Involvement of a Golgi-resident GPI-anchored Protein in Maintenance of the Golgi Structure[□]

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The Golgi apparatus consists of a series of flattened cisternal membranes that are aligned in parallel to form stacks. Cytosolic-oriented Golgi-associated proteins have been identified that may coordinate or maintain the Golgi architecture. Here, we describe a novel GPI-anchored protein, Golgi-resident GPI-anchored protein (GREG) that has a brefeldin A-sensitive Golgi localization. GREG resides in the Golgi lumen as a *cis*-oriented homodimer, due to strong interactions between coiled-coil regions in the C termini. Dimerization of GREG as well as its Golgi localization depends on a unique tandem repeat sequence within the coiled-coil region. RNA-mediated interference of GREG expression or expression of GREG mutants reveals an essential role for GREG in maintenance of the Golgi integrity. Under these conditions, secretion of the vesicular stomatitis virus glycoprotein protein as a marker for protein transport along the secretory pathway is inhibited, suggesting a loss of Golgi function as well. These results imply the involvement of a luminal protein in Golgi structure and function.

INTRODUCTION

In mammalian cells, the Golgi apparatus consists of a series of flattened cisternal membranes that are aligned in parallel to form stacks (Shorter and Warren, 2002). Occupying a central position along the secretory pathway, the Golgi apparatus plays a pivotal role in protein processing and sorting to various destinations (Pfeffer and Rothman, 1987; Marsh and Howell, 2002). Besides the secretory pathway, the Golgi exchanges membrane components with several other subcellular organelles, including endosomes, caveosomes, autophagosomes, and lipid droplets (Mallard *et al.*, 1998; Legesse-Miller *et al.*, 2000; Nichols *et al.*, 2001; Litvak *et al.*, 2002). An emerging role of the Golgi is its involvement in various cellular processes such as transcription, apoptosis, and mitosis via signaling pathways mediated by Ras proteins, protein kinases, and G proteins (Helms, 1995; Helms *et al.*, 1998;

DeBose-Boyd *et al.*, 1999; Lane *et al.*, 2002; Sutterlin *et al.*, 2002; Bivona *et al.*, 2003; Nardini *et al.*, 2003; Preisinger *et al.*, 2004).

These distinct Golgi functions operate within a structure that is unique among subcellular organelles. The structure of the Golgi is robustly retained against a large flux of lipids and proteins. Despite this high degree of organization, Golgi stacks are extremely dynamic structures and imbalances in membrane transport can result in reversible fragmentation or disappearance of the Golgi morphology, as observed for example during mitosis (Seemann *et al.*, 2002) or experimentally induced conditions (Lippincott-Schwartz *et al.*, 1989). However, little is known about the proteins involved in maintenance of the Golgi structure and the structure–function relationship of the Golgi apparatus.

A cohort of cytosolically oriented molecules including matrix proteins (Short and Barr, 2003) and cytoskeletal elements (Allan *et al.*, 2002) precisely coordinates the dynamic properties of the Golgi (Donaldson and Lippincott-Schwartz, 2000; Rios and Bornens, 2003). By spatial and temporal organization in membrane domains, these proteins contribute to the structural and functional properties of the organelle (Zerial and McBride, 2001). Besides protein complexes, biological membranes contain other types of microenvironments. These environments include lipid-enriched microdomains or lipid rafts that are enriched in cholesterol, sphingolipids, and a subset of proteins such as specific signaling molecules (Brown and London, 1998; Helms and Zurzolo, 2004; Mukherjee and Maxfield, 2004; Simons and Vaz, 2004). Lipid rafts are small submicroscopic domains that can cluster to

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Abbreviations used: GIC, Golgi-derived detergent-insoluble complex; GREG, Golgi-resident GPI-anchored protein.

gether to function as platforms and to execute functions in membrane trafficking and signaling (Simons and Toomre, 2000; Helms and Zurzolo, 2004). Most raft proteins found to date localize to the cytosolic face of the membrane. In contrast, at the extracellular leaflet of the plasma membrane, only GPI-anchored proteins and few transmembrane proteins have been identified. Little is known about the transduction of signals across membranes mediated by raft components on either site of the membrane. General properties of lipid rafts such as interactions of interdigitated lipids may contribute to coupling both membrane leaflets. However, transmembrane proteins seem necessary to efficiently couple GPI-anchored receptor signals with specific lipid-anchored signaling molecules in the inner leaflet raft (Devaux and Morris, 2004; Kusumi *et al.*, 2004).

Many raft proteins are resistant to solubilization by the non-ionic detergent Triton X-100 in a cholesterol-sensitive manner (Simons and Toomre, 2000). We previously isolated Golgi-derived low-density detergent-insoluble complexes (GICs) from Chinese hamster ovary (CHO) Golgi membranes (Gkantiragas *et al.*, 2001). Several GIC proteins localize to brefeldin A-sensitive compartments, and all the proteins identified so far have a cytosolic orientation (Gkantiragas *et al.*, 2001; Eberle *et al.*, 2002). Here, we report the characterization of a novel protein in Golgi-derived lipid-enriched microdomains that is involved in the structural maintenance of the Golgi apparatus. Unlike other proteins required for maintaining the Golgi architecture, this protein has a luminal orientation and is linked to the membrane by use of a GPI anchor.

MATERIALS AND METHODS

Cells, DNA Constructs, Antibodies, and Reagents

Total membranes were prepared as described previously (Helms *et al.*, 1998). Phosphatidylinositol-specific phospholipase C (PI-PLC) (Sigma-Aldrich, St. Louis, MO) treatment and phase separation with Triton X (TX)-114 (Sigma-Aldrich) were performed as described by Bordier (1981). Phosphatidylinositol glycan class L (PIG-L) deficient and reconstituted CHO cell lines were grown and maintained as described previously (Abrami *et al.*, 2001). Cells were transfected with *N*-acetylglucosamine-transferase-I (NAGT-I)-green fluorescent protein (GFP) chimera (kindly provided by G. Warren, Ludwig Institute for Cancer Research, New Haven, CT) (Shima *et al.*, 1997). SDS-PAGE and Western blot analysis were carried out according to standard procedures. Antibodies used for Western blotting and immunofluorescence included the following: M2 monoclonal antibody (mAb) against Flag (Sigma-Aldrich), monoclonal anti-GM130 (BD Biosciences, San Jose, CA), monoclonal anti-folate receptor (FolR) (a gift from Dr. Silvana Canevari, Istituto Nazionale Tumori, Genova, Italy), polyclonal anti-Caspase-2L (C-20) and caveolin-1 (N-20) (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-p23 antibody as described previously (Sohn *et al.*, 1996), α -tubulin (kindly provided by Wei Yao, EMBL, Heidelberg, Germany), mannosidase II (Mann II) (Chemicon International, Temecula, CA), GRASP65 (a gift from Dr. Lowe, University of Manchester, Manchester, United Kingdom) and GM130 (BD Biosciences). Primary antibody labeling was then visualized in immunofluorescence by incubation with Alexa Fluor 488 anti-rabbit IgG or Alexa Fluor 568 anti-mouse IgG antibodies (Invitrogen, Carlsbad, CA). Anisomycin was purchased from Calbiochem and 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) from Sigma-Aldrich. Anti GFP-A6455 (polyclonal rabbit serum) was obtained from Invitrogen.

Isolation and Sequence Analysis of Golgi-Resident GPI-anchored Protein (GREG)

Based on the reported peptide sequence (Gkantiragas *et al.*, 2001), we designed two degenerate oligonucleotides: 5'-CA (A/G)GA (A/G)CA (A/G)GA (A/G)GC (A/C/G/T)CA (A/G)AT (A/C/T)AA (A/G)-3' and 5'-(C/T)TT (A/G/T)AT (C/T)TG (A/C/G/T)GC (C/T)TC (C/T)TG (C/T)TC (C/T)TG-3'. By polymerase chain reaction (PCR) using the above-mentioned degenerate primers in combination with the vector arm primers, a 1.0-kb fragment was amplified from a CHO cDNA library (Stratagene, La Jolla, CA). This fragment was randomly labeled with [α -³²P]dCTP (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) and used as probes to screen the same cDNA library by colony hybridization. Out of 1×10^6 , 40 positive clones were obtained. Screened by PCR, four clones with longer inserts were

completely sequenced. All these four clones contain the same open reading frame (ORF).

GPI anchorage processing was predicted using big-PI predictor at University of Vienna (Vienna, Austria) (http://mendl.imp.univie.ac.at/gpi/gpi_server.html). Multiple sequence alignment was done at European Bioinformatics Institute (<http://www.ebi.ac.uk/clustalw/>) and decorated with BoxShade 3.21 at Swiss EMBnet node (http://www.ch.embnet.org/software/BOX_form.html).

DNA Constructs and Transfection

Constructs were cloned into the EcoRI/XhoI or EcoRI/NotI sites of expression plasmid pcDNA3 (Invitrogen). To generate GREG-Flag, a single Flag-tag was inserted between 181Q and 182N by PCR. For constructing Δ GPI-GREG and 23TM-GREG, a fragment without the C-terminal GPI-signal peptide (SP) (amino acid residue [AA] 1-183) was first amplified by PCR. This fragment was fused with either a single Flag-tag or the transmembrane domain of p23 together with a Flag-tag at the C-terminal end (RVLYSFISFMFLIGLAT-WQVFYLRiggleDYKDDDDK, residues in small cap are spacers). To generate a GPI-anchored form of both N-terminal (AA 1-110) and C-terminal (AA 109-211), GREG-Flag cDNA was digested with BstYI. The resulting two fragments were ligated with either the signal peptide of GREG (C-terminal) or the GPI-SP linked with a single Flag-tag (N-terminal). For making the Δ EQ construct, a fragment from amino acid 139-211 of GREG-Flag was amplified and ligated with the N-terminal fragment without the Flag-tagged GPI-SP. For GPI-anchored GREG-GFP (wt-GREG-GFP), a GFP fragment (XhoI/BsrGI) from pEGFP-N1 was ligated with a pair of the GPI-SP oligonucleotides (BsrGI/NotI) and Δ GPI-GREG in pcDNA3 (XhoI/NotI), thus removing the Flag-tag. To construct cc-GREG, a fragment from amino acid 50-183 of wt-GREG was amplified and cloned into pcDNA3. All constructs were verified by DNA sequencing. Cells were maintained in α -minimal essential medium (CHO) or DMEM (normal rat kidney [NRK]) media (Invitrogen) supplemented with 10% fetal calf serum and 10 mg ml⁻¹ penicillin and streptomycin, and they were transfected with the appropriate constructs using Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions. Stable cell clones from CHO or NRK cells were selected with 0.6 mg ml⁻¹ G-418 (Geneticin) (Calbiochem, San Diego, CA) and maintained in appropriate media with 0.2 mg ml⁻¹ G-418.

RNA Interference (RNAi)

We had 25-nucleotide-modified synthetic RNA probes (small interfering RNA [siRNA]) custom synthesized by Stealth RNAi Technology, Invitrogen. Primer sequences used were as follows: sense: 5'-UGGCCUUGGUAUUC-CCGCUCAUCAU-3' and antisense: 5'-AUGAUGAGCGGGAUAACCAAG-GCCA-3'. As a control, scrambled oligonucleotides were used that are based on the above-mentioned sequences: sense: 5'-UGGUUGGUUUAACCGCUCA-CUCCCAU-3' and antisense: 5'-AUGGGAGUGAGCGGUAACCAACCA-3'. Transient transfection of siRNA probes was achieved using the Lipofectamine 2000 transfection reagent (Invitrogen).

Immunofluorescence Microscope

After appropriate treatment, cells either stably or transiently transfected with the GREG constructs, treated with siRNA, were prepared for indirect immunofluorescence according to standard procedures. Briefly, cells were fixed in phosphate-buffered saline (PBS) containing 3.5% paraformaldehyde for 30 min. After quenching with 50 mM NH₄Cl in PBS, cells were permeabilized with 0.5% TX-100 in PBS for 10 min and subsequently blocked in PBS containing 2% bovine serum albumin for 1 h. Cells were stained with DAPI for 10 min immediately before mounting in Fluoromount G (Biozol, Eching, Germany). A Zeiss LSM510 with appropriate filters was used, and images were collected and processed with LSM510 software (Carl Zeiss, Jena, Germany). For cells stained with DAPI, images were taken with the use of a Zeiss inverted fluorescence microscope equipped with a cooled charge-coupled device camera. All images were adjusted with Adobe Photoshop 5.5 (Adobe Systems, Mountain View, CA) and edited with Canvas 8.0.4. PIG-L deficient and reconstituted CHO cell lines were fixed with 3% paraformaldehyde, and images were acquired using a 100 $\times$ lens on a Zeiss Axiophot equipped with a Hamamatsu cooled camera by using the Openlab acquisition software (Improvision, Lexington, MA).

ts-O45-G Transport Assay

For assaying ts-O45-G transport, cells transfected with siRNA were incubated for 48 h and then overlaid with recombinant adenoviruses encoding ts-O45-G-cyan fluorescent protein (CFP) (Keller *et al.*, 2001). After 60 min, adenoviruses were washed away and ts-O45-G was accumulated in the endoplasmic reticulum (ER) by incubating the cells at 39.5°C for 24 h. Thereafter, cells were shifted to 32°C to release ts-O45-G from the ER in the presence of cycloheximide. Sixty minutes after the temperature shift, cells were fixed with 3% paraformaldehyde, and ts-O45-G on the cell surface was detected by immunostaining with a VG antibody recognizing ts-O45-G at the plasma membrane (PM). Cell nuclei were labeled with Hoechst 33342 diluted to a final concentration of 0.1 μ g/ml. Analogous experiment was done under the conditions of Δ GPI-GREG overexpression, except that the cells were transfected

aerolysin, which specifically recognizes GPI-anchored proteins in blot overlay experiments (Hong *et al.*, 2002). We observed that aerolysin bound to a diffuse protein band comigrating with the GREG protein (data not shown).

To directly test GPI anchorage of GREG, we treated solubilized Golgi membranes with PI-PLC followed by TX-114 phase separation (Figure 1c). GREG partitions in the detergent phase and runs as a smear from 35,000 to 60,000 on SDS-PAGE (see Figure 6a), but it can be recovered in the aqueous phase after PI-PLC treatment (Figure 1c), which specifically removes the hydrophobic GPI anchor (Ikezawa *et al.*, 1976; Low and Finean, 1977). P23, a type I transmembrane protein (Sohn *et al.*, 1996), partitions in the detergent phase in a PI-PLC-independent manner (Figure 1c). These data confirm that GREG is a GPI-anchored protein.

GREG Is a Golgi-Resident Protein

The identification of a GPI-anchor came as a surprise, because most if not all GPI-anchored proteins characterized to date have a steady-state localization at the plasma membrane (Nosjean *et al.*, 1997; Mayor and Riezman, 2004). GREG, however, was isolated from an enriched Golgi fraction, and preliminary data showed its localization to the Golgi complex (Gkantiragas *et al.*, 2001).

To investigate the subcellular localization of GREG in more detail, we established CHO and NRK cell lines constitutively expressing a GREG-Flag construct (Figure 2a). GREG-Flag colocalizes with the Golgi marker Man II (Figure 2b). In agreement with our previous observations (Gkantiragas *et al.*, 2001), the Golgi localization of GREG-Flag is BFA sensitive (Figure 2b). Extensive treatment of cells with cycloheximide (8 h; 100 $\mu\text{g}/\text{ml}$) did not chase GREG out of the Golgi, suggesting that it is not in transit to the plasma membrane (Supplemental Figure 1). The Golgi localization of GREG was confirmed in subcellular fractionation studies that separated plasma membranes from endogenous membranes by Nycodenz density gradient centrifugation (Jenne *et al.*, 2002). As shown in Figure 2c, both wt-GREG and GREG-Flag cosediment with GM130, an early Golgi marker (Nakamura *et al.*, 1995). In contrast, FolR, a GPI-anchored protein that primarily localizes to the plasma membrane (Varma and Mayor, 1998), is found in fractions 2 and 3 of the Nycodenz gradient. The amount of FolR present in fractions 7 and 8 is due to its recycling between the plasma membrane and endosomes (Sabharanjak *et al.*, 2002). To assess the localization of GREG at the ultrastructural level, ultrathin cryosections of wt-GREG-GFP-expressing CHO cells were immunogold labeled with anti-GFP (Figure 3). GREG was clearly found associated with the Golgi stacks (Figure 3a), in which it was homogeneously distributed over the individual cisternae and from *cis*- to *trans*-Golgi. Additional label was found in the vesicular tubular clusters (Figure 3b) that may represent a fenestrated cisternal membrane or vesicle tubular clusters between the ER and Golgi (Klumperman, 2000). The *trans*-Golgi network was virtually devoid of label (Figure 3c). Collectively, these data suggest that GREG resides at early Golgi compartments and does not localize to the plasma membrane.

A possible Golgi-targeting motif was identified by sequence comparison with BST-2 proteins (Ishikawa *et al.*, 1995; Ohtomo *et al.*, 1999; Kupzig *et al.*, 2003). Neither the human and mouse BST-2 nor DAMP-1 contains the EQ tandem repeat (Figure 1a), and both localize to the cell surface. When the tandem repeat is deleted from the protein, GREG occurs at the plasma membrane (Supplemental Fig. 2). The tandem repeat was, however, not sufficient to retain lumenally expressed soluble GFP in the Golgi (data not shown).

Figure 2. GREG is a Golgi resident protein. (a) Schematic representation of GREG-Flag construct. (b) Subcellular localization of GREG-Flag. Cells stably expressing GREG-Flag were treated with or without 5 μM brefeldin A for 15 min and processed for immunofluorescence by using antibodies against Flag and Man II. Images shown are representative confocal sections. (c) Biochemical analysis of GREG localization to intracellular membranes. CHO cells constitutively expressing FolR or GREG-Flag were treated with cycloheximide (50 $\mu\text{g}/\text{ml}$) for 5 h at 37°C. Cells were homogenized in 25 mM Tris-Cl, pH 7.4, and 130 mM KCl by passing through a 25-gauge needle, and the postnuclear supernatants were fractionated on a Nycodenz velocity gradient as described previously (Jenne *et al.*, 2002). Fractions were collected from top to bottom and analyzed by Western blotting by using antibodies against the SEA peptide, Flag, FolR, and GM130. The band observed in fraction 2 with the Flag antibody is non-specific, because it also occurs in untransfected cells (data not shown). In addition, the molecular weight is different from GREG-Flag. A cross-reactivity of the M2 Flag antibody was also observed by others (Schafer and Braun, 1995).

Involvement of GREG in Maintenance of Golgi Structure

A structural role of GREG was investigated by knockdown experiments using RNA interference techniques to block the expression of GREG. On transfection of cells with double-strand RNA, GREG-Flag expression was significantly reduced in most cells, as observed by immunofluorescence microscopy and Western blot analysis (data not shown). The Golgi structure of cells that had no detectable levels of GREG-Flag was fragmented (data not shown). Fragmentation of the Golgi was

Figure 3. GREG localizes to the Golgi stack. CHO cells expressing GFP-GREG were labeled with anti-GFP (10-nm gold particles) for ultrastructural localization studies. (a and b) GFP-GREG is found in the Golgi complex (G) and associated membranes. Within a Golgi stack, GFP-GREG distributes from *cis* to *trans*. The asterisk in b points to a transverse section of a fenestrated cisternal membrane. (c) At the *trans*-side of the Golgi complex, the clathrin-coated membranes of the TGN (arrows) are devoid of GFP-GREG. E, endosome. Bars, 200 nm.

also observed when GREG expression was suppressed using stabilized synthetic 25-nucleotide RNA probes (siRNA) (Figures 4a and 7a). Only in the presence of GREG-specific siRNAs, the subcellular distribution of various Golgi markers including Mann II and GM130 was affected (Figure 4a). Other Golgi markers such as COPI subunits, p24, and KDEL receptor were affected as well (data not shown). The efficiency of protein knockout was confirmed by Western blot analysis using specific and scrambled probes (Figure 4b). These results (Figures 4 and 7a) show that GREG is essential in maintaining the Golgi structure.

Similar to other GPI-anchored proteins, GREG can be recovered in an insoluble membrane fraction in cold non-ionic detergents, which are thought to represent lipid rafts or microdomains (Supplemental Fig. 3). A GREG mutant lacking the GPI-anchor (Δ GPI-GREG; Figure 5a) or with the GPI-anchor replaced for the transmembrane domain of p23, a type I transmembrane protein at the Golgi complex (Sohn *et al.*, 1996) (23TM-GREG; Figure 4a) partitions in the detergent-soluble fraction (Supplemental Fig. 3). These data show that the GPI moiety of GREG causes association with microdomains in Golgi membranes. Expression of Δ GPI-GREG resulted in partial colocalization with the Golgi marker mannosidase II (Figure 5b). In addition, partial fragmentation of the Golgi complex was observed in cells expressing the Δ GPI-GREG construct. Expression of 23TM-GREG also generates a scattered Golgi structure (Figure 5b). Quantitative analysis demonstrates that 12 h after transfection with either GPI anchor-deficient mutant construct, ~50% of cells revealed fragmented Golgi structures (Figure 5c). These results indicate that the GPI anchor of GREG is functionally required and that the mature protein is involved in maintenance of the Golgi structure. The disassembly of the Golgi apparatus driven by mutant GREG proteins occurs in an apoptosis-independent manner (Supplemental Fig. 4, a and b). In addition, microtubule integrity is not significantly affected in cells that express Δ GPI-GREG and fragmented Golgi structures remain preferentially associated with or in proximity to microtubules (Supplemental Fig. 4c).

Figure 4. Involvement of GREG in maintenance of the Golgi structure. (a) wt-CHO cells were transfected with control or GREG-specific siRNA, incubated for 68 h, and subsequently analyzed by immunofluorescence by using antibodies against GM130 and mannosidase II to determine disruption of the Golgi apparatus. Nuclei were identified using DAPI. (b) Analysis of knockdown of GREG expression by Western blotting. CHO cells constitutively expressing GREG-Flag (6-well) were transfected with the indicated RNAi probes (100 pmol) for the indicated times (0, 48, and 72 h). Post-nuclear supernatants (50 μ g) were analyzed for the presence of GREG-Flag (bottom) and v-ATPase (top) by Western blotting by using the respective antibodies.

Figure 5. Expression of GREG mutants disrupts the Golgi apparatus. (a) Schematic representation of GREG mutants. (b) CHO cells were transiently transfected with ΔGPI-GREG and 23TM-GREG for 12 h. The cells were analyzed for Flag and Mann II by immunofluorescence. (c) Quantification of cells containing punctuate Golgi structures or no Golgi signal. The percentage of cells with abnormal Golgi structures over cells under four separate scopes was plotted. Shown are the results from one of four individual experiments.

Expression of ΔGPI-GREG leads to multiple morphological changes in the Golgi region as observed by immunoelectron microscopy (EM), showing the localization of ΔGPI-GREG (10-nm gold particles) and Giantin (15-nm gold particles) as a marker for Golgi membranes (Supplemental Fig. 5). Quite remarkable is the formation of tightly stacked, sometimes circular membranes. In addition to membrane stacking, accumulations of vesicles containing ΔGPI-GREG are seen. These morphological changes support the observations made by immunofluorescence.

If a GPI-dependent membrane anchorage is essential for GREG function, then a PIG-L CHO cell line (Abrami *et al.*, 2001), deficient in biosynthesis of the GPI-anchor, is predicted to have an altered Golgi morphology. Indeed, a dramatic increase in nonelongated Golgi structures is observed in PIG-L

CHO cells (90% fragmented Golgi, compared with 50% in PIG-L recomplemented CHO cells (Supplemental Fig. 6).

Structural Requirements of GREG Localization

SDS-PAGE and Western blot analysis of GREG revealed an apparent molecular mass of 35,000 to 60,000 (Figure 6a, lane 1), which is larger than expected. Treatment of solubilized Golgi membranes with an *N*-glycanase (PNGase F) resulted in a band of about 28,000 (Figure 6a, lane 2). In consideration of a theoretical M_r of 15,000 the mature protein is likely to be a (homo)dimer, resistant to SDS. In agreement with the deglycosylation results, a protein with an apparent molecular mass of 25 and 28 kDa can be purified from *Pichia pastoris* upon expression of a His-tagged construct with both SPs deleted (coiled-coil domain of GREG; ccGREG) (Figure 6a, lane 3).

The structural requirements for GREG dimerization were further investigated by mutational analysis of GREG by using LexA-based yeast-two-hybrid interaction system (Brent and Finley, 1997). The entire ccGREG or GREG domains (see Figure 6b, left) were fused to the LexA DNA-binding domain (bait) and the B42 transcriptional activation domain (prey). Cotransformation with bait and prey plasmids expressing ccGREG (residues N50-T178) fusion proteins resulted in strong expression of β -galactosidase as indicated by blue colonies when grown on X-Gal-containing medium (Figure 6b). These results underline that GREG interacts with itself and has an intrinsic potential to form homodimers. The area of interaction was mapped to the C-terminal half of the protein, because ccGREG interacts with CT-GREG (L116-T178) but not with NT-GREG (N50-Q118). Expression of bait and prey CT-GREG fusion proteins was sufficient to retain the interaction (Figure 6b), indicating that GREG forms *cis*-oriented (parallel) dimers. When the EQ-repeat was omitted from ccGREG or CT-GREG, the interaction with ccGREG and/or CT-GREG was not observed anymore, indicating an essential role of the EQ repeat in dimer formation of GREG. Because omission of the EQ-repeat results in plasma membrane localization of GREG (Supplemental Fig. 2), these data imply the involvement of dimer formation in GREG localization to the Golgi complex.

GREG Affects Protein Secretion of Vesicular Stomatitis Virus Glycoprotein (VSV G) Protein to the Plasma Membrane

The structure–function relationship of the Golgi remains largely unknown and fragmentation of the Golgi in smaller units that are scattered through the cytoplasm not necessarily implies a inhibition of exocytic protein transport (Kondylis *et al.*, 2005; Sutterlin *et al.*, 2005).

To study a possible effect of GREG on protein secretion, CHO cells were transfected with a temperature-sensitive mutant of CFP-tagged VSV G protein (ts-O45-G), a widely used marker for visualization of the secretory pathway (Scales *et al.*, 1997). Under permissive conditions, this protein is secreted via the secretory pathway and accumulates at the plasma membrane (Figure 7a, control). Treatment of cells with GREG-specific siRNA resulted in a strong inhibition of protein secretion through the secretory pathway, and the VSV G protein accumulates in structures that also contain mannosidase II, suggesting a block at the Golgi apparatus (Figure 7a, GREG siRNA). Cotransfection of CHO cells with Flag-tagged GREG mutants that affect the Golgi morphology, including ΔGPI-GREG, resulted in inhibition of secretion as well and a partial accumulation of VSV G protein at the perinuclear region (Figure 7b). Quantitation over a large number of cells revealed a 44% (GREG siRNA) and 23% (ΔGPI-GREG) inhibition (Figure 7c). The observed in-

Figure 6. GREG is involved in flattening of Golgi cisternae. (a) Isolated CHO Golgi membranes (15 μ g) were incubated in the absence (lane 1) and presence (lane 2) of 1000 U of PNGase in detergent for 3 h at 37°C. Samples were analyzed by Western blot by using a peptide-antibody against GREG. Expression of cc-GREG. cc-GREG was cloned as described in *Materials and Methods* and expressed in *P. pastoris* cells. His-tagged cc-GREG (lane 3) was purified from the lysate (lane 4) by using nickel-nitrilotriacetic acid affinity chromatography and analyzed by SDS-PAGE stained with Coomassie brilliant blue. In the purified fraction, two bands are observed. Both bands reacted to a peptide antibody against GREG, indicating that GREG has been partially proteolytically cleaved by a protease activity in *P. pastoris*. (b) Yeast two-hybrid analysis of GREG. Left, schematic representation of wt-GREG and the various domains of GREG that were amplified by PCR and cloned into both bait (pLexA) and prey (pB42AD) plasmids to study mutual interactions by using the LexA-based yeast two-hybrid system; numbering refers to amino acid residues. EQ, tandem repeat 3x[EQAQIK]. Right, EGY48 (pSH18-34) yeast strains carrying bait and prey plasmids expressing different GREG-domains. Yeast two-hybrid constructs are described in *Materials and Methods*. The results shown were obtained after growth for 3 d at 30°C and are representative of three independent experiments. Control constructs: POS, positive interaction (GAPR-1/GAPR-1) (Serrano *et al.*, 2004); NEG, negative interaction (nucleolin/nucleolin).

hibition is comparable with other strong inhibitors and established effectors of the secretory pathway applied to the same assay system (Starkuviene *et al.*, 2004). For comparison, down-regulation of established components of the secretory pathway, like β -COP, GM130, and Sec31, inhibits ts-O45-G by 50% in HeLa cells by using the same assay system (Erfle *et al.*, 2004). Down-regulation of β -COP and β prime-COP in 3T3 fibroblasts inhibits ts-O45-G secretion by 32 and 40% (Starkuviene, unpublished data), respectively.

DISCUSSION

Golgi Targeting of GREG

Here, we have described a Golgi resident GPI-anchored protein involved in maintenance of Golgi structure. Little is known about the targeting of proteins to the Golgi complex. The transmembrane domain (Bretscher and Munro, 1993; Yuan and Teasdale, 2002) as well as protein-protein and

protein-lipid interactions (Munro, 2002, 2004) have been implicated in the dynamic Golgi localization. The GPI moiety is not expected to contribute to a Golgi localization, because all GPI-anchored proteins identified so far localize to the plasma membrane and the endosomal system (Mayor and Riezman, 2004). Therefore, GREG must be actively retained in the Golgi. We showed that the EQ repeat is essential for Golgi localization. This observation is in agreement with the finding that homologues of GREG, none of which contain the EQ-repeat, were found to be present at the plasma membrane (Ohtomo *et al.*, 1999; Kupzig *et al.*, 2003). Because the EQ-repeat is also essential for dimerization of GREG (Figure 6b), our data suggest that dimerization and possibly oligomerization is essential for Golgi localization. Oligomerization has been implicated in the dynamic Golgi localization of p24 proteins (Jenne *et al.*, 2002). Oligomerization of proteins into large protein complexes (a so-called Golgi matrix) (Munro, 1998; Gillingham and Munro, 2003)

Figure 7. GREG is required for protein transport along the secretory pathway. (a) Trafficking of ts-O45-G is inhibited under the conditions of GREG down-regulation. wtCHO cells were transfected with control or GREG-specific siRNA, incubated for 48 h, and subsequently infected with the recombinant adenoviruses encoding ts-O45-G-CFP (see *Materials and Methods*). After an additional 24 h of incubation at the restrictive temperature, ts-O45-G-CFP was released from the ER by the temperature shift, and its arrival to the PM was monitored by the immunostaining. In cells transfected with siRNA to down-regulate GREG, ts-O45-G arrival to the PM is markedly reduced. (b) Trafficking of ts-O45-G is inhibited under the conditions of overexpression of ΔGPI-GREG. wtCHO cells were infected with the recombinant adenoviruses encoding ts-O45-G-CFP, incubated at the restrictive temperature for 16 h and subsequently transfected with a plasmid encoding ΔGPI-GREG and incubated for 8 h. In cells expressing ΔGPI-GREG, ts-O45-G-CFP trafficking was reduced. Asterisks indicate transfected cells. (c) Quantitative evaluation of ts-O45-G-CFP trafficking under the conditions of GREG down-regulation and overexpression of ΔGPI-GREG. Secretion rate of ts-O45-G-CFP was expressed as a ratio between the fraction of protein that has reached the PM in 60 min to the total intracellular amount of ts-O45-G-CFP (y-axis) (see *Materials and Methods*). Transport rate of ts-O45-G-CFP in nontreated cells was assigned to be 100%. For each condition 50–200 cells were taken for analysis. Bars indicate standard deviations. Similar results were obtained with different sets of specific stealth RNAi against GREG and a mix of the different RNA probes.

has also been suggested to contribute to Golgi localization. In this respect it is interesting to note that due to its GPI-anchor, GREG has an affinity for lipid rafts. GPI-anchored proteins are able to oligomerize into high-molecular-weight complexes, affecting raft partitioning (Paladino *et al.*, 2004). Vice versa, raft partitioning of proteins can modulate the oligomeric status of a protein (Simons and Toomre, 2000; Anderson and Jacobson, 2002; Helms and Zurzolo, 2004). Our data suggest that oligomerization may be a general mechanism of Golgi localization, applicable not only to cytosolic proteins but also to luminal proteins.

Similar to other GIC proteins, GREG redistributes in the cell 10–15 min after addition of BFA. This suggests that GIC proteins, including GREG, localize to early (*cis-trans*) Golgi membranes (Klausner *et al.*, 1992) and that GREG loses its Golgi localization concomitant with the dispersal of Golgi membranes.

A Luminal and GPI-anchored Protein Involved in Golgi Structure and Function

The identification of a luminal protein involved in maintenance of Golgi structure raises the question how a luminal

protein could be involved in maintenance of Golgi structure. Most likely, transmembrane signaling must occur to allow interaction(s) with the protein machinery at the cytosolic site of the membrane. A similar situation occurs at the plasma membrane where GPI-anchored receptors such as the folate receptor (Nosjean *et al.*, 1997) have to transmit signals across the membrane, yet they span the membrane only halfway. Little is known about the coupling of outer and inner membrane leaflet components. Based on biophysical properties of lipids in model membranes, transmembrane proteins are required for efficient coupling of inner and outer membrane molecules (Devaux and Morris, 2004). Examples for functional coupling of GPI-anchored proteins with transmembrane proteins in signaling processes have been found (Schmitt-Verhulst *et al.*, 1987; Klein *et al.*, 1997). In lipid rafts at the Golgi complex, all proteins identified so far have a cytosolic orientation except GREG (this study) and flotillin-1 (Gkantiragas *et al.*, 2001). The topology of flotillin-1 is not entirely clear (Morrow and Parton, 2005), but it may be suited for transmembrane signaling. An involvement of lipid rafts in GREG function is consistent with the observed fragmentation of Golgi structure when the GPI anchor of GREG has been replaced with a transmembrane anchor (Figure 5). These data suggest that lipid-enriched microdomains may play a role in maintenance of the Golgi architecture, either by its involvement in the oligomerization of GREG at the luminal leaflet of the membrane and/or by its involvement in transmembrane signaling.

From the immunofluorescence data, it seems that some of the mutant GREG protein is localized to the ER. This could be an artifact due to high overexpression and/or slow folding. Alternatively, a significant pool of mutant-GREG does reside in the ER, in contrast to wt-GREG that resides in the Golgi. This may be (as for many Golgi proteins) the result of a change in an equilibrium partitioning between the ER and Golgi. Several GIC proteins cycle in the early secretory pathway (Gkantiragas *et al.*, 2001; Eberle *et al.*, 2002). Therefore, it is feasible that mutant-GREG binds to wt-GREG forming a stable dimer, thus affecting its Golgi localization. Irrespective of the mechanism, mutant GREG could bind to Golgi-interacting partners, dragging these proteins out of the Golgi as well, affecting the Golgi morphology.

GREG and the Golgin Family of Proteins

Several characteristics of GREG are reminiscent of the Golgin family of proteins (Barr and Short, 2003; Gillingham and Munro, 2003). These include a Golgi localization, the involvement in maintenance of Golgi structure, the predicted long coiled-coil structure, and the presence as a parallel homodimer. Furthermore, blast searching reveals that several unknown proteins contain a stretch of similar EQ repeats (e.g., EQEEKIR in XP_496037 and XP_498421). Interestingly, these proteins share significant homology with the Golgin family of proteins containing a long coiled-coil. In contrast to Golgins identified so far, however, GREG has a luminal orientation. In addition, GREG does not contain a GRIP domain that has been identified at the C terminus of some Golgin proteins and that is involved in Golgi targeting (Barr, 1999; Kjer-Nielsen *et al.*, 1999; Munro and Nichols, 1999).

The function of Golgins is to bind and link adjacent membranes, either in tethering of vesicles to membranes or between cisternae (Barr and Short, 2003). Therefore, a more speculative possibility to explain the involvement of GREG in Golgi structure and function may relate to its similarity to Golgin proteins. In this model, opposing membranes within a single cisterna are stabilized by the formation of protein

complexes that contain GREG in a *trans*-configuration. In a *trans*-configuration, these protein complexes could either stabilize opposing membranes or act as a sensing device, monitoring the distance between opposing membranes.

Structure-Function Relationship of the Golgi

The structural organization of the Golgi varies among species. In mammalian cells, stacks of cisternae that are interconnected form a single organelle. In fly, plants, and *Pichia pastoris*, Golgi stacks are not interconnected and are dispersed in the cytoplasm. In *S. cerevisiae*, the Golgi is not stacked and single cisternae are observed. Yet, under these varying morphological conditions, the function of the Golgi in anterograde and retrograde transport along the secretory pathway is fully functional. The smallest functional unit of the Golgi remains to be determined. Expression of GREG mutants results both in fragmentation and inhibition of anterograde protein transport, suggesting that the observed fragmentation also results in loss of function of the Golgi complex and that GREG fulfills an essential function in the Golgi complex. Golgi morphology and function are, however, often strongly related and difficult to discriminate from each other. In this respect, it is interesting to note that in the GPI-deficient cell line, protein secretion must continue to stay viable. This may suggest that in these cells, the primary effect of GREG is on Golgi morphology and that GREG is not essential for protein secretion. In agreement with this is that on close inspection of the Golgi structures in the PIG-L cells that are labeled with NAGTI-GFP, many circular or ring-like structures are observed. These structures are reminiscent of the changes in Golgi structure observed in EM that are induced by expression of Δ GPI-GREG. Our results indicate that cytosolic as well as luminal proteins at the Golgi complex act in concert to maintain a unique Golgi structure. Dynamic integration of the various signals involved defines the overall Golgi structure.

The sequence shown in this article has been deposited at GenBank (accession nos. AY272060 and AAQ16301).

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