The GRIP domain is a targeting sequence found in a family of coiled-coil peripheral Golgi proteins. Previously we have demonstrated that the GRIP domain of p230/golgin245 is specifically recruited to tubulovesicular structures of the trans-Golgi network (TGN). Here we have characterized two novel Golgi proteins with functional GRIP domains, designated GCC88 and GCC185. GCC88 cDNA encodes a protein of 88 kDa, and GCC185 cDNA encodes a protein of 185 kDa. Both molecules are brefeldin A-sensitive peripheral membrane proteins and are predicted to have extensive coiled-coil regions with the GRIP domain at the C terminus. By immunofluorescence and immunoelectron microscopy GCC88 and GCC185, and the GRIP protein golgin97, are all localized to the TGN of HeLa cells. Overexpression of full-length GCC88 leads to the formation of large electron dense structures that extend from the trans-Golgi. These de novo structures contain GCC88 and co-stain for the TGN markers syntaxin 6 and TGN38 but not for α2,6-sialyltransferase, β-COP, or cis-Golgi GM130. The formation of these abnormal structures requires the N-terminal domain of GCC88. TGN38, which recycles between the TGN and plasma membrane, was transported into and out of the GCC88 decorated structures. These data introduce two new GRIP domain proteins and implicate a role for GCC88 in the organization of a specific TGN subcompartment involved with membrane transport.

The trans-Golgi network (TGN) is a highly dynamic and complex membrane network that represents a major protein sorting compartment of the secretory pathway. From the TGN, proteins are shunted into distinct transport carriers for transport to the plasma membrane, regulated secretory granules, different populations of endosomes, or earlier compartments of the secretory pathway (1, 2). A fundamental, unresolved question in cell biology is how the Golgi apparatus is formed and in particular how the TGN maintains a highly dynamic tubulovesicular structure and generates the diverse populations of TGN-derived transport carriers. A number of Golgi matrix proteins have been identified, for example GRASP55 and Golgin-45, which may be involved in the maintenance of the medial-Golgi structure (3, 4). Little, however, is known about a matrix associated with the trans-Golgi responsible for generating or maintaining the tubulovesicular structure of the TGN (5–7). There is growing evidence that components of the Golgi matrix interface with components of the trafficking machinery (8, 9). Unique sets of accessory molecules dictate the budding of transport vesicles and their subsequent docking on specific membrane domains. Many of these proteins are peripheral membrane proteins that are recruited to the donor membrane in a highly regulated manner. For example, on the TGN the AP-1 adaptor complex is involved in the formation of clathrin-coated vesicles whereas other heterotetrameric adaptors, AP-3 and AP-4, and the GGA (Golgi-localizing, ARF-binding) proteins are recruited to distinct TGN domains involved in the formation of both clathrin and non-clathrin-coated vesicles (10, 11). However, the specific membrane association of many of these components and the basis for the domain-specific organization of the TGN is poorly defined.

Recently we and others (12–14) have identified a family of Golgi coiled-coil peripheral membrane proteins in animal and yeast cells that contain a conserved ~42-amino acid sequence at the C terminus, called the GRIP domain. The level of sequence identity between the GRIP domains is only modest; however, functional assays have demonstrated that a number of different GRIP sequences can specifically target reporter molecules to the Golgi apparatus. By analysis of chimeric green fluorescent proteins (GFP), the GRIP domain has been shown to be both necessary and sufficient for Golgi targeting in animal cells (12–15). Furthermore, the GRIP domain of one family member, p230, has been shown to target specifically to the TGN of mammalian cells (16). In addition, we have also identified recently a coiled-coil protein with a GRIP sequence in protozoan parasites and shown that the GRIP domain was specifically recruited to the TGN in the trypansomatid parasite, Leishmania mexicana (17). The identification of GRIP domain proteins associated with the TGN of this very primitive protozoan eukaryotic cell suggests that these coiled-coil proteins play a fundamental role in the organization and/or function of this highly dynamic Golgi compartment. In Saccharomyces cerevisiae, only one coiled-coil protein with...
a GRIP domain has been identified. This protein, Imh1p, is a suppressor of a temperature-sensitive yeast strain that lacks a functional GTPase Rab6 homologue, Ypt6p (18, 19). In contrast a number of proteins with potential GRIP domains have been identified in mammalian cells, including the two characterized Golgi proteins, p230/golgin245 and golgin97. p230/golgin245 binds to the TGN in a brefeldin A-sensitive manner (15, 20). Recent studies have shown that endogenous p230 and a GFP chimera containing the p230-GRIP sequence binds to a subdomain of the TGN that forms highly dynamic tubular vesicular extensions that have the characteristics of transport carriers (16, 20). Furthermore, the GFP-p230 chimera has been shown to be associated with a population of in vitro generated vesicles that lack markers (β-COP, γ-adaptin, Rab6, and p200/myosin II) associated with other TGN-derived vesicles (16). The extended coiled-coil structure of the GRIP domain proteins links them to the broader family of Golgi coiled-coil proteins known as golgins, which are proposed to function as molecular tethers in the docking of transport vesicles with a target membrane and in the maintenance of Golgi structure (21).

In addition to p230 and golgin97, data base searches also identified two other putative human coiled-coil proteins with GRIP domains, referred to previously as GCC1p (Golgi local) and KIAA0336 (13). By the analysis of GFP fusion proteins, the GRIP domain of GCC1p was shown to function as a Golgi targeting signal in transfected cells. The predicted GCC1p sequence was derived from a DNA cosmid sequence (p1572c101) and required prediction of exon/intron boundaries (13). The human expressed sequence tag sequence, KIAA0336 (GenBank™ accession number AB002334), is predicted to encode a coiled-coil protein of 1583 amino acids. The GRIP domain of KIAA0336 has not yet been analyzed experimentally; however it is predicted to contain the highly conserved Tyr (or Phe) residues at positions 4 and 12 of the domain, aromatic residues that are essential for the GRIP domain to function in Golgi targeting. These findings raised a number of questions. First, are GCC1p and KIAA0336 GRIP proteins? Second, in view of the modest sequence similarity between GRIP domains, are the different GRIP domains localized to the same or different regions of the Golgi apparatus? Third, how many mammalian GRIP proteins are found in one particular cell type? Finally, do all the mammalian GRIP proteins have similar functions? In the present study we report on the full-length sequences of both novel human GRIP proteins, which we now call GCC88 (for GCC1p) and GCC185 (for KIAA0336), and demonstrate that they are both localized to the TGN in HeLa cells, along with the other two GRIP family members p230 and golgin97. Altogether, our data show that four different brefeldin A-sensitive GRIP proteins are associated with the TGN of mammalian cells. We further show that GCC88 overexpression results in a major perturbation of a domain of the TGN associated with the membrane transport of TGN38.

**EXPERIMENTAL PROCEDURES**

**Antibodies—**Human autoantibodies to p230 have been described (22). The 9E10 mouse monoclonal antibody specific for the myc epitope has been described (22). The P5D4 mouse monoclonal antibody that recognizes the VSV-G epitope was described by Kries (24). Rabbit polyclonal antibodies to β-COP were kindly provided by Dr. R. Teasdale (University of Queensland, Brisbane, Queensland, Australia). A monoclonal antibody to GFP, purchased from Roche Molecular Biochemicals, was used at dilutions of 1/1000 for immunoblotting. Monoclonal antibodies to golgin97, syntaxin 6, GM130, and TGN38 were purchased from Transduction Laboratories (Lexington, KY). For electron microscopy, a polyclonal anti-GFP (Molecular Probes) was used at 1/500 dilution. Sheep anti-rabbit IgG-FITC, sheep anti-mouse IgG-FITC, and sheep anti-human IgG-FITC were purchased from Silenus Laboratories (Melbourne, Victoria, Australia), goat anti-rabbit IgG-Texas Red, goat anti-mouse IgG-Texas Red, and mouse anti-golgin97 (Golgi Science Inc.) were purchased from Molecular Probes. Horseradish peroxidase-conjugated rabbit anti-mouse Ig and porcine anti-rabbit Ig were obtained from DAKO corporation (Carpineta, CA).

**Cell Culture and Transfection—**HeLa, COS, and normal rat kidney (NRK) cells were maintained as monolayers in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 5% fetal calf serum, 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin in a humidified 37 °C incubator with 10% CO2. Stable HeLa cells expressing pC6.2-sialytransferase (SialylT) tagged at the C terminus with a VSV-G epitope (25) were generously supplied by Dr. T. Nilsson and were grown in the above medium supplemented with 500 μg/ml G418 (Invitrogen). Transient transfections of cells were performed using FuGENE transfection reagent (Roche Molecular Biochemicals) as described previously (18).

**Reverse Transcriptase Polymerase Chain Reaction and Cloning of GCC88—**HeLa cell RNA was isolated using the RNAqueous kit according to the manufacturer’s instructions (GeneWorks). cDNA was prepared from HeLa RNA using Superscript (Invitrogen) and oligo(dT) (Promega) primer. GCC88 cDNA was amplified as four overlapping fragments that collectively spanned the entire length of the open reading frame predicted from the genomic sequence. Fragment 1 (bases 1–783) was amplified using the primers CCGGAATTCACATGAGGAGTTCGTTGCTCCTCCCTCCTG. Fragment 2 (bases 715–1293) was amplified using the primers CCGGAATTCCTATCGAAGTCCAGACCA. The GCC88 C199aa polypeptide was then cleaved from a fusion protein comprising the GRIP domain, derived from a PCR product, was subcloned into the pGEX-6P-3 (GST-GCC88 C199aa). GCC88 C199aa polypeptide was then cleaved from the pGEX-6P-3 plasmid into the pCMU-3xMyc (supplied by Dr. Rohan Teasdale) plasmid. The sequence encoding the C-terminal 82 amino acids of GCC185 was subcloned from the pEGFP-GCC185GRIP. The sequence encoding the C-terminal 199 amino acids of GCC88 fused to the C terminus of GST was produced by first generating a fusion protein comprising the GCC88 GRIP domain, derived from a PCR product, was subcloned into the pCMU-3xMyc (supplied by Dr. Rohan Teasdale) plasmid. The sequence encoding the C-terminal 199 amino acids of GCC88 fused to the C terminus of GST was produced by first generating a fusion protein comprising the GCC88 GRIP domain, derived from a PCR product, was subcloned into the pCMU-3xMyc (supplied by Dr. Rohan Teasdale) plasmid. The sequence encoding the C-terminal 82 amino acids of GCC185 was subcloned from the pEGFP-GCC185GRIP. The sequence encoding the C-terminal 199 amino acids of GCC88 fused to the C terminus of GST was produced by first generating a fusion protein comprising the GCC88 GRIP domain, derived from a PCR product, was subcloned into the pCMU-3xMyc (supplied by Dr. Rohan Teasdale) plasmid. The sequence encoding the C-terminal 82 amino acids of GCC185 was subcloned from the pEGFP-GCC185GRIP. The sequence encoding the C-terminal 199 amino acids of GCC88 fused to the C terminus of GST was produced by first generating a fusion protein comprising the GCC88 GRIP domain, derived from a PCR product, was subcloned into the pCMU-3xMyc (supplied by Dr. Rohan Teasdale) plasmid. The sequence encoding the C-terminal 82 amino acids of GCC185 was subcloned from the pEGFP-GCC185GRIP. The sequence encoding the C-terminal 199 amino acids of GCC88 fused to the C terminus of GST was produced by first generating a fusion protein comprising the GCC88 GRIP domain, derived from a PCR product, was subcloned into the pCMU-3xMyc (supplied by Dr. Rohan Teasdale) plasmid.
Figure 1. Analysis of the sequences of GCC88 and GCC185. A, alignment of the GRIP sequences of g1572c101 (GCC1p/GCC88), KIAA0336 (GCC185), golgin97, and p230. Asterisks indicate identity, and single and double dots represent degrees of similarity. B, predicted amino acid sequence of GCC88 based on cDNA sequence. The GRIP domain is shaded.

Identification of Two Novel Golgi Proteins with Functional GRIP Domains, GCC88 and GCC185.—Previously, we established that the defined Golgi proteins, p230/golgin245 and golgin97, contained functional GRIP domains (15). Data base searches identified two other putative human proteins with similar GRIP domains that are predicted to contain extensive coiled-coil regions. One of the putative proteins was predicted from genomic sequences and referred to previously as GCC1p (Golgi localized coiled-coil protein). We established that the GRIP sequence of this putative protein represented a functional Golgi targeting domain, as a fusion with GFP was efficiently targeted to the Golgi of transfected COS cells (13). As the predicted GCC1p protein was based on the sequence of a putative gene within the human genomic cosmid g1572c101 (GenBank™ accession number HSAC0000357), requiring prediction of intron/exon boundaries, we have analyzed the sequence of this protein. cDNA clones of GCC1p were obtained by reverse transcriptase PCR using HeLa cell RNA as described under “Experimental Procedures.” 5′ sequence of the mRNA was obtained using rapid amplification of cDNA ends. Together the clones comprise 2522 bp, and an open reading frame of GCC1cDNA spans an in-frame ATG initiation codon at nucleotide position 194 bp and a TGA termination at nucleotide position 2519 bp. The open reading frame encodes a protein of 775 amino acids with an expected molecular mass of 87.8 kDa (Fig. 1B); therefore we now call this protein GCC88. A plot of hydrophilicity suggests GCC88 is a predominantly hydrophilic structure, with no evidence for a signal sequence or hydrophobic transmembrane domain, consistent with a peripheral membrane protein. Analysis of the sequence for coiled coils showed that a considerable portion (>80%) of the molecule had a high probability of assuming a coiled-coil structure, typical of other GRIP proteins. There are a number of interruptions in the predicted coiled-coil structure, suggesting GCC88 adopts a rod-like structure with periodic flexible joints. Non-coiled-coil regions of the molecule include a ~75 residue segment close to the N terminus and the C-terminal GRIP domain (Fig. 1C).

The biotinylated protein was expressed in bacteria, and the bacterial cell lysate was fractionated by centrifugation and sucrose gradient ultracentrifugation. After separation, the fractions containing the Golgi proteins were combined and concentrated. The resulting supernatant was centrifuged at 100,000 × g for 60 min. The resulting microsome pellet was resuspended in hypotonic-sucrose buffer (250 mM sucrose, 10 mM HEPES, pH 7.4), containing Complete Protease Inhibitors (Roche Molecular Biochemicals, Germany). Cells were resuspended in 500 μl of hypotonic-sucrose buffer and passed 20 times through a 26-gauge needle. Intact cells and nuclei were removed by centrifugation at 2,700 × g for 10 min, and the resulting supernatant was centrifuged at 100,000 × g for 60 min. The resulting microsome pellet was resuspended in hypotonic sucrose buffer. Equivalent proportions of each fraction were analyzed by SDS-PAGE and immunoblotting.

Membrane Fractionation—Subconfluent monolayers of HeLa cells were detached by scraping into PBS and washed in chilled hypotonic-sucrose buffer (250 mM sucrose, 10 mM HEPES, pH 7.4), containing Complete Protease Inhibitors (Roche Molecular Biochemicals, Germany). Cells were resuspended in 500 μl of hypotonic-sucrose buffer and passed 20 times through a 26-gauge needle. Intact cells and nuclei were removed by centrifugation at 2,700 × g for 10 min, and the resulting supernatant was centrifuged at 100,000 × g for 60 min. The resulting microsome pellet was resuspended in hypotonic sucrose buffer. Equivalent proportions of each fraction were analyzed by SDS-PAGE and immunoblotting.

Brefeldin A Treatment—Cell monolayers were treated with 5 μg/ml brefeldin A (Calbiochem) diluted in complete DMEM at 37 °C. Following treatment, cells were washed three times in PBS, fixed, and processed for immunofluorescence.

Immunoblotting—Cell extracts were dissolved in reducing sample buffer and subjected to SDS-PAGE. Proteins were then transferred to nitrocellulose membrane, and the membrane was blocked with 5% skim milk powder in PBS for 2 h. Antibodies were diluted in PBS containing 5% (w/v) skim milk powder and incubated on the membrane for 1 h, followed by three 10-min washes in 0.05% Tween 20/PBS. Membranes were then incubated with peroxidase-conjugated anti-mouse or rabbit antibodies, as appropriate, and bound antibodies were detected by enhanced chemiluminescence (PerkinElmer Life Sciences) as described (20).

Immunogold Labeling and Electron Microscopy.—For immunogold labeling, confluent monolayers of HeLa cells were fixed with 4% paraformaldehyde (electron microscopy grade; ProSciTech) diluted in complete DMEM at 37 °C and processed for immunofluorescence. The resulting microsome pellet was resuspended in hypotonic sucrose buffer (250 mM sucrose, 10 mM HEPES, pH 7.4), containing Complete Protease Inhibitors (Roche Molecular Biochemicals, Germany). Cells were resuspended in 500 μl of hypotonic-sucrose buffer and passed 20 times through a 26-gauge needle. Intact cells and nuclei were removed by centrifugation at 2,700 × g for 10 min, and the resulting supernatant was centrifuged at 100,000 × g for 60 min. The resulting microsome pellet was resuspended in hypotonic sucrose buffer. Equivalent proportions of each fraction were analyzed by SDS-PAGE and immunoblotting.

Brefeldin A Treatment—Cell monolayers were treated with 5 μg/ml brefeldin A (Calbiochem) diluted in complete DMEM at 37 °C. Following treatment, cells were washed three times in PBS, fixed, and processed for immunofluorescence.

Immunoblotting—Cell extracts were dissolved in reducing sample buffer and subjected to SDS-PAGE. Proteins were then transferred to nitrocellulose membrane, and the membrane was blocked with 5% skim milk powder in PBS for 2 h. Antibodies were diluted in PBS containing 5% (w/v) skim milk powder and incubated on the membrane for 1 h, followed by three 10-min washes in 0.05% Tween 20/PBS. Membranes were then incubated with peroxidase-conjugated anti-mouse or rabbit antibodies, as appropriate, and bound antibodies were detected by enhanced chemiluminescence (PerkinElmer Life Sciences) as described (20).

Immunogold Labeling and Electron Microscopy.—For immunogold labeling, confluent monolayers of HeLa cells were fixed with 4% paraformaldehyde (electron microscopy grade; ProSciTech), containing 0.2M sucrose buffer. Equivalent proportions of each fraction were analyzed by SDS-PAGE and immunoblotting.

Brefeldin A Treatment—Cell monolayers were treated with 5 μg/ml brefeldin A (Calbiochem) diluted in complete DMEM at 37 °C. Following treatment, cells were washed three times in PBS, fixed, and processed for immunofluorescence.
The second putative human protein with a predicted GRIP domain was based on the sequence of an expressed sequence tag, KIAA0336, and is predicted to encode a protein of 1583 amino acids (calculated molecular mass of 185 kDa) with extensive coiled-coil domains spanning almost the entire length of the protein and a potential non-coiled-coil GRIP domain at its C terminus (Fig. 1C). We call this protein GCC185. The lack of a signal sequence or transmembrane domain is consistent with it also being a peripheral membrane protein. In view of the low sequence similarity between the GRIP domain sequences (Fig. 1A) it is important to determine whether the GRIP domain of GCC185 is a functional Golgi targeting sequence. The cDNA encoding the 200 C-terminal residues of KIAA0336 was isolated by reverse transcriptase PCR of HeLa cell RNA, and the sequence of the GRIP domain was shown to be identical to that found in the data base. We constructed a chimeric cDNA encoding the non-coiled-coil C-terminal domain of GCC185 (82 residues), which includes the GRIP domain, fused to the C terminus of GFP. COS cells transfected with the GFP-GCC185-GRIP construct showed targeting of GFP fluorescence to the Golgi region of the transfected cells, as indicated by the concentrated juxtanuclear fluorescence, with a similar staining pattern to GFP-GCC88GRIP and GFP-p230GRIP. Therefore, even though the sequence similarity between the GRIP domains is modest, the GRIP sequence of GCC185 is a functional Golgi targeting motif.

To determine whether the two novel human proteins are bona fide Golgi proteins, we generated rabbit antisera to the C-terminal domains of either GCC88 or GCC185 as described under “Experimental Procedures.” Because the antibodies were generated to sequences that included GRIP domains, we were cognizant of the possibility for cross-reactivity of the antisera with other members of the GRIP family. Therefore, we assessed the ability of the antisera to cross-react with GRIP domains of other mammalian members of the family (p230, golgin97, GCC88, and GCC185). Fusions with GFP were analyzed to avoid cross-reactivity of antibodies with the GST. COS cells were transiently transfected with GFP constructs, and cell extracts were immunoblotted with the antisera. As expected, the antisera raised to GCC88 (Fig. 2A) reacted strongly to the 47-kDa GFP-GCC88C199aa and importantly no cross-reactivity was observed with GFP-GCC185GRIP, GFP-p230GRIP, or GFP-golgin97GRIP fusion protein (Fig. 2A). Blotting with anti-GFP antibodies demonstrated the presence of abundant levels of each fusion protein in the membrane blot (Fig. 2A). Likewise, antibodies to GCC185 showed a strong reactivity to the 35-kDa GFP-GCC185GRIP fusion protein but no cross-reactivity to fusion proteins containing GRIP sequence of p230, golgin97, or GCC88 (Fig. 2B). The band observed at 32 kDa with the anti-GCC185 serum, which does not correspond to sizes of any of the GFP fusion proteins, is nonspecific.

Immunofluorescence of transfected COS cells was also performed to ensure the two antisera did not cross-react with other GRIP domains in native rather than denatured proteins. COS cells transfected with GFP-GCC88GRIP stained strongly with the anti-GCC88 antibody, and the anti-GCC88 staining co-localized with the GFP fluorescence, demonstrating that the antibody was binding to the GFP fusion protein. On the other hand, COS cells expressing GFP-GCC185GRIP, GFP-p230GRIP, or GFP-golgin97GRIP showed no staining with the anti-GCC88 antibody (not shown). Similarly, the anti-GCC185 antibody recognized the GFP-GCC185GRIP fusion protein in transfected COS cells but showed no cross-reactivity with the other three GFP-GRIP fusion proteins (not shown). Collectively, these data demonstrate that the anti-
GCC88 and anti-GCC185 antibodies are specific for the proteins against which they were raised.

Immunoblotting of HeLa cell extracts with anti-GCC88 antibody detected a single specific component of 105 kDa, an apparent molecular mass slightly larger than the predicted 88 kDa. Fractionation of HeLa cells showed that the 105-kDa component was associated with both the membrane and cytosolic fraction, indicative of a peripheral membrane protein (Fig. 3A). To ensure that the cDNA encoding GCC88 we had isolated was full-length, we expressed the GCC88 cDNA in COS cells and immunoblotted cell extracts with anti-GCC88 antibody. In untransfected COS cells, a weak band of 105 kDa was detected, and in extracts of transfected cells we detected a highly abundant component of identical size (105 kDa), showing that the GCC88 cDNA encoded a protein of identical size as the endogenous GCC88 (Fig. 3C). The additional, smaller components, detected in the transfected cell extracts, probably represent degradation products of the overexpressed GCC88.

Anti-GCC185 antibodies detected a specific component of ~175 kDa in HeLa cell extracts by immunoblotting, and like GCC88, was also associated with both membrane and cytosolic fractions (Fig. 3B). A size of 175 kDa is consistent with the expected size of GCC185. The additional band observed at 130 kDa was considered nonspecific as it was also detected in the control blots with pre-immune serum and varied in intensity between samples. The anti-GCC185 antibodies also recognized a specific 175-kDa component in COS cells transfected with the full-length GCC185 (Fig. 3C). These data confirm that the antibody raised to the GST fusion protein specifically recognizes endogenous GCC185 and that the cDNA clone is full-length.

Four Human GRIP Proteins Are Localized to the TGN of HeLa Cells—By immunofluorescence, anti-GCC88 antibodies showed strong juxtanuclear staining of HeLa cells (Fig. 4, A and B), a typical staining of the Golgi apparatus. Staining of HeLa cells with rabbit antibodies to GCC88 and human autoantibodies to p230 showed that GCC88 co-localized extensively with the anti-IG conjugates and the irrelevant primary antibody. Similar results were obtained irrespective of the order of the primary antibodies. Bar, 10 μm.

Fig. 4. Endogenous GCC88, GCC185, and golgin97 colocalize with p230 in HeLa cells. Semiconfluent HeLa cell monolayers were fixed, permeabilized, and stained for GCC88 and p230 (A), GCC88 and GM130 (B), GCC185 and p230 (C), and golgin97 and p230 (D), using rabbit anti-GCC88, human anti-p230 antibody, mouse monoclonal anti-GM130, rabbit anti-GCC185, and mouse monoclonal anti-golgin97 antibody. Bound antibody was detected with either anti-rabbit IgG-Texas Red or anti-rabbit Ig-FITC or Alexa594- or FITC-anti-human IgG. A–C, monolayers were fixed in paraformaldehyde and in cold methanol (D). Confocal images were collected with identical iris settings. Superimposed images (Overlay) reveal regions of co-localization. Control incubations demonstrated no cross-reactivity between the anti-IG conjugates or between the anti-IG conjugates and the irrelevant primary antibody. Similar results were obtained irrespective of the order of the primary antibodies.
same region (Fig. 5B). myc-GCC185 was also found associated with tubulovesicular structures in the TGN, consistent with the immunofluorescence data (Fig. 5C). Antibodies to golgin97 were unsuitable for immunogold labeling of cryosections. GFP labeling of cryofixed GFP-golgin97 GRIP-transfected HeLa cells was located to one side, the TGN side, of the Golgi stack associated with tubulovesicular structures (Fig. 5D). The majority of the Golgi cisternae were unlabeled. We have shown previously (16, 20) that p230 and the p230 GRIP domain are localized to the TGN. Taken together, these data show that all four GRIP proteins are localized to the trans-Golgi/TGN.

Golgi Membrane Binding of GCC88 and GCC185 Is Brefeldin A-sensitive—Previously, we showed that p230 is slowly dissociated from HeLa Golgi membranes in the presence of brefeldin A (20). We have compared the dissociation rate of p230, GCC88, and GCC185 from Golgi membranes in the presence of this drug. Immunofluorescence staining was carried out on HeLa cells treated with brefeldin A for various times. There was no effect on the perinuclear localization of GCC88 after a 5-min brefeldin A treatment; by 15 min of treatment, however, there was a reduction in perinuclear staining of GCC88, together with an increase in the cytoplasmic staining (Fig. 6). Similar rates of dissociation of GCC185 from Golgi membranes were observed in the presence of brefeldin A (not shown). In contrast, and as expected, β-COP was mostly dissociated from Golgi membranes within 2 min of treatment (Fig. 6). The relatively slow rate of dissociation of GCC88 and GCC185 is similar to that observed previously for p230 and distinguishes these proteins from most other brefeldin A-sensitive peripheral membrane Golgi proteins that dissociate from the membrane rapidly.

Overexpression of Full-length GCC88 Results in an Abnormal Structure of a Domain of the TGN—In the course of experiments expressing full-length GCC88 we noted that although low levels of expression showed GCC88 localized to the typical juxtanuclear Golgi pattern, at higher levels of expression GCC88-labeled structures began to extend out from the Golgi region. The size of these structures was dependent on the level of expression of GCC88. Fig. 7 shows a series of three-dimenional reconstructions of Z-series images to illustrate the topology of these structures and the relationship to the level of GCC88 protein. The GCC88-labeled structures extend from the Golgi, and at high levels of protein, the GCC88 structures totally enclose the nucleus (see Fig. 7). By immunofluorescence...
these GCC88-labeled structures resemble the appearance of “cauliflowers.” The transfected HeLa cells containing the cauliflower-like structures were viable, as determined by propidium iodide staining 48 h after transfection. The formation of the cauliflower phenotype was not observed with the GFP-GCC88GRIP fusion protein. Overexpression of GFP-GCC88GRIP results in saturation of Golgi membrane binding sites, and excess fusion protein is found in the cytoplasm. In contrast the high levels of full-length GCC88 appear to be inevitably recruited to the abnormal structures. To exclude the possibility that the myc epitope was contributing to the formation of the abnormal structures we examined COS cells expressing untagged GCC88, as well as FLAG-tagged and GFP-tagged GCC88. Substitution of the myc tag with either FLAG or GFP resulted in the formation of the structures, as did expression of untagged full-length GCC88 (not shown).

Next we examined the localization of a range of markers in cells overexpressing GCC88, to identify molecules that were affected by the cauliflower phenotype. GM130 staining was unaffected in cells expressing high levels of GCC88, demonstrating that the Golgi stack was not perturbed (Fig. 8A). The resident TGN membrane protein, SialylT, was examined using a stable HeLa cell line expressing VSV-G epitope-tagged SialylT (25). The SialylT expressing HeLa cells were transiently transfected with GCC88. In transfected cells expressing low levels of GCC88, there was almost complete co-localization of GCC88 and SialylT, consistent with the TGN location of GCC88 (Fig. 8B, arrows). In transfected cells displaying a mild cauliflower phenotype SialylT remained tightly perinuclear and showed little overlap with GCC88 (Fig. 8B, inset). In transfected cells with a more extreme cauliflower phenotype the SialylT staining pattern showed a more punctate staining pattern; however, the SialylT staining pattern did not overlap with the GCC88-labeled structures (Fig. 8B). These results indicate

**Fig. 7. Overexpression of GCC88 results in a morphological phenotype.** HeLa cells were transfected with myc-GCC88, fixed, permeabilized, and stained with anti-myc monoclonal antibody followed by FITC-anti-mouse Ig. Nuclei were stained with propidium iodide. Shown are a series of three-dimensional reconstructions of Z-series images of transfected cells expressing low to high levels of GCC88. Bar, 10 μm.

**Fig. 8. Distribution of Golgi markers in HeLa cells expressing high levels of GCC88.** HeLa cells were transiently transfected with myc-GCC88, fixed, permeabilized, and co-stained for GCC88 and GM130 (A), p230 (C), golgin97 (D), or β-COP (E). In B, HeLa cells, stably expressing SialylT fused to the VSV-G epitope, were transiently transfected with myc-GCC88, fixed, permeabilized, and co-stained for GCC88 and TGN38. GCC88 was detected with rabbit anti-GCC88 antibodies followed by Texas Red-goat anti-rabbit IgG. GM130, epitope-tagged SialylT, golgin97, β-COP, and TGN38 were detected with mouse monoclonal antibodies, and bound antibodies were detected with FITC-labeled sheep anti-mouse Ig. In C, p230 was detected with human anti-p230 antibodies and FITC-goat anti-human Ig. In each case, the marker was labeled first and GCC88 was labeled second. Shown are images of transfected cells expressing high levels of GCC88. Superimposed images (Overlay) reveal regions of co-localization. In B, arrows indicate cells expressing low levels of GCC88. Control incubations demonstrated no cross-reactivity between the anti-Ig conjugates and the irrelevant primary antibody. Bars, 10 μm.
that the TGN resident membrane protein SialylT is not recruited into the GCC88-labeled structures.

Dual labeling of high expressing GCC88 cells for endogenous p230 and golgin97 showed considerable co-staining, whereas, the coat protein β-COP retained its typical staining pattern and was not localized to the GCC88-labeled cauliflower structures (Fig. 8, C–E). To investigate whether molecules that recycle through the TGN were incorporated into the cauliflower structures we stained GCC88-transfected NRK cells for the membrane protein TGN38. At low levels of GCC88 expression, extensive co-localization of GCC88 and TGN38 was observed (not shown). This result was expected as TGN38 is predominantly located within the TGN (27). Significantly, at high levels of GCC88 expression, TGN38 was also localized within the cauliflower structures (Fig. 8F), clearly demonstrating that the GCC88-labeled structures include recycling membrane proteins.

By electron microscopy, myc-tagged GCC88 transfected cells displayed electron dense structures in close proximity to the trans-face of the Golgi, and in some cells these structures were found to extend some distance from the Golgi stack (Fig. 9, A and B). Immunolabeling for GCC88 with anti-myc antibodies revealed GCC88 decorated tubulovesicular structures associated with the TGN, as well as giving heavy labeling of the electron dense structures adjacent to the TGN membranes. Both the tubulovesicular structures and the electron dense structures were co-labeled with syntaxin 6, confirming that the GCC88-labeled structures are either TGN membranes or derived therefrom. The large electron dense structures are highly ordered and label very strongly with either anti-myc antibodies.

![Fig. 9. Overexpression of GCC88 leads to the formation of aberrant structures extending from the TGN.](image)

HeLa cells were transfected with myc-GCC88 and cells fixed with glutaraldehyde and processed for cryoelectron microscopy. Ultra thin cryosections were double labeled with monoclonal antibodies to myc and rabbit polyclonal antibodies to GCC88 (A) or double labeled with monoclonal antibodies to myc and to syntaxin 6 (syn6) (arrows) (B) or labeled with antibodies to syntaxin 6 or myc alone (C and D, respectively). Antibodies were detected with 5–10 nm protein A gold particles. Inset in A shows lower magnification of electron dense GCC88-labeled structures. Note the labeling of structures that are in close proximity to the TGN. Bars, 100 nm.
or anti-GCC antibodies (Fig. 9A). Labeling of GCC88 was observed predominantly on the outside of the high density protein arrays. The structures are composed of highly ordered electron dense arrays, and lipid bilayers were not readily visible in these structures. The high level of immunogold labeling indicates that the electron dense structures in these transfected cells are because of close packing of GCC88 molecules.

**Cauliflower Phenotype Is Dependent on Coiled-coil Regions and the N Terminus of GCC88**—Overexpression of GFP-GCC88GRIP does not result in the generation of abnormal phenotype, rather at high levels of expression of the GRIP fusion protein the membrane binding sites are saturated, and excess GFP fusion protein is found distributed throughout the cytoplasm (Fig. 10). To determine whether the N-terminal domain is required for the formation of the cauliflower structures, a deletion mutant was constructed lacking the 279 N-terminal residues. The myc-GCC88<sub>280-775</sub> mutant showed Golgi localization, as expected, but high levels of expression showed no evidence of abnormal Golgi staining, and the GCC88 deletion mutant was localized both on the Golgi and in the cytoplasm, analogous to the behavior of the GFP-GRIP fusion protein (Fig. 10). Therefore, the non-coiled-coil N-terminal domain is required to generate the abnormal phenotype.

**TGN38 Traffics through GCC88-labeled Structures**—The localization of TGN38 to the GCC88-labeled cauliflower structures may represent a pool of TGN38 molecules that are blocked in transport or alternatively may represent TGN38 molecules that are in transit through a subdomain of the TGN. To determine whether the cauliflower structures can receive membrane cargo molecules, we traced TGN38 molecules from the cell surface of NRK cells to the TGN by incubation of live cells with anti-TGN38 monoclonal antibody. After the incubation, cells were washed to remove unbound antibody, and the TGN38/antibody complexes were detected using FITC-anti-mouse Ig. Incubation of NRK cells with anti-TGN38 antibodies on ice showed low level staining of cell surface TGN38 and no co-localization of TGN38 and the GCC88-stained structures (Fig. 11A). Incubation of untransfected cells with anti-TGN38 antibody at 37 °C showed co-localization of TGN38 with endogenous GCC88, confirming the movement of TGN38 from the cell surface to the TGN (Fig. 11F). Incubation of GCC88-transfected NRK cells with anti-TGN38 antibody at 37 °C for 10 min resulted in the localization of the antibody-TGN38 complex within GCC88-labeled cauliflower structures (Fig. 11B). Further incubation at 37 °C for 90 min showed an increase in antibody-TGN38 complex detected within the GCC88 structures (Fig. 11C). On the other hand, incubation of the cells at 37 °C for 90 min with an irrelevant monoclonal antibody showed only a low level internalization of the antibody, demonstrating that the uptake of anti-TGN38 antibody was specific (Fig. 11E). This result demonstrates that TGN38 is transported from the cell surface into the subdomain of the TGN that has been perturbed by the overexpression of full-length GCC88.

To determine whether TGN38 can be transported out of the cauliflower structures, live transfected NRK cells were loaded with antibody to TGN38, incubated at 37 °C for 90 min to chase the antibody-TGN38 complexes into the TGN/cauliflowers, washed to remove unbound antibody in the medium, and then incubated further for 45 min in the presence of bafilomycin A1.

**Fig. 10. Aberrant structures induced by high levels of GCC88.** Confocal fluorescence images of fixed COS cells transfected with GFP-GCC88<sub>GRIP</sub>, myc-GCC88<sub>279-775</sub>, or myc-GCC88<sub>280-775</sub> as indicated. myc-GCC88 products were detected with anti-myc monoclonal antibody followed by FITC-anti-mouse Ig. Transfected cells were fixed 48 h after transfection. Bars, 10 μm.

**Fig. 11. Cell surface TGN38 is transported into enlarged GCC88-labeled structures.** NRK cells were transfected with myc-GCC88. The cells were then washed in PBS and incubated in serum-free medium containing anti-TGN38 (1.25 μg/ml) (A–D) or a control monoclonal antibody (E) on ice for 10 min (A), 37 °C for 10 min (B), or 37 °C for 90 min (C and E). In D, NRK cells transfected with myc-GCC88 were incubated with anti-TGN38 antibodies for 90 min at 37 °C and then washed and incubated further for 45 min at 37 °C in serum-free medium containing 0.2 μM bafilomycin A1. F, untransfected cells were incubated with anti-TGN38 antibody at 37 °C for 90 min. Monolayers were fixed and permeabilized, and the TGN38-Ig complex was detected with FITC-anti-mouse Ig and stained for GCC88 using rabbit anti-GCC88 antibodies followed by Texas Red goat anti-rabbit IgG. Bars, 10 μm.
If antibody-TGN38 complexes can be transported out of the GCC88-labeled structures, their subsequent transport from endosomes should then be blocked by bafilomycin A1 (28). Incubation of antibody-loaded GCC88-transfected cells in the presence of bafilomycin A showed a significant reduction of TGN38 in the cauliflower structures with additional staining in endosomes (Fig. 11D). This result indicates that the TGN38 molecules that had been transported into the GCC88-labeled structures could be transported from these structures to the plasma membrane and then internalized into endosomes. Thus, the GCC88 decorated cauliflowers appear to represent a subdomain of the TGN that can accommodate movement of membrane cargo molecules.

**DISCUSSION**

Based on the presence of putative Golgi targeting GRIP sequences, we have identified two novel human trans-Golgi/TGN proteins, designated GCC88 and GCC185. Both proteins have structural features similar to other members of the GRIP family of proteins, namely extensive coiled-coil regions throughout the polypeptide and the GRIP domain located at the C terminus. We have demonstrated that the GRIP domains of GCC88 and GCC185 are functional Golgi targeting sequences and furthermore, that these proteins and another member of the GRIP family, golgin97, are localized, together with p230, to the trans-Golgi/TGN. Thus we can conclude that there are four human proteins with GRIP domains associated with the TGN of HeLa cells. Overexpression of GCC88 induced a dramatic enlargement of a domain of the TGN through which the membrane protein TGN38 could recycle, suggesting that GCC88 may function to maintain the organization of a TGN domain involved with membrane transport.

GCC88 was initially identified based on a GRIP sequence in the data base. Here we isolated cDNA clones and established that the clones encode the full-length sequence of GCC88, as rabbit antibodies raised against a bacterial fusion protein recognized an endogenous protein in HeLa and COS cell extracts of the same size as that encoded by the cloned cDNA. Likewise, we established that the human cDNA clone (HG1120; gene name KIA0A336) encodes a protein of the same size as the endogenous GCC185 protein, confirming that the clone is full-length. GCC88 and GCC185 were shown to be bona fide Golgi proteins with similar membrane binding characteristics as p230. GCC88 and GCC185 are peripheral membrane proteins as both (1) are associated with both cytosol and membrane fractions, (2) dissociate from Golgi membranes in the presence of brefeldin A, and (3) lack a signal sequence or membrane-spanning domain. The relatively slow rate of brefeldin A-induced dissociation of GCC88 and GCC185 from Golgi membranes is very similar to the behavior of p230 (20, 22) and clearly distinguishes the GRIP proteins from many other brefeldin A-sensitive proteins, such as β-COP, that dissociate rapidly (<2 min) after treatment. The membrane binding of the different GRIP proteins therefore appears to be regulated in a similar, as yet undefined, manner. The kinetics of GRIP protein dissociation from the Golgi indicates that the action of brefeldin A on the G protein, ARF, occurs considerably upstream from GRIP protein dissociation or that there are additional undefined targets of brefeldin A.

The sequence similarity between the GRIP domains is only modest. The data presented here and elsewhere (12–14) collectively show that four different human GRIP sequences are functional Golgi targeting signals. In view of the modest sequence similarity between GRIP domains it was possible that different GRIPs may have distinct fine specificities and may be localized to different regions of the Golgi. By immunofluorescence and immunogold labeling we demonstrated that GCC88, GCC185, and the GRIP domain of golgin97 are all localized to the trans-Golgi/TGN. A TGN localization for GCC88 and golgin97 is consistent with earlier studies that showed that GFP fused to the GRIP domain of GCC88 (previously GCC1p) or golgin97 was able to displace endogenous p230 from Golgi membranes when present at high levels in transfected cells (15). The finding here that the four GRIP proteins are indeed localized to the TGN raises the possibility that the different family members may compete for the same TGN membrane determinants. The mechanism of GRIP binding to Golgi membranes appears to be highly conserved throughout evolution as GRIP sequences are also functional in yeast (14) and the primitive eucaryotic organism, Leishmania (17). However, the membrane determinants for GRIP domains remain to be identified.

The function of GRIP proteins has not been clearly defined. The predominance of coiled-coil structures is highly suggestive of rod-like molecules and furthermore links the GRIP proteins to other coiled-coil proteins of the golgin family that are proposed to function in the organization of the Golgi and as molecular tethers in the docking of transport vesicles with target membranes (21). Recent studies have shown that endogenous p230 and a GFP fusion protein containing the p230-GRIP sequence are recruited to a subdomain of the TGN that forms dynamic tubular extensions that have the characteristics of transport carriers (16). In addition, GFP-p230 GRIP associates with a population of *in vitro* generated vesicles (16), consistent with a potential role of p230 as a tethering molecule for vesicle docking or for attachment of the transport carriers to the cytoskeleton. A membrane transport role has also been implicated for Imh1p, a GRIP protein from *S. cerevisiae* (19).

Surprisingly, the overexpression of GCC88 in transfected cells induced considerable morphological change, namely a dramatic enlargement of Golgi-associated structures. The development of these cauliflower structures was independent of cell type and the epitope tag present on the full-length GCC88. The abnormal structures, which extended from the trans-face of the Golgi apparatus, contained large amounts of GCC88 protein that appeared as regularly packed arrays in electron micrographs. Although no membrane bilayers could be seen in the arrays in cryosections, the presence of TGN38 and syntaxin 6 clearly indicates the inclusion of membrane in these structures. The high content of GCC88 on these structures indicates that full-length GCC88 may be able to self-associate to form highly ordered oligomers, possibly via their coiled-coil regions, on the surface of the membrane, in contrast to the GFP fusion protein containing only the GRIP domain, which readily saturates the Golgi membrane binding sites. Analysis of deletion mutants showed that the N-terminal domain of GCC88 is required to induce the morphological change in the TGN. The requirement for the non-coiled-coil N-terminal domain raises the possibility that the interaction of full-length GCC88 with other molecules is necessary to generate the perturbation of the TGN. Overall, our data show that the organization of the TGN is dependent on maintaining the appropriate level of GCC88 in the cell.

At the light microscopic level the GCC88-labeled structures resemble abnormal outgrowths of endoplasmic reticulum membranes produced by overexpression of the cis-Golgi membrane protein p23/24 (29). At the ultrastructural level, however, there is little similarity between the p23 and GCC88 structures. Significantly, both abnormal structures are examples of how compartments can be morphologically perturbed by changing levels of critical organizational proteins.

Overexpression of GCC88 did not affect the entire TGN compartment as the membrane resident protein SialylT did not co-localize with the cauliflower structures whereas TGN38 can...
move into and from the GCC88 decorated structures. These findings imply that resident TGN proteins may be sorted into domain(s) distinct from membrane molecules in transit through the TGN. The finding that a resident TGN protein is excluded from the abnormal structures suggests that the GCC88 decorated cauliflowers are derived from TGN domains associated with membrane transport. Other studies have also indicated that the TGN exists as subdomains; for example the TGN-associated proteins p230 and p200/myosin II are found on distinct tubulovesicular structures (20); however, the underlying molecular basis for maintaining the complex network of tubular structures is poorly understood. The behavior of GCC88 suggests that this molecule may be involved in maintaining the organization of a subdomain of the TGN.

The basis for the formation of the cauliflower structures remains unclear. The GCC88 decorated structures are not sensitive to brefeldin A, suggesting that GCC88 overexpression may result in oligomers that are insensitive to G protein regulation, thereby perturbing dissociation of GCC88 from the membrane. Of note is that overexpression of other Golgi molecules involved in linking membranes to the cytoskeleton, such as CLIPR-59 and GMAP-210, also dramatically affects Golgi morphology (30, 31). One possibility is that GCC88 is involved in maintaining the tubular network of the TGN compartment via interactions with the membrane and cytoskeleton. High levels of GCC88 may result in drawing TGN membranes from a tightly packed network into an open structure as a result of enhanced interactions with the cytoskeleton. Such an open structure would occupy a larger unit volume of the cell compared with a compact tubular network, consistent with the appearance of the GCC88-labeled structures. Thus, GCC88 may function as a TGN matrix protein to help maintain the tubular extensions of the TGN, structures likely to be required for efficient sorting and transport. The cauliflower-like structures present in cells expressing high levels of GCC88 clearly allow the transport of cargo, but it is possible that the efficiency of sorting and/or transport may be affected. Further experiments to determine the rates of recycling and exocytosis from the TGN will be required to address these possibilities.

Why are four different GRIP proteins expressed in one cell type? Each of the GRIP proteins may have a different function, or alternatively, they may be overlap between the functions of the four members. Further experiments defining the molecular basis for the generation of abnormal TGN structures by GCC88 should provide insight into the precise role of GCC88 and other GRIP proteins in maintaining the complex organization of the TGN.

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REFERENCES


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