# Mammalian GRIP domain proteins differ in their membrane binding properties and are recruited to distinct domains of the TGN

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## Summary

**The four mammalian golgins, p230/golgin-245, golgin-97, GCC88 and GCC185 are targeted to *trans*-Golgi network (TGN) membranes by their C-terminal GRIP domain in a G-protein-dependent process. The Arf-like GTPase, Arl1, has been shown to mediate TGN recruitment of p230/golgin245 and golgin-97 by interaction with their GRIP domains; however, it is not known whether all the TGN golgins bind to Arl1 and whether they are all recruited to the same or different TGN domains. Here we demonstrate differences in membrane binding properties and TGN domain recruitment of the mammalian GRIP domain proteins. Overexpression of full-length GCC185 resulted in the appearance of small punctate structures dispersed in the cytoplasm of transfected cells that were identified as membrane tubular structures by immunoelectron microscopy. The cytoplasmic GCC185-labelled structures were enriched for membrane binding determinants of GCC185 GRIP, whereas the three other mammalian GRIP family members did not colocalize with the GCC185-labelled structures. These GCC185-labelled structures included the TGN resident protein α2,6 sialyltransferase and excluded the recycling TGN protein, TGN46. The Golgi stack was unaffected by overexpression of GCC185. Overexpression of both full-length GCC185 and GCC88 showed distinct and nonoverlapping structures. We also show that the GRIP domains of GCC185 and GCC88 differ in membrane binding properties from each other and, in contrast to p230/golgin-245 and golgin-97, do not interact with Arl1 in vivo. Collectively these results show that GCC88, GCC185 and p230/golgin245 are recruited to functionally distinct domains of the TGN and are likely to be important for the maintenance of TGN subdomain structure, a critical feature for mediating protein sorting and membrane transport.**

Key words: Golgins, GRIP domain, Tethers, trans-Golgi network, Arl1

## Introduction

The *trans*-Golgi network (TGN) is an extensive membrane network on the distal face of the Golgi apparatus. The TGN gives rise to membrane carriers for anterograde and retrograde transport of newly synthesized cargo proteins heading to the plasma membrane or to other intracellular organelles, as well as receiving incoming traffic from endocytic and recycling pathways (Farquhar and Palade, 1998; Nelson and Yeaman, 2001; van Vliet et al., 2003). The TGN is a highly dynamic subcompartment of the Golgi, with the ability to form many tubules (Lippincott-Schwartz et al., 1998; Lippincott-Schwartz et al., 2000). Real-time imaging and ultrastructural analysis have indicated that the TGN may have specialized sorting domains and vesicle budding exit sites, which give rise to distinct populations of coated vesicle carriers and uncoated vesicles and tubules (Ladinsky et al., 1994; Hirschberg et al., 1998; Brown et al., 2001; Keller et al., 2001; Marsh et al., 2001; White et al., 2001; Ladinsky et al., 2002; Wylie et al., 2003). An unresolved issue is how the TGN maintains its highly dynamic tubulovesicular structure in spite of the considerable membrane flux into and from this compartment.

A phelora of sorting events occurs in the TGN, including the segregation of cargo from resident Golgi proteins and the sorting of cargo proteins to a variety of different membrane carriers (Keller and Simons, 1997; Nelson and Yeaman, 2001; Marsh and Howell, 2002). Evidence is emerging to suggest that the TGN may be organized into distinct subdomains, with each domain having a unique role in the sorting and transport activity of this complex compartment (Gleeson et al., 2004). Functional subdomains of the TGN could be established by recruitment of distinct arrays of proteins complexes, for example by small G proteins, coats and adaptors, and tethers, as in the case for the establishment of the multiple domains of the early endosome (Pfeffer, 2003). However, more information is required on the biogenesis of specific subdomains of the TGN.

Large coiled-coil golgins, such as p115 and GM130, have been shown to be important as tethering molecules and in the

biogenesis of membranes of the Golgi stack (Seemann et al., 2000; Pfeffer, 2001; Short et al., 2001; Barr and Short, 2003; Gillingham and Munro, 2003). The recent identification of several golgins specifically associated with the TGN (Brown et al., 2001; Luke et al., 2003b) raises the possibility that these extended fibrous molecules may also play important roles regulating TGN biogenesis. The discovery of the family of TGN golgin proteins stemmed from the identification of a modestly conserved ~45 residue Golgi targeting sequence located at the C-terminus, called the GRIP domain (Barr, 1999; Kjer-Nielsen et al., 1999a; Munro and Nichols, 1999). Mammalian GRIP domain proteins include a 230 kDa peripheral membrane protein (p230 also known as golgin-245), shown to be specifically associated with buds/vesicles of TGN membranes (Kooy et al., 1992; Fritzler et al., 1995; Erlich et al., 1996; Gleeson et al., 1996), golgin-97 (Griffith et al., 1997), GGC88 and GGCC185 (GCC for Golgi localised Coiled-Coil protein) (Luke et al., 2003b).

The Golgi membrane binding of each of the four mammalian GRIP domain proteins is G protein dependent (Luke et al., 2003b; Gleeson et al., 2004). Recruitment of at least two of the mammalian GRIP domain proteins to the TGN is mediated through an interaction with Arl1, a member of the ARF/Arl small G protein family. Arl1 was initially shown to interact with golgin-97 and p230/golgin-245 in yeast two-hybrid analyses (van Valkenburgh et al., 2001), and subsequent studies from a number of laboratories showed that activated, GTP-bound Arl1 interacts directly with the GRIP domains of not only golgin-97 and p230/golgin-245 but also the sole yeast GRIP domain protein, namely Imh1p (Gangi Setty et al., 2003; Jackson, 2003; Lu and Hong, 2003; Panic et al., 2003b). Furthermore, the interaction with Arl1 is directly responsible for the Golgi recruitment of GRIP domain proteins in both mammalian cells and yeast (Gangi Setty et al., 2003; Jackson, 2003; Lu and Hong, 2003; Panic et al., 2003b). Structural studies of the Arl1-GTP complex with the GRIP domain of p230/golgin-245 have revealed that the isolated GRIP domain forms a homodimer that interacts with two Arl1-GTP molecules (Panic et al., 2003a; Wu et al., 2004). The membrane recruitment of Arl1 is in turn dependent on another member of the Arl small G protein family, namely Arl3/ARFRP1 (Gangi Setty et al., 2003; Panic et al., 2003b; Behnia et al., 2004). However, it was not clear from these earlier studies if Arl1 interacts with the other two human GRIP proteins, GCC88 and GCC185. Of relevance to this current study is that yeast two-hybrid analysis indicated that Arl1 does not interact very efficiently with the GRIP domains of either GCC88 or GCC185 (Lu and Hong, 2003).

The importance of TGN golgins is highlighted by the finding that GRIP domain proteins are conserved throughout evolution, as functional GRIP targeting sequences have been identified in yeast, plants and protozoan parasites (Munro and Nichols, 1999; McConville et al., 2002; Gilson et al., 2004). Given the molecular characteristics of the GRIP domain family it is likely that they play a role similar to the golgins of the Golgi stack, as either matrix components and/or in vesicular tethering (Gleeson et al., 2004). Recent evidence indeed supports a role for TGN golgins in these functions. Overexpression of GCC88 in transfected cells was found to result in a major perturbation of a domain of the TGN, indicating a role in maintaining the structure of the TGN (Luke et al., 2003b). In addition, p230/golgin245 and the yeast GRIP domain protein, Imh1p, have been implicated in maintaining normal endosome to TGN traffic (Li and Warner, 1996; Tsukada et al., 1999; Yoshino et al., 2003).

The four mammalian GRIP domain proteins are localized to the TGN of HeLa cells (Gleeson et al., 1996; Luke et al., 2003b). In view of the fact that the sequence similarity between GRIP domains is only modest it is possible that different GRIP domains may have distinct targeting specificities and be localized to different subdomains of the TGN. Here we have explored this possibility and have shown that GCC88 and GCC185 are recruited to different functional domains of the TGN and have identified resident and cargo molecules that segregate specifically with either GCC88 or GCC185. We also show that the GRIP domains of GCC88 and GCC185 differ in their membrane binding characteristics compared with golgin-97 and p230. In contrast to golgin-97 and p230, neither GCC88 nor GCC185 interacts with Arl1 in vivo. Our data shows the existence of distinct domains of the TGN, demonstrates the complexity of the TGN membrane structure and provides markers for distinct regions of the TGN.

## Materials and Methods

### Antibodies

Human autoantibodies to p230 (Kooy et al., 1992) and the 9E10 mouse monoclonal antibody specific for the myc epitope (Evan et al., 1985) have been described previously. The P5D4 mouse monoclonal antibody that recognizes the VSV-G epitope was described by Kries (Kries, 1986). Rabbit polyclonal antibodies to β-COP were kindly provided by R. Teasdale (University of Queensland, Brisbane). A monoclonal antibody to green fluorescent protein (GFP), purchased from Boehringer-Mannheim, was used at dilutions of 1/1000 for immunoblotting. Monoclonal antibodies to golgin-97, GM130 and TGN46 were purchased from Transduction Labs (Lexington, KY). Sheep anti-rabbit Ig-FITC, sheep anti-mouse Ig-FITC and sheep anti-human IgG-FITC were purchased from Silenus laboratories (Melbourne, Australia); goat anti-rabbit IgG-Texas Red, goat anti-rabbit IgG-Alexa Fluor™ 568, goat anti-mouse IgG-Texas Red, goat anti-mouse IgG-Alexa Fluor™ 568, goat anti-human IgG-Alexa Fluor™ 594 and Cy5 labelled goat anti-rabbit IgG were from Molecular Probes (Eugene, OR). Horse-radish peroxidase-conjugated rabbit anti-mouse Ig was obtained from DAKO corporation (Carpinteria, CA). Rabbit antibodies to GCC88, GCC185 have been described previously (Luke et al., 2003b).

### Cell culture and transfection

HeLa and COS were maintained as monolayers in Dulbecco's modified Eagles medium (DMEM), supplemented with 5% fetal calf serum, 2 mM glutamine and 100 μg/ml penicillin/streptomycin, in a humidified 37°C incubator with 10% $CO_2$. Stable HeLa cells expressing α2,6 sialyltransferase (SialylT) tagged at the carboxy terminus with a VSV-G epitope (Rabouille et al., 1995) were grown in the above medium supplemented with 500 μg/ml G418 (Gibco BRL, Australia).

Transient transfections of cells were performed using FuGENE transfection reagent (Boehringer Mannheim) as previously described (Brown et al., 2001).

### DNA constructs

pCIneoGCC88, GFP-GCC88$_{GRIP}$, GFP-GCC185$_{GRIP}$, GFP-p230$_{GRIP}$, GFP-golgin97$_{GRIP}$, myc-tagged GCC185, GFP-GCC88 and GFP-GCC185 have been described previously (Kjer-Nielsen et al., 1999a;

Kjer-Nielsen et al., 1999b; Luke et al., 2003b). GFP-Arl1, and myc-SNX3-Arl1(Q71L) constructs in mammalian expression vectors have been described previously (Lu et al., 2001; Lu and Hong, 2003).

### Immunofluorescence

Cells were processed for immunofluorescence as previously described (Kjer-Nielsen et al., 1999b) and examined by confocal microscopy using a Bio-rad MRC-1024 imaging system. For dual labelling, images were collected independently to ensure there was no spillover of fluorescence between channels.

### Immunoblotting

Cell extracts were dissolved in reducing sample buffer and subjected to SDS-PAGE. Proteins were then transferred to PVDF membranes, and the membrane dried. Antibodies were diluted in PBS containing 5% (w/v) skim milk powder and incubated on the membrane for 1 hour, followed by three 10 minute washes in 0.05% Tween-20/PBS. Membranes were then incubated with peroxidase-conjugated anti-mouse and bound antibodies detected by enhanced chemiluminescence (NEN) as described previously (Gleeson et al., 1996).

### Immunogold labelling and electron microscopy

For immunogold labelling, subconfluent monolayers of untransfected or transfected COS cells were washed with PBS and fixed with buffered 4% paraformaldehyde (EM grade, ProSciTech, Australia), containing 0.2 M sucrose, then scraped from the dishes and resuspended in 10% gelatin and infiltrated with 15% poly-vinyl-pyrrolidone (Sigma) in 1.7 M sucrose before snap freezing. Preparation of ultrathin cryosections and immunogold labelling were carried out as previously described (Brown et al., 2001). Sections were labelled with antibodies to myc. Antibodies were detected with 10 nm Protein A-gold conjugates (gift from J. Slot, University of Utrecht).

## Results

### Overexpression of GCC185 results in disruption of the TGN

In the course of experiments expressing full-length GCC185 we noted that while low levels of expression showed full-length GCC185 localized to the typical juxtanunclear Golgi pattern, at apparently higher levels of expression GCC185-labelled structures were detected throughout the cytoplasm. A variety of phenotypes were detected, ranging from slight dispersal of GCC185-labelled structures around the Golgi region to a punctate vesicular staining throughout the cytoplasm with a lack of staining in the typical Golgi region (Fig. 1A; see also Fig. 3 for comparison with Golgi markers). The GCC185 fluorescently labelled cytoplasmic structures were up to approximately 700 nm in size. The transfected cells containing the punctate structures were viable, as determined by propidium iodide staining 48 hours after transfection. Both myc- and GFP-tagged full-length GCC185, as well as untagged full-length GCC185, gave the same phenotype in HeLa and COS cells, indicating that the perturbation was independent of the epitope tag and cell line. The generation of the cytoplasmic structures was dependent on the full-length molecule and was not observed with overexpression of solely the GRIP domain of GCC185.

In many of the transfected cells the locations of the GCC185 punctate structures were observed in close proximity with the Golgi region (see Fig. 1A). To explore the relationship between the GCC185-labelled cytoplasmic structures and TGN membranes, we analysed the intracellular distribution of the membrane determinants for GCC185$_{GRIP}$ in these transfected cells. COS cells were co-transfected with myc-tagged full-length GCC185 and GFP-tagged GCC185$_{GRIP}$. In cells expressing both constructs, GFP-GCC185$_{GRIP}$ no longer exhibited tight perinuclear localization. Rather, GFP-tagged

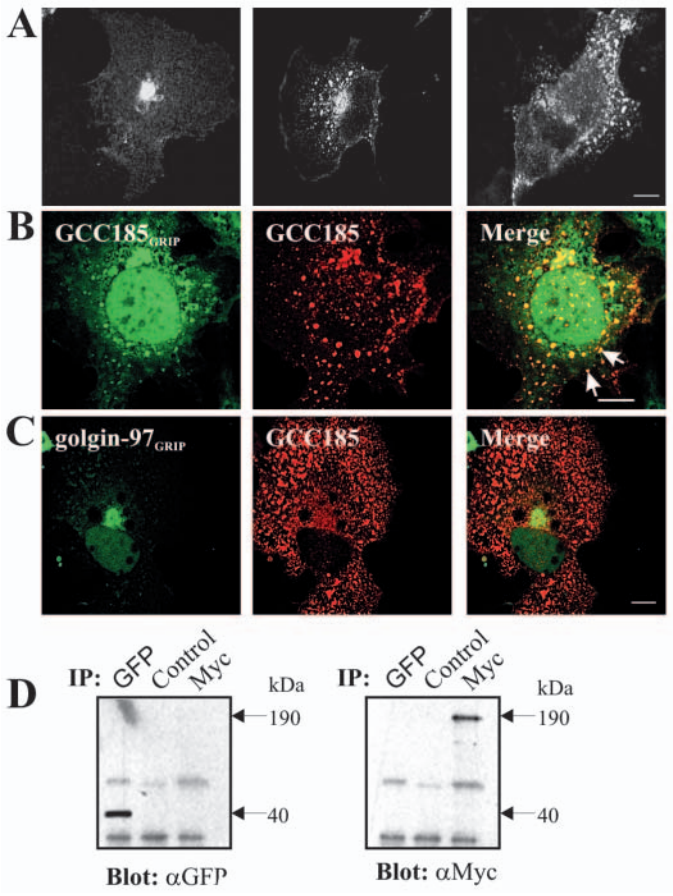


**Fig. 1.** Expression of full-length GCC185 results in dispersal of GCC185-labelled structures. (A) COS cells were transfected with myc-GCC185, fixed, permeabilized and stained with anti-myc monoclonal antibodies and FITC-anti-mouse Ig. Shown are three images expressing different levels of GCC185. (B) COS cells were co-transfected with myc-GCC185 and GFP-GCC185$_{GRIP}$ constructs, fixed, permeabilized and stained with anti-myc monoclonal antibody and Alexa 568 anti-mouse IgG. Superimposed images (merge) reveal regions of colocalization (arrows). (C) COS cells were co-transfected with myc-GCC185 and GFP-golgin97$_{GRIP}$ constructs, fixed, permeabilized and stained with anti-myc monoclonal antibody and Alexa 568 anti-mouse IgG. (D) COS cells were co-transfected with constructs encoding GFP-GCC185$_{GRIP}$ and myc-GCC185. Total extracts were prepared after 48 hours transfection as described in Materials and Methods, and lysates were either immunoprecipitated (IP) with anti-GFP antibody (GFP), anti-myc monoclonal antibody (Myc) or with an irrelevant monoclonal antibody (Control). Immune complexes were collected and subjected to SDS-PAGE under reducing conditions. After transfer to PVDF membranes, membranes were immunoblotted with anti-GFP antibody or anti-myc antibody as indicated using a chemiluminescence detection system. Bars: 10 μm.

$GCC185_{GRIP}$ was distributed throughout the cytoplasm in vesicular-like structures that extensively colocalized with the full-length myc-GCC185-labelled structures (Fig. 1B). In transfected cells expressing high levels of GFP-$GCC185_{GRIP}$, the GFP fusion protein was also detected diffusely throughout the cytoplasm and nucleus (Fig. 1B), indicating that the recruitment to the vesicular-like structures is saturable. However, co-transfection with myc-tagged full-length GCC185 and GFP-tagged golgin-$97_{GRIP}$ resulted in the typical Golgi localization of GFP-golgin-$97_{GRIP}$ with no colocalization with the GCC185-labelled cytoplasmic structures (Fig. 1C). These results indicate that the membrane determinants for $GCC185_{GRIP}$ are relocated to the vesicular-like structures dispersed throughout the cytoplasm. An alternative explanation for the colocalization of full-length GCC185 and GPF-$GCC185_{GRIP}$ is that they may interact to form oligomers in vivo. However, we do not consider this likely as our previous studies have shown that the isolated GRIP domains of other golgins do not form oligomers with either the endogenous GRIP domain protein or overexpressed full-length molecules (Kjer-Nielsen et al., 1999b). In addition, immunoprecipitation of the myc-tagged full-length GCC185 and GFP-tagged $GCC185_{GRIP}$ transfected cells with anti-myc antibody did not result in coprecipitation of the GFP fusion protein (Fig. 1D), indicating that the full-length GCC185 and the GFP-$GCC185_{GRIP}$ fusion protein are independently recruited to the structures found distributed throughout the cytoplasm.

The nature of the GCC185 structures in transfected COS cells was examined by immunogold electronmicroscopy. Immunolabelling for GCC185 with anti-myc antibodies revealed GCC185 decorated tubulovesicular structures that in some cells were found widely scattered throughout the cytoplasm and were detected in the cell periphery in close proximity to the plasma membrane (Fig. 2). These labelled membrane structures were distant from the Golgi stacks observed in the sections. The immunogold labelled cytoplasmic membrane structures were often observed as tubular structures of ~200 nm in length or as vesicular/tubular profiles (Fig. 2). No labelling was observed of untransfected cells (not shown). The distribution of the GCC185-labelled membrane structures in these transfected COS cells is consistent with the cytoplasmic stained structures observed by immunofluorescence.

## GCC185 is recruited to a TGN domain distinct from other TGN golgins

We next addressed the question of whether overexpression of full-length GCC185 affected the distribution of other Golgi stack and TGN markers. In cells expressing high levels of GCC185, the *cis*-Golgi marker GM130 was unaffected (Fig. 3), indicating that the Golgi stack is not perturbed. Likewise, neither β-COP nor the TGN membrane protein TGN46 was affected by the dispersal of GCC185-labelled structures throughout the cytoplasm (Fig. 3), indicating that the affect of GCC185 overexpression was restricted to a region of the TGN.

To determine whether other members of the GRIP protein family were also associated with the GCC185-labelled cytoplasmic structures in transfected cells, dual labelling for endogenous p230, golgin97 and GCC88 was carried out. These three mammalian GRIP proteins retained their juxtanuclear Golgi localization in the GCC185 transfected cells (Fig. 4A), and very little colocalisation of endogenous p230, golgin97 or GCC88 was observed with the cytoplasmic GCC185-labelled structures. Similar findings were observed with GFP- or myc-tagged GCC185 in either COS and HeLa cells. Therefore, p230, golgin97 and GCC88 are clearly excluded from the GCC185-labelled membranes.

Our previous studies have shown that overexpression of GCC88 also results in an abnormal phenotype (Luke et al., 2003b). In contrast to the cytoplasmic

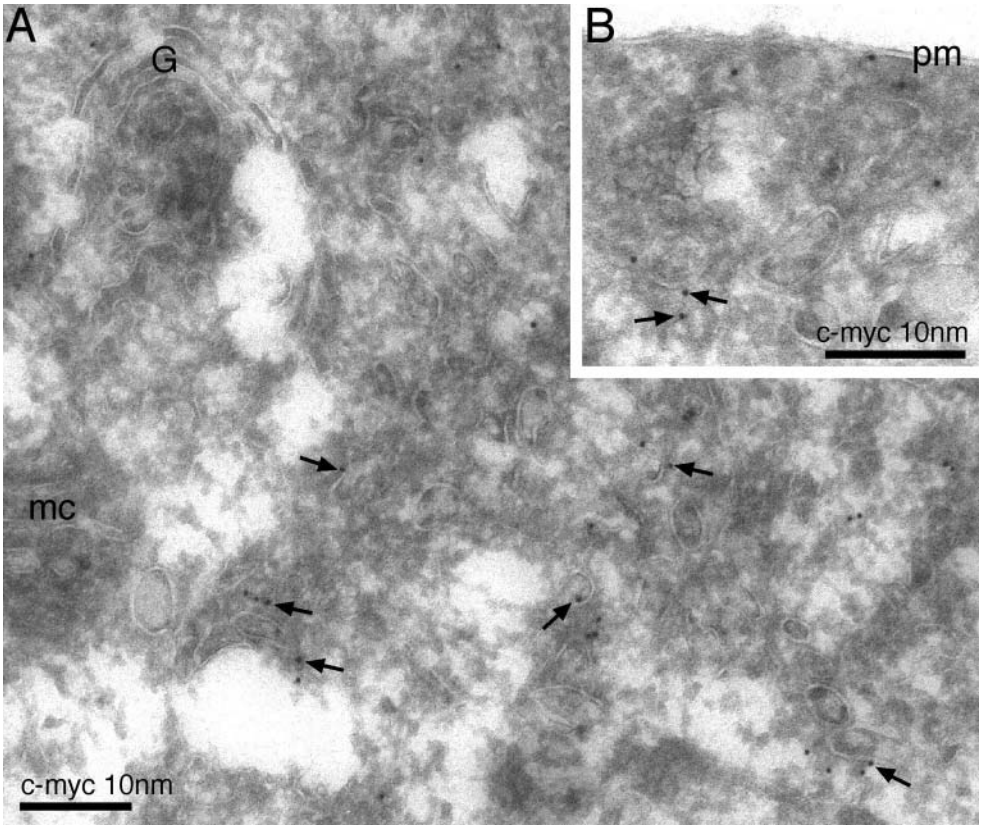


**Fig. 2.** Overexpression of GCC185 leads to the formation of GCC185 labelled tubular structures throughout the cytoplasm. COS cells were transfected with myc-GCC185, fixed with glutaraldehyde and processed for cryoelectron microscopy. Ultra-thin cryosections were labelled with monoclonal antibodies to myc. Antibodies were detected with 10 nm protein A gold particles (c-myc 10 nm). Note the labelling on tubulo-vesicular structures (arrows) scattered throughout the cytoplasm that get close to the plasma membrane (B). G, Golgi; mc, mitochondria; pm, plasma membrane. Bars, 200 nm.

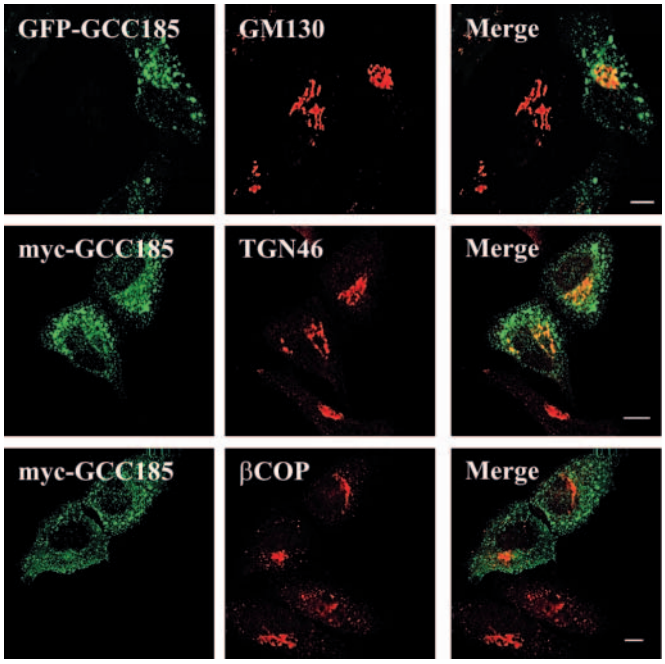


**Fig. 3.** Distribution of Golgi markers in HeLa cells expressing high levels of GCC185. HeLa cells were transiently transfected as indicated, with either GFP-GCC185 or myc-GCC185, fixed, permeabilized and co-stained for GM130, TGN46 or β-COP. GM130 was detected with mouse monoclonal antibody and Alexa 568 goat anti-mouse IgG, TGN46 with sheep anti-TGN46 and FITC-donkey anti-sheep Ig, β-COP with rabbit anti-β-COP and Alexa 568 goat-anti-rabbit IgG. Images of transfected cells expressing high levels of GCC185 and also superimposed images (merge) are shown. Control incubations demonstrated no cross-reactivity between the anti-Ig conjugates and the irrelevant primary antibody. Bars, 10 μm.

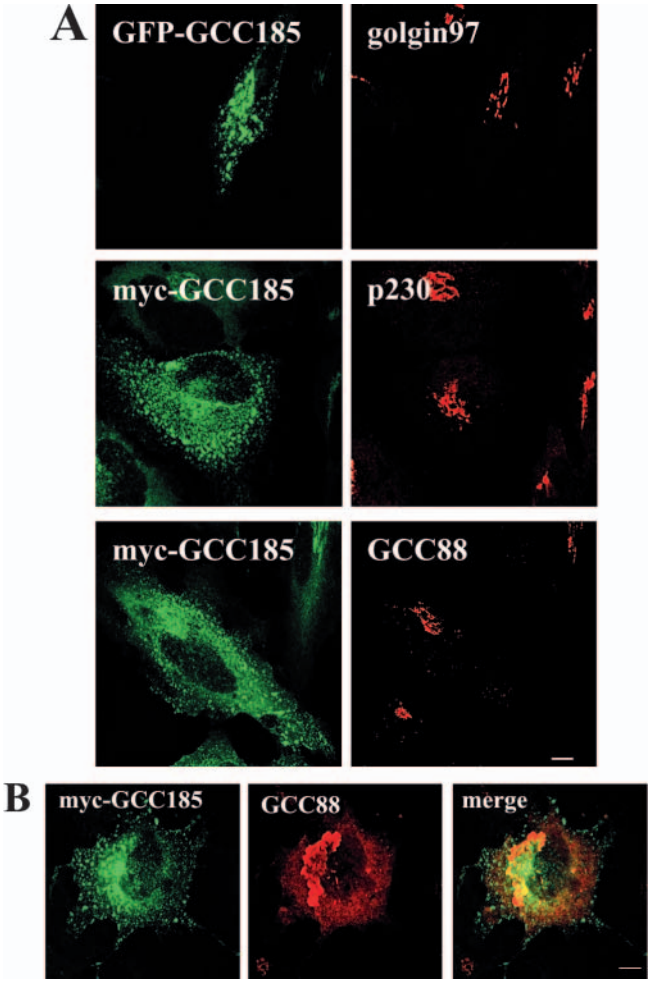


**Fig. 4.** GCC185 is recruited to structures distinct from the other three TGN golgins. (A) HeLa cells were transiently transfected as indicated, with either GFP-GCC185 or myc-GCC185, fixed, permeabilized and co-stained for endogenous golgin-97, p230 or GCC88. Golgin-97 was detected with a mouse monoclonal antibody and Alexa 568 goat anti-mouse IgG, p230 detected with human anti-p230 antibodies and Alexa 594 goat anti-human IgG and GCC88 detected with rabbit anti-GCC88 antibodies followed by Alexa 594 goat anti-rabbit IgG. (B) COS cells were transfected with myc-GCC185 and untagged GCC88, fixed, permeablized and stained with rabbit anti-GCC88 followed by Alexa 568 goat anti-rabbit IgG, and anti-myc monoclonal antibody followed by FITC goat anti-mouse Ig. Shown are images of transfected cells expressing high levels of both myc-GCC185 and GCC88. Bar, 10 μm.

tubulovesicular structures resulting from GCC185 overexpression, high levels of full-length GCC88 results in structures extending from the Golgi that resemble 'cauliflowers' when viewed by immunofluorescence (Luke et al., 2003b). The generation of two distinct phenotypes by GCC88 and GCC185 was used to further assess the different localizations of GCC185 and GCC88. COS cells were transfected with myc-tagged full-length GCC185 and full-length GCC88, fixed and stained with anti-myc monoclonal antibody and rabbit anti-GCC88 antibody. Cells expressing high levels of both GCC185 and GCC88 clearly showed both the cauliflower and cytoplasmic punctate structures. Each structure was labelled with either GCC88 or GCC185, and very little colocalisation of the two GRIP domain proteins was observed (Fig. 4B). Distinct structures were also observed with GFP-GCC185 and myc-tagged GCC88, where only one antibody was required to determine the relative localization of both golgins (not shown). Therefore, as the two abnormal structures in these transfected cells was GRIP-protein specific, it indicates that each structure is due to perturbation of a distinct subdomain of the TGN.

### Sialyltransferase segregates into GCC185-labelled structures in transfected HeLa cells

The localization of the resident TGN membrane protein α2,6 sialytransferase (SialylT) in cells with high levels of full-length GCC185 was examined using a stable HeLa cell line expressing VSV-G epitope-tagged SialylT. In cells with GCC185-labelled cytoplasmic structures, the localization of SialylT was substantially perturbed. Instead of a typical tight perinuclear staining, SialylT showed a much more extended staining pattern in the Golgi region (Fig. 5A) and overlapped substantially with the GCC185 structures found throughout the cytoplasm (Fig. 5A). All transfected cells analysed that displayed cytoplasmic GCC185 structures showed colocalization with SialylT. This result contrasts with the recycling membrane protein, TGN46, where no colocalization was observed between TGN46 and the cytoplasmic GCC185-

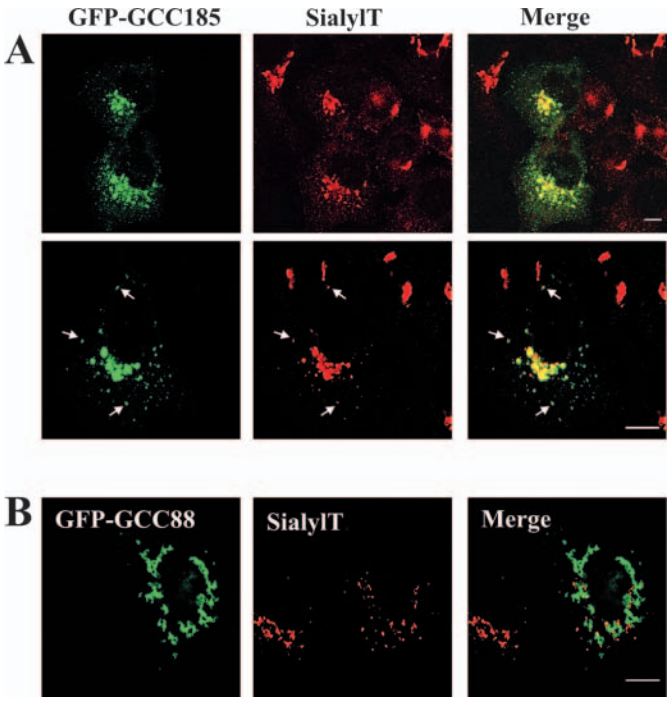


**Fig. 5.** The TGN resident membrane protein, sialyltransferase, segregates into GCC185-labelled structures. HeLa cells, stably expressing SialylT fused to the VSV-G epitope, were transiently transfected with (A) GFP-GCC185 or (B) GFP-GCC88, fixed, permeabilized and stained for VSV-G epitope tagged SialylT with mouse monoclonal antibodies and Alexa 568 anti-mouse IgG. Superimposed images (merge) are shown. Arrrows in A indicate structures in the periphery of the cell, labelled with both GFP-GCC185 and SialylT. Bar, 10 μm.

labelled structures (Fig. 3). Therefore, overexpression of GCC185 results in the perburation of a domain of the TGN that includes the resident TGN protein SialylT but excludes the recycling membrane protein TGN46. By contrast, overexpression of full-length GCC88, which generates cauliflower-type structures, resulted in some dispersal of SialylT from a tight perinuclear localization; however, as previously reported (Luke et al., 2003b) there was no colocalization of SialylT with the GCC88-labelled structures (Fig. 5B), showing that the inclusion of SialylT with the GCC185 labelled structures was specific for this particular TGN golgin.

## Competitive Golgi membrane binding by GRIP-domain proteins

In view of recruitment of the GRIP domain proteins to different TGN domains, we then investigated whether there are differences in the membrane binding specificities of the different GRIP domains. Golgi membrane binding of each of the four mammalian GRIP domains is G protein dependent, and p230/golgin245 and golgin97 have both been shown to interact with the small G protein, Arl1 (Lu and Hong, 2003; Panic et al., 2003a; Wu et al., 2004). However, it is not known if GCC88 and GCC185 are recruited to the TGN by the same Arl1-dependent mechanism as p230 and golgin-97. We have previously shown that overexpression of the p230 GRIP domain results in displacement of endogenous p230 and golgin-97 from TGN membranes (Kjer-Nielsen et al., 1999a), which is probably due to competition for a limited number of binding sites. Using this in vivo competition assay, we have carried out an analysis of the four mammalian members of the GRIP protein family. COS cells were transfected with GFP-p230$_{GRIP}$ and stained with mouse monoclonal anti-golgin-97, rabbit anti-GCC88 and rabbit anti-GCC185 to determine if these proteins are also displaced by high levels of GFP-p230$_{GRIP}$. Endogenous p230, golgin-97 and GCC88 were all displaced by high levels of GFP-p230$_{GRIP}$ as indicated by a loss of Golgi staining; however, GCC185 was not displaced from the Golgi by high levels of GFP-p230$_{GRIP}$ (Fig. 6A). The images shown in Fig. 6 are representative of over 50 cells transfected with each construct. Similar results were obtained with a HeLa cell line stably expressing GFP-p230$_{GRIP}$ under the control of the tetracycline responsive promoter (not shown).

Reciprocal experiments were also performed. As expected, high levels of GFP-golgin-97$_{GRIP}$ displaced endogenous p230 (not shown). Thus, p230 and golgin-97 GRIP domains compete for Golgi membrane binding, consistent with their interaction with Arl1. As for GFP-p230$_{GRIP}$, high levels of GFP-golgin-97$_{GRIP}$ also did not displace GCC185 from the Golgi (Fig. 6B). Further, overexpression of either GFP-GCC185$_{GRIP}$ or GFP-GCC88$_{GRIP}$ did not displace endogenous p230 from the Golgi apparatus (Fig. 6C). These results indicate differences in the behaviour of GCC185 and GCC88 compared with p230 and golgin-97. In particular, the lack of competitive membrane binding between GCC185 and p230 strongly indicates that the GCC185 GRIP domain may be recruited to Golgi membranes differently from the Arl1-mediated GRIP domains of p230 and golgin-97.

We have also investigated whether GCC88 and GCC185 compete for the same or different membrane binding sites. GFP-GCC185$_{GRIP}$ did not displace endogenous GCC88, and likewise, GFP-GCC88$_{GRIP}$ did not displace endogenous GCC185 (Fig. 6D), indicating that these two TGN golgins may interact with different membrane binding sites.

## Arl1 interacts with p230 and golgin-97 but not GCC88 and GCC185

Because the GRIP domains of GCC88 and GCC185 failed to displace endogenous p230 and golgin-97 from Golgi membranes, these two GRIP domains might not bind Arl1. We therefore investigated whether Arl1 can directly interact with GCC88 or GCC185 in mammalian cells. An Arl1 construct that is targeted to early endosomes was used and then the affect of redirecting Arl1 to the early endosomes on the localization of endogenous GRIP domain proteins was assessed. Wild-type Arl1 colocalizes with p230 to the Golgi region of HeLa cells (Fig. 7A). A GTP restricted mutant of Arl1 (Arl1Q71L) was fused to the early endosome molecule, SNX3. SNX3 contains a phosphatidylinositol-3-phosphate binding Phox domain that is responsible for specific recruitment to the early endosome (Xu et al., 2001). As expected, myc-tagged SNX3-Arl1(Q71L) fusion protein was localized to endosomal structures in transfected HeLa cells (Fig. 7B). Endogenous p230 was relocated to endosomal structures in cells expressing myc-SNX3-Arl1(Q71L), and very little p230 was detected within

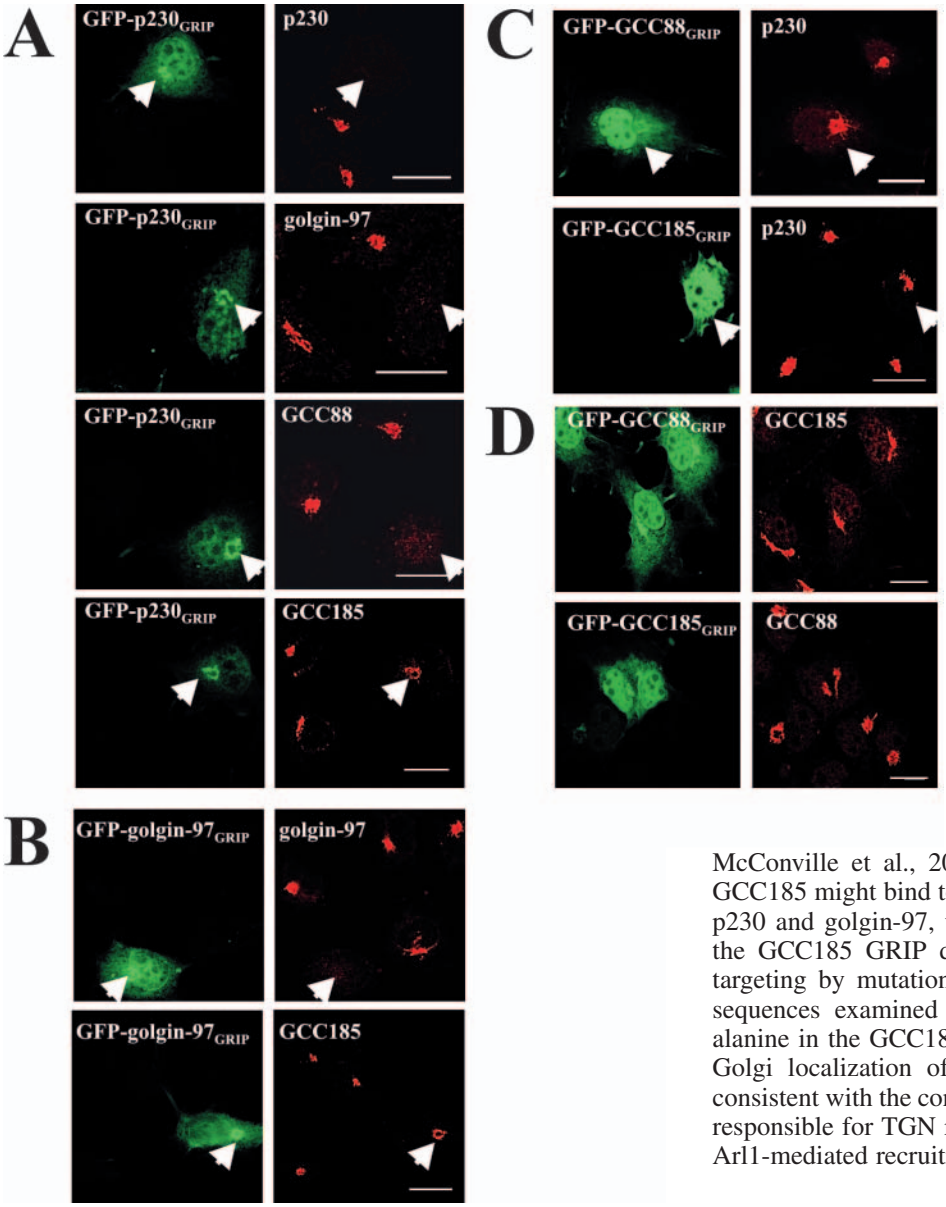


**Fig. 6.** Differential displacement of endogenous GRIP proteins by expression of GFP-GRIP fusion proteins.
(A,B) Displacement of endogenous GRIP proteins by GFP-p230$_{GRIP}$ or GFP-golgin-97$_{GRIP}$. COS cells were transfected with (A) GFP-p230$_{GRIP}$ or (B) GFP-golgin-97$_{GRIP}$, fixed and stained for each of the endogenous golgins using mouse and rabbit antibodies and detected with TRITC conjugated secondary antibodies as indicated.
(C,D) Localization of endogenous p230 (C) and endogenous GCC185 and GCC88 (D) in cells overexpressing GFP-GCC88$_{GRIP}$ and GPF-GCC185$_{GRIP}$. COS cells were transfected with constructs encoding either GFP-GCC88$_{GRIP}$ or GFP-GCC185$_{GRIP}$ fusion proteins, as indicated. Cells were fixed and stained with human anti-p230 followed by anti-human-Ig-TRITC in C, and rabbit anti-GCC88 or rabbit GCC185 followed by Alexa 568 goat anti-rabbit IgG in D. Arrows indicate the Golgi region in cells expressing GFP-GRIP fusion proteins in paired images of the same field. Green and red images were collected separately. Bars, 20 μm.

the Golgi region. The endosomal relocation of p230 is consistent with previous findings and indicates that an interaction with Arl1-GTP is directly responsible for membrane recruitment of this GRIP domain protein. However, the localization of endogenous GCC88 and GCC185 was unaffected in cells expressing myc-SNX3-Arl1(Q71L) as both these golgins remained Golgi localized with no evidence of an endosomal localization (Fig. 7B). These results show that the GRIP domains of GCC88 and GCC185, unlike those of p230 and golgin-97, do not interact with Arl1 in vivo.

### Conserved tyrosine of the GRIP domain is not required for membrane binding of GCC185

A conserved tyrosine (position 2177 of p230) is a feature of all GRIP domains identified to date. Mutation of this nonvariant tyrosine residue to alanine in p230 and several other GRIP domains results in loss of Golgi targeting (Barr, 1999; Kjer-Nielsen et al., 1999a; Munro and Nichols, 1999; McConville et al., 2002). As the data above indicated that GCC185 might bind to different membrane determinants from p230 and golgin-97, we investigated whether the tyrosine of the GCC185 GRIP domain is also essential for membrane targeting by mutation to alanine. In contrast to other GRIP sequences examined to date, replacement of tyrosine with alanine in the GCC185$_{GRIP}$ had no affect on the efficiency of Golgi localization of GFP-GCC185$_{GRIP}$ (Fig. 8), a finding consistent with the conclusion that the identity of the G protein responsible for TGN recruitment of GCC185$_{GRIP}$ differs from Arl1-mediated recruitment of p230$_{GRIP}$.

## Discussion

The four mammalian GRIP domain proteins are all localized to TGN membranes via the GRIP targeting sequence. HeLa cells express the four TGN golgins; however, it is not known if each has a specific function or if their functions are redundant. To gain further insight into the role of the TGN golgins in maintaining TGN structure and function, we have analysed their membrane binding properties in more detail. As there is only a modest level of sequence similarity between different GRIP domains it could not be assumed that all four mammalian GRIP domains were able to interact with Arl1. Here we have shown that the GRIP domains from GCC88 and GCC185 do not bind to Arl1 in vivo and are probably recruited to TGN membranes independently of Arl1. Further, we have shown that GCC88 and GCC185 are recruited to different subdomains of the TGN from p230/golgin245 and golgin-97. The GCC185 subdomain is enriched for the resident membrane protein α2,6 sialyltransferase, whereas the GCC88 subdomain excludes this TGN protein. These results highlight the

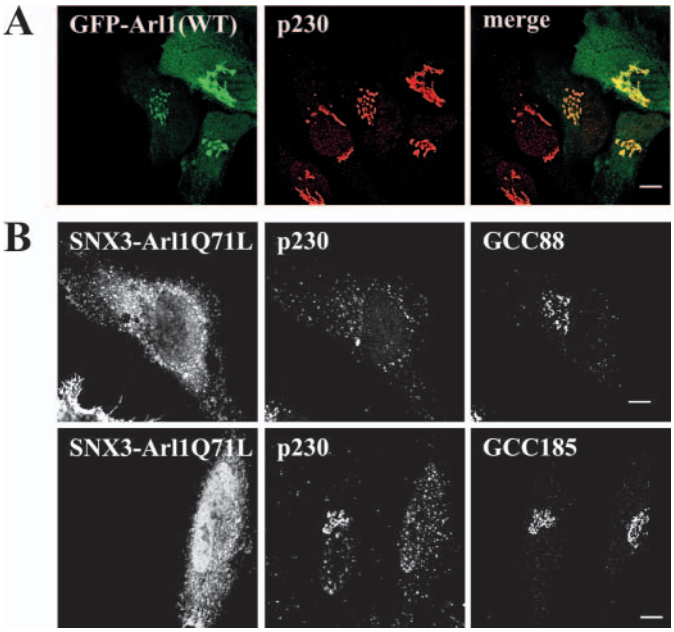


**Fig. 7.** Targeting of Arl1(Q71L) to endosomes results in recruitment of p230, but not GCC88 or GCC185 to endosomal membranes. HeLa cells were transiently transfected with either (A) GFP-Arl1(WT) or (B) myc-SNX3-Arl1(Q71L), fixed and permeabilized.
(A) Transfected cells were stained for p230 with human anti-p230 antibodies followed by Alexa 594 goat anti-human IgG.
(B) Transfected cells were co-stained for myc-SNX3-Arl1(Q71L) and p230 and either GCC88 or GCC185, as indicated. myc-SNX3-Arl1(Q71L) was detected with mouse anti-myc monoclonal antibodies and Alexa 568 goat anti-mouse IgG, p230 detected with human anti-p230 antibodies and FITC-sheep anti-human IgG, and GCC88 and GCC185 detected with rabbit anti-GCC88 or anti-GCC185 antibodies followed by Cy5-labelled goat anti-rabbit IgG. Transfected cells expressing high levels of myc-SNX3-Arl1(Q71L) resulted in recruitment of endogenous p230 onto endosomal structures, whereas both GCC88 and GCC185 retained their normal Golgi localization. Bars, 10 μm.

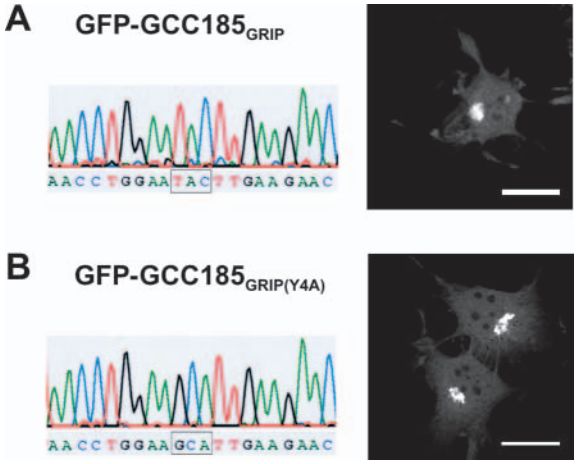


**Fig. 8.** Invariant tyrosine of the GRIP domain is not required for Golgi targeting of $GCC185_{GRIP}$. (A) DNA sequence profile of GFP-$GCC185_{GRIP}$ with the TAC coding for the invariant tyrosine highlighted, and an image of COS cells transfected with GFP-$GCC185_{GRIP}$. (B) DNA sequence profile of GFP-$GCC185_{GRIP\ (Y4A)}$ showing the GCA substitution (boxed), with an image of COS cells transfected with GFP-$GCC185_{GRIP}$ (Y4A). Bars, 20 μm.

complexity of the TGN and indicate that the TGN golgins are likely to have roles uniquely associated with specific subdomains of this compartment.

Using several approaches we have shown that the GRIP domains of GCC88 and GCC185 have different membrane binding properties from the GRIP domains of golgin-97 and p230/golgin-245. First, the association of the membrane binding sites for $GCC185_{GRIP}$, but not for golgin-$97_{GRIP}$, with cytoplasmic structures generated by overexpression of the full-length GCC185 molecule shows that membrane binding determinants for GCC185 GRIP are likely to differ from other TGN golgins. Second, the inability of the GRIP domains of GCC185 and GCC88 to displace either p230 or golgin-97 shows differences in their membrane binding interactions. Third, the inability of Arl1 fused to SNX3 to redirect GCC88 and GCC185 to endosomes clearly shows that these two GRIP domain proteins are unable to bind Arl1 in vivo. Our finding that the GRIP domains of GCC185 and GCC88 do not bind to Arl1 in vivo is consistent with published yeast two-hybrid analysis, in which GTP-restricted Arl1 interacted poorly with the GRIP domains of either GCC88 or GCC185 (Lu and Hong, 2003). Pull-down assays, however, had indicated that, at high concentrations, the GRIP domains of GCC88 and GCC185 can bind to glutathione beads loaded with GST-Arl1(Q71L) (Panic et al., 2003a). In view of the concentrations of the two species used in this latter assay, low-affinity interactions may be detected that are not physiologically relevant. GCC88 and GCC185 behaved differently in the in vivo competition assays in that GCC88, but not GCC185, was displaced by an excess of $p230_{GRIP}$, whereas an excess of $GCC88_{GRIP}$ did not affect endogenous p230. This observation may be due to a weak interaction between $p230_{GRIP}$ and the G protein that interacts with GCC88.

These findings argue that neither GCC88 nor GCC185 can bind strongly to Arl1. Because the GRIP domains of GCC185 and GCC88 bind to TGN membranes in a G-protein-dependent manner (Luke et al., 2003b), we propose that G proteins other than Arl1 are necessary for the membrane recruitment of these two golgin family members. The Arl family includes at least ten human members, and the localization and function of many of these members have not been established (Clark et al., 1993; Pasqualato et al., 2002). It is possible that the GRIP sequences of GCC88 and GCC185 may interact with these other members of the Arl family. At this stage we cannot exclude the possibility that Arl1 may be indirectly required for GCC88 and GCC185 Golgi localization. It is difficult to firmly establish whether GCC88 and GCC185 can be recruited to the Golgi in the absence of Arl1, as the expression of the dominant negative mutant of Arl1 results in extensive fragmentation of the Golgi apparatus (Lu et al., 2001).

The structure of the Arl1-GTP complex with the GRIP domain of p230/golgin-245 has recently been solved by two groups (Panic et al., 2003a; Wu et al., 2004). The isolated GRIP domain forms a homodimer that interacts with two Arl1-GTP molecules. Two of three anti-parallel α-helices of each GRIP domain make contacts with an Arl1-GTP molecule (Panic et al., 2003a). An invariant tyrosine residue was shown by

mutagenesis to be critical for Golgi targeting of several GRIP domains (Kjer-Nielsen et al., 1999a; Munro and Nichols, 1999; McConville et al., 2002). From the Arl1-GTP/GRIP structure, the invariant tyrosine residue of the GRIP domain occupies a hydrophobic pocket of Arl1 and plays a major role in defining the specificity of the interaction. Our finding that the invariant tyrosine in the GRIP domain of GCC185 can be mutated to an alanine without loss of Golgi targeting is further evidence of a difference in G protein specificity between GCC185 and other members of the family. The GRIP domain of GCC88, however, does require the invariant tyrosine for Golgi targeting (Kjer-Nielsen et al., 1999a); given that neither GCC88 nor GCC185 bind Arl1 in vivo a probable explanation for these data is that these two GRIP domains each interact with different G proteins.

Overexpression of full-length GCC185 resulted in fragmentation of membrane structures derived from the TGN. The development of these cytoplasmic structures was independent of cell type and the epitope tag present on the full-length GCC185. The size of the punctate structures by immunofluorescence was similar to the size of the GCC185-labelled cytoplasmic structures observed by immunoelectron microscopy. GCC185-labelled cytoplasmic structures were often tubular, indicating that GCC185 is either recruited to existing tubular extensions of the TGN and/or playing a role in the formation of TGN tubules. The inclusion of SialylT into the GCC185-labelled fragmented structures, and exclusion of TGN46 and the three other TGN golgins, indicates that these GCC185-decorated structures represent a distinct functional domain of the TGN in which cargo and resident Golgi proteins have been sorted and segregated. The exclusion of TGN46 from GCC185-specific structures also indicates that this golgin is unlikely to be involved in endosome-to-TGN recycling, as has been suggested for some of the other GRIP domain proteins in yeast (Tsukada et al., 1999) and mammals (Yoshino et al., 2003; Lu et al., 2004). The GCC185-labelled cytoplasmic structures are likely to be generated from the tubular extensions of the TGN. GCC185 may promote an exaggerated extension of tubular structures within a subdomain and these tubular extensions then break away from the main TGN compartment. As the overexpression of the isolated GRIP domain of GCC185 alone does not result in membrane fragmentation, it is likely that the full-length GCC185 molecule binds to several downstream effector molecules, some of which may mediate the dynamic process of tubulation.

Our earlier work showed that the overexpression of GCC88 in transfected cells also induced considerable morphological change, namely a dramatic enlargement of Golgi-associated structures that arise from the TGN (Luke et al., 2003b). Although the membrane resident protein SialylT does not colocalize with the ‘cauliflower’ structures resulting from GCC88 overexpression, TGN38 can move into and from the GCC88-decorated enlarged structures (Luke et al., 2003b). These findings imply that GCC185 and GCC88 define two distinct subdomains of the TGN, the former associated with SialylT and the latter with TGN38. Overexpression of p230/golgin-245 and golgin-97 also results in perturbation of the TGN (Luke et al., 2003a). Furthermore, simultaneous expression of GCC88 and golgin-97 resulted in distinct, nonoverlapping golgin-positive structures (Gleeson et al., 2004). GRIP domains in isolation do not cause a perturbation of TGN structure and our recent studies of live imaging of different GRIP domains, tagged with CFP and YFP, in the same cell supports the conclusion that GRIP domains are recruited to distinct regions of the TGN (J. Lock, F. Houghton, J.L.S. and P.A.G., unpublished observations).

The behaviour of the TGN golgins suggests they could play a role in maintaining the structure of the TGN. The TGN golgins, along with the adaptors and coats of the TGN, may define distinct subdomains of the TGN that are critical for the specific sorting and transport functions of this compartment (Gleeson et al., 2004). A key step in the establishment of subdomains is likely to be the binding and activation of different G proteins. Activated G proteins could induce the formation of subdomains by recruitment of molecules that associate to form a unique complex. For example, once golgins are recruited to TGN membranes, further interactions with other components, as yet unidentified, could stabilize a distinct subdomain with specific morphological, biochemical and functional characteristics. It will be important now to define the nature of the interactive partners with the individual members of the TGN golgins.

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