4. Discussion

Presented in this thesis is the isolation and characterization of a novel protein component of GICs. By use of degenerate primers in PCR, combined with colony hybridization, the complete coding sequence of the gene encoding this protein was obtained. The nascent translated polypeptide, named GREG, has 203aa with a calculated molecular mass of 22.8kD. After removal of the sugars, mature GREG migrates as a 28kD protein band on SDS-PAGE. Purification and characterization of the overexpressed truncated protein without hydrophobic segments at both the N- and C-terminus, respectively, confirmed that the complete coding sequence has been obtained. The mature form of GREG is predicted to be a coiled-coil protein, indicating that interactions with other proteins or with itself could be important for its function. The ability of GREG to be involved in protein-protein interactions is demonstrated by expression of GREG in both CHO and yeast cells.

In this thesis stable cell clones were selected from both NRK and CHO cells which express FLAG-tagged GREG. Tagged GREG localizes to the Golgi in both CHO- and NRK-originated cells. This localization is sensitive to BFA. Its Golgi localization was confirmed by sub-cellular fractionation experiments on density gradients.

GREG shows homology to human BST-2, a plasma membrane protein. Mapping the Golgi localization motif showed a requirement of three so-called EQ repeats which are absent in human BST-2 and which explains the Golgi-specific localization of GREG. Despite a prerequisite, the EQ tandem repeat as a Golgi targeting motif is not sufficient for Golgi localization and must be combined with other signals.

Several lines of evidence demonstrate that GREG is modified with a GPI-anchor. Therefore, this thesis describes the identification of GREG as the first GPI-anchored protein residing at the Golgi complex. Expression of GPI anchor-deficient forms of GREG, either without or with a membrane-spanning domain instead, leads to Golgi fragmentation. This effect on Golgi morphology does not originate from apoptosis or de-polymerization of the microtubule cytoskeleton.

A role of GREG in maintaining the Golgi structure was confirmed by dsRNAi-mediated suppression of GREG expression.

To fulfill its function in maintenance of the Golgi structure, GREG requires partitioning into GICs, Golgi-specific lipid rafts. Its Golgi-resident nature could keep GICs away from the prototype of lipid rafts sorted to other compartments like the plasma membrane. GREG, GAPR-1 and Caveolin have a tendency to dimerize or oligomerize and stoichometric amounts are present in GICs. A simple model is proposed regarding GIC formation.

4.1 GREG, a GPI-anchored protein resident at the Golgi complex

By IF using an antibody against an endogenous peptide, GREG was demonstrated to localize to the early Golgi complex (Gkantiragas et al., 2001). These observations were confirmed in both CHO- and NRK-cell lines constitutively expressing the FLAG-tagged wt protein GREG (Fig. 3-13). In agreement with previous observations (Gkantiragas et al., 2001), no signals of GREG-Flag could be visualized at the plasma membrane. The Golgi localization of GREG was further confirmed by sub-cellular fractionation of both wt cells and stably transfected cell lines (Fig. 3-14). On velocity gradients, GREG was enriched in fractions containing GM130, a cis-Golgi matrix protein. It did not behave like FolR, a GPI-anchored protein which cycles between the plasma membrane and endosomes and which becomes enriched in plasma membrane fractions after treatment with cycloheximide. Although it can be detected in lighter fractions, GREG does not appear in plasma membrane fractions. Together, these data establish GREG as a Golgi-resident protein.

4.1.1 Golgi-resident proteins

In general, Golgi-resident proteins are involved in two functions of the Golgi apparatus. One of these functions is to modify proteins and lipids as they pass through the organelle (Munro, 2001). Proteins responsible for this purpose are luminal enzymes including glycosidases and glycosyltransferases. These

enzymes synthesize a huge variety of complex polysaccharides that are linked to glycoproteins, both on N- and O-glycans, or glycolipids. Similar proteins are responsible for generating glycosphingolipids. The other function of the Golgi complex is to receive proteins and lipids from the ER and to sort them, after completion of sequential modifications by Golgi enzymes, into distinct carriers destined to different locations (Simons & Mellman, 1992). This process requires another class of Golgi-resident proteins, which are engaged in tethering carriers, catalyzing membrane fusion, recruiting vesicle coats, packaging particular sets of proteins and lipids into distinctive transport intermediates, and pinching off and targeting these intermediates to their appropriate compartments. These proteins generally face the cytosol. Other Golgi-resident proteins either facilitate or regulate the above described functions. These proteins include signalling proteins at the cytoplasmic side, structural proteins, and other luminal non-enzymatic proteins.

Unlike in the ER, very few soluble luminal proteins have been characterized in the Golgi complex. Indeed, only Cab45, a calcium-binding protein, has been identified so far (Scherer et al., 1996). In general, Golgi-resident proteins are either integral or peripheral membrane proteins. The latter class of proteins generally faces the cytosol, except two EF-hand calcium-binding proteins, CALNUC (which shows similarity to calreticulin) (Lin et al., 1998) and p54/NEFA (Morel-Huaux et al., 2001). Membrane association of the luminal CALNUC could be mediated via its acidic domain (pI 4.9), its C-terminal hydrophobic region (15aa), or a lipid anchor. Interestingly, evidence has been presented that both a cytosolic as well as a Golgi-luminal pool of CALNUC exists. The mechanism to produce two pools of CALNUC remains unclear. The cytosolic pool does not originate from membrane leakage during Golgi preparation (Lin et al., 2000). Cytosolic oriented CALNUC can interact with Gαi3 which requires immobilization of Gαi3 to the Golgi membrane (Weiss et al., 2001)

Topologically, GREG is oriented to the Golgi lumen (Fig. 3-5). Unlike other Golgi proteins, GREG is attached to the Golgi membrane via a GPI moiety

(Fig. 3-12). Evidence for this modification of GREG was obtained from two independent lines of evidence: i) Overexpression of tagged GREG revealed an increased signal detected by aerolysin, which specifically binds to the glycan core of the GPI moiety; and ii) ability to be released from membranes by PI-PLC. Together with its Golgi-resident nature, this thesis characterizes GREG as the first Golgi-resident GPI-anchored protein.

4.1.2 Signals for Golgi targeting

In eukaryotic cells, GPI anchorage is a fairly common posttranslational modification (Udenfriend & Kodukula, 1995). After attachment with a GPI moiety in the ER, the anchored proteins are destined to the cell surface through the classical secretory pathway (Muniz & Reizman, 2000). In polarized epithelial cells, the GPI anchor and the N-glycans on the protein are determinants to target these proteins to the apical cell surface (Benting et al., 1999). GREG is both glycosylated (Fig. 3-6) and linked with a GPI anchor (Fig. 3-12), but reveals a BFA-sensitive Golgi localization (Fig. 3-13 & Gkantiragas et al. 2001). Because the two known determinants do not direct GREG to the cell surface, an additional signal to trap GREG in the Golgi must exist.

Signals responsible for targeting a protein to the Golgi can be divided into two groups, retrieval and retention signals (Nilsson & Warren, 1994, Munro, 1998). A retrieval signal serves as a rescue mechanism to capture a protein when it escapes from the organelle. An active retrieval mechanism has been demonstrated to operate on most ER-resident proteins (Teasdale & Jackson, 1996). In the case of Golgi proteins, a retrieval signal applies to some TGN proteins. This signal locates to the cytoplasmic tail of TGN proteins, which contains a short tyrosine-containing motif. Such a motif initially specifies endocytosis from cell surfaces (Matter et al. 1992). This signal may then direct diversion from endosomes and subsequent return of proteins containing this motif to the TGN (Reaves et al. 1993, Alconada et al. 1996, Teuchert et al. 1999). Another example of a protein using a retrieval signal to ensure location at the Golgi complex is GPP130, an early-Golgi integral membrane protein. The

retrieval signal in GPP130 is its luminal coiled-coil segments (Bachert et al. 2001, Puri et al. 2002). GREG contains structural segments similar to GPP130, but no evidence thus far shows that GREG cycles between the Golgi apparatus and other compartments (Fig. 3-3 & -17). Nevertheless, it cannot be excluded that GREG has a dynamic Golgi localization.

The best understood one of retention signals is the transmembrane domain of some glycosyltransferases that trap these proteins to the Golgi complex through protein-lipid interactions (Machamer 1993, Opat et al., 2001). Besides the membrane-spanning domain, other motifs are involved in Golgi localization of some luminal Golgi enzymes (Vowels & Payne, 1998). These motifs may include protein-protein interactions between the luminal domains of Golgi-resident proteins (Nilsson et al. 1995). Their precise Golgi targeting signals are, however, unknown. The p24 family of cargo receptors was found to directly interact with the Golgi matrix proteins GRASP-55 and GRASP-65 (Barr et al. 2001). This interaction may facilitate the Golgi retention of these proteins. Other mechanisms are, however, also operative, like homo- or hetero-dimerization among family members of the p24 protein family (Jenne et al. 2002). Indeed, dimerization of p24 proteins is essential for their binding to Golgi matrix proteins (Barr et al. 2001).

Protein-protein interactions, mediated by coiled-coil interactions therefore seem to be involved in Golgi targeting. A GRIP motif has been defined as a Golgi targeting signal via an as yet unknown mechanism (Kjer-Nielsen et al., 1999, Munro & Nichols, 1999, Brown et al., 2001, McConville et al., 2002, Luke et al., 2003). This motif, located to the C-terminal end, is conserved among a family of Golgi coiled-coil peripheral membrane proteins, like Golgin-97 (Kjer-Nielsen et al., 1999, Munro & Nichols, 1999). All these retention signals are, however, either located in the cytosol or buried in the membrane bilayer. In contrast, GREG is anchored to the membrane via a GPI moiety and has a luminal orientation.

In case of Golgi luminal proteins, Golgi targeting is poorly understood. Cab45 was proposed to recycle via its C-terminal HEEF sequence using a mechanism similar to the KDEL retrieval system. If so, a receptor for this

sequence must exist to participate in recycling of Cab45. Thus far, such a receptor has not been identified (Scherer et al., 1996). NEFA/p54 might gain Golgi localization by direct interaction with Golgi integral membrane proteins, like Man II (Morel-Huaux et al., 2001). With respect to CALNUC, its Golgi targeting signal has not yet been identified. Given the sequence similarity to calreticulin, the lack of a KDEL sequence might somehow contribute to its Golgi localization (Lin et al., 1998, 2000). No sequence similarity was found between GREG and these Golgi luminal proteins.

4.1.3 Requirement of the EQ repeats for Golgi localization of GREG

A human GREG-related protein, BST-2, localizes to the plasma membrane (Ishikawa et al., 1995). Differences in localizations between GREG and BST-2 might be due to different cell types or species, as appears to be the case for Flotillin-1 which shows a Golgi-specific localization in CHOwt, NRK and PC12 cells (Gkantiragas et al., 2001), whereas a plasma membrane localization was observed in differentiated neuronal cells (Lang et al., 1998, Morrow et al., 2002). However, ectopically expressed BST-2 was also delivered to the cell surface in CHO cells (Ohtomo et al., 1999), suggesting that the different localization between BST-2 and GREG must be attributed to differences in the primary structure. Sequence analysis revealed that the EQ tandem repeat is absent in BST-2 (Fig. 3-9). Deletion of only the EQ tandem repeat drives GREG out of the Golgi complex to the plasma membrane (Fig. 3-15), consistent with observations of ectopically expressed BST-2 in CHOwt cells. Thus, the EQ tandem repeat confers Golgi localization to GREG. However, the EQ tandem repeat is not sufficient to retain a soluble luminal protein (GFP) at the Golgi complex, and therefore additional signals must be present.

Since the C-terminal half of GREG (including the EQ motif) localizes to the Golgi complex (Fig. 3-15), the additional Golgi targeting motif is expected to reside in this part of the protein. Given the recruitment of GREG into lipid microdomains, the EQ tandem repeat may act as a Golgi targeting motif for e.g. GPI-anchored proteins. This speculation is further supported by the subcellular

location of 23TM-GREG, which localizes predominantly to the ER (Fig. 3-15d). More experiments are needed to verify this hypothesis.

All human and mouse proteins related to GREG identified to date do not contain the EQ tandem repeat, which could raise doubts concerning the existence of a Golgi-located GREG isoform in other species. Recently, a rat homologue (DAMP1) appeared in the databases. Like human BST-2, DAMP1 does not contain the EQ repeat and localizes to lipid rafts at the plasma membrane (unpublished data). By IF, however, GREG was identified as a Golgi-resident protein in a rat cell line (NRK cells). It was recognized by antibodies against the endogenous ALI peptide of GREG (Gkantiragas et al. 2001) which is not present in DAMP1 (Fig. 3-9). This strongly implicates the existence of GREG in cells or tissues other than CHO cells.

4.1.4 Is the EQ repeat a universal Golgi targeting signal?

Interestingly, several proteins were retrieved when searching the Genebank databases with the EQ tandem repeat using the Retrieve Short Nearly Exact Matches program. All these proteins have in common to contain a long stretch of similar tandem repeats, such as EQEGQVR in XP_091107.5, EQEEMLR in XP_165407.1, EQEKQMR in XP_208786.1, EQEEKIR in BAC05084.1 and EQEERLR in BAC05050.1. Furthermore, all these proteins are predicted to be related to Golgin-97, a member of the Golgin family of proteins which locate to the cytoplasmic site of the Golgi complex. This family of proteins is believed to participate in maintenance of the Golgi architecture by stitching together cisternae aligned in parallel and in tethering transport vesicles (Pfeffer, 2001, Short et al., 2001, Diao et al., 2003). The presence of these similar tandem repeats implicates that GREG and these matrix proteins may share a similar targeting mechanism, which is distinct from a conserved GRIP motif that was also found among the matrix proteins (Kjer-Nielsen et al., 1999, Munro & Nichols, 1999, Brown et al., 2001, McConville et al., 2002, Luke et al., 2003).

4.1.5 Involvement of the SNARE-like domain in Golgi targeting

How could the EQ tandem repeat function as a universal Golgi targeting signal for proteins with opposite membrane orientations? Golgi targeting by the EQ repeat may involve its surrounding amino acids as well. Sequence analysis with the web-based SMART program revealed that the EQ tandem repeat locates to the core of a SNARE-like domain (Fig. 3-4), a special type of coiled-coil motif (Burkhard et al., 2001). Despite the impossibility of GREG to function as a SNARE protein, such a domain might participate in Golgi targeting through protein-protein interactions, which has been proposed for the Golgi localization of p54/NEFA.

Coiled-coil motifs as a Golgi localization signal are well described in GPP130 (Bachert et al. 2001). Two such domains in the Golgi lumen confer *cis*-Golgi localization to GPP130, whereas the third domain directs it to endosomes. Deletion of the endosome-targeting coiled-coil domain traps GPP130 in the Golgi complex (Bachert et al. 2001). The authors argued, however, that coiled-coil structures are not sufficient for Golgi localization, since chimeric proteins in which the endogenous coiled-coil domain was replaced with another coiled-coil segment, predicted to interact strongly with other coiled-coil proteins, localized to the cell surface. It was proposed that modifications within the coiled-coil region might be crucial for Golgi localization. Sequence alignment revealed about 50% similarity between one of the two Golgi determinants in GPP130 (AA 41 – 101aa) and the EQ repeat-containing SNARE-like domain of GREG (AA 101 – 160) (Fig. 4-1). These similarities suggest that the SNARE-related EQ repeat-containing domain might mediate Golgi targeting. Further experiments should be performed to challenge this hypothesis.

4.1.6 Is oligomerization involved in Golgi localization of GREG?

A common feature shared by GREG and several other Golgi-resident proteins is their predicted long coiled-coil sequences, which might trigger oligomerization. Coiled-coil domains are believed to mediate the oligomerization among subunits

of protein complexes (Burkhard et al., 2001). Golgi localization somehow involves an oligomerization among several Golgi-resident proteins. Large aggregates formed among Golgi proteins could prevent these proteins from entering into transport carriers, thereby achieving Golgi retention. Some Golgi enzymes might exploit this mechanism, as indicated by observations that the Golgi enzyme mannosidae II stably associates with N-acetylglucosaminyl-transferase I (Nilsson, et al., 1993, 1994, 1996). In this model, the retained protein must have a strong tendency to interact with other proteins or with itself, and should contain motifs facilitating such interactions to occur (Colley, 1997). A coiled-coil sequence fulfills this requirement. These interactions could also play a role in GREG localization to the Golgi complex since high molecular mass complexes containing GREG were shown to exist (Fig. 3-11 & -12). The N-terminal half of GREG which contains a coiled-coil motif, however, does not localize to the Golgi complex but is transported to the plasma membrane (Fig. 3-15). This is consistent with the speculation that coiled-coil structures are not sufficient for Golgi targeting (Bachert et al., 2001). An additional signal, such as the EQ motif in GREG, is required as well.

```
GREG     DSLEKKVSQIQEKQALIQEQEAQIKEQEAQIKEQEAQIKEQKAHIQEQQVRIQKLEGE
GPP130   RKAEAVALKYQQHQESLSAQLQVVYEHRSRLEKSLQKERLEHKKAKEDFL-VYKLEAQ
          . *  . : *::*  :. *   : *:.::::::.  : : :: : :*: : : ***.:
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Figure 4-1 Sequence alignment of the SNARE-related domain with the Golgi-localization motif in GPP130. Sequence alignment was based on the web-based SMART program at EMBL. The aligned coiled-coil motif of GPP130 was described by Bachert et al. (1999). Acidic residues are in blue, basic in pink, polar in dark green and non-polar in red.

The C-terminal domain of GREG was shown to colocalize with the Golgi marker Man II (Fig. 3-15). On SDS-PAGE, this domain migrated as 8, 16 and 24kD bands (Fig. 4-2), suggesting that oligomerization occurs (the predicted molecular mass based on amino acid sequence is 5kD). Deletion of the EQ tandem repeat did not affect its dimerization or tetramerization (Fig. 3-16). Self-

oligomerization apparently is not sufficient to cause Golgi localization. This is in agreement with e.g. the kin recognition model, which does not require only self-oligomerization (Nilsson et al., 1994). In GREG, the EQ tandem repeat may somehow mediate interactions with other Golgi proteins. Thus, oligomerization, together with the EQ tandem repeat might target GREG to the Golgi complex.

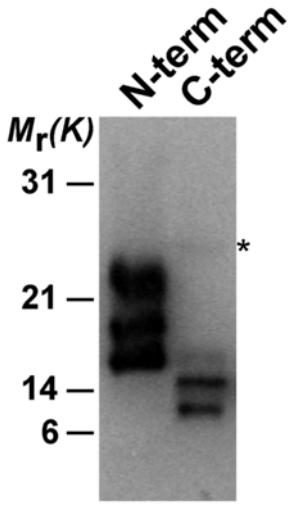

Figure 4-2 Oligomerization of C-term proteins. Total membranes were prepared from CHO cells transiently expressing either N-term (containing both N-glycosylation sites) or C-term (without any N-glycosylation site) and analyzed by Western blot. The 3 bands in the lane containing N-term proteins are due to glycosylation rather than oligomerization. Asterisk indicates a weak band in the lane containing C-term proteins.

Overall, the Golgi apparatus is a highly dynamic organelle with continuous flow of membranes and proteins. Anterograde intra-Golgi transport requires retrograde transport to offset the loss of membrane surface and components of earlier compartments. Certain Golgi enzymes have a dynamic presence within the stack despite their distinct localizations. The dynamic nature of these proteins may facilitate their localization to different subcompartments (Nickel & Wieland, 1998).

Its resistance to Endo H digestion (data not shown) suggests that the localization to early Golgi compartments of GREG should be dynamic. However, GIC proteins including GREG are segregated from COP I vesicles (Gkantiragas et al., 2001). How GIC proteins such as GREG obtain their early Golgi localization should be further investigated.

4.2 Involvement of GREG in maintenance of the Golgi structure

In principal, GPI-anchored proteins are delivered to the cell surface and function there as receptors, enzymes, cell adhesion molecules, etc (Udenfriend & Kodukula, 1995). GREG is an exception to this rule. Unlike other Golgi-resident proteins, GREG is linked with a GPI moiety. Its unusual localization is likely to mirror a functional role of GREG in the Golgi apparatus. This seems to be the case, as inhibition of its expression by dsRNAi indeed fragments the Golgi apparatus (Fig. 3-18).

4.2.1 Is GREG a structural protein at the Golgi apparatus?

How the Golgi apparatus preserves its identity and unique compartmental organization amid huge membrane fluxes is not clear. Golgi matrix proteins together with cytoskeleton are required to maintain its biochemical and structural polarity (Warren & Malhotra, 1998, Seeman et al., 2000, Shorter & Warren, 2002). Stacking the Golgi cisternae must involve interactions among these matrix proteins sitting on the outer (cytoplasmic) membrane surface of opposite cisternae filled with a distinct set of enzymes (Pfeffer, 2001). Indeed, blocking protein-protein interactions of these matrix proteins leads to an accumulation of Golgi vesicles (Lowe et al., 1998, Seemann et al., 2000). Depletion of certain matrix proteins disseminates the Golgi complex (Short et al., 2001). All these proteins, either peripheral or integral, face the cytosol.

In contrast to Golgi matrix proteins, GREG is not exposed to the cytosol. However, its luminal orientation does not exclude a structural role. To facilitate its modification and processing function, the Golgi apparatus may require a high

volume:surface ratio. Flattened cisternae are well suited for this purpose. Matrix proteins, that stitch the cisternae together over long membrane surfaces, could be involved in cisternal flattening. Other proteins, such as luminal proteins may also be involved in maintenance of a flattened state of a cisterna. GREG is a coiled-coil protein that may form trans-dimers or trans-oligomers between opposing membranes within a cisterna to stabilize its flattened structure. This could support the observed fragmentation of the Golgi complex induced by depletion of GREG by dsRNAi or by expression of ΔGPI-GREG, a putative soluble protein (Fig. 3-18 & -19). In the latter case, mutant proteins are expected to compete with endogenous GREG, inhibiting formation of trans-GREG pairs. However, the membrane-spanning version of GREG also causes fragmentation of the Golgi complex (Fig. 3-19). This mutant protein could principally function similar to the GPI-anchored version, since the membrane-spanning GREG could also form transpairs within a cisterna. Therefore participation of GREG in maintenance of Golgi integrity as a structural protein should involve other factors as well, such as its localization to lipid-enriched microdomains. Alternatively, GREG is not directly involved in maintenance of the Golgi complex as a structural protein but rather indirectly via signaling cascades from the lumen across the membrane to the cytosolic face of the membrane, allowing interactions with known or unknown regulators of the Golgi structure. These possibilities will be discussed below.

4.2.2 Does GREG function via heterotrimeric G proteins?

Coupling to regulatory factors are likely occur in GICs because the membrane-spanning version of GREG (23TM-GREG) is excluded from these microdomains (Fig. 3-20). In one scenario, GREG would transmit some signals to the cytoplasm via Golgi-specific lipid rafts. Any factors impeding recruitment of GREG into lipid rafts would terminate this signal flow, which is essential for maintaining the structure and/or function of the Golgi complex.

Several α subunits of heterotrimeric G proteins were found to locate at the Golgi apparatus and to cooperatively regulate the Golgi structure (Jamora et al.,

1997, Yamaguchi et al. 2000, Nagahama et al. 2002). Activation of trimeric G proteins by the drug ilimaquinone led to vesiculation of the Golgi complex. This effect of ilimaquinone involves free Gβγ heterodimers (Jamora et al., 1997), which activates PKD, an isoform of PKC, promoting uncontrolled membrane fission at the TGN (Jamora et al., 1999). Heterotrimeric G proteins are well known to relay signals at the plasma membrane by coupling cell surface receptors with intracellular effectors (Pierce et al. 2002). Such receptors have not yet been identified at the Golgi complex, but non-classical signalling cascades are likely to exist (Helms, 1997). Both GREG and heterotrimeric G proteins are enriched in GICs (Gkantiragas et al., 2001) and are both engaged in structural maintenance of the Golgi complex. It is tempting to speculate that GREG may function through activation of heterotrimeric G proteins.

GPI-anchored proteins can activate heterotrimeric G proteins at the plasma membrane. In macrophages, cross-linking a GPI-anchored protein (CD14) with its ligand LPS induces lower levels of membrane-associated trimeric G proteins, particularly the Gαi3 subunit (Markhlouf et al., 1998). IP combined with an *in vitro* kinase assay revealed that Gαi and Gαo subunits of trimeric G proteins were activated (Solomon et al., 1998). Inhibition of trimeric G proteins with mastoparan protects LPS-induced endotoxic shock.

A common feature of GPI-anchored proteins and heterotrimeric G proteins is their association with membranes via a lipid moiety. However, they are anchored to the opposite side of the membrane. Topologically, direct protein-protein interactions between them are impossible. An important question is how exoplasmic oriented GPI-anchored proteins can activate G proteins on the other side of membranes. Communication between lipid rafts on opposite sides of the membranes is possible as demonstrated by cross-linking of GPI-GFP at the outer leaflet of the plasma membrane, which results in the redistribution of ras-GFP on the inner leaflet (Prior et al. 2003).

At the molecular level, one possibility would be an interaction between the lipid moiety of GPI-anchored proteins and fatty acid moieties of heterotrimeric G proteins. Such an interaction was described in caveolin-1 null

cells, in which GPI-anchored proteins are retained at the Golgi complex (Sotgia et al. 2002). Ectopic expression of caveolin-1 restored the cell surface expression of GPI-anchored proteins. Further investigations pointed to the requirement of palmitoylation in caveolin-1 for this complementation (Sotgia et al. 2002).

Similar to LPS binding to CD14, the ΔGPI-GREG mutant may act as a "ligand" when binding to endogenous GREG. In agreement with this hypothesis, this theoretically soluble mutant is not secreted. Its presence in cell culture media could not be detected and it was found tightly associated with membranes (Fig. 3-19b & -20). One result of activation of Golgi-located heterotrimeric G proteins is vesiculation of the Golgi complex as discussed above. Similarly, the Golgi complex is fragmented upon expression of ΔGPI-GREG, supporting the possibility that GREG could function through heterotrimeric G proteins. The Golgi structure was, however, still disrupted when this mutant was expressed in the presence of pertussis toxin (data not shown). Pertussis toxin catalyzes the ADP-ribosylation of heterotrimeric G protein α subunits in the region where the α subunits interact with seven membrane-spanning receptors (Fields & Casey, 1997). Nevertheless, non-classical signalling cascades could be involved in GPI-anchored protein mediated activation of G proteins.

One alternative signalling cascade could be the activation of trimeric G proteins by GREG via an adaptor protein. This protein should contain a luminal and cytoplasmic domain, connected by a transmembrane segment. Transmembrane proteins are not very abundant in lipid rafts. Based on the current protein composition of GICs, only Flotillin-1 has been identified as a possible transmembrane protein (Gkantiragas et al. 2001), although some data exist that it may form a hairpin loop, similar to caveolins and stomatins (Morrow et al. 2002). Flotillin-1 has been shown to be involved in signaling, i.e. the transmembrane signaling of insulin, evoking glucose uptake (Baumann et al., 2000). Flotillin-1 could be co-precipitated together with subunits of trimeric G proteins in immunoprecipitation of GREG-Flag using anti-FLAG antibodies (Fig. 3-25). A direct protein-protein interaction between GREG and Flotillin-1 could not be concluded from these experiments. This interaction could have been

induced by the raft properties of these proteins during detergent-solubilization of the membranes.

4.2.3 Alternative signaling pathways, possibly mediating GREG function

Dominant negative GREG mutants or inhibition of GREG expression could also affect alternative signal cascades regulating the Golgi structure. Only recently, casein kinase II was identified to interact with GAPR-1 (Serrano & Helms, personal communication). Interestingly, phosphorylation of p115 at serine 941 within its C-terminal acidic region was mediated by casein kinase II or a casein kinase II-like kinase (Dirac-Svejstrup et al., 2000). P115 is involved in several transport steps including ER to Golgi (Lupashin et al., 1996, Sapperstein et al., 1996, Barlowe 1997, Alvarez et al., 1999) and intra-Golgi (Seemann et al., 2000). It tethers COP I-coated vesicles with Golgi membranes by linking Giantin on vesicles with GM130 on the Golgi (Soennichsen et al., 1998). The interaction between p115 and Golgins (GM130 and Giantin) involves the C-terminal acidic region of p115. Phosphorylation of p115 at the serine 941 by casein kinase II enhances its binding to GM130 and Giantin and is essential for reassembly of Golgi cisternae (Dirac-Svejstrup et al., 2000). Inhibition of GREG expression or expression of dominant negative mutants might affect the function of other GIC proteins such as GAPR-1, which regulates membrane association of casein kinase II, thereby influencing the phosphorylation state of p115, affecting the structural maintenance of the Golgi complex.

In apoptotic cells, caspase-dependent cleavage of Golgi matrix proteins leads to fragmentation of the Golgi apparatus (Chiu et al., 2002, Lane et al., 2001, Mancini et al., 2000). Could caspase-mediated signalling be involved in the functional role of GREG? The evidence presented in this thesis seems to exclude this hypothesis: upon expression of mutant GREG proteins, scattered Golgi structures were visualized with normal nuclear staining and active forms of caspases were not observed (Fig. 3-21).

Maintenance of the Golgi structure and localization of this organelle around the centrosome requires an intact microtubule cytoskeleton (Allan et al.

2002). Some drugs, like nocodazole, trigger depolymerization of microtubules and this treatment results in fragmentation of the Golgi ribbon structure and redistribution to the cell periphery (Allan et al. 2002). Morphological data, however, revealed no major differences in microtubule staining between wild-type cells and those expressing ΔGPI-GREG and containing fragmented Golgi structures (Fig. 3-22). This suggests that disruption of the Golgi apparatus by expression of the GPI anchor-deficient GREG mutants is independent of microtubule integrity.

4.3 GREG as an essential component of Golgi-specific lipid rafts

Consistent with the presence of wt GREG in GICs, GREG-Flag was predominantly found in lipid-enriched microdomains whereas non-GPI-anchored GREG mutants were largely excluded from these microdomains (Fig. 3-20). This suggests that raft-association of GREG is mediated by the GPI moiety. Prolonged expression of GPI-deficient GREG mutants in cells resulted in degradation of GIC proteins (Fig. 3-23), suggesting that GREG is an essential component of GICs.

4.3.1 Biogenesis of GICs

All lipid-enriched microdomains identified so far have relatively similar lipid compositions, enriched in sphingolipids and sterols. One of the most important issues about lipid rafts is how these microdomains are formed. In general, forces driving raft formation are considered to be derived from weak and transient interactions between lipids. Chelated between long saturated acyl-chains of sphingolipids are cholesterol molecules which condense the packaging of sphingolipid molecules (Simons & Ikonen, 2000). These microdomains are unstable, until proteins partition to these domains and sphingolipids receive their head groups at the Golgi complex. Upon incorporation of lipid modified proteins, like GPI-anchored, doubly acylated, or palmitoylated proteins, these microdomains become increasingly resistant to detergent extraction (Harder &

Simons, 1997). Protein incorporation may facilitate raft clustering through protein-protein and/or protein-lipid interactions (Harder & Simons, 1997).

Since all precursors of lipid rafts are synthesized in the ER, and GPI-anchored proteins are sorted at the exit site of the ER in yeast, pre-GIC formation is herein proposed to occur at the ER. The precursors of GICs are sensitive to detergent extraction. Upon arrival at the Golgi complex, sphingolipids receive their head groups, which enhance the lateral packing of sphingolipids. In addition, modifications of GIC proteins such as GREG are completed in the Golgi apparatus. Despite the presence of its coiled-coil nature, GREG seems to form oligomers only after maturation at the Golgi complex. Indeed, only a band of about 36kD was detected in ER fractions (Fig. 3-7b), while higher molecular weight forms were revealed in Golgi membranes (Fig. 3-11). At this organelle, the precursors of GICs can cluster together and become more ordered, increasing their resistance to detergent-solubilization.

4.3.2 A model of GICs

A simple model for the formation of GICs is proposed and summarized in Fig. 4-3. It should be noted that only four subunits of v-ATPase (belonging to v1 domain) were included in this model according to the primary identification of GICs (Gkantiragas et al., 2001). This model is based on the following observations:

GREG might exist in the cell as a homodimer or oligomer. Yeast two-hybrid data implicate that GAPR-1 may also exist as a dimer (Serrano & Helms, unpublished data), which was also observed in cross-linking experiments (Eberle et al. 2002). Oligomers were reported to exist among caveolin-1 molecules, which involve the scaffold domain and the membrane-spanning domain (Song et al., 1997). The C-terminal domain, where palmitoylation occurs (Uitenbogaard & Smart, 2000), confers detergent insolubility to oligomerized caveolin-1 (Song et al., 1997). The α subunits of heterotrimeric G proteins and GAPR-1 contain a caveolin-binding motif (ΦXΦXXXXΦ, where Φ is aromatic amino acid Trp, Phe or Tyr) (Okamoto et al., 1998, Eberle et al., 2002). Flotillin-1 was reported to form

a hetero-dimer or –oligomer with caveolin (Volonte et al. 1999). In addition, subcomplexes exist after disrupting the lipid scaffold of GICs (Gkantiragas et al. 2001), suggesting that protein-protein interactions occur among GIC proteins (at least for Flotillin-1, caveolin-1, GAPR-1 and the B subunit of v-ATPase). Finally, when isolated as detergent-resistant membranes, the protein components of GICs are present in stoichometric amounts (Gkantiragas et al., 2001). These data suggest that at least two molecules of caveolin-1, GAPR-1, Flotillin-1 and GREG are present in GICs, as shown in the model.

Of course, these microdomains may be highly dynamic. Under certain conditions, selected proteins can move into these microdomains, while others move out. As discussed above, GREG, perhaps together with other raft proteins, might represent a core complex of GICs. Similar mechanism might be employed to retain GICs at the Golgi complex. Retention of the GREG-containing core complex at the Golgi may be instrumental in the functioning of lipid rafts at the early Golgi complex.

4.3.3 GICs as distinct microdomains with distinct function(s)

The distinct identity of GICs compared to microdomains at the cell surface might be reflected in the lipid composition. GICs were found to be more enriched in sphingomyelin than total DRMs. This is surprising given the higher concentration of sphingolipids at the plasma membrane (Gkantiragas et al., 2001). However, it is difficult to distinguish these two types of lipid rafts solely based on lipid compositions. Thus far, the BFA-sensitive Golgi localization of the protein components of GICs is the strongest evidence to support that GICs are Golgi specific microdomains rather than precursors for lipid rafts at the cell surface. Besides, a core complex seems to exist, which may represent the Golgi identity of GICs. The proteins in this core complex may therefore determine the functions of these microdomains at the Golgi complex.

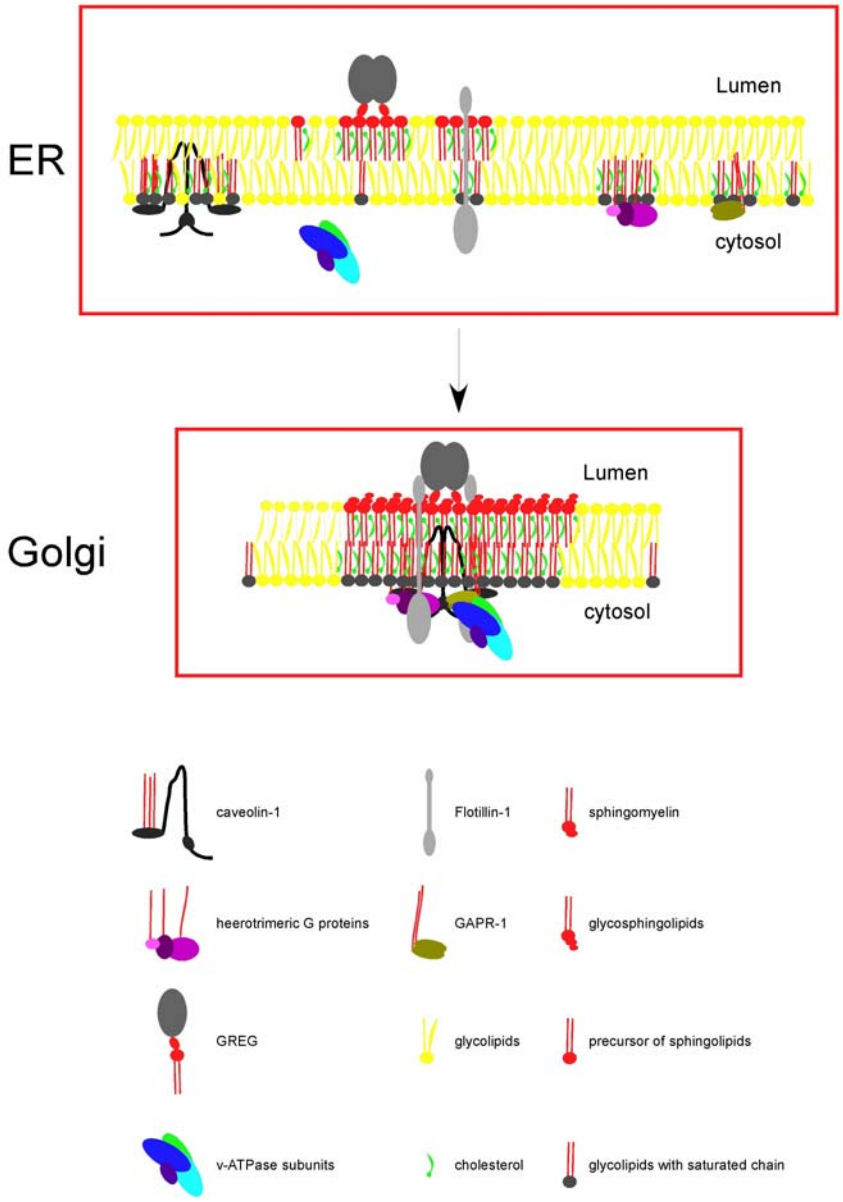

Figure 4-3 A model for the generation of microdomains at the Golgi complex. Precursors of microdomains are formed at membranes of the ER due to relatively weak interactions between sphingolipid precursors and cholesterol. Upon arrival at the Golgi, head groups are added onto sphingolipids, resulting in increasingly packaging of sphingolipids. Meanwhile, more cholesterol molecules intercalate between saturated acyl chains of sphingolipids, further enhancing the packaging forces of sphingolipids. These forces bring together the individual precursors of GICs, allowing occurrence of protein-protein interactions among protein components to form stable GICs.

As discussed above and in the Introduction, several protein components of GICs are also present in phagosomes (Garin et al. 2001). It was shown that Flotillin-1 is integrated into phagosomes during the maturation process. These findings implicate that GICs may serve as a sorting platform at early Golgi compartments. Such prediction is strengthened by the recent finding that GAPR-1 can associate with phagosomes upon cycloheximide treatment (Kaloyanova & Helms, unpublished data). This issue is under current investigations.

GICs may not only mediate membrane sorting at the early Golgi, but, and perhaps most importantly, regulate membrane trafficking and signal cascades as well. Indeed, observations of regulating intra-Golgi transport by heterotrimeric G proteins led to the identification of GICs (Helms et al., 1998). Consistent with this, signal molecules such as heterotrimeric G proteins are enriched in GICs (Gkantiragas et al., 2001). Extraction of small amounts of cholesterol from Golgi membranes drastically inhibits intra-Golgi transport, possibly reflecting regulation of membrane trafficking via GICs.

In summery, GREG is a Golgi-resident GPI-anchored and therefore a luminal protein. GREG is involved in regulation of the Golgi structure and lipid-enriched micrdomains may serve as a platform to mediate this function of GREG.