2. Introduction

In eukaryotic cells, many cellular events occur in different separate membrane-enclosed compartments, or organelles, e.g. the Golgi apparatus or lysosomes. A distinct set of proteins give these organelles not only their unique structure but also their distinct functions. Correct delivery of proteins to their appropriate locations is therefore essential for the identity of an organelle. Hence, one of the most important events of the cell is to sort proteins to their appropriate compartments. Such events are considered to occur, to a large extent, through the highly conserved secretory pathway, an elaborate network of membrane-bound citernal, tubulo-vesicular and vesicular compartments including the ER, the Golgi apparatus and transport vesicles (Rothman 1994).

Along the secretory pathway *en route* to their final compartments, secretory proteins and many lipids are first synthesized in the ER. After being properly folded and assembled with the aid of ER chaperones, newly synthesized proteins are packaged into COP II coated vesicles, either selectively (Bannykh et al. 1998, Barlowe 2002) or by default (Rothman & Wieland 1996). Upon arrival at the so-called ER-Golgi intermediate compartment (ERGIC), anterograde cargo is sorted away from escaped ER and vesicular machinery proteins which are returned back to the ER in COP I-dependent and –independent fashions (Klumperman 2000) and transported to the Golgi apparatus. In the Golgi, sorting events continually occur through the different Golgi cisternae, acting as a distillation tower, progressively separating the secretory cargo from residents of the ER and Golgi cisternae (Rothman, 1981, Rothman & Wieland, 1996). After completion of sequential modifications by Golgi enzymes, cargo at the *trans*-Golgi network (TGN) is sorted into distinct carriers destined for different compartments, such as lysosomes and the plasma membrane (Allan & Balch, 1999).

The central station of the secretory pathway is the Golgi apparatus, which is involved in posttranslational modifications and sorting of newly synthesized proteins and lipids to their ultimate compartments (Warren & Malhotra, 1998). The Golgi apparatus is built up of a set of membrane-bound cisternae which

together form a polarized stack (Rambourg & Clermont, 1990, Ladinsky et al. 1999, Marsh et al. 2001). In accordance, each cisterna contains a specific set of resident enzymes responsible for the sequential modifications of anterograde cargo, including proteins and lipids, by these enzymes. Bound at either face of the Golgi stack are extensive tubulovesicular networks: the CGN and the TGN. After post-translational modifications, the CGN receives newly biosynthesized cargo from the ER. The TGN sorts proteins and lipids to their final destinations. At the rims of distinct cisternae are small COPI-coated vesicles which are involved in recycling Golgi-resident enzymes and transporting forward-directed cargo (Pelham 2001) (Fig. 2-1). This unique structure of the Golgi apparatus is highly conserved through eukaryotic evolution (Shorter & Warren, 2002).

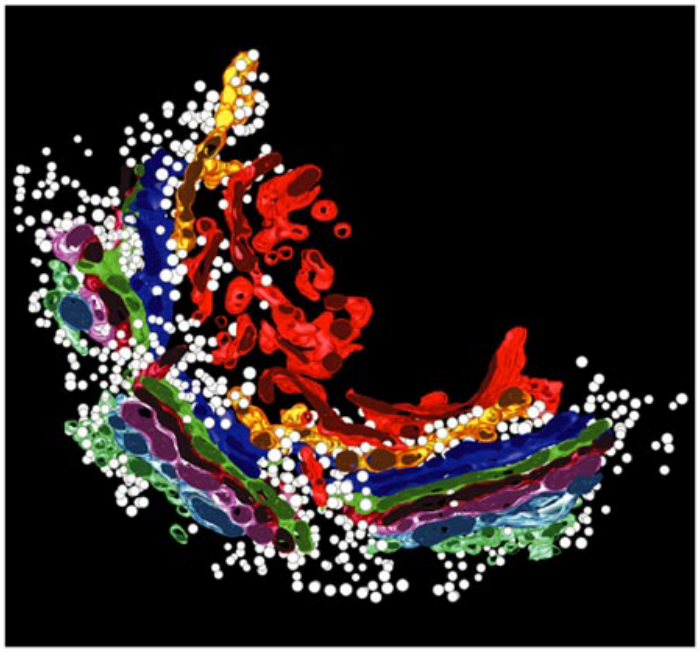

Figure 2-1 The Golgi apparatus. This 3D Golgi structure is reconstructed according to tomography scans of plastic-enbedded specimens prepared from fast-frozen/freeze-substituted HIT-T15 cells in an electron microscope. The Golgi ribbon in this model consists of 5 cisternae – in light blue, purple, dark green, dark blue and dark yellow, respectively (*cis* to *trans*) - between *cis* (light green) and *trans* (red) vesiculotubular clusters (corresponding to the CGN and the TGN). Small non-clathrin coated and uncoated vesicles are shown in white. (cited from Marsh & Howell, Nat Rev Mol Cell Biol, 2002, 3: 789-95).

2.1 Formation of polarized Golgi stacks

An unanswered but fundamental question is how cells form a polarized Golgi complex. Biogenesis of the Golgi complex involves entrance and retention of the components synthesized in the ER and assembly of peripheral membrane complexes at the cytoplasmic face of the Golgi membrane (Shorter & Warren, 2002). Golgi components synthesized in the ER are delivered to the CGN and sorted to their proper cisterna by protein-protein (Nilsson et al. 1993, 1994, 1996, Barr et al. 2001) and/or protein-lipid (Munro, 1998) interactions. The Golgi complex is the main site of sphingolipid biosynthesis within the cell and acts as a buffer between the glycerolipid-enriched ER and sphingolipid- and sterol-enriched plasma membrane (Holthuis et al. 2001). It has long been recognized that the lipid composition changes from one side of the Golgi stack to the other, which may help establish its polarity (Holthuis et al. 2001).

The Golgi apparatus is disassembled into small Golgi clusters during mitosis (Shima et al. 1997, Jokitalo et al. 2001). Under these conditions, separation of Golgi enzymes due to localization to different cisternae is maintained in these mitotic Golgi clusters (Shima et al. 1997). An underlying matrix is involved in the organization of the complex architecture of the Golgi apparatus. Indeed, this Golgi matrix persists in cells treated with BFA, whereas Golgi enzymes are absorbed into the ER by tubule-mediated ER-Golgi retrograde transport (Sciaky et al. 1997). Upon BFA treatment, elements of this matrix are distributed in clusters proximate to but distinct from ER exit sites (Prescott et al. 2001, Seemann et al. 2002). By movement along microtubules (Fath et al. 1997), matrix elements can also be evenly distributed between daughter cells (Seemann et al. 2002). In summary, this matrix represents a fundamental unit, necessary for structure and inheritance of the Golgi apparatus. The question how this matrix protein complex builds up a polarized Golgi is under intense investigations.

The Golgi matrix was initially identified as a detergent-resistant complex that contains Golgi enzymes (Slusarewicz et al. 1994). It is a proteinaceous scaffold comprising of the GRASP family of Golgi stacking proteins, the Golgin family of long coiled-coil peripheral and integral membrane proteins, and a

spectrin/ankyrin framework (Shorter & Warren, 2002). The best characterized matrix proteins are GM130, GRASP65 and Giantin (Linstedt & Hauri, 1993, Nakamura et al. 1995, Barr et al. 1997). GRASP65, a *cis*-Golgi surface protein required for stacking the *cis*-Golgi *in vitro* (Barr et al. 1997), acts as a receptor for a coiled-coil protein GM130, targeting it to the Golgi complex (Barr et al. 1998). GM130 serves as a receptor for p115, another coiled-coil protein which is required for docking of transport vesicles to the *cis*-Golgi (Nakamura et al. 1997). Giantin is another receptor for p115 (Sonnichsen et al. 1998). Unlike GM130, which is exclusively localized to Golgi membranes (Nakamura et al. 1997), Giantin is also incorporated into COP I vesicles (Soennichsen et al. 1998). Therefore, the current view on the vesicle tethering role of p115 is that it mediates the connection between Giantin on vesicles and GM130 on Golgi membranes. Likewise, p115 could function to stack cisternae by bridging GM130 on a cisterna and Giantin on an opposing cisterna. How GRASP65 is involved in stacking Golgi cisternae remains elusive. In view of its oligomeric nature, the cisternae to which it is anchored via its N-terminal lipid moiety could be linked to the adjacent cisterna containing GM130 (Barr et al. 1998) and/or other Golgi proteins with a short cytoplasmic tail (Barr et al. 2001) by direct protein-protein interaction. It is also possible that a GRASP65 *cis*-dimer on a cisterna forms a *trans*-oligomer with the *cis*-dimer on the opposite cisternal membrane.

Recently, Golgin-84, a *cis*-Golgi protein proposed to be involved in stacking Golgi cisternae, was shown to bind rab1 but not to Golgi matrix proteins (Diao et al. 2003). Another report claims that Golgin-84 is present in COP I vesicles and interacts with CASP at the Golgi complex (Satoh et al. 2003). The connection between Golgin-84 and CASP is similar to the interaction between Giantin and GM130. However, a docking protein like p115 has not been identified to bridge the interaction between Golgin-84 and CASP. The rab1 GTPase appears to regulate the interaction between the above mentioned proteins because both GM130 and p115 together with Golgin-84 are effectors of rab1 (Allan et al. 2000, Moyer et al. 2001, Weide et al. 2001, Diao et al. 2003, Satoh et al. 2003). These proteins may represent a blueprint of the *cis*-Golgi.

GRASP55, a *medial*-Golgi matrix protein, is required to stack the Golgi complex *in vitro* (Shorter et al. 1999). Its distinct localization may implicate that it is responsible for stacking the *medial*-Golgi (Pfeffer, 2001a, 2003). In agreement, GRASP55 interacts with Golgin-45, which is essential for Golgi structure and function, but not with GM130, a *cis*-Golgi protein (Short et al. 2001a). These matrix proteins may define the *medial*-Golgi. Interestingly, Golgin-45 interacts with rab2 but not rab1 (Short et al. 2001). Both rab1 and rab2 are involved in ER-Golgi transport (Pfeffer 2001b). Since rab1 participates in the docking of vesicles to the *cis*-Golgi, it is tempting to speculate that rab2, if it functions in intra-Golgi transport, will target vesicles to the *medial*-Golgi. It has been suggested (Pfeffer 2001b) that a gradient of interactions between cisterna-specific Golgi matrix proteins could itself provide the basis to establish the functional and morphological polarity of the Golgi complex. It is also proposed that rabs regulate the formation of a polarized Golgi structure by regulating the recruitment of specific effectors, i.e. Golgi matrix proteins (Pfeffer, 2003). If so, similar GRASP proteins are expected to exist at the *trans*-Golgi to interact with Golgin proteins other than Golgin-45 and GM130 and/or CASP. Likewise, a corresponding rab GTPase should also exist to regulate interactions and dock vesicles specifically to the *trans*-Golgi.

The above described discussions are based on the prediction that formation of a Golgi occurs on a pre-existing template, the Golgi matrix. The central point of this prediction claims that the Golgi is an autonomous organelle. This view, however, is challenged by observations that under certain conditions absorption of Golgi enzymes, together with matrix proteins, into the ER is observed with concomitant disassembly of the Golgi structure (Zaal et al. 1999, Prescott et al. 2001, Ward et al. 2001). Formation of a Golgi apparatus is therefore proposed to be dependent on continuous membrane input from the ER.

Irrespective of the model, the Golgi itself is a highly dynamic organelle and its unique structure is maintained amidst multiple membrane fission and fusion events. In view of this, passage of cargo through the Golgi is discussed below.

2.2 Intra-Golgi transport

A hotly debated issue regarding the Golgi complex is how cargo is transported through this organelle. The controversy focuses on whether the COPI-coated vesicles mediate forward cargo transport.

In the early studies, COPI-coated vesicles were found to contain the anterograde cargo VSV G protein which is targeted to plasma membranes (Balch et al. 1984). These and other observations resulted in formation of the vectorial transport model. This model claims that the Golgi cisternae exist as stable and pre-formed structures having unique compositions in terms of a gradient enzyme activities (Dunphy & Rothman, 1983) and sphingolipids and cholesterol (Orci et al. 1981). Findings from yeast genetics that COP I vesicles also mediate Golgi-ER retrograde transport (Letourneur et al. 1994) raised the question how a COPI vesicle could serve as mutiple cargo carriers, both in backward and forward directions. By electroimmuno-histochemistry, two distinct sets of COP I vesicles were revealed in the cell (Orci et al. 1997). One set contained only retrograde-directed cargo KDEL receptor while the other set contained the forward-directed cargo proteins pro-insulin and VSV G protein. These two populations of COPI-coated vesicles, which account for at least 80% of the vesicles budded from every level of the Golgi stack, can be faithfully reproduced in a cell-free Golgi budding system (Orci et al. 1997). Possibly, the existence of isoforms of coatomer components (for γ– as well as ε–subunits) may provide an answer to this issue. There are at least three different combinations of coatomer subunit complexes which may define three different populations of COP I vesicles (Reinhard and Wieland, personal communication). In addition, at the *cis*-Golgi, two vesicle tethering systems may exist: GM130-Giantin and CASP-Golgin-84. It seems possible that one system tethers forward-directed vesicles while the other system tethers backward-directed vesicles. Thus, COP I vesicles could act as shuttles between different cisternae in both directions (Rothman & Wieland, 1996, Nickel & Wieland, 1998).

Certain macromolecular complexes, such as glycoprotein scales in algae and pre-collagen aggregates in fibroblasts, which are much larger than COPI-

coated vesicles, nonetheless transverse the Golgi stack (Beams & Kessel, 1968, Becker & Melkonian, 1996, Bonfanti et al. 1998, Martinez-Menarguez et al. 2001, Mironov et al. 2001). These aggregates are retained within cisternae, distinct from being enclosed in some kind of megavesicles (Volchuk et al. 2000). These observations were taken as evidence to revive the cisternal progression/maturation model, originally proposed in 1960s. This model describes *de novo* formation of cisternae at the *cis*-face and disassembly at the *trans*-face (Beams & Kessel, 1968). In this model, anterograde cargo transport is mediated by a progressive cisternal flow from *cis* to *trans*. Vesicles are used to balance the loss of Golgi proteins and membranes from earlier cisternae by retrograde transport to these early cisternae. However, the rate of aggregate movement is far slower than that of most proteins like VSV G and even bulk lipids in studied cells (Bonfanti et al. 1998, Pelham & Rothman 2000). Furthermore, large aggregates are relatively rare and may be restricted to special cell types. A unifying model was proposed that integrated both vesicular transport and cisternal maturation: large nondiffusable cargo is transported by cisternal progression mechanism while small molecules may move faster across the Golgi stack via vesicular carriers (Pelham & Rothman, 2000, Volchuk et al. 2000). This issue remains controversial and still conflicting data are reported. Recently, evidence was presented that small cargo proteins, like VSV G protein, can move across the Golgi stack without leaving the cisternal lumen and travel together with pre-collagen aggregates at a comparable rate in the same cell (Mironov et al. 2001). These small cargo proteins were dramatically depleted from vesicular carriers (Mironov et al. 2001, Martinez-Menarguez et al. 2001, Dahan et al. 1994). Other groups, in the same type of cells, reported opposite findings and showed that Golgi enzymes are excluded from COPI vesicles (Cosson et al. 2002).

A third model describes intra-Golgi transport by lateral diffusion through membrane continuities or tubules (Mironov et al. 1997). In fact, tubules are a well-characterized feature of the Golgi complex, whose stacks are interlinked with tubuloreticular networks (Rambourg & Clermont, 1990). Membrane

continuities have been implicated in the diffusion of Golgi enzymes within intact Golgi in living cells (Cole et al. 1996, Marra et al. 2001). Golgi tubules are dynamic structures and are affected by local lipid compositions (Weigert et al. 1999, de Figueiredo et al. 1999). However, such connections are absent or rare in the Golgi from other organisms like yeast (Rossanese et al. 1999). In addition, vesicles and tubules appear to co-exist in the pathway (Ladinsky et al. 1999). To what extent tubules contribute to membrane transport between Golgi cisternae still remains to be clarified (Marsh & Howell, 2002, Storrie & Nilsson, 2002).

2.3 The Golgi as a platform to coordinate signalling cascades

Transport processes are tightly regulated by small G proteins such as ARFs. Besides promoting the recruitment of coatomer, activated ARF1 can locally alter lipid compositions of membranes by activation of PLD (Powner & Wakelam, 2002), which produces phosphatidic acid (PA). Recruitment and activation of phosphatidylinositol phosphate kinase by ARFs (Honda et al. 1999, Skippen et al. 2002) increases the level of PIP2. Both PA and PIP2 can in turn facilitate vesicle budding and fusion, thereby promoting vesicular trafficking. ARF1 may also regulate reorganization of cytoskeleton surrounding the Golgi apparatus and regulate cell growth by interaction with cdc42 (Stamnes, 2002). These and other findings indicate that the Golgi complex may serve as a platform not only to spatially and temporally regulate membrane transport but also to coordinate signalling cascades and preserve its unique structure perhaps through these processes.

Several α subunits of heterotrimeric G proteins are localized to the Golgi complex. Activation of heterotrimeric G proteins by fluoroaluminate promotes stable association of both ARF1 and coatomer to the Golgi membrane (Finazzi et al. 1994) and inhibits intra-Golgi transport (Helms et al. 1998). The Gβγ heterodimer, on the other hand, inhibits the recruitment of ARF1 to membranes (Donaldson et al. 1991). This discrepancy may be due to different combinations between Gβγ and α subunits of trimeric G proteins. Heterotrimeric G proteins also modulate the dynamic interaction of PKA II with the Golgi

apparatus (Martin et al. 1999), thereby affecting ARF binding to Golgi membranes (Martin et al. 2000). Alternatively, production of free Gβγ heterodimer by activation of trimeric G proteins with drug ilimaquinone leads to activation of PKD, an isoform of PKC, promoting uncontrolled membrane fission at the TGN (Liljedahl et al. 2001). Golgi-located heterotrimeric G proteins not only help balancing membrane fission and fusion events at the Golgi complex, but also participate in the regulation of other signal cascades involving PI(3) kinase, cdc42 etc. These events contribute to the regulation of the Golgi structure.

2.4 Lipid-enriched microdomains

Many signalling cascades are thought to occur in subdomains of the plasma membrane called lipid-enriched microdoamains or lipid rafts (Brown 2002). Lipid rafts are cholesterol- and sphingolipids-enriched microdomains. Forces governing raft formation arise from transient and weak interactions among lipids, especially glycosphingolipids and cholesterol. Glycospingolipids are biophysically propone to lateral cohesion, involving van der Waals interations and hydrogen bonds between their head groups and sphingosine backbones (Harder & Simons, 1997). In addition, glycosphingolipids contain longer and more saturated acyl chains than glycerolipids. The length of the fatty acid chain in ceramide, the backbone of sphingolipids, ranges from 16 to 26 carbon atoms while that of glyerolipids is generally 16 to 22 (Dickson, 1998, Eisenkolb et al. 2002). The average double bonds in a sphingomyelin molecule are 0.1 to 0.35 whereas phosphocholine (PC) contains 1.1 to 1.5. A double bond formed within the acyl chain frequently generates a kinked structure. These features further increase the tendency of glycosphingolipids to segregate from the mobile PC-rich phase and to form a more tightly packed phase (Barenholz & Thompson, 1980).

Cholesterol, another type of lipid that is enriched in lipid rafts, preferentially interacts with sphingolipids (Sankaram & Thompson, 1990), although it can also interact with glycerolipids (Brown, 1998). This preference of cholesterol may be attributed by interaction of the entire long and fully saturated

acyl backbones of sphingolipids with the planar steroid ring of cholesterol. Also, hydrogen-bond formation between the 3-β-hydroxyl group of cholesterol and the ceramide group of sphingolipids may contribute to the preferential interaction of cholesterol with sphingolipids. Cholesterol plays an essential role in promoting sphingolipid lateral segregation. Real-time analysis using atomic force microscope showed that addition of cholesterol increases the size of sphingomyelin-rich domains, while depletion of cholesterol results in disappearance of lipid rafts in model systems (Lawrence et al. 2003).

At the plasma membrane, sphingolipids are strongly enriched in the outer leaflet of the bilayer. This is exemplified by different isoforms of ras proteins, which have different affinities for lipid rafts at the inner leaflet of the plasma membrane (Apolloni et al. 2000, Prior et al. 2001, 2003). This raises the question how the exoplasmic lipid rafts can interact with opposite lipid rafts at the inner leaflet. This may be achieved by the long acyl chains of sphingolipids penetrating into the inner leaflet containing more saturated phospholipids (Boggs & Koshy, 1994), thereby affecting the assemblage of lipids at the inner leaflet. Cholesterol may also intercalate between the fatty acid chains of the inner leaflet lipids. At low concentrations, cholesterol can form transbilayer tail-to-tail dimers in lipid bilayers (Mukherjee & Chattopadhyay, 1996). This local organization of cholesterol is also observed in sphingolipid-containing membranes (Rukmini et al. 2001). If this is also true in living cells, cholesterol could serve as a bridge to connect lipid-enriched microdomains between two membrane leaflets.

In model membrane systems, lipids with different melting temperatures (Tm) are prone to separate into different phases. Most common are the fluid phase (also called the liquid-disordered or -crystalline (lc) phase), rich in kinked unsaturated acyl chains, and the solid-like gel phase, rich in long, highly saturated and ordered acyl chains. Addition of cholesterol to membrane bilayers led to discovery of a new phase, the liquid-ordered (lo) phase in which acyl chains are tightly packed as in the gel phase, but have a high degree of lateral mobility (Brown & London 2000). Sphingolipid- and cholesterol-rich microdomains are proposed to exist in the lo phase or a state with similar

properties. This characteristic supports the resistance to extraction at low temperatures with nonionic detergents like Triton X-100 and CHAPS. Under physiological conditions, microdomains can also be isolated by using the polyoxyethylene ether Brij 98 (Drevot et al. 2002). Due to enrichment in lipids, these microdomains migrate to a low density by equilibrium density-gradient centrifugation. Biochemical characterization revealed that GPI-anchored and dual-acylated proteins are enriched in these lipid-enriched microdomains (Simons & Ikenon, 2000). These acyl chains exhibit features typical for raft lipids as they are long and saturated (Shenoy Scaria et al. 1993 & 1994, Rodgers et al. 1994). Besides these lipid-modified proteins, lipid rafts recruit a limited set of membrane-spanning proteins including VIP17/MAL (Zacchetti et al. 1995, Cheong et al. 1999) and influenza viral hemagglutinin (Scheiffele et al. 1997). A model of lipid rafts is shown in Fig. 2-2.

The presence of signaling molecules, (e.g. Src, heterotrimeric G proteins), suggests that lipid rafts function as a platform to coordinate signal cascades by recruiting certain signaling molecules while excluding others (Simons & Toomre, 2000). With increasing evidence, lipid rafts are believed to participate in several important cellular events, such as membrane sorting, signal transduction, and host-pathogen interactions (Ikonen 2001, Kenworthy 2002, Simons & Ehehalt 2002).

2.5 Golgi-derived lipid rafts

Recent findings demonstrate that lipid-enriched microdomains are not limited to the plasma membrane. In yeast, Bagnat et al found that rafts are already formed in the ER (Bagnat et al. 2000). In mammalian cells, three groups independently reported that caveolin accumulated in ER-originated membrane-bound lipid droplets (Ostermeyer et al. 2001, Fujimoto et al. 2001, Pol et al. 2001) and resisted detergent extraction (Pol et al. 2001). These observations indicate that lipid rafts are present in intracellular organelles, in agreement with earlier reports (Sevlever et al. 1999). Proteomic analysis of phagosomes revealed that raft-associated proteins exist in phagosomes (Garin et al. 2001), strongly implicating the

presence of lipid rafts in these endomembranes. These microdomains do not originate from plasma membranes because association of Flotillin-1 occurs during phagosomal maturation (Dermine et al. 2001).

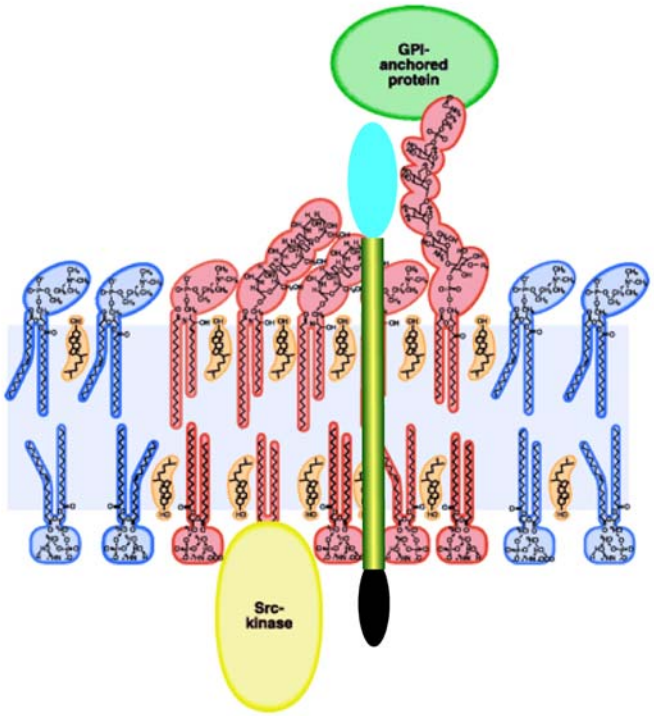

Figure 2-2 A model of lipid rafts. This model is based on Simons & Ikonen (Science, 2000, 290: 1721-6). Lipids in the raft region are shown in red and those in the non-raft region are in blue. Cholesterol, in orange, preferentially partitions into the raft region. Shown in this model are a GPI-anchored protein attached to the sphingolipid-enriched exoplasmic leaflet, a doubly-acylated Src to the cytoplasmic leaflet which is rich in glycerolipids with saturated fatty acid chains, and a transmembrane protein.

Studies related to the inhibitory role of Golgi-located heterotrimeric G proteins in intra-Golgi transport led to the characterization of sphingomyelin-enriched microdomains from the Golgi complex, termed GICs for Golgi-derived detergent-insoluble complexes (Helms et al. 1998, Gkantiragas et al. 2001). Detailed characterizations suggest a unique identity of these microdomains at

the Golgi complex rather than a precursor function for lipid rafts at the plasma membrane.

Compared to total detergent-resistant membranes derived from total cell lysates (DRMs), the protein to lipid ratio does not significantly differ from GICs. However, GICs are more enriched in sphingomyelin. This is remarkable because Golgi membranes have less sphingomyelin (as percentage of total phospholipids) than plasma membranes (Gkantiragas et al. 2001). In contrast to DRMs, the protein composition of GICs contains a relatively simple set of ten major proteins (Fig. 2-3). Subunits of heterotrimeric G proteins, especially the αi subclass of G proteins and β subunits, are highly enriched in GICs (50 to 100 fold as compared to their donor Golgi membranes). This finding is consistent with initial investigations of trimeric G proteins in regulating intra-Golgi transport and may implicate a role for GICs in coordinating signal cascades at the Golgi apparatus. Caveolin-1 and flotillin-1, both lipid raft markers, are also present in GICs.

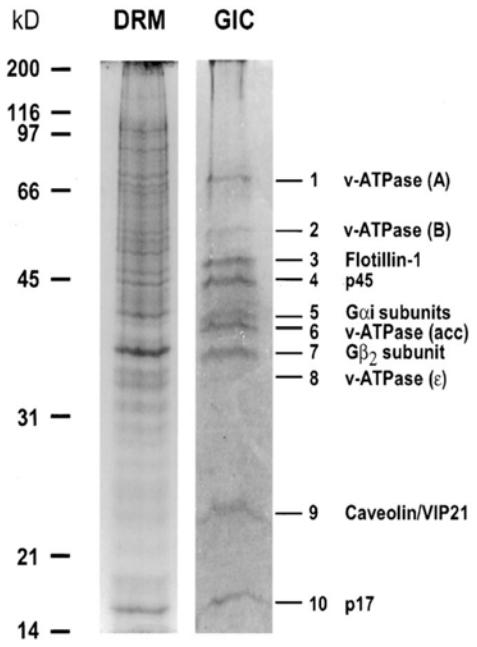

Figure 2-3 Comparison of the protein components of DRM and GICs. Both DRM and GIC were prepared from CHO cells and analyzed by SDS-PAGE and detection with Coomassie blue. Proteins identified in GICs are shown on the right. (Gkantirags et al. *Mol Biol Cell* 2001, 12: 1819-83).

Microsequencing identified subunits of the vacuolar ATPase (Gkantiragas et al. 2001), which may regulate and maintain the luminal pH of the Golgi apparatus. Recently, the V0 domain of the v-ATPase has been implicated in vacuolar membrane fusion (Peters et al. 2001). The presence of v-ATPase subunits therefore suggests that GICs may be involved in regulation of the luminal pH of the Golgi apparatus and of membrane fusion events by interaction between V1 and V0 domains.

GAPR-1, a protein that belongs to the superfamily of plant pathogenesis-related proteins family 1 (PR-1), was identified from the 17kD band of GICs (Eberle et al. 2002). PR proteins participate in the plant defense system against pathogen invasion (Stintzi et al. 1993). It has been hypothesized that GAPR-1 may possess a similar function in mammals. The Golgi localization, however, raised the question how in the animal kindom GAPR-1 could be involved in the defense against pathogens. Recently, GAPR-1 was found to associate with phagosomes (Kaloyanova & Helms, personal communication). This finding is in agreement with the proposed immunological function of GAPR-1. Associated with phagosomes, GAPR-1 is unlikely to directly contact pathogens (as homologues do in plant) due to its cytosolic orientation. An alternative possibility is that GAPR-1 may function as a sorting marker to phagosomes. This hypothesis is currently investigated. Similar to other GIC proteins (caveolin-1, flotillin-1 and trimeric G proteins), GAPR-1 may also participate in signaling cascades. In support of this hypothesis, GAPR-1 was found to bind to casein kinase II (Serrano & Helms, personal communication), which may modulate the Golgi structure by phosphorylating p115 (Dirac-Svejstrup et al. 2000, Brunati et al. 2001).

What could be the function of microdomains at the Golgi complex? Extraction of cholesterol with cyclodextrin led to inhibition of *in vitro* intra-Golgi transport (Stueven et al. submitted). Removal of cholesterol by cyclodextrin from living cells resulted in partial vesiculation of the Golgi apparatus (Hansen et al. 2000). On the other hand, increasing cholesterol also inhibits *in vitro* intra-Golgi transport (Stueven et al. submitted) and fragments the Golgi complex as well

(Grimmer et al. 2000). It was further shown that lipid rafts were affected by these treatments and that raft partitioning of selected raft proteins was affected. It remains to be shown whether the observed effects on Golgi morphology and intra-Golgi transport is directly or indirectly caused by changing raft properties.

2.6 GPI-anchored proteins

In eukaryotic cells, lipid modification with glycosylphosphatidylinositol (GPI) is believed to be a ubiquitous modification (Udenfriend & Kodukula, 1995). Proteins containing this modification, called GPI-anchored proteins, are involved in a variety of cell functions, e.g. uptake of nutrients (receptor) and immobilization of a cell at a certain locus (cell adhesion). Besides an alternative means of membrane attachment, the GPI anchor also plays an important role in several processes, such as in sorting GPI-anchored proteins to the apical cell surface (Lisanti et al. 1990) and in transmitting signals (Robert & Gazzinelli 2000). Mutants defective in GPI synthesis are embryonic lethal in protozoa (Takeda & Kinoshita, 1995). Mammalian cells can survive without GPI anchors, but a number of disorders are observed (Karadimitris & Luzzatto, 2001).

GPI anchorage is a posttranslational process that occurs in the ER. Two independent metabolic pathways are involved in generating a GPI-anchored protein. One is the biosynthesis of the GPI moiety, and the other is the processing of the nascent protein and covalent attachment of the GPI moiety to the protein (Ferguson, 1999). Biosynthesis of the GPI anchor takes place on the cytoplasmic face of the ER. The GPI anchor is then translocated into the lumen, resulting in association with the inner leaflet of the lipid bilayer. By use of a cell-free system to study GPI biosynthesis, at least four physically associated proteins were demonstrated to participate in this process (Watanabe et al. 1998). The core structure of the GPI moiety, which is conserved across all thus-far studied species, consists of two fatty acid tails inserted in the membrane leaflet and a complex head group, comprising a phosphodiester-linked inositol, a glucosamine linked to inositol, a linear chain of three mannose sugars linked to glucosamine and a phosphoethanolamine linked to the terminal mannose

residue (McConville & Ferguson, 1993). The protein is attached to the GPI anchor via an amide bond between the C-terminal residue of the protein moiety and the amino group of the phosphoethanolamine. At least two protein components are required to complete the attachment of the GPI anchor *en bloc* to the protein (Ferguson, 1999). Fig. 2-4 highlights the basic structure of a GPI anchor.

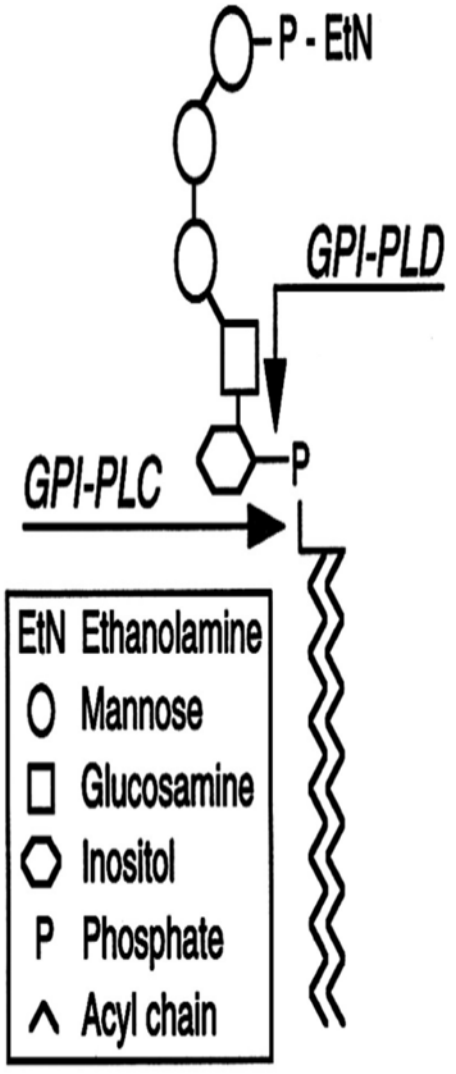

Figure 2-4 The conserved core structure of the GPI anchor and the cleavage sites of PI-PLD (GPI-PLD) and PI-PLC (GPI-PLC). Some GPI anchors contain an additional fatty acid (in most cases, palmitate) linked to inositol that results in insensitiveness to PI-PLC.

A protein destined for GPI anchor attachment contains an N-terminal signal peptide that directs it to the ER. There the N-terminal signal peptide is removed. Also, a cleavable C-terminal hydrophobic peptide must exist in the protein. Between these two hydrophobic peptides, the remainder of the peptide sequence is essentially hydrophilic (Udenfriend & Kodukula 1995). Besides these features, there must be suitable amino acid residues at or near the ω site (C-terminal peptide cleavage site). In principal, a small amino acid (serine, glycine, alanine, aspartate and asparagine) appears at the ω and ω+1 site (Micanovic et al. 1990). Only alanine, glycine and serine are found at the ω+2 site in the GPI-anchored proteins identified so far. There appears to be no selectivity with respect to other amino acids near the ω site (Udenfriend & Kodukula, 1995).

Low & Finean (1977) and Ikezawa (1976) independently found that the enzyme alkaline phosphatase (AP) can be released from the plasma membrane of intact cells by treatment with bacterial PI-PLC. Subsequent studies showed that several other proteins, including acetylcholinesterase (Low & Finean, 1977), Thy-1 (Low & Kincade, 1985) and trypanosomal variant surface glycoproteins (VSG) (Cross, 1984), behaved similarly and were shown to be linked to membranes in the same way as AP, i.e. by a GPI anchor (Fugerson et al. 1985). Sensitiveness to PI-PLC thereby became a standard test for the presence of GPI-anchored proteins (Ferguson, 1999). However, not all GPI-anchored proteins are sensitive to PI-PLC. This could be due to the presence of an additional fatty acid (usually palmitate) in the GPI moiety (Roberts et al. 1988). This acyl modification occurs on the inositol ring (Treumann et al. 1995). During biosynthesis, almost all mammalian GPI-intermediates are inositol acylated, but after transfer to the protein moiety, some or all of the GPI anchors are deacylated at the inositol ring (Chen et al. 1998). Besides PI-PLC sensitivity, other methods have been developed to detect the presence of GPI-anchored proteins. A standard feature of all GPI-anchored proteins is an ethanolamine group to bridge the GPI moiety to the COOH terminus of the protein via an amide linkage (McConville & Ferguson, 1993). Therefore GPI-anchorage can be determined by detecting the presence of a metabolically labeled ethanolamine group in the isolated protein.

Several pore-forming toxins, like aerolysin secreted by *Aeromonas hydrophila* (Fivaz et al. 2001), interact with their target cells by binding to the glycan core of GPI-anchored proteins on the cell surfaces. The ability of proteins to bind aerolysin is a frequently used method to determine the presence of GPI-anchored proteins (Hong et al. 2002, Fivaz et al. 2002, Abrami & van der Goot, 1999).

2.7 Trafficking of GPI-anchored proteins

After the attachment of a GPI anchor, GPI-anchored proteins are subject to sorting processes along the biosynthetic and endocytic pathways. After synthesis, these proteins are delivered to the cell surface through the classical secretory pathway. Upon arrival at the cell surface, some GPI-anchored proteins recycle between the plasma membrane and endomembranes, most likely via both clathrin-dependent and -independent endocytosis (Mayor et al. 1998, Nichols et al. 2001). The folate receptor (FolR), a well characterized GPI-anchored protein, is taken up into a transferring-positive endocytic compartment and is temporarily retained in recycling endocytic compartments (Mayor et al. 1998). This retention requires the intactness of lipid rafts (Chatterjee et al. 2001). Nichols et al. reported that other GPI-anchored proteins (GPI-GFP and CD59) recycle between plasma membranes and the TGN in a way independent of the clathrin-interacting endocytic machinery and rab5 (Nichols et al. 2001). Reduced cholesterol levels inhibited the delivery of GPI-GFP to the Golgi complex (Nichols et al. 2001). Further investigations showed that these proteins (GPI-GFP and CD59) accumulated in caveolin-1-positive endosomes (Nichols 2002), also called caveosomes which were previously shown to be involved in the trafficking of SV40 virus from the plasma membrane to the ER (Pelkmans et al. 2001).

In polarized epithelial cells, GPI-anchored proteins are predominantly delivered to the apical cell surfaces. Fischer rat thyroid epithelial cells are, however, an exception (Zurzolo et al. 1993). Conversion of the VSV G protein from a transmembrane protein to a GPI-anchored form changed the basolateral expression to apical expression. Replacement of the GPI anchor of AP with the transmembrane domain of VSV G altered its expression from apical to

basolateral surfaces (Brown et al. 1989). Fusion of the consensus sequence for GPI-attachment to a bacterial enzyme directed it to the apical cell surfaces in transgenic mice (Ali et al. 1996). These findings establish that the GPI anchor serves as a sorting signal.

Sorting to the apical surface requires, however, a second determinant that works cooperatively in the sorting process. Soole and colleagues engineered a GPI-anchored bacterial endoglycanase, but found that this GPI-anchored enzyme was missorted to the basolateral surface in Caco-2 cells (Soole et al. 1995). A GPI-linked heparan sulfate proteoglycan does not show a polarized expression in Caco-2 and MDCK cells. However, complete removal of the heparan sulfate glycosylation sites from the protein moiety resulted in exclusive apical expression (Mertens et al. 1996). In MDCK cells, GPI-anchorage of unglycosylated and unpolarized secreted growth hormone did not increase the apical delivery. Rather, addition of the N-glycans to the GPI-anchored hormone led to predominant apical expression (Benting et al. 1999). The apical sorting effect of N-glycans was not affected by disruption of its raft-association (Benting et al. 1999).

GPI-anchored proteins are found to be associated with lipid rafts, as determined by their detergent-insolubility in nonionic detergent at low temperatures. This association could originate from direct interaction of the GPI moiety with cholesterol and sphingolipids (Schroeder et al. 1994). Indeed, insertion of GPI-anchored proteins in artificial sphingolipid- and cholesterol-enriched liposomes led to a dramatic increase in resistance to detergent extraction (Morandat, et al. 2003, Schroeder et al. 1998). Vice versa, extraction of cholesterol from microdomains with β-cyclodextrin resulted in solubility of GPI-anchored proteins (Ilangumaran & Hoessli, 1998, Schroeder et al. 1998). Raft-association is essential for the trafficking of GPI-anchred proteins to their final destinations. Cholesterol is required for selective delivery of GPI-anchored proteins to the plasma membrane (Hannan & Edidin, 1996). Inhibition of ceramide synthesis leads to mis-location of GPI-anchored proteins in polarized

MDCK cells and primary hippocampal neurons (Mays, et al. 1995, Ledesma et al. 1998).

In summary, the GPI anchor plays a role in protein sorting and this effect may include raft partitioning. An important question is then at which transport steps GPI-anchored proteins are sorted. Proteins containing an uncleaved signal peptide for GPI attachment are retained in a post-ER compartment (Moran & Caras, 1992). Mutations leaving the C-terminal peptide uncleavable allowed transport of the unanchored and uncleaved protein to the Golgi apparatus *in vivo* (Horvath et al. 1994). However, in yeast the unanchored proteins were not packaged into the newly formed ER-derived vesicles (Doering & Schekman, 1996). These experiments implicate that sorting occurs in the early stages of the secretory pathway and that the GPI anchor could act as an ER exit signal or that the uncleaved peptide functions as a retention signal. In yeast, GPI-anchored proteins were already sorted at the ER exit sites (Muniz et al. 2001) and resistant to detergent extraction (Bagnat et al. 2000). However, in mammalian cells, GPI-anchored proteins obtain their resistance to cold nonionic detergent extraction only upon arrival at the Golgi complex (Brown & Rose, 1992), implicating that rafts may form at the Golgi complex in mammalian cells. This difference in detergent solubility of lipid rafts between yeast and mammalian cells may arise from differences in the structural particularities of the GPI anchor and sphingolipids in the different species.

One difference between mammals and yeast is the length of the acyl group in sphingolipids (Dickson et al. 1998) and in the GPI anchor (McConville & Ferguson, 1993). In yeast, ceramide contains a long fatty acid chain (26 carbon atoms), in contrast to C18-C24 in mammalian cells. This could favor the clustering of ceramides in the ER. Also, in the ER the lipid moiety of the GPI anchor can be remodeled from short fatty acid tails (diacylglycerol) to long fatty acid chains (ceramide or diacylglycerol with long acyl chains, C26) (Reggiori et al. 1997, Sipos et al. 1997). This remodeling of the GPI anchor may affect the segregation of GPI-anchored proteins from other secretory cargo at ER exit sites in yeast (Muniz et al. 2001). Consistent with this speculation, yeast mutants for

fatty acid chain elongation (*elo2* and *elo3*) are defective in transport of GPI-anchored proteins from the ER to the Golgi complex (David et al. 1998).

Another difference is that the occurrence of a hydroxyl group in the fatty acid chain of ceramide is common in yeast but rare in mammals (Dickson, 1998). These polar groups could be used to form hydrogen bonds between adjacent ceramides or between ceramides and GPI anchors. Therefore, the efficiency of clustering of ceramide and GPI-anchored proteins in the ER may be higher in yeast than in mammals. In mammalian cells, ceramides are clustered in the Golgi apparatus where they are converted into sphingolipids, whose head groups are involved in the formation of hydrogen bonds. This may explain the ability of GPI-anchored proteins to resist detergent extraction upon Golgi passage in mammalian cells.

2.8 Purpose of the thesis

Recently, lipid-enriched microdomains at the Golgi complex (GICs) were described. Several protein components showed a BFA-senstive Golgi localization (Gkantiragas et al. 2001). The early Golgi localization identified GICs as Golgi-specific microdomains rather than being precursors for lipid rafts at the plasma membrane. Enrichment of heterotrimeric G proteins implicates a role of GICs in coordination of signal transduction processes at the Golgi complex. The function of these Golgi microdomains remains, however, unknown. Several protein components of GICs are also present in phagosomes (Garin et al. 2001). It was shown that Flotillin-1 associated with phagosomes during the maturation process. These findings could establish a connection between Golgi rafts and phagosomes. Such a connection is strengthened by the recent finding of GAPR-1, a homologue of PR proteins, can also associate with phagosomes (Kaloyanova and Helms, unpublished data). Movement to phagosomes may be part of its predicted function in innate immunity.

A major 45kD protein component of GICs, together with Flotillin-1 and GAPR-1, shows a dynamic Golgi localization in the presence of BFA (Gkantiragas et al. 2001). Characterization of this unknown protein will offer more insights into the functions and the unique properties of these distinct Golgi lipid rafts. This thesis will focus on the identification and functional characterization of this novel protein, which includes isolation of the cDNA, confirmation of its Golgi localization, and functional characterization of its potential role at the Golgi complex.