NTA agarose beads for 1 h at 4°C. The beads were washed three times in 500 μl of RIPA and eluted in 100 μl RIPA containing 25 mM EDTA. The CD-MPR was detected by SDS-PAGE and autoradiography.

**Transport and Enzyme Assays**

The in vivo transport assay was adapted to HEK-293 cells as described previously (Sincock et al., 2003), except that cells were transfected with GCC185 or Golgin-97 siRNA for 72 h before initiation of 2 h of transport. The in vitro transport assay was also adapted to HEK-293 cells (Itin et al., 1997). In some cases, purified GST or GST-GCC185 protein was added at 94 μg/ml before 90 min of transport at 37°C. To examine tyrosine sulfation of endogenous proteins, in vivo transport was conducted with cells transfected with either GCC185 siRNA or Golgin-97 siRNA for 72 h before initiation of 2 h of transport. Equal quantities of whole cell lysates were run on a 6% SDS-PAGE gel and the signal quantified by autoradiography. Transferrin uptake was measured in HeLa cells seeded onto six-well plates, transfected with GCC185 siRNA for 72 h. Cells were washed three times with PBS followed by incubation with 5 μg/ml Alexa Fluor 594 transferrin in PBS buffer A (1 mM MgCl2, 1 mM CaCl2, and 0.2% BSA in PBS) for 90 min at 4°C. Cells were washed a further three times with PBS and then incubated in DMEM media containing 7.5% fetal bovine serum for either 5 or 15 min at 37°C. Surface bound transferrin was washed away with ice-cold citrate buffer containing 25.5 mM citric acid, 24.5 mM Na citrate, and 280 mM sucrose. After a final wash in PBS buffer A, the cells were processed for immunofluorescence as described above. Images were quantified using ImageJ software. Hexosaminidase was measured according to Riederer et al. (1994) except that cells were incubated in 1 ml of DMEM media lacking phenol red plus 10 mM mannose 6-phosphate.

**RESULTS**

We carried out a two-tiered, yeast two-hybrid screen to identify novel Rab9 effectors. Clones (1 × 108) were selected for interaction with an activated, Rab9-GTP protein (Rab9 Q66L) in preference to a Rab9-GDP–preferring mutant, Rab9 S21N. In the past, this approach has yielded physiologically relevant binding partners, such as a protein named p40 (Diaz et al., 1997). One of the positive clones obtained in the present screen encoded 342 C-terminal residues of a protein named GCC185 (Figure 1; Luke et al., 2003). GCC185 contains a GRIP domain at its C terminus, and the remainder of the 185,000 Mw protein is predicted to form a long, extended coiled-coil (Figure 1).

Two independent methods were used to confirm that the interaction between Rab9 and GCC185 was direct. In the first, the 342 C-terminal residues of GCC185 (1343-1684) were expressed in bacteria as a His-tagged protein. This protein was incubated with untagged Rab9 or Rab5 (Figure 2A); bound proteins were collected by Ni-NTA column chromatography and detected by immunoblot. As shown in Figure 2B, Rab9 showed significant binding to the 342 C-terminal residues using this method, whereas Rab5 did not. The second approach used Rab proteins preloaded with [35S]GTPγS or [3H]GDP to facilitate detection. Bound proteins were eluted, and subsequent immunoblot analysis revealed specific interaction of Rab9 but not Rab1 with cytosolic GCC185. Thus, the Rab9 binding site is available for...
Rab interaction within the context of the native, full-length protein.

**TGN Localization of GCC185**

A hallmark of GRIP domain proteins is their ability to bind Arl1 GTPase. Indeed, the three-dimensional structure of the Golgin-245 GRIP domain bound to Arl1 has been determined previously (Panic et al., 2003a; Wu et al., 2004). But there has been some controversy regarding the capacity of GCC185 to bind Arl1. A His-tagged, 110 residue, GCC185 C-terminal construct was shown to bind GST-Arl1 GTPase with preference for the GTP-bound conformation (Panic et al., 2003a). However, Derby et al. (2004) failed to relocalize GCC185 from the TGN in cells expressing an endosome-associated Arl1-hybrid protein construct. Moreover, GCC185 was shown to occupy a domain of the TGN that is distinct from the other mammalian GRIP domain-containing Golgin proteins (Derby et al., 2004), suggesting that it may use a distinct mechanism to localize to the Golgi.

As shown in Figure 3A, we failed to detect binding of His-tagged, active Arl1 protein to glutathione agarose-immobilized GCC185. In contrast, significant binding of Arl1-GTP to immobilized Golgin-245 C terminus was readily detected. From these experiments, we conclude that Arl1 interacts much less strongly with GCC185 than with Golgin-245. It is possible that the dimeric nature of the GST-Arl1 construct used by others (Panic et al., 2003a) revealed a weaker binding interaction than we were able to detect using His-tagged Arl1 protein.

GRIP domain-containing proteins contain an invariant tyrosine residue that is key for Arl1 binding and in many cases, Golgi localization. Mutation of the corresponding residue (Tyr1618) in the GST-tagged GCC185 C terminus did not interfere with Rab9 binding (Figure 3B), suggesting that Rab9 binds GCC185 via a molecular interaction other than that used for typical Arl1 interaction. Future work will determine the precise binding interface of Rab9 GTPase with GCC185 C-terminal sequences.

If GCC185 does not bind tightly to Arl1, how is it localized to the Golgi complex? Most Rab9 is present on late endosomes, but a small amount is detected on the TGN (Lombardi et al., 1993). Therefore, we tested whether the Golgi localization of GCC185 was in any way dependent on Rab9 protein. Cellular depletion of Rab9 by using siRNA (Ganley et al., 2004) did not in any way seem to alter the TGN localization of GCC185 (Figure 3C). Unfortunately, attempts to alter the fraction of GCC185 that was membrane associated by Rab9 overexpression were not possible to interpret, because this resulted in a significant pool of unprenylated, cytosolic Rab9.
that could have bound to cytosolic GCC185. Nevertheless, these data show that Rab9 is not a primary determinant of GCC185’s Golgi association. It is possible that another Rab protein or another member of the large family of Arl proteins mediates GCC185 Golgi localization.

GCC185 Is Required for Endosome-to-Golgi Transport

Rab9 is required for the transport of MPRs from late endosomes to the TGN (Lombardi et al., 1993; Riederer et al., 1994). The established localization of GCC185 at the TGN was intriguing in that it suggested a possible role for GCC185 as a tether for Rab9-positive, MPR-containing transport vesicles (Barbero et al., 2002) en route to the Golgi from late endosomes. If true, cellular depletion of GCC185 would be predicted to block this transport pathway and lead to aberrant trafficking of proteins cycling between endosomes and the TGN. We therefore used two distinct siRNAs to deplete cells of GCC185 protein to investigate its function.

First, loss of GCC185 altered the morphological appearance of the Golgi ribbon (Figure 4). Golgin-245 is another TGN-associated GRIP domain-containing protein that shows labeling that is slightly distinct but overlapping with GCC185 (Luke et al., 2003; Derby et al., 2004; Figure 4). Golgin-245 staining is sometimes more extended than resident Golgi enzymes but is representative of the TGN as determined by electron microscopy (Luke et al., 2003; Derby et al., 2004; Figure 4). Golgin-245 is another TGN-associated GRIP domain-containing protein that shows labeling that is slightly distinct but overlapping with GCC185 (Luke et al., 2003; Derby et al., 2004; Figure 4). On depletion of GCC185, Golgin-245 labeled less elongated structures that nevertheless, remained on one side of the nucleus (Figure 4, top). This suggested a slight alteration in the Golgi ribbon, which was confirmed by electron microscopy of GCC185-depleted cells (Figure 4, bottom). In depleted but not control cells, clusters of Golgi mini-stacks were readily detected in individual sections. A similar change in Golgi morphology was recently reported in cells missing Golgin-245 (Yoshino et al., 2005), TATA modulatory factor (Fridmann-Sirkis et al., 2004) as well as the so-called COG complex, another putative Golgi tether (Zolov and Lupashin, 2005; for review see Puthenveedu et al., 2006). In all of these studies, Golgi morphology is reminiscent of what is observed when cells are treated with nocodazole; however, unlike nocodazole, the Golgi fragments remain localized on one side of the nucleus.

As shown in Figure 5, siRNA depletion of GCC185 led to a major alteration in the steady-state, cellular distribution of MPRs as determined by deconvolution light microscopy. MPRs are normally present in perinuclear late endosomes and to a lesser extent, in the TGN of HeLa cells. On depletion of GCC185, MPRs lost their perinuclear localization and were detected in significantly more dispersed, vesicular structures (Figure 5). The distribution of MPRs was quantified using a series of concentric rings centered upon the peak intensity of MPR staining. The amount of MPR staining detected within the inner circle, middle ring, and outer ring was then compared for cells of similar size. This analysis confirmed a significant difference in the distribution of MPRs between cells of similar size, in the presence or absence of GCC185 protein.

In all cases, MPRs seemed to be more broadly dispersed than any of the Golgi markers examined (compare Figure 5 with 4). For example, furin, a protein thought to cycle between endosomes and the Golgi, was not detected in structures other than those occupied by residual GCC185 (Supplemental Figure 1). A staining pattern resembling that observed for furin (and Golgin-245; Figure 4) was also seen for the Golgi protein p115 in GCC185-depleted cells (Supplemental Figure 2).

To determine whether the MPR-containing accumulated vesicles contained Rab9 protein, we depleted GCC185 from...
BS-C-1 cells stably expressing GFP-Rab9 (Figure 6A). In control BS-C-1 cells, a small amount of the total GFP-Rab9 is present in small structures in the periphery (Figure 6A, left), and in HeLa cells, it colocalizes there with Rab5 and EEA1 in early endosomes (Aivazian et al., 2006). In control cells peripheral zones containing 550 GFP-Rab9 vesicles, 29 vesicles contained MPR; 13 of these vesicles (45%) also contained Rab9. As was shown in Figure 5, control cells contained little peripheral MPR staining.

In GCC185-depleted cells (6A, right), peripheral zones containing 550 GFP-Rab9 vesicles, 29 vesicles contained MPR; 13 of these vesicles (45%) also contained Rab9. As was shown in Figure 5, control cells contained little peripheral MPR staining.

In GCC185-depleted cells (6A, right), peripheral zones containing many more MPR-positive structures: among 470 GFP-Rab9 vesicles, we counted 181 MPR-positive vesicles. Of these MPR-positive vesicles, 99 (55%) also contained GFP-Rab9 protein (Figure 6B). The MPR-positive vesicles seemed slightly larger in the GCC185-depleted cells (Figure 6A, right) compared with MPR+ or GFP-Rab9-positive vesicles in control cells. This is likely because these vesicles are much brighter than the few MPR- or more abundant, GFP-Rab9-positive peripheral vesicles in control cells. In summary, more than half of the peripheral MPR-containing vesicles in GCC185-depleted cells also contain Rab9.

The MPR positive, peripheral vesicles in GCC185-depleted cells were not early endosomes, by the criterion that they lacked EEAI protein (Figure 6C, Hela cells). The vesicles did not resemble late endosomes or lysosomes in terms of their small, apparent uniform size or distribution in these cells; the residual, perinuclear MPR staining (Figure 5) suggested that significant numbers of late endosomes remained intact. Depletion of a protein needed for vesicle consumption but not formation would be expected to result in the accumulation of transport intermediates. Together, these data are consistent with GCC185 depletion leading to a block in recycling, and possible accumulation of transport intermediates that are unable to dock at the Golgi.

**GCC185 Is Needed for MPR Recycling**

The dispersal of MPRs in cells lacking GCC185 (Figure 5) indicated that this protein may be needed for MPR recycling to the TGN. Because the Golgi complex was functional under these conditions (see below), we could use biochemical tests to determine whether MPR transport to the TGN was blocked—either in vivo or in vitro (Itin et al., 1997; Carroll et al., 2001). Briefly, our assays take advantage of a TGN-specific enzyme, tyrosine sulfotransferase. We use cells expressing CD-MPRs that contain an N-terminal 6-histidine tag and a consensus sequence for tyrosine sulfation derived from the cholecystokinin precursor. For in vivo analysis, late endosome-localized MPRs are accumulated in a nonsulfated form by incubation of cells in sulfate-free media containing a sulfotransferase inhibitor. The inhibitor is then removed, and transport is measured by monitoring the action of tyrosine sulfotransferase on CD-MPRs (in the presence of [35S]sulfate) that occurs upon their arrival at the TGN. This process is time, temperature, and cytosol-dependent and requires Rab9 and other components needed for a typical vesicular transport process (Itin et al., 1997).

Intact cells depleted of ~90% GCC185 (Figure 7A, top, inset) showed significant inhibition (~75%) in the transport of CD-MPRs to the TGN (Figure 7A). In contrast, siRNA depletion of the related Golgin-97 inhibited transport only 25%. These data demonstrate that GCC185 is required for the transport of MPRs from late endosomes to the TGN in
living cells. As an independent confirmation of a role for GCC185 in MPR transport, we used wild-type cell extracts to test whether excess, soluble GCC185 C terminus would function as a dominant-negative inhibitor of in vitro transport. In this case, we used an assay very similar to that described for intact cells, except that the cells are broken and reactions are supplemented with cytosol and an ATP-regenerating system (Itin et al., 1997). Addition of a polypeptide representing the 110 C-terminal–most amino acids of GCC185 led to significant inhibition of MPR transport to the TGN in vitro, relative to control reactions to which GST alone was added (Figure 7B). The inhibition observed may be due to titrating out an available binding partner on the TGN or to masking available Rab9 sites so that endogenous GCC185 cannot interact. In either case, these combined data strongly support a role for GCC185 in MPR transport to the TGN.

It was important to confirm the biochemical functionality of the Golgi complex after depletion of GCC185, to ensure the reliability of our conclusions. First, the Golgi complex supported the transport and processing of secretory cargo. We detected no difference in the acquisition of complex oligosaccharides by newly synthesized CD-MPRs in control and GCC185-depleted cells (Supplemental Figure 3). More importantly, to confirm that TGN export of proteins was not defective, we monitored the plasma membrane arrival of a newly synthesized hybrid protein comprised of VSV-G fused to YFP. Cells were pulse labeled with [35S]methionine for 30 min and chased for either 45 or 120 min at 37°C. Cell surface proteins were then immunoprecipitated by incubation of intact cells with antibody at 4°C, washing away unbound antibody, followed by detergent solubilization, and GCC185-depleted cells (Supplemental Figure 3). More importantly, to confirm that TGN export of proteins was not defective, we monitored the plasma membrane arrival of a newly synthesized hybrid protein comprised of VSV-G fused to YFP. Cells were pulse labeled with [35S]methionine for 30 min and chased for either 45 or 120 min at 37°C. Cell surface proteins were then immunoprecipitated by incubation of intact cells with antibody at 4°C, washing away unbound antibody, followed by detergent solubilization,
protein A-agarose incubation, SDS-PAGE, and PhosphorImager analysis. As shown in Figure 8A, the rate with which VSV-G-YFP arrived at the surface was essentially indistinguishable between control and GCC185-depleted cells. No cell surface protein was detected in cells held on ice rather than at 37°C, as would be expected for a vesicular transport process. Thus, despite a slight dispersion of Golgi markers and generation of Golgi mini-stacks, Golgi function did not seem compromised in cells depleted of GCC185.

In addition, the ability of cells to add sulfate to endogenous tyrosine acceptors was only slightly inhibited upon GCC185 depletion (Figure 8B). Finally, the specificity of the inhibition of endosome to Golgi transport was further confirmed by analyzing the ability of cells to endocytose fluorescent transferrin. As shown in Figure 8C, GCC185-depleted cells displayed wild-type transferrin endocytic capacity. Thus, it seems that only endosome-to-Golgi transport is blocked upon loss of GCC185 protein.

Several methods have been previously used to block MPR transport to the TGN in living cells. This includes expression of a Rab9 S21N dominant-negative mutant (Riederer et al., 1994), antisense oligonucleotide, or siRNA depletion of Rab9 or TIP47 proteins (Diaz and Pfeffer, 1998; Ganley et al., 2004), and expression of various TIP47 dominant-negative mutants (Carroll et al., 2001; Hanna et al., 2002; Sincock et al., 2003). These blocks in transport are usually accompanied by compensatory up-regulation of MPRs, enhanced MPR turnover, and in some cases, increased levels of lysosomal enzymes. We therefore investigated the broader consequences of GCC185 depletion, as an independent clue to the function of this protein.

In support of a role for GCC185 in MPR transport, absolute cellular levels of CI-MPRs increased approximately twofold after 72 h of GCC185 siRNA treatment (Figure 9A). Under these conditions, the levels of Rab9 were unchanged (our unpublished data). The effect was specific for GCC185 depletion, because Golgin-97 depletion did not influence the levels of CI-MPRs in these cells (Figure 9B). It is important to note that Golgin-97 depletion decreased GCC185 levels 37% (Figure 9B). This may in part explain the 23% inhibition of MPR transport detected upon Golgin-97 depletion in living cells (Figure 7A).

GCC185-depleted cells displayed an increase in the amount of newly synthesized MPRs and the rate of their turnover (Figure 9, C and D). When cells were metabolically labeled for 1 h, chased in complete medium for 3 h to allow CI-MPRs to fold and transit completely through the Golgi complex, and then chased for additional time to monitor receptor turnover (Figure 9C), we noted that at time 0, GCC185-depleted cells contained more newly synthesized and processed CI-MPR protein. In addition, the MPRs were more rapidly degraded in cells depleted of GCC185 protein (Figure 9D). Together, our data show that GCC185 is intimately involved in the normal life cycle of MPRs in vivo.

We estimate an overall difference in the CI-MPR degradation rate of about twofold (Figure 9D) and an increase of ~2.8-fold for CI-MPR synthesis (Figure 9C). Because of the long half-life of MPRs, we had to start the metabolic labeling after only 24 h of GCC185 depletion. The steady-state levels (Figure 9A) are measured after 72 h of depletion. We think it is likely that these rates change between 24 and 72 h of GCC185 depletion, yielding an overall twofold change in CI-MPR levels at steady state.

Finally, we noted that cells lacking GCC185 displayed increased secretion of hexosaminidase (Figure 10). Control and depleted cells contained similar total amounts of hexosaminidase, yet GCC185-depleted cells secreted twice as much hexosaminidase as the controls. Hexosaminidase is normally captured by mannose 6-phosphate receptors in the Golgi for transport to prelysosomes. The inability of MPRs to sort this lysosomal enzyme in the Golgi is consistent with MPR sequestration in a post-Golgi compartment. In summary, these data confirm our conclusion that GCC185 is needed for MPRs to accomplish efficient lysosomal enzyme delivery.
DISCUSSION

We have shown here that the Golgi-associated coiled-coil protein GCC185 is a specific binding partner of the Rab9 GTPase and is needed along with Rab9, both in vitro and in vivo, for the transport of MPRs from late endosomes to the TGN. GCC185 is a member of the Golgin family of putative tethering factors. These proteins all contain long, extended stretches of coiled-coil forming sequences, and soluble members of this family associate with the Golgi complex via membrane-associated binding partners. Golgin tethers have been implicated in the process of vesicle docking; they are also proposed to play a role in Golgi cisternal assembly (for review, see Short et al., 2005). The close relationship between the subcellular localizations of membrane-bound GCC185 and sialyltransferase (Luke et al., 2003) is consistent with GCC185 being localized to just the right place to receive MPRs returning to the site of sialyltransferase at the TGN where they are known to become resialylated (Duncan and Kornfeld, 1988).

We have shown here that the interaction of the putative TGN tether GCC185 with the Rab9 GTPase is not used to localize the majority of GCC185 protein in cells. Indeed, siRNA depletion of Rab9 did not change the Golgi association of GCC185. Thus, Rab9 interaction with GCC185 may represent an interaction important for transport vesicle targeting, rather than Golgin localization. There are several examples of specific Rab effectors, including golgins, that do not require a Rab protein for their steady-state localization. For example, a mutant form of EEA1 that binds phosphatidylinositol 3-phosphate but not Rab5 is targeted to early endosomes as efficiently as the wild-type protein (Lawe et al., 2002). Likewise, Golgin-45 uses GRASP55 for Golgi association, yet it is a Rab2 effector (Short et al., 2001). Moreover, the integral membrane protein Golgin-84 interacts with Rab1 (Diao et al., 2003; Satoh et al., 2003). Thus, there are numerous examples of Rab interactions that do not necessarily drive protein localization.

Models for GCC185 Function in MPR Transport

The simplest model for a TGN-localized, Rab9 binding partner would be for the Golgi-bound protein to engage a Rab9-decorated transport vesicle. Yet, the Rab9 binding site we have identified is located near the C terminus of GCC185 that is likely to be oriented adjacent to the TGN membrane. It is difficult to imagine how a membrane-proximal binding site would be of benefit in an initially, long-distance tethering process. Several alternative models resolve this dilemma.

First, GCC185 may have an additional binding site for Rab9 at its N terminus. A second possibility is equally plausible: there is a significant pool of cytosolic GCC185 protein (~50%) that seems much fainter in fluorescence micrographs because it is not concentrated in the perinuclear region. Because Rab9 does not localize GCC185 to the Golgi, we favor a model in which Rab9 transport vesicles interact first with cytosolic GCC185. In yeast exocytosis, for example, a cytosolic exocyst tether is thought to bind transport vesicles and participate in their docking at the plasma membrane. Moreover, p115 is thought to act as a tether from a soluble pool. Thus, such a model is not without precedent.

If cytosolic GCC185 first engages the transport vesicle-associated Rab9 protein, the GCC185-decorated transport vesicle may engage another tethering protein at the TGN—either GCC185 that is already localized there, or another protein. All tethers must release their bound vesicles, to enable a vesicle to fuse with its target. Nothing is known about tether release, and it will be of interest to determine where GCC185 engages Rab9, and how this pairing is released.

Our data support a model in which GCC185 uses a protein other than Rab9 to achieve its localization at the TGN. Because Rab9 binding to GCC185 did not require a conserved Tyr residue that is an important determinant of typical Arl1 binding, Rab9 may bind GCC185 by interaction with different amino acids than those used by Arl1. The protein construct we have used in our studies contains 110 residues of GCC185, and experiments are in progress to map the precise Rab9 interaction domain within this construct. It remains possible that another member of the large family of Arl proteins uses the GRIP domain of GCC185 to mediate TGN binding. Perhaps this interaction competes with Rab9, allowing release of GCC185 from a docked vesicle, and subsequent fusion.

Interference with the Rab9-dependent pathway for MPR transport from late endosomes to the Golgi increases the total number of MPRs within such cells (Riederer et al., 1994; Ganley et al., 2004). Similar changes were seen here in cells depleted of GCC185, consistent with a role for this protein in MPR recycling from late endosomes. In contrast, MPR levels remained constant in cells depleted of Golgin-97. Independent evidence that GCC185 is needed for efficient lysosomal sorting comes from our finding that cells lacking GCC185 showed hypersecretion of normally lysosomally targeted hexosaminidase.
Recently, Lu et al. (2004) and Yoshino et al., (2005) implicated Golgin-97 and Golgin-245, respectively, in early/recycling endosome-to-TGN transport of Shiga toxin. Unlike the Rab9-dependent MPR transport from late endosomes that we study, endosome to TGN transport of Ricin is independent of Rab9 (Iversen et al., 2001), and early endosome-to-TGN transport of Shiga toxin requires Rab6A (Mallard et al., 2002) and Rab11 instead (Wilkie et al., 2000). In their very thorough biochemical study, Lu et al., (2004) showed that purified Golgin-97 GRIP domain inhibited Shiga toxin transport to the Golgi in vitro, and expression of that GRIP domain inhibited transport in vivo. siRNA depletion of >90% of Golgin-97 led to a ~35% block in Shiga toxin transport from early endosomes to the TGN in those cells. Yoshino et al. (2005) used morphological localization as a measure of Shiga toxin transport to the TGN, which was inhibited in cells lacking Golgin-245.

In our experiments, GCC185 depletion led to >75% inhibition of MPR recycling from late endosomes in living cells under conditions in which Golgin-97 depletion only inhibited MPR transport 23%. Although GCC185 depletion in the HEK-293 cells used for transport assays did not influence Golgin-97 levels (our unpublished data), it should be noted that Golgin-97 depletion in this cell line decreased GCC185 levels 37% (Figure 9B). This decrease could be responsible for the 23% transport inhibition that we observed under these conditions (Figure 7A). Nevertheless, the residual GCC185 remaining in the Golgin-97–depleted reactions was sufficient to support MPR transport to the TGN. We conclude that most MPR transport represents a GCC185-mediated process; a small proportion may use Golgin-97.

In summary, we have shown that GCC185 acts in concert with Rab9 to ensure efficient transport of MPRs from endosomes to the Golgi complex. An important challenge for the future will be to determine how GCC185 is able to bind to Rab9 in a manner that enables transport vesicles, but not entire late endosome compartments, to dock productively at the trans-Golgi network.

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