Proteins mediating intra- and intercellular transport of lipids and lipid-modified proteins

A Ferry-Tale of Lipids

Eiwitten verantwoordelijk voor het intra- en intercellulaire transport van lipiden en lipide-gemodificeerde eiwitten

(met een samenvatting in het Nederlands)

Proefschrift

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Abbreviations

apo	apolipoprotein
BFA	brefeldin A
C_6-NBD	*N*-6-NBD-aminohexanoyl
CerS	ceramide synthase
CERT	ceramide transfer protein
DES1/2	desaturase
EPC	ethanolphosphorylceramide
FAPP2	four-phosphate adaptor protein
GalCer	galactosyl β1-1 ceramide
GalCS	galactosylceramide synthase
GCS	glucosylceramide synthase
GlcCer	glucosyl β1-1 ceramide
GLTP	glycolipid transfer protein
GM3	sialyllactosylceramide
GM3S	GM3 synthase
GPI-	glycosylphosphatidylinositol-
GPI-PLC	glycosylphosphatidylinositol-specific phospholipase C
GPI-PLD	glycosylphosphatidylinositol-specific phospholipase D
GSL	glycosphingolipid
HDL	high density lipoprotein
3KSR	3-ketosphinganine reductase
LacCer	galactosyl β1-4 GlcCer, lactosylceramide
LCS	lactosylceramide synthase
LDL-R	low density lipoprotein receptor
Nir2	Pyk2 N-terminal domain-interacting receptor 2
OSBP	oxysterol binding protein
PAPST	3'-phosphoadenosine 5'-phosphosulfate transporter
PI(4)P	phosphatidylinositol-4-phosphate
PNS	post nuclear supernatant
SGalCer	galactosylceramide sulfate
SGCS	chimeric protein consisting of an HA-tagged PAPST and SGalCer synthase
SM	sphingomyelin
SMS1	sphingomyelin synthase 1
SPT	serine palmitoyl transferase
SR-BI/II	scavenger receptor class B I/II

Table of contents

Scope of this thesis

Biological life is based on a high diversity of bioactive molecules and their defined chemical reactions and interactions. Through the formation of membranes, cells separate their contents from the environment and create discrete compartments, in which biochemical reactions can take place in an organized and controlled manner. Membranes are composed of lipids, which do not only provide a simple scaffold, but are also actively involved in biochemical reactions and regulate the localization and activity of enzymes and proteins that assist these processes. To fulfill this diversity of functions, cells actively synthesize a wide range of different lipids. These lipids interact non-covalently with other lipids and proteins in membranes, but they can also be attached covalently to a protein moiety bestowing it with hydrophobicity and the ability to associate to membranes. Such lipid-modifications allow proteins to exist as water soluble or insoluble forms, one mechanism to regulate their localization, their interaction with other proteins and lipids and consequently their function. Nonetheless, many aspects of how cells organize their membranes in terms of lipids and proteins are poorly understood. The object of this thesis is the transport of membrane constituents, one important parameter to understand their molecular function. In particular, sphingolipids and lipid-modified proteins will be studied.

The first part of this thesis focuses on the transport of sphingolipids. **Chapter 1** reviews the mechanisms of sphingolipid transport, with an emphasis on lipid transfer proteins. They have the ability to solubilize and transport lipids in a monomeric fashion by shielding them from the hydrophilic environment. It is discussed, how these proteins are regulated and how they can contribute to the generation of unique membrane lipid compositions of the various membranes.

Glycosphingolipids constitute a huge family within the class of sphingolipids and are the major contributers to the diversity of lipids because of the variation in their head groups. Most of the complex glycosphingolipids are based on glucosylceramide. **Chapter 2** describes one of the two identified glycolipid transfer proteins (GLTP, Brown and Mattjus, 2007). Applying biophysical methods, we investigated how GLTP can interact with membranes and which membrane characteristics facilitate lipid uptake. It was elucidated that a surface exposed tryptophan residue (Trp142) is involved in membrane binding of GLTP. In addition it was found, that the activity of GLTP reflects the concentration of glucosylceramide. Together with the fact that GLTP hardly associates with membranes *in vivo*, this may implicate that it could be a sensory protein instead of being a genuine transfer protein.

The fate of glucosylceramide *in vivo* was studied in **Chapter 3**. Glucosylceramide is synthesized at the cytosolic side of the Golgi but to be converted to more complex glycosphingolipids, it has to be translocated to the lumenal leaflet of the membrane.

Where this translocation takes place and how it is mediated is not known. Using a cell surface assay for the presence of glucosylceramide at the exoplasmic leaflet, in combination with an artificial modification of glucosylceramide in the ER and *in vitro* translocation assays, we showed that glucosylceramide can translocate at several locations in the cell, namely across the ER membrane, post-Golgi compartments and to a minor extent directly at Golgi membranes. Surprisingly, the major site where glucosylceramide translocates to be converted to higher glycosphingolipids was found to be the ER membrane. Therefore one question was, how glucosylceramide reaches this location. It turned out that FAPP2, another glycolipid transfer protein (D'Angelo et al., 2007; Godi et al., 2004), is involved in this transport step. How exactly glucosylceramide passes the membrane is still not understood. The physical properties of the ER membrane may allow spontaneous translocation but Golgi and post-Golgi membranes appear to be much more rigid and would require a protein mediated transport step. The ABC-transporter MDR1 has been proposed to facilitate translocation of glucosylceramide (van Helvoort et al., 1996), but we now found that it was not involved.

The second part of this thesis enters into the question how lipid-modified proteins can be transported through the extracellular space. Complex, multicellular organisms require the development of different cell types, which have to communicate with each other in order to coordinate specialized functions. For communication, cells use proteins that are passively or actively transported to elicit responses in target cells. Some of these proteins happen to be lipid-modified. On the one hand this seems to attach them very tightly to membranes, but on the other hand lipid-modifications may allow proteins to enter special transport routes. **Chapter 4** discusses several possibilities how these hydrophobic proteins can be assembled onto carriers. Special attention is drawn to lipoprotein particles as carriers for lipid-modified proteins. These large globular structures transport lipids throughout the circulation of complex organisms. They are surrounded by a phospholipid monolayer, which resembles the exoplasmic leaflet of the plasma membrane. To study the transport of lipid-modified proteins via lipoprotein particles not only gives insight into underlying mechanisms regarding these proteins, but may also serve as a model of how membrane lipids can be transferred from cells to lipoproteins.

In *Drosophila* lipoprotein particles have been described to be potential carriers of different lipid-modified proteins (Panakova et al., 2005). In **Chapter 5** we investigated whether this process is present in mammals using cultured cells as models. Members of the family of Wnt proteins are lipid-modified and act as morphogens. Therefore, Wnt has to be released from producing cells into the extracellular space, form a concentration gradient and elicit concentration-dependent responses in the neighboring cells. We found that the release of Wnt is mediated by lipoprotein par-

ticles, in particular by HDL, which requires the cell surface receptor SR-BI/II. Furthermore in specific cell types, Wnt can associate with lipoprotein particles expressed by the cells themselves.

Other lipid-modified proteins are GPI-anchored proteins. In *Drosophila* also these proteins are associated with lipoprotein particles (Panakova et al., 2005). However, in mammals a phospholipase D is very abundant in serum that specifically cleaves off the protein moiety from the GPI-anchor (GPI-PLD, Hoener et al., 1990; Huang et al., 1990). This protein is found to be inactive at cellular surfaces. In **Chapter 6** we show that GPI-PLD can cleave GPI-anchored proteins present on lipoprotein particles but not from cell membranes. When GPI-PLD activity is inhibited, for example by bacterial lipids, GPI-anchored proteins were stably present on lipoprotein particles from cells. Therefore under certain conditions lipoprotein particles may be carriers for GPI-anchored proteins in mammals as well.

In summary, this thesis presents a broad spectrum of mechanisms how hydrophobic molecules can be transported through aqueous phases.

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Sphingolipid management by an orchestra of lipid transfer proteins

Based on

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Abstract
The various membranes in eukaryotic cells display unique lipid compositions. Despite important discoveries in lipid research over the past decades, the basic principles of how cells define their membrane compositions are essentially unknown. Cells must sense the concentration of each lipid, integrate these signals and regulate the activity of their metabolic enzymes and transport routes to dynamically meet their needs in terms of membrane composition. Sphingolipids constitute a lipid category that is essential for eukaryotic life and appears to be key to the differences in lipid composition. Here we discuss recent findings that assign an important role to lipid transfer proteins in the regulation of sphingolipid metabolism, organization and function.

Introduction
The past decades have witnessed the identification of most eukaryotic enzymes and proteins involved in lipid synthesis, degradation and transport. Still, the question remains, how eukaryotic cells sense and control the lipid composition of their membranes. For this, it is necessary to identify the sensors and find out how they connect to - and regulate - the metabolic enzymes. In addition, those enzymes need to be localized and, finally, we must understand how cells employ and regulate the lipid transport machineries to ensure the specificity in lipid transport between the cellular organelles (van Meer, 2005).

Sphingolipids comprise a small but vital fraction of membrane lipids in eukaryotes. They arose parallel to the appearance of eukaryotic life and are conserved from yeast to mammals. In these organisms the expression of phosphosphingolipids is indispensable for cell growth and survival (Wells and Lester, 1983; Hanada et al., 1992; Geta Tafesse et al., 2007). In contrast, glycosphingolipids are not required for the survival of individual cells (Ichikawa et al., 1994) but essential for the development of multicellular organisms and the differentiation of distinct cell types. Mice deficient in glucosylceramide synthase, the first enzyme in complex glycolipid synthesis, die at an early embryonic stage (Yamashita et al., 1999) and mice deficient in galactosylceramide synthase show severe defects in brain function and in the male reproductive system (Bosio et al., 1996b; Coetzee et al., 1996a; Coetzee et al., 1998), emphasizing the role of galactolipids in myelination and spermatogenesis. Complex glycosphingolipids were implied in cell-cell contacts and signaling events (Lisanti et al., 1994; Hakomori and Igarashi, 1995; Hakomori et al., 1998). Furthermore, glycolipids are highly enriched at the apical side of intestinal epithelial cells where they protect the cell against the harsh chemical environment and the action of lipases (Simons and van Meer, 1988). Individual sphingolipids are known to be signaling molecules, and are important mediators of survival, stress response and apoptosis (Hannun and Obeid, 2008). Not only the expression of sphingolipids is important, but also their degradation. This is reflected in the incidence of sphin-

golipid storage disorders that are often accompanied by severe phenotypes (Futerman and van Meer, 2004; Jeyakumar et al., 2005; Sabourdy et al., 2008).

For a large part, the functions of sphingolipids have been related to the enormous structural variability of their headgroups which allows a nearly unlimited number of specific interactions with glycoproteins and other glycosphingolipids. On the other side, sphingolipids possess a unique organization potential due to their high affinity for each other. Because of their saturated nature and the possibility for hydrogen bonding, together with cholesterol they can form microdomains within a glycerophospholipid environment, that are more ordered and rigid and thicken the membrane. Some proteins preferentially insert into these membrane domains, called lipid rafts, resulting in distinct specialized membrane environments. Therefore lipid rafts can be utilized to sort proteins during vesicular transport, or as platforms to recruit proteins, for example in signaling (Grassme et al., 2007; Sengupta et al., 2007; van Meer et al., 2008).

Notwithstanding our increased insights in sphingolipid structure and function, the individual processes in which sphingolipids are involved and the mechanisms by which they affect cellular functions have been poorly elucidated. The function of a lipid depends on its local concentration in time. Thereby, it can contribute to a defined membrane environment or directly interact with protein binding partners. To unravel the role of the manifold number of different sphingolipids, it is important to study their localization within the cell. As the direct observation of sphingolipids *in vivo* is difficult due to the lack of biosensors (Hoetzl et al., 2007), we must rely on information obtained from studying their metabolism and transport. Here we want to discuss recent advances in lipid research with respect to sphingolipid metabolism: the enzymes that are involved in their formation and transport. In particular, we want to highlight how cells orchestrate lipid transfer proteins in the creation of unique membrane lipid compositions and discuss mechanisms of lipid sensing.

1. Enzymes of sphingolipid synthesis

Sphingolipid synthesis is organized along the secretory pathway, indicating the functional significance of sphingolipids for membrane and protein transport. The reactions underlying the formation of sphingolipids have been known already for many years and to date almost all enzymes of sphingolipid synthesis in mammals have been cloned, although of some only the yeast homologs are known (Figure 1 Dickson, 2008). Less is known concerning the overall regulation of sphingolipid metabolism, which is partially due to the lack of a grand scheme for the physiological functions of the various sphingolipids: it has remained unclear of which lipid the concentration has to be regulated, at what location and under which conditions.

Ceramide synthesis

The precursor for the complex sphingolipids is ceramide and its synthesis is localized to the cytosolic leaflet of the ER, whereas most sphingolipid species are synthesized in the Golgi. Ceramide formation is divided into four steps and starts with the condensation of L-serine and palmitoyl-CoA to 3-ketosphinganine. This reaction is catalyzed by serine palmitoyl transferase (SPT). Three subunits of SPT have been identified in eukaryotes (Buede et al., 1991; Nagiec et al., 1994; Weiss and Stoffel, 1997; Hornemann et al., 2006). They are present in an oligomeric complex (Hornemann et al., 2007). To what extent SPT3 contributes to *de novo* synthesis of 3-ketosphinganine is not clear, as it is differentially expressed in tissues (Hornemann et al., 2006). 3-Ketosphinganine reductase forms sphinganine (3KSR, Beeler et al., 1998), which is acylated by (dihydro)ceramide synthase (CerS, known as longevity assurance gene: LASS, D'mello et al., 1994; Guillas et al., 2001; Schorling et al., 2001). In human and in mouse six different genes for CerS are found, that display specificity for the fatty acid chain lengths (Pewzner-Jung et al., 2006). The introduction of a double bond at the sphinganine C4,5 by dihydroceramide desaturase 1 (DES1, Ternes et al., 2002) finally yields ceramide containing sphingosine as the sphingoid backbone. Most ceramide species in mammals contain sphingosine, but in skin, brain, intestine and kidney the sphinganine in dihydroceramides can be hydroxylated at C4 (phytoceramide), a reaction catalyzed by the bifunctional desaturase/hydroxylase DES2 in mouse or by Sur2 in yeast (Haak et al., 1997; Ternes et al., 2002). Actually, most ceramide may be directly synthesized from sphingosine derived from sphingolipid breakdown in the lysosomes, via the salvage pathway (Kitatani et al., 2008).

Ceramide is readily converted to glycolipids or sphingomyelin

In kidney and intestinal epithelial cells as well as in myelinating cells (part of the) ceramide is consumed in the lumen of the ER for the formation of galactosylceramide by galactosylceramide synthase (GalCS, Bosio et al., 1996a; Coetzee et al., 1996b). Hereby the galactose headgroup is transferred from UDP-galactose to ceramide. UDP-galactose is imported into the lumen of the ER by UDP-galactose transporter (UGT). This transporter is normally localized to the *trans* side of the Golgi and supplies substrates for the glycosylation of proteins and lipids. However, in cells expressing galactosylceramide synthase, UGT is retained in the ER via a direct protein-protein interaction (Sprong et al., 2003). At the cytosolic side of the Golgi glucosylceramide is formed by glucosylceramide synthase (GCS, Ichikawa et al., 1996) through the addition of the glucose headgroup from UDP-glucose to ceramide. Because of its relatively small headgroup ceramide can flip into the lumenal leaflet of the Golgi membrane, where it is consumed by sphingomyelin synthase which transfers the headgroup from phosphatidylcholine forming sphingomyelin (SMS1, Huitema et al., 2004). At the exoplasmic leaflet of the plasma membrane a second sphingomyelin synthase is involved in the re-synthesis of sphingo-

myelin from ceramide formed at the cell surface (SMS2, Huitema et al., 2004). Both enzymes are required for cell growth and homeostasis. The sphingomyelin synthase-related enzyme (SMSr, Huitema et al., 2004) may form ethanolaminephosphorylceramide, a minor lipid in mammals.

Complex glycosphingolipids

The next glycosylation step in the formation of glycolipids is the tranfer of galactose from UDP-galactose to glucosylceramide. This reaction occurs in the lumen of the Golgi and is facilitated by lactosylceramide synthase (LCS, Nomura et al., 1998). Further addition of headgroups results in complex glycosphingolipids of e.g. the lacto-, globo- or ganglioseries (For an overview of glycosphingolipids we direct the interested reader to http://sphingolab.biology.gatech.edu/). For the addition of sugar headgroups not only the expression and localization of sugar transferases is important, but also the abundance of the activated sugar precursor which requires the presence of its specific transporter. Cells expressing GalCS sometimes synthesize one or two complex glycosphingolipids based on galactosylceramide. In addition, part of the galactosylceramide is sulfated to sulfatide (3-sulfogalactosylceramide) by cerebroside sulfotransferase (CST, Honke et al., 1997; Hirahara et al., 2000).

Generation of signaling sphingolipids

The sphingolipid species found to be signaling lipids are either formed by the *de novo* pathways or by degradation (Figure 2). Under normal conditions ceramide formed *de novo* is converted to more complex sphingolipids, but under stress conditions it can accumulate, which leads to apoptotic signaling (Obeid et al., 1993; Grassme et al., 2007). Alternatively sphingomyelin can be degraded to ceramide by the action of acid (lumenal) or neutral (cytosolic surfaces) sphingomyelinases (Schuchman et al., 1991; Hofmann et al., 2000; Tani and Hannun, 2007; Zeidan and Hannun, 2007). Acid ceramidase in the lysosomes (Koch et al., 1996) and neutral ceramidase at the plasma membrane (Hwang et al., 2005) deacylate ceramide to sphingosine. Phosphorylation of sphingosine and ceramide to sphingosine-1-phosphate and ceramide-1-phosphate by their cognate kinases (Stoffel et al., 1970; Kohama et al., 1998; Sugiura et al., 2002) turns them into anti-apoptotic mediators. Together, these lipids constitute the sphingostat, a metabolic regulatory system that integrates all sorts of exogenous and endogenous input signals and balances apoptosis and proliferation (Hannun and Obeid, 2008). The hydrolysis of sphingosine-1-phosphate to ethanolamine-phosphate and palmitaldehyde by the lyase abrogates signaling, and is the final step in sphingolipid degradation (Saba et al., 1997).

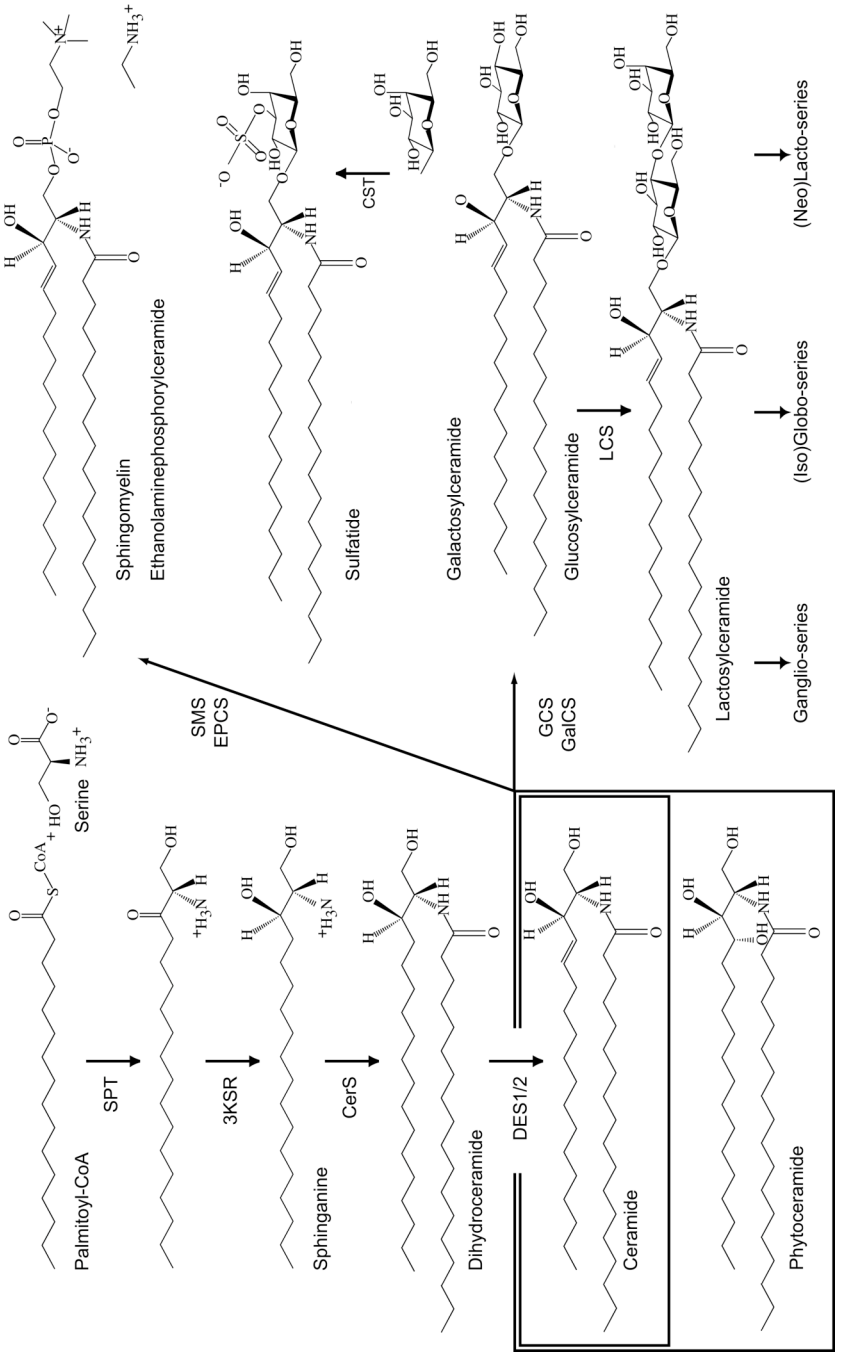
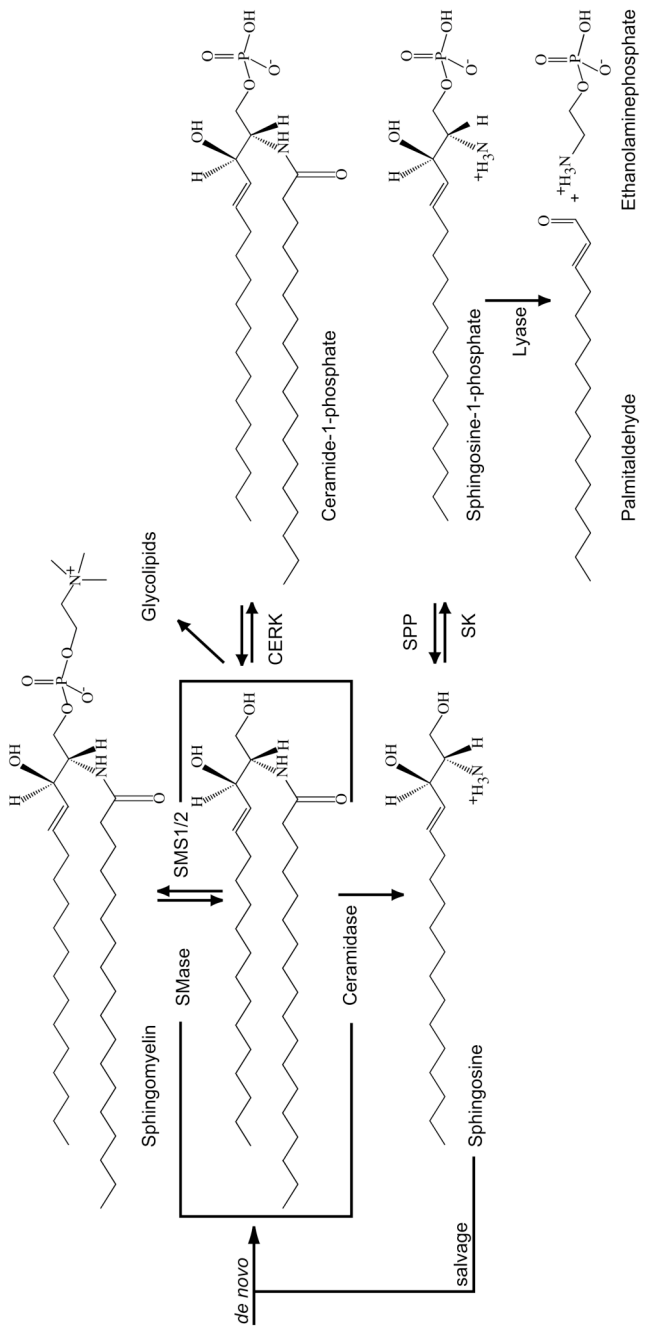

Palmitoyl-CoA
Serine
SPT
3KSR
Sphinganine
CerS
Dihydroceramide
DES1/2
Ceramide
Phytoceramide
SMS
EPCS
Sphingomyelin
Ethanolaminephosphorylceramide
CST
Sulfatide
GCS
GalCS
Galactosylceramide
Glucosylceramide
LCS
Lactosylceramide
Ganglio-series
(Iso)Globo-series
(Neo)Lacto-series

Figure 1: Mammalian sphingolipid synthesis
The committed step in sphingolipid synthesis is the condensation of serine with palmitoyl-CoA to 3-ketosphinganine, which is reduced to sphinganine and acylated to dihydroceramide. Six (dihydro)ceramide synthase genes in human and mouse prefer acyl-CoAs of different chain lengths. Usually, dihydroceramide is converted to ceramide by introducing a double bond at the sphinganine C4. In some tissues dihydroceramide is hydroxylated instead, to form phytoceramide, important in skin, but also found in brain, intestine and kidney. Ceramides can contain hydroxylated fatty acids, mainly in myelin galactosylceramide species. Ceramide is converted to glycolipids, sphingomyelin or the less abundant ethanolaminephosphorylceramide. Galactosylceramide, mainly found in myelin and epithelial cells of kidney and intestine, can be sulfated to sulfatide. Glucosylceramide is (partially) converted to lactosylceramide, which is generally only found as an intermediate and further glycosylated. For further information about lipid structures or species visit www.lipidmaps.org or www.sphingomap.org. SPT: serine palmitoyl transferase; 3KSR: 3-ketosphinganine reductase; CerS: (dihydro)ceramide synthase; GCS: glucosylceramide synthase; DES1/2: desaturase/hydroxylase; GalCS: galactosylceramide synthase; SMS: sphingomyelin synthase; EPCS: ethanolaminephosphorylceramide synthase; CST: cerebroside sulfotransferase; LCS: lactosylceramide synthase

Figure 2: Generation of signaling sphingolipids
Signaling pools of ceramide are either formed by *de novo* synthesis in the ER or through degradation of sphingomyelin at the cell surface, probably discriminating between different locations of ceramide signaling. Ceramide can be phosphorylated to ceramide-1-phosphate, or degraded to sphingosine, which can be phosphorylated to sphingosine-1-phosphate, or reutilized. SM deacylase: sphingomyelin deacylase; SMase: sphingomyelinase; CERK: ceramide kinase; SK: sphingosine kinase; SMS1/2: sphingomyelin synthase; SPP: sphingosine-1-phosphate phosphatase.


Glycolipids
CERK
Ceramide-1-phosphate
SPP
SK
Sphingosine-1-phosphate
Lyase
Palmitaldehyde
Ethanolaminephosphate
Sphingomyelin
SMase
SMS1/2
Ceramidase
Sphingosine
de novo
salvage

2. Lipid transfer proteins in sphingolipid metabolism

The local concentration of a lipid depends on its synthesis and degradation, but lipids are not confined to the membranes where they are synthesized. Hence, lipid transport is an important parameter in defining a membrane environment. Because lipids are building blocks of membranes, there is a continuous bidirectional lipid flux between organelles by vesicular transport. However, in addition, eukaryotes contain various proteins in their cytosol that can solubilize lipids and transport them in a monomeric fashion across the aqueous phase. Although some of these lipid transfer proteins have been well characterized *in vitro,* their actual *in vivo* function remains largely unclear (Wirtz, 2006). The newest evidence suggests that facilitated monomeric exchange via lipid transfer proteins may occur only over short distances at so-called membrane contact sites. There, it may be responsible for the rapid lipid exchange that has been measured between the mitochondrial inner and outer membrane (Simbeni et al., 1990; Ardail et al., 1991) and between the mitochondrial outer membrane and the ER (Shiao et al., 1995; Achleitner et al., 1999), membranes that are not connected by vesicular transport.

Finally, cell membranes are asymmetric with one bilayer leaflet facing the cytosol and the opposite surface oriented towards the lumen of the organelle or the extracellular environment. Except for the ER membrane, which appears to have a symmetrical lipid distribution with rapid transbilayer equilibration of the common membrane lipids, the membranes of the secretory and endocytotic recycling routes display an asymmetric lipid distribution, with an enrichment of the glycerophospholipids phosphatidylserine and phosphatidylethanolamine in the cytosolic and the sphingolipids and phosphatidylcholine in the non-cytosolic membrane leaflet (van Meer et al., 2008). The lipid asymmetry results from asymmetric synthesis, and from a lack of free transbilayer movement for the common lipids in combination with active, unidirectional transport of specific lipids across the bilayer. In general, lipids containing small uncharged headgroups like diacylglycerol, ceramide and cholesterol rapidly translocate spontaneously, whereas lipids with large hydrophilic headgroups need a protein machinery to move across. The best characterized active translocation is that of phosphatidylserine and phosphatidylethanolamine towards the cytosolic surface by P4 P-type ATPases (Daleke, 2007); the effect on lipid asymmetry of translocation in the opposite direction by ABC transporters is unclear, because they seem to act as extruders rather than translocases (van Meer et al., 2006).

Sphingomyelin synthesis is regulated by the ceramide transfer protein CERT

Sphingolipid synthesis is compartmentalized in ER and Golgi. While ceramide synthesis takes place at the ER, the subsequent sphingolipids - except galactosylceramide - are synthesized in the Golgi. Consequently, for conversion to sphingomyelin and glucosylceramide ceramide must be transported from ER to Golgi. An obvi-

ous transport mechanism would be the vesicular secretory pathway, and this route appears to be followed by a ceramide pool that is converted to glucosylceramide. However, numerous studies have indicated the involvement of a non-vesicular pathway in sphingomyelin synthesis (Hanada et al., 2007), culminating in the discovery of the cytosolic ceramide transfer protein CERT (Hanada et al., 2003).

The identification of CERT as a cytosolic protein required for sphingomyelin synthesis (Hanada et al., 2003) provided mechanistic insight in the vesicle-independent transport of ceramide from ER to Golgi. CERT is expressed as two splice variants $CERT_L$ and the more frequently expressed CERT, which lacks an internal 26 amino acid sequence. Both restore the wild-type phenotype in CERT mutant cells. The lipid transfer activity lies in the steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domain of CERT. Its structure explains the lipid specificity (Kudo et al., 2008): ceramide is harbored in a hydrophobic cavity too narrow for lipids with bulky head groups. Furthermore, only ceramides containing a fatty acid ≤C18 fit adequately; longer fatty acyl chains are protected less efficiently, in line with the preferred ceramide species transferred by CERT *in vitro* (Kumagai et al., 2005).

The activity of CERT is mainly regulated by its localization, which depends on three factors: phosphatidylinositol-4-phosphate (PI(4)P) binding at the *trans*-Golgi (Hanada et al., 2003), VAMP-associated protein (VAP) binding at the ER (Kawano et al., 2006) and phosphorylation (Fugmann et al., 2007; Kumagai et al., 2007). CERT binds to the Golgi via its PH-domain that recognizes PI(4)P like some other lipid transfer proteins. Transfer activity can be blocked by the point mutation G67E in the PH domain that inhibits binding to PI(4)P (Hanada et al., 2003) or by inhibiting PI 4-kinase IIIβ, the kinase mainly responsible for PI(4)P synthesis at the Golgi (Toth et al., 2006). The association of CERT to the ER is mediated by VAP binding via CERT's FFAT motif (two phenylalanines in an acidic tract, Kawano et al., 2006). VAPs may act as a protein interaction platform in the ER, and, in general, protein-protein interaction with VAPs can also occur in a FFAT-independent manner. The dual binding specificity of CERT may locate the complex to ER-*trans* Golgi membrane contact sites (Mogelsvang et al., 2004), which would greatly stimulate its transfer efficiency (Figure 3).

Several serines and threonines in CERT can be phosphorylated. The region between the PH domain and the START domain contains a serine repeat with the typical sequence SXX' (Raya et al., 1999; Kumagai et al., 2007). This motif is recognized by casein kinase I, when a phosphorylated pSer/pThr is already present. Serine 132, the first serine in the serine repeat, could serve as such a residue and indeed it was found to be phosphorylated independently by protein kinase D (PKD, Fugmann et al., 2007). Phosphorylation impedes membrane binding, transfer activ-

ity (Kumagai et al., 2007) and sphingomyelin synthesis. In the phosphorylated form, the PH domain and the START domain of CERT bind each other into an inactive conformation. Dephosphorylation of CERT was shown to be mediated by protein phosphatase 2Cε (PP2C, Saito et al., 2007) and restores membrane binding. Interestingly, PP2Cε is a binding partner for VAP-A as well. Phosphorylation of CERT by PKD may serve as a kind of feed-back control for vesicular transport: PKD is recruited by diacylglycerol to the Golgi, where it is phosphorylated and activated by protein kinase Cη (PKC, Diaz Anel and Malhotra, 2005), and promotes vesicular transport (Baron and Malhotra, 2002). PKD phosphorylates and activates PI 4-kinase IIIβ to form PI(4)P (Hausser et al., 2005) and thereby recruits CERT to the Golgi (Toth et al., 2006). Diacylglycerol formation via sphingomyelin synthesis then recruits more PKD in a positive feedback loop. At the same time, PKD phosphorylates CERT, which turns off lipid transfer, diacylglycerol production and PKD recruitment. CERT is a member of the 15 member START domain family of proteins including lipid transfer proteins for cholesterol (StAR and MLN64), phosphatidylcholine (PCTP) and phosphatidylcholine and phosphatidylethanolamine (StarD10), all of which are regulated by phosphorylation (Soccio and Breslow, 2003; see Fugmann et al., 2007).

The localization of CERT and sphingomyelin synthase to the *trans* side of the Golgi (Halter et al., 2007; Hanada et al., 2007) may explain the preferential usage of ceramides transferred by CERT for sphingomyelin synthesis. To what extent glycolipid synthesis depends on CERT is less clear (Hanada et al., 2003; Perry and Ridgway, 2006; Halter et al., 2007). It probably depends on whether the glucosylceramide synthase is located on the *cis*- (Futerman and Pagano, 1991) or *trans*-side of the Golgi (Halter et al., 2007), which may be cell-type specific. The contribution of the CERT pathway may also be tissue specific because CERT is differentially expressed (Raya et al., 1999; Raya et al., 2000). CERT may be required for the formation of ceramide-1-phosphate at the Golgi (Boath et al., 2007; Lamour et al., 2007).

Complex glycosphingolipid synthesis requires transmembrane translocation of glucosylceramide

The higher glycosphingolipids are synthesized in the Golgi lumen by the stepwise addition of sugars to glucosylceramide, which itself is synthesized at the cytosolic face of the Golgi (Ichikawa et al., 1996). Thus, not only the activated sugars but also glucosylceramide must be translocated towards the Golgi lumen. Because of the relatively large hydrophilic headgroup of glucosylceramide, translocation across the membrane is most likely protein-mediated. ABC-transporters that translocate fluorescent glucosylceramide (van Helvoort et al., 1996) were proposed as candidate flippases (De Rosa et al., 2004), but natural glucosylceramide appeared not to

be a substrate (Halter et al., 2007). Moreover, as stated above, ABC-transporters with lipid substrates function as exporters rather than *bona fide* flippases (van Meer et al., 2006). Although glucosylceramide might translocate across the Golgi membrane directly, we have presented evidence that glucosylceramide flips across the ER membrane instead, and that the majority of the lipid flipped in the ER is directed to complex glycosphingolipid synthesis (Halter et al., 2007). Glucosylceramide flip across the ER is a tempting hypothesis, because the conditions of the ER membrane facilitate lipid flip-flop. The ER membrane contains unsaturated lipids and has low amounts of sphingolipids and cholesterol, resulting in a high membrane fluidity. Moreover, the ER is the location of membrane protein translocation, causing a high concentration of transmembrane domains. In addition, signal peptides cleaved from secretory or membrane proteins reside in the membrane (Lyko et al., 1995), and may facilitate lipid flip (Boujaoude et al., 2001). Unexpectedly, glucosylceramide was also found to translocate across post-Golgi membranes by a mechanism that depended on the activity of the proton-ATPase. However, this process did not contribute to higher glycosphingolipid synthesis (Halter et al., 2007) and must serve some other function.

Synthesis of higher glycosphingolipids is also regulated by lipid transfer proteins
Important information on the itinerary of newly synthesized glucosylceramide from the cytosolic aspect of the Golgi to the site of complex glycosphingolipid synthesis in the Golgi lumen came from the finding that this process depends on the cytosolic protein FAPP2 (D'Angelo et al., 2007; Halter et al., 2007). FAPP2 (four phosphate adaptor protein 2, Godi et al., 2004) has a domain with homology (36% identical, 56% similar) to the cytosolic glycolipid transfer protein GLTP (Abe et al., 1982; Metz and Radin, 1982). The GLTP crystal structure (Malinina et al., 2004) showed that, compared to the START domain found in CERT and its family members, the GLTP domain harbors the lipid in a rather open “sandwich” conformation, that may allow potential protein binding-partners of glycosylceramide to bind to the GLTP-lipid complex. Both GLTP and FAPP2 transport a variety of glycolipids *in vitro* (Brown and Mattjus, 2007; D'Angelo et al., 2007). *In vivo*, glucosylceramide synthesized on the cytosolic surface of the Golgi and galactosylceramide, synthesized in the ER lumen - but having free access to the cytosolic surface of the ER (Burger et al., 1996) - are likely substrates. The proteins should not have access to the complex glycosphingolipids, which reside in the non-cytosolic leaflet of the Golgi and endocytotic membranes.

Via its PH domain with specificity for PI(4)P FAPP2 binds to the *trans*-Golgi. Binding also involves ARF1 (Godi et al., 2004). The reduction of conversion of newly synthesized glucosylceramide to complex glycolipid upon FAPP2 knockdown implies an involvement of FAPP2 in glucosylceramide transport from its site of synthesis to its site of translocation (D'Angelo et al., 2007; Halter et al., 2007).

Whereas D'Angelo et al. (D'Angelo et al., 2007) favored glucosylceramide synthesis at the *cis*-Golgi, FAPP2-mediated transport to the *trans*-Golgi and translocation across a *trans*-Golgi membrane, our evidence suggested glucosylceramide synthesis in the *trans*-Golgi followed by FAPP2-mediated retrograde transport to, and translocation across, the ER (Halter et al., 2007); further experiments are required to settle this issue. FAPP2 activity may be regulated by phosphorylation. It contains several serine and threonine residues that could serve as phosphorylation sites.

Originally, FAPP2 (and FAPP1) was found to be important for vesicular transport from the Golgi to the plasma membrane (Godi et al., 2004; Vieira et al., 2005; D'Angelo et al., 2007). We observed no effect of FAPP2 knockdown on protein secretion nor on transport of the complex glycosphingolipid GM3 to the surface of melanocytes (Halter et al., 2007) and also the lack of glycosphingolipids in melanocyte mutant cells did not affect protein transport to the cell surface (Ostermeyer et al., 1999). How FAPP2 is involved in protein transport to the (apical) plasma membrane of certain cells remains to be resolved.

Since reasonable amounts of PI(4)P are also found at the plasma membrane (Di Paolo and De Camilli, 2006), FAPP2 might be involved in the reported glucosylceramide transport to this location (Warnock et al., 1994). Indeed, FAPP2 knockdown reduced transport of newly synthesized glucosylceramide to the cell surface. Also a GLTP knockdown had a partial effect on this transport (Halter et al., 2007). In contrast, the GLTP knockdown did not affect complex glycosphingolipid synthesis (Tuuf and Mattjus, 2007). Genome analysis revealed a second GLTP gene in humans, but it is not transcriptionally active (Zou et al., 2008). In fungi and plants GLTP homologs have been implicated in apoptosis (Saupe et al., 1994; Brodersen et al., 2002). However, the function of GLTP in mammalian cells still remains to be explored.

Vesicular transport of sphingolipids

After synthesis in the Golgi lumen, sphingomyelin and complex glycosphingolipids do not have access to the cytosolic leaflet and therefore can be transported exclusively by vesicular transport. As sphingolipids and cholesterol are enriched at the plasma membrane, they must be preferably transported by anterograde routes (van Meer, 1989). Indeed, they were found to be largely excluded from retrograde carriers (Brügger et al., 2000). In epithelial cells transport of sphingolipids is specialized, because of the presence of two distinct plasma membrane compartments. In these cells glycosphingolipids are enriched at the apical side, whereas the basolateral side displays a normal plasma membrane composition (van Meer and Simons, 1988). How glycosphingolipids are sorted in apical transport routes is poorly understood, but it must involve their lateral enrichment at the budding site of the apical precursor vesicles; their large capacity for hydrogen bonding may induce their seg-

regation from other membrane lipids in the lumenal leaflet of the Golgi membrane (van Meer and Simons, 1988). Such an enrichment has been observed for lactosylceramide during recycling through the lumen of endosomes (Sharma et al., 2003), and may also occur during the budding of vesicles (or viruses) away from the cytosol into the lumen of late endosomes (Wubbolts et al., 2003; Trajkovic et al., 2008) or into the extracellular medium. Evidence has accumulated over the years that lateral segregation on short time- and length-scales is a property of the plasma membrane surface, and that small domains or rafts can coalesce when they are stabilized by physical parameters like curvature or membrane stress, or by a change in lipid composition like sphingomyelin hydrolysis and ceramide production (van Meer et al., 2008).

Sphingolipid transfer proteins in the endo-lysosomal lumen

Proteins with lipid transfer activity *in vitro* have also been purified from the lumen of the endo/lysosomes. In general, these proteins turn out to be involved in presenting glycosphingolipids to the degradative soluble enzymes (Kolter and Sandhoff, 2005). These saposins are apparently most efficient in mobilizing glycosphingolipids from the intralumenal vesicles in multivesicular endosomes (Locatelli-Hoops et al., 2006). A different function of the saposins in the endo/lysosomal lumen appears to be the insertion of glycolipids into the antigen binding groove of the antigen presenting CD1b and -1d proteins (Winau et al., 2004; Yuan et al., 2007). A soluble CD1 molecule, CD1e, now turns out to be a dedicated glycolipid transfer protein involved in loading antigenic lipids onto CD1b (Tourne et al., 2008). The cholesterol transporter Niemann-Pick type C2 protein (Babalola et al., 2007) has been reported to transport a glycolipid (Schrantz et al., 2007), which however could not be confirmed (Konrad Sandhoff, Bonn, pers. commun.).

3. Regulation of local lipid composition

To create a specific lipid environment, lipid metabolism and transport must be coordinated, but the basic layout of how the cell orchestrates the activity of metabolic enzymes is not known. Many reports in the literature connect specific tissues, conditions, signaling pathways and molecules to the occurrence and (local) concentration of a specific sphingolipid, and the results reflect the common complex regulation at the transcriptional and post-transcriptional level of the enzymes of sphingolipid metabolism.

Crosstalk between ER and Golgi

A special feature of the sphingolipid system is the control of substrate supply via lipid transfer proteins, one of which (CERT) works between ER and Golgi and may be present at contact sites between these organelles (Figure 3). It attaches via its PH domain to the *trans*-Golgi via a molecules that is itself highly regulated: PI(4)P. On the ER side, CERT binds to VAPs in the ER via a FFAT motif. In addition,

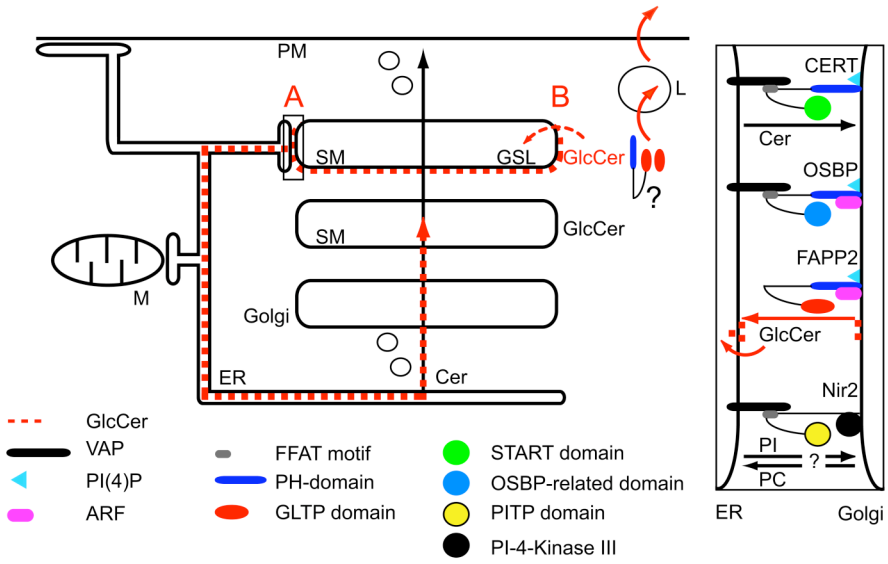


Figure 3: **Integration of sphingolipid transport at ER-Golgi contact sites**
Newly synthesized ceramide (Cer) is transported from ER to Golgi by CERT or vesicles for sphingomyelin (SM) and glucosylceramide (GlcCer) formation. GlcCer must translocate into the lumen, where it is converted to complex glycosphingolipids (GSL). (A) One major pathway is GlcCer transport to the ER by the lipid transfer protein FAPP2. There it translocates and is transported by vesicles to the Golgi, where it is converted to lactosylceramide and GM3 (Halter et al., 2007). One prediction of this model is that GlcCer is concentrated in the anterograde vesicle pathway from the ER, which may exclude phosphatidylinositol (PI) and, in the Golgi, ceramide. (B) Translocation directly at the Golgi has also been proposed (D'Angelo et al., 2007), but occurred less efficiently than at ER membranes *in vitro* (Halter et al., 2007). GlcCer can also be transported through the cytosol, maybe by FAPP2 or GLTP, to the plasma membrane (PM) or lysosomes (L), where it translocates to the lumenal side. Cytosolic transfer proteins have no access to SM and GSL in the lumen. Many lipid transfer proteins share the START domain for lipid transfer activity, others have the OSBP-related domain. Glycolipid transfer proteins have a GLTP domain, which displays a novel folding motif. Transport by lipid transfer proteins may occur efficiently at membrane contact sites. The lipid transfer proteins CERT and OSBP bind to two organelles, the ER in a VAP-dependent manner through a FFAT motif and the Golgi via their PH domain for PI(4)P which is enriched in the *trans*-Golgi. PI(4)P levels at the Golgi are regulated by the PI/PC exchange protein Nir2, which binds to the ER via its FFAT motif. FAPP2 binds to the Golgi via its PH domain and transports GlcCer to the ER. OSBP and FAPP2 also interact with the small GTPase ARF on the Golgi. Together, these characteristics interconnect the metabolism of phosphoglycerolipids, sphingolipids and cholesterol and link these to vesicular transport.

CERT is regulated via phosphorylation by different types of kinases. Surprisingly, OSBP a lipid transfer protein of a different family, the 12 member ORP family (Perry and Ridgway, 2006), shares with CERT its binding to PI(4)P on the trans-Golgi, its regulatory phosphorylation and its FFAT-domain-mediated binding to the ER. Also phosphatidylinositol/phosphatidylcholine-binding/transfer protein Nir2 of the 3 member Nir family is phosphorylated (Litvak et al., 2002) and binds to ER VAPs by its FFAT domain (Amarilio et al., 2005). OSBP was required for sterol-dependent activation of CERT (Levine and Munro, 2002; Perry and Ridgway, 2006), while Nir2 recruits the PI 4-kinase IIIβ to the Golgi (Aikawa et al., 1999) producing the PI(4)P needing for Golgi-binding of both CERT and OSBP. This stresses the point that we are dealing with a complex lipid transport system at the ER-Golgi interface that integrates the metabolic regulation of multiple lipid classes, sphingolipids, glycerophospholipids and sterols. Via a variety of kinases, the various transfer proteins are embedded in the cellular signalome.

In addition to PI(4)P, the binding of OSBP to the *trans*-Golgi involves ARF (Perry and Ridgway, 2006). Thus, FAPP2 has the same binding site as OSBP on the *trans*-Golgi, which may suggest that FAPP2 transports at the same *trans*-Golgi-ER contact sites. PH-domains can dimerize (Klein et al., 1998) and thereby enhance binding to the cognate phosphoinositide at the membrane. However, it is not clear whether this occurs also among lipid transfer proteins. The FAPP2 homolog FAPP1 is a protein of unknown function, which contains a PI(4)P PH domain but lacks the GLTP domain. *In vivo*, over-expression of PH-domains competes with binding of other PH-domain containing proteins (Godi et al., 2004). On the other hand, FAPP1 could dimerize with a lipid transfer protein like FAPP2 and thus enhance its binding properties. Remarkably, ARF1 on the trans-Golgi is an essential part of the binding; also the membrane attachment of ARF is regulated, which links FAPP binding to vesicular traffic.

Lipid transfer proteins: composition sensors?

One missing element in the lipid regulation network are the sensors for the bulk lipid composition. It has been suggested that some of the lipid transfer proteins with their respective specificities would actually be the sensors. For example, PITPb is specifically bound to the Golgi (van Tiel et al., 2002) and has an *in vitro* transfer activity towards phosphatidylinositol and phosphatidylcholine but also to sphingomyelin (de Vries et al., 1995); instead of acting as a transfer protein, it might sense the concentration of sphingomyelin in the cytosolic surface (which should normally be very low). Because of GLTP's high affinity for glycosphingolipids (Neumann et al., 2007) and taking into account that the amount of GLTP binding to membranes reflects the glycosphingolipid concentration *in vitro* (Rao et al., 2005), GLTP might act as a sensor for glycolipids instead of being a genuine transfer protein. However,

it has remained unclear how the sensor would pass on the signal to an effector that would translate the signal into a change of membrane composition.

4. Perspectives

Under all conditions, cells appear to maintain the unique lipid composition and transbilayer organization of their various membranes, which therefore seem essential for eukaryotic cells. For this, the concentration of individual lipid species has to be sensed and translated into synthesis, degradation or transport. However, how this is achieved is only understood in a few specific cases. The best characterized system is the regulation of cholesterol homeostasis via the SREBP pathway. Hereby, a decrease in cholesterol concentration in the ER releases a membrane-bound transcription factor into the nucleus to activate genes involved in cholesterol metabolism (Brown and Goldstein, 1999). This pathway cannot only be activated by proteins containing sterol sensing domains (SSD) but also by the sterol transfer proteins OSBP and the related ORPs (Olkkonen et al., 2006), which also act as sterol sensors. By its sterol-dependent activation of CERT OSBP forms a regulatory link between sterol and sphingolipid metabolism. Elevated levels of 25-hydroxy cholesterol lead to increased synthesis of sphingomyelin (Perry and Ridgway, 2006).

For sphingolipids only little is known about the sensing mechanisms. It is an interesting notion, that CerS, except CerS1, contain Hox-domains (Pewzner-Jung et al., 2006). Hox-domains are related to homeobox proteins, transcription factors involved in developmental regulation and in CerS they are not essential for enzymatic activity (Mesika et al., 2007). This system displays some marked similarities to the SREBP system and suggests that CerS could be sphingolipid sensors, but so far experimental evidence is missing.

To gain further insight into sphingolipid biology and to fully understand the phenotypes related to mutations in proteins involved in sphingolipid metabolism and transport, it is important to identify their interaction partners *in vivo*. The unraveling of the dynamic structural and functional interactions of the lipid transfer poteins and their embedding in the cellular signaling networks will greatly contribute to an understanding of how eukarotic cells use sphingolipids for their vital functions.

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Glycolipid Transfer Protein

Clear structure and activity, but enigmatic function

Based on

Neumann, S., M. Opacic, R.W. Wechselberger, H. Sprong, and M.R. Egmond. 2008. ***Adv Enzyme Regul***. 48:137-51

Introduction

Glycosphingolipids comprise a small (typically 5%-10% by weight) but vital fraction of membrane lipids in eukaryotes (Holthuis *et al.,* 2001). They provide the plasma membrane with chemical and mechanical stability and take part in fundamental biological processes including differentiation, cell-cell interaction, and transmembrane signaling. For these lipids, as for most lipids in general, local metabolism and selective transport are important determinants (Sprong *et al.,* 2001). Little is known about these processes, but it is clear that several key players in the organization and control of sphingolipid composition remain to be identified. A protein purified from bovine spleen cytosol specifically transferring glycolipids was already described more than twenty years ago (Metz and Radin, 1980; Radin and Metz, 1982). An absolute specificity was found for glycolipids containing a β-linked sugar to the hydrophobic backbone (Yamada *et al.,* 1986), as they are found in natural glycosphingolipids. Glycolipid transfer protein (GLTP) is a water-soluble protein of 24 kDa size that has been studied extensively *in vitro* (Abe et al., 1982; Metz and Radin, 1982; Abe and Sasaki, 1985; Gammon et al., 1987; Brown et al., 1990). Very recently, its structural and *in vitro* functional properties were reviewed in detail (Brown and Mattjus, 2007). GLTP has been isolated from different sources ranging from spinach chloroplasts to mammalian brain, liver and kidney (Sasaki, 1985; Brown et al., 1990; Sasaki, 1990). GLTP orthologs in fungi and plants were found to be involved in programmed cell death (Saupe et al., 1994; Brodersen et al., 2002; Mattjus et al., 2003) but their function in mammals is largely unknown. The activity of GLTP is preserved after expression in *E. coli* (Lin et al., 2000; Godi et al., 2004; Malinina et al., 2004; Rao et al., 2004) allowing for detailed structure-function studies *in vitro.*

Crystal structures of GLTP in the absence and presence of bound glycolipid were solved revealing a new folding motif among lipid-binding proteins (Malinina *et al.,* 2004). In combination with mutational studies the structures provided new insights into glycolipid binding (Malakhova *et al.,* 2005). The sugar head group of the glycolipid is specifically recognized by a network of hydrogen bonds involving Trp96 while the acyl chains are embedded in a hydrophobic tunnel flanked by α-helices. It remains to be investigated, however, in what way GLTP binds to membranes and how glycolipids are being transferred from their membrane environment into the binding pocket of GLTP and subsequently donated to acceptor membranes.

In this study we investigated the mechanism of lipid uptake by GLTP. With the help of fluorescent techniques (Li *et al.,* 2004) in combination with photochemically induced dynamic nuclear polarization (photo-CIDNP) (Kaptein et al., 1978; Mok and Hore, 2004) and Chemical Shift Perturbation methods (Bax et al., 1990; Otting,

1993; Zuiderweg, 2002) we have studied interaction of GLTP with liposomes and conditions of lipid uptake.

Materials and Methods

Materials

Chemicals were from Sigma (St. Louis, MO) and used in the highest purity available, unless indicated otherwise. Silica TLC plates were from Merck (Darmstadt, Germany), organic solvents were from Riedel de Haën (Darmstadt, Germany), [N-methyl-^{3}H] choline, 1,2 –dipalmitoyl, and [9,10–^{3}H]–palmitic acid were from Amersham (Buckinghamshire, UK). Pyrene-hexanoic acid was from Molecular Probes (Eugene, OR). Porcine glucosylceramide (GlcCer), L-α-phosphatidylcholine (egg PC) and cholesterol were purchased from Sigma-Aldrich. Pyrene-labeled glucosylceramide (GlcCer*pyr*) was synthesized from pyrene-hexanoic acid and 1-ß-D-glucosylsphingosine (Sigma-Aldrich) as described (Kishimoto, 1975), purified by two-dimensional thin layer chromatography (TLC) as described previously (Sprong *et al.,* 2000), and quantified spectrophotometrically at λ_{ex} = 342nm and λ_{em} = 378nm. Deuterium oxide (^{2}H, 99.9%) was obtained from Cambridge Isotope Laboratories, Inc.

Expression and purification of GLTP

The N-terminal 6*His-tag plasmid pQE9-GLTP encoding bovine GLTP was a kind gift from Peter Mattjus (West, 2004). The plasmid was transformed into *E.coli* BL21 cells and grown in LB medium at 37°C until OD_{600} 0.6. Expression of the His-tagged GLTP was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM. The cells were incubated for 2h at 37°C, harvested and lysed by incubation with (1 mg/ml) lysozyme in lysis buffer (50 mM NaH_2PO4, 300 mM NaCl, 10 mM imidazole 1 mg/ml PMSF pH 8.0) followed by sonication. Cell debris was removed by centifugation and the supernatant was subjected to affinity chromatography using Ni-NTA (Quiagen). The protein was eluted with elution buffer (50 mM NaH_2PO4, 300 mM NaCl, 250 mM imidazole and 10% glycerol pH 7.5), and the buffer was exchanged to phosphate buffer (25 mM phosphate, 1 mM DTT, 150 mM NaCl, pH 6.8) to a final concentration of imidazole of 0.1 mM using Amicon centrifugation columns (Millipore). The purity was confirmed by analysis by SDS-PAGE and comassie stain and estimated to be at least 98% pure. Protein concentration was calculated from optical density measurements at 280 nm using ε_{280nm}=13 $M^{-1}cm^{-1}$. Transfer activity measurements under standard assay conditions (Mattjus *et al.,* 1999) yielded a second order rate constant of $8*10^4$ $M^{-1}s^{-1}$ at 20 °C. Mutant GLTP W142Y was a kind gift of Peter Mattjus (Helsinki, Finland).

For expression and purification of ^{15}N labeled GLTP pQE9-GLTP was transformed into *E.coli* BL21 cells and grown in minimal M9 medium (containing 0.5 g/L $^{15}NH_4Cl$) at 37°C until the cell density OD_{600} reached 0.5. Purification of the ^{15}N-labeled GLTP was carried out essentially as described for the unlabeled protein.

Vesicle preparations
In titrations of GLTP: Vesicles used in the titration experiments contained a fixed amount of cholesterol (40%), while the total molar fraction of GlcCer and egg-PC was kept at 60%. The molar fraction of GlcCer was varied from 0-30 mol%. Lipids were mixed in chloroform /methanol, or ethanol solution, dried under a stream of nitrogen and hydrated in Tris buffer (20 mM Tris, 5mM EDTA, 200mM NaCl, pH 7.4). The dispersion was subjected to 6-7 freeze-thaw cycles to get a uniform distribution of buffer solutes between lipid bilayers. After each thawing cycle at 60°C the lipid dispersion was thoroughly mixed. The lipid suspension was then extruded by 11 passes through 400 nm polycarbonate membrane, and 11 passes through 200 nm polycarbonate membrane using a hand held extruder (Avanti). For preparation of ^{3}H-labeled vesicles trace amounts of ^{3}H-labeled PC or GlcCer were added to the lipid mixture before lipid drying under nitrogen.

In titrations of vesicles with GLTP: Vesicles were prepared with fixed amounts of egg PC (30 mol%), cholesterol (40 mol%) and GlcCer (25 mol%). In addition these vesicles contained 5 mol% GlcCer*pyr.* These vesicles were made essentially as described above for unlabeled vesicles.

Intrinsic fluorescence emission of GLTP
Intrinsic fluorescence emission measurements of GLTP were described before (Li *et al.,* 2004). Fluorescence measurements were performed using a PTI - Photon Technology International fluorimeter. Excitation and emission slot widths were set both to 2 nm. Experiments were performed using an excitation wavelength of λ_{ex} = 285 nm at 37 ± 0.5°C with constant stirring. Emission was monitored from 300 nm to 500 nm. The concentration of GLTP in all the measurements was 0.5 μM. Vesicles were added in varying amounts (ranging from 5 μM to 160 μM total lipid) to fresh protein solutions. Difference spectra of all fluorescence measurements were obtained by subtracting the spectra of GLTP loaded with vesicles from spectra of GLTP itself recorded each time prior to the addition of vesicles and corrected for dilution.

Vesicle integrity
GLTP (0.25 mM) was incubated with ^{3}H-labeled vesicles (composition and total lipid concentration were varied) followed by ultracentrifugation at 4°C for 1h at 100,000 x g (Optima ^{TM} MAX Ultracentrifuge, Beckman Coulter, rotor TLA 55). Ra-

dioactivity was measured for supernatant and pellet separately using a Liquid Scintillation Analyzer (Packard, TRI-CARB 2300TR)

Photo-CIDNP NMR

Surface exposed aromatic residues in proteins can be highlighted using photo-CIDNP NMR (Kaptein et al., 1978; Mok and Hore, 2004). These NMR measurements were performed on a Bruker Avance 500 MHz spectrometer. An Argon laser was used as light source (Spectra Physics, Stabilite 2017). The output power was 5 W. The light beam was directed to the sample in the NMR tube using an optical fiber. The photo-CIDNP radical reaction was initiated with FMN (Riboflavin 5′-monophosphate sodium salt dihydrate) as a laser-reactive dye. The irradiation leads to the generation of peptide-dye radical pairs involving dye-accessible (and therefore surface-exposed) Tyr, Trp and His residues. The laser light was gated into pulses of 400 ms using a computer controlled mechanical shutter. Spectra were recorded after a short delay of 5 ms. The re-cycle delay of the experiments was 6 s. Light and dark spectra were recorded in the same experiment in interleaved scans in order to maintain as much of the same conditions for the two experiments as possible. For each spectrum a total number of 64 scans was accumulated. The final photo-CIDNP data were obtained by subtraction of the dark from the light spectra. Apo-GLTP (0.25 mM) was monitored with 0.26 mM FMN. The effect of different types of vesicles (0% GlcCer, 40% cholesterol, 60% egg PC and 30% GlcCer, 40% cholesterol, 30% egg PC) was studied as well. Before monitoring by NMR, GLTP was harvested after incubation with each type of vesicles (2.5 mM total lipid) by centrifugation for 1h at 100 000 g as described before. As a control 1D NMR and photo-CIDNP spectra of FMN (0.26 mM in 25 mM phosphate, 150 mM NaCl, 1 mM DTT, pH 6.8) were recorded. Experiments were carried out at 20°C and 40°C.

Chemical shift perturbation experiments

The chemical shifts of amide protons are very sensitive to their chemical and structural environment (Otting, 1993). Therefore, ^{1}H-^{15}N correlation NMR experiments (HSQC, (Bax *et al.,* 1990)) can be used to monitor chemical shift changes in the course of a titration or addition of a ligand and thereby reveal an interaction or binding surface on a protein (Zuiderweg, 2002). Uniformly ^{15}N labeled GLTP was dissolved in phosphate buffer (25 mM phosphate, 150 mM NaCl, 1 mM DTT, pH 6.8, containing 9% D_2O) at a concentration of 0.3 mM. GLTP was studied with or without GlcCer. HSQC spectra were recorded at 20°C before and after addition of GlcCer using on a Bruker Avance 600 MHz spectrometer.

CD measurements

CD spectra of GLTP itself and GLTP pre-incubated with vesicles containing PC/cholesterol with or without 30 mol% GlcCer were monitored at temperatures

ranging from 20°C – 60°C with 1°C/min increments at a constant wavelength of 220 nm. Protein samples were pre-scanned at 20°C between 190 - 280 nm. All measurements were performed using a Jasco J-810 spectropolarimeter equipped with a CDF-426S temperature controller

Results

GLTP has a high affinity for glucosylceramide

The glycolipid transfer activity of GLTP from donor to acceptor vesicles *in vitro* is routinely monitored using a fluorescence based resonance energy transfer assay (Abe et al., 1984; Mattjus et al., 1999; Sahoo et al., 2000). Self quenching due to excimer formation of pyrene-labeled lipids is also used to study lipid transport (Sahoo et al., 2000; Somerharju, 2002). We applied this technique to study single turnover transfer of partially pyrene-labeled glucosylceramide (GlcCer*pyr* and GlcCer mixed in 1:5 molar ratio) from donor vesicles to GLTP. Addition of GLTP to a solution of vesicles (9.8 µM total lipid) containing fixed amounts of partially pyrene-labeled GlcCer (30 mol%), egg PC (30 mol%) and cholesterol (40 mol%) leads to an increase in fluorescence intensity at 378 nm (Figure 1). This increase takes place within seconds after mixing protein and vesicles and remains stable thereafter. It is assumed that the fluorescence increase results from complex formation of GLTP with GlcCer. The amount of GLTP-GlcCer complex (X) is then related to the measured fluorescence emission intensity F_{em} using equation [1]:

$$F_{em} = F_{em(0)} + C[X] \qquad [1]$$

where $F_{em(0)}$ is the fluorescence emission intensity before addition of GLTP and C is a constant. An apparent binding constant (Kd) for GlcCer taken up by GLTP is obtained by solving equation [2],

$$Kd = [([E_{tot}] - [X]) \,.\, ([GlcCer] - [X])] / [X] \qquad [2]$$

allowing only one physically possible root as solution:

$$[X] = 0.5\ a.(1-\sqrt{1-(4[E_{tot}][GlcCer])/a^2}\ ) \qquad [3]$$

where $a = Kd+[E_{tot}]+[GlcCer]$ and E_{tot} is total GLTP concentration in µM. While the total concentration of GlcCer is known, the concentration of glycolipid available for GLTP, [GlcCer] in equations 2 and 3 is considered as an unknown.

Substitution of equation [3] into [1] gives a value for the function F_{emcalc}. The solid line in Figure 1 represents the best fit to the data by nonlinear regression where F_{em}-F_{emcalc} is minimised while varying Kd, C and [GlcCer]. Based on this analysis, a replot of the concentration of GLTP-GlcCer complex as a function of free GLTP (inset Figure 1) demonstrates the high affinity of GLTP for GlcCer, estimated at Kd

= 2.5 10^{-2} µM for various concentrations of vesicles containing 30 mol% GlcCer. This Kd value represents the concentration of free GLTP when half of the available GlcCer is bound to the protein. Interestingly, another result of this analysis is the finding that only 50% of total GlcCer present in the vesicles is available for uptake by GLTP.

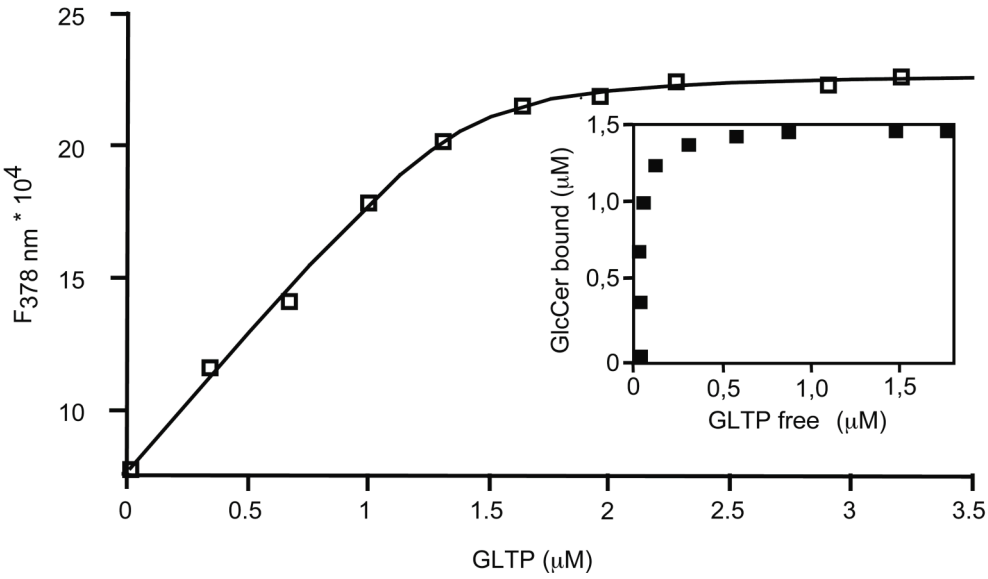


Figure 1: Titration of unilamellar vesicles (9.8 µM total lipid) containing 25 mol% GlcCer, 5 mol% GlcCer*pyr*, 30 mol% egg PC and 40 mol% cholesterol with GLTP. Fluorescence intensities are measured at 378 nm after rapid mixing of vesicles with GLTP. Inset: Replot of the calculated GLTP-GlcCer concentrations as a function of free GLTP concentrations.

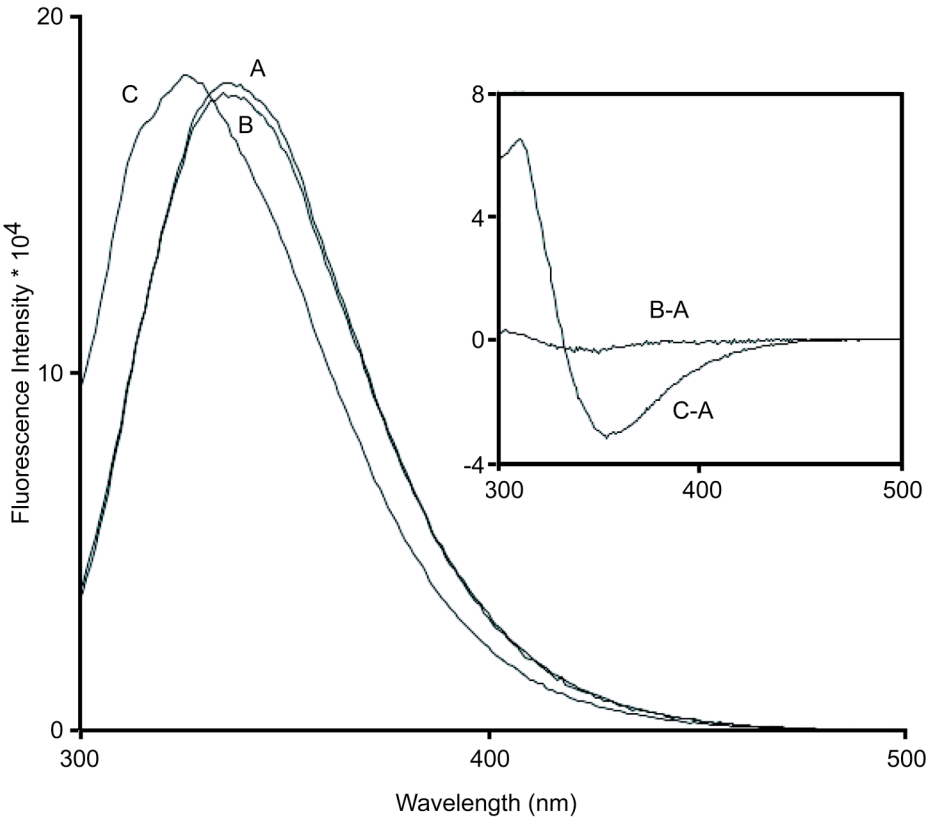


Figure 2: Fluorescence emission spectra of GLTP (0.5 µM) in the absence of vesicles. A: apo-GLTP, B: GLTP after treatment with vesicles containing egg PC (60 mol%) and cholesterol (40 mol%), C: GLTP after treatment with vesicles containing 30 mol% GlcCer, egg PC (30 mol%) and cholesterol (40 mol%). Inset: difference spectra for B and C relative to A.

Intrinsic fluorescence of GLTP changes in response to the molar fraction of GlcCer in vesicles

The emission maxima of apo-GLTP and GLTP treated with vesicles containing egg PC/cholesterol were both found at 340 nm, while for GLTP treated with vesicles containing 30 mol% of GlcCer this maximum was shifted to 328 nm (Figure 2). The inset highlights the spectral changes observed by taking difference spectra before and after treatment with vesicles. Such difference spectra were also recorded in the presence of vesicles (Figure 3), both for vesicles containing no GlcCer (panel A), or with 30 mol% GlcCer (panel B) or various concentratios of GlcCer (Figure 4). Fluorescence signals monitored at 340 nm increase linearly with higher vesicle concentrations. However, this increase was found to arise from light scattering off the vesicles, as similar linear changes were observed in the absence of GLTP (data not shown). While also these titrations do not provide evidence for GLTP binding to

the vesicles, an affinity constant for GlcCer can be obtained by plotting the intercepts of the straight lines fitted to the data as a function of mol% GlcCer (inset Figure 4). From the hyperbolic function fitted to the data an apparent affinity constant of 9 ± 3 mol% was calculated for GlcCer. Taken together with the observation that only 50% of total GlcCer can be accessed by GLTP for the vesicles used, the true affinity constant should be 4.5 mol%.

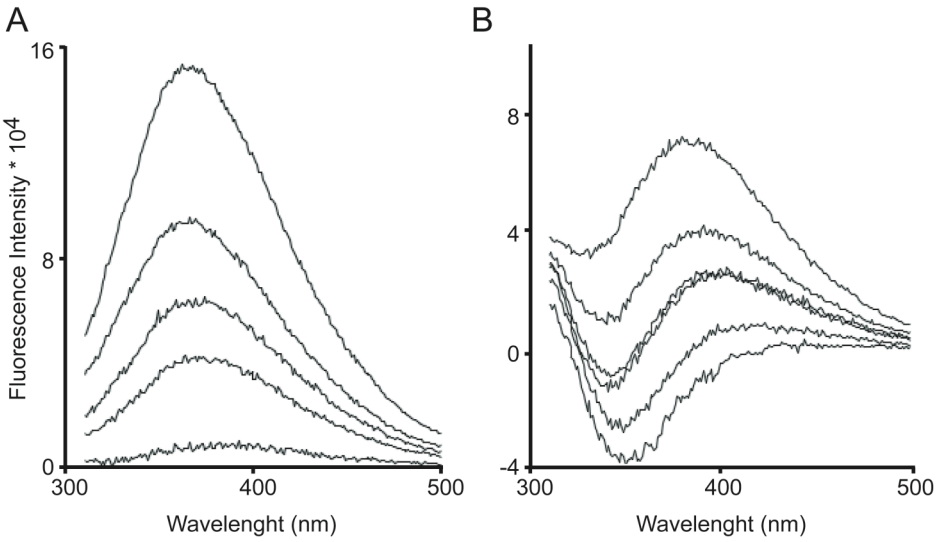


Figure 3: Fluorescence difference spectra obtained by titration of GLTP (0.5 μM) with increasing amounts of vesicles. Panel A titration with vesicles (total lipid 10, 45, 65, 100, 155 μM) containing 60 mol% egg PC and 40 mol% cholesterol panel B: titration with vesicles (total lipid:10, 24, 45, 65, 100, 155 μM) containing 30 mol% GlcCer, 30 mol% egg PC and 40 mol% cholesterol.

Vesicular integrity depends on GLTP concentration

In order to check the integrity of the vesicles before and after addition of GLTP trace amounts of either ^{3}H-labeled GlcCer or PC were added to vesicles. Vesicles at all compositions were pelleted almost exclusively (91 ± 3%) after centrifugation in the absence of GLTP. Strikingly, in the presence of a high concentration of GLTP (100 μM) and total lipid (1 mM), 76% of the lipid remained in solution (Table 1). At low concentrations of GLTP (0.5 μM) and total lipid (50 μM) such as used in the fluorescence experiments we found that vesicles only containing PC were not affected by the protein.

A solvent exposed tryptophan residue is involved in binding membrane lipids

Further studies were needed to identify amino acid residues in GLTP that are likely involved in the interaction of the protein with vesicular membrane lipids. In principle, solvent exposed aromatic residues (Trp, Tyr or His) can be identified using photo-CIDNP NMR. Resonance signals of these residues could become enhanced after interaction with a laser excited dye, but are attenuated when lipid molecules compete with the dye for interaction with the aromatic residues. Photo-CIDNP NMR spectra were taken of wild type GLTP (Figure 5A), of GLTP species incubated with vesicles (2.5 mM total lipid), either composed of GlcCer / egg PC / cholesterol (30/30/40 mol%, respectively; Figure 5B) or egg PC/ cholesterol (60/40 mol%, Figure 5C).

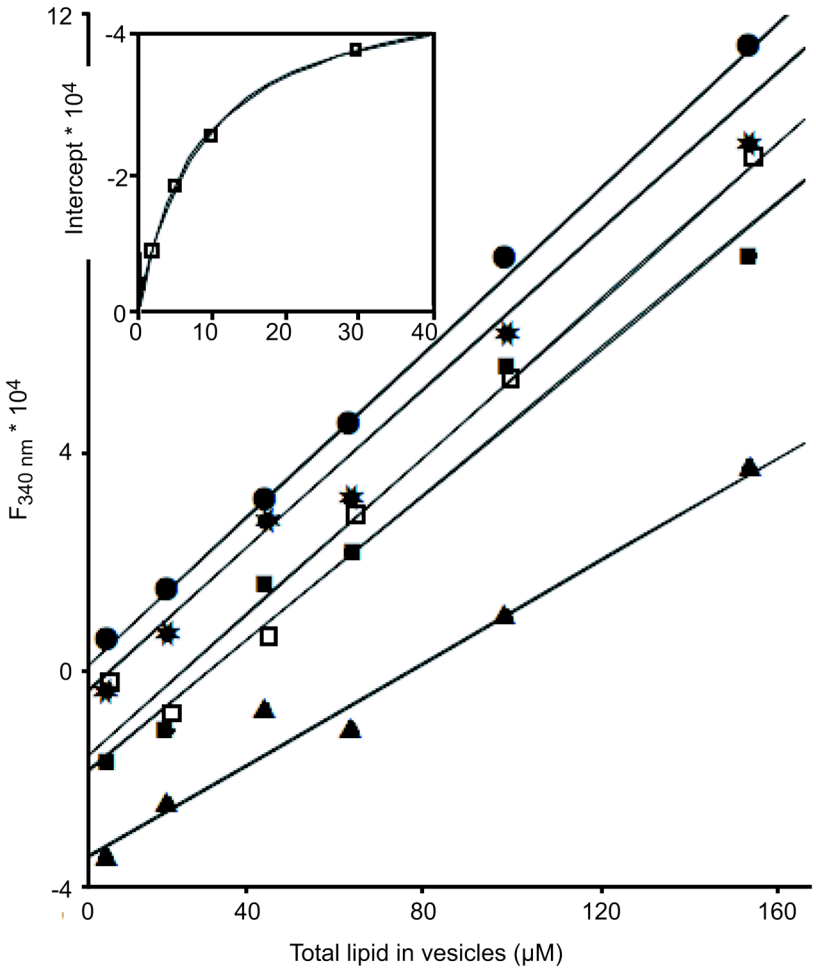


Figure 4: Titration studies of GLTP (0.5 µM) with vesicles containing several mol fractions of GlcCer (0mol% ●; 2 mol% ✷;5 mol% □; 10 mol% ■ ;30 mol% ▲). Total lipid concentrations were varied from 5 µM to 160 µM. Straight lines were fitted to data points taken from fluorescence difference spectra at 340nm. Inset: replot of intercepts derived from the straight lines as a function of GlcCer content in vesicles added. Data are fitted to a hyperbolic function

Table 1: Radioactivity (% ^{3}H-labeled lipid in solution and pellet) measured after centrifugation of mixtures containing GLTP and vesicles.

Vesicle composition + 40% cholesterol	Total lipid (mM)	GLTP (µM)	% ^{3}H in solution	% ^{3}H in pellet
60% egg [^{3}H]PC	0.05	0	6 (± 1)	94 (±1)
60% egg [^{3}H]PC	0.05	1	7 (±2)	93 (±2)
60% egg [^{3}H] PC	1	100	76 (±8)	24 (±8)
30% GlcCer/ 30% egg [^{3}H] PC	0.05	0	12 (±3)	88 (±3)
30% [^{3}H] GlcCer/ 30% egg PC	1	0	10	90
30% [^{3}H] GlcCer/ 30% egg PC	1	100	87	13

After centrifugation (1h, 100,000g) spectra were recorded for the GLTP species at 0.25 mM. For both kind of vesicles the resonance positions of all peaks in the spectra remain the same as for apo-GLTP. The resonances observed at 6.8, 6.9, 7.75, and 7.8 ppm are assigned to the single highly exposed Trp142 in GLTP (Mok and Hore, 2004) both in apo-GLTP and in GLTP containing glycolipid. Solvent exposure of Trp residues in GLTP was derived using the program DSSP (Kabsch and Sander, 1983). To confirm that the CIDNP signal derived from Trp142, mutant GLTP W42Y was used in the CIDNP experiment (Figure 5D) which did not give rise to a signal. A small resonance at 10.2 ppm is assigned to the indole N1H of Trp142. This resonance is no longer visible in the spectrum for GLTP incubated with vesicles containing egg PC /cholesterol only (Figure 5C). The narrow line widths of the Trp142 resonances in this 24 kDa protein point to a rather high flexibility of this exposed residue. Interestingly, two highly solvent exposed Tyr residues (Tyr153 and Tyr157) are not observed by photo-CIDNP. In an attempt to observe exposure of aromatic residues other than Trp142 photo-CIDNP NMR studies of GLTP species were carried out at elevated temperatures. However, already at 40 °C the protein was found to denature and precipitate due to aggregation. This de-

naturation was rather modest for GLTP treated with vesicles containing 30 mol% GlcCer.

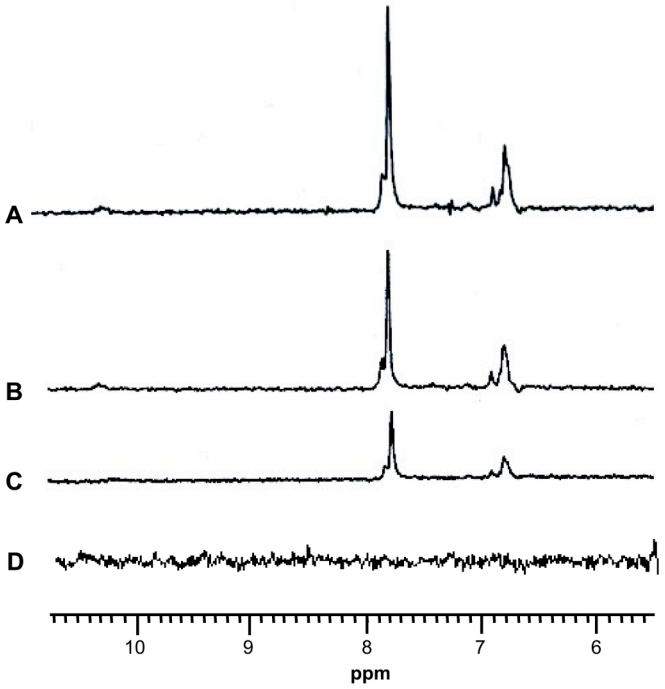


Figure 5: Photo-CIDNP spectra of **A:** apo-GLTP **B:** GLTP incubated with vesicles (2.5 mM total lipid) containing GlcCer (30 mol%), egg PC (30 mol%) and cholesterol (40 mol%) or **C:** with vesicles (2.5 mM total lipid) containing egg PC (60 mol%) and cholesterol (40 mol%). D: Photo-CIDNP spectrum of mutant GLTP W142Y. Protein concentrations were 0.25 mM for all.

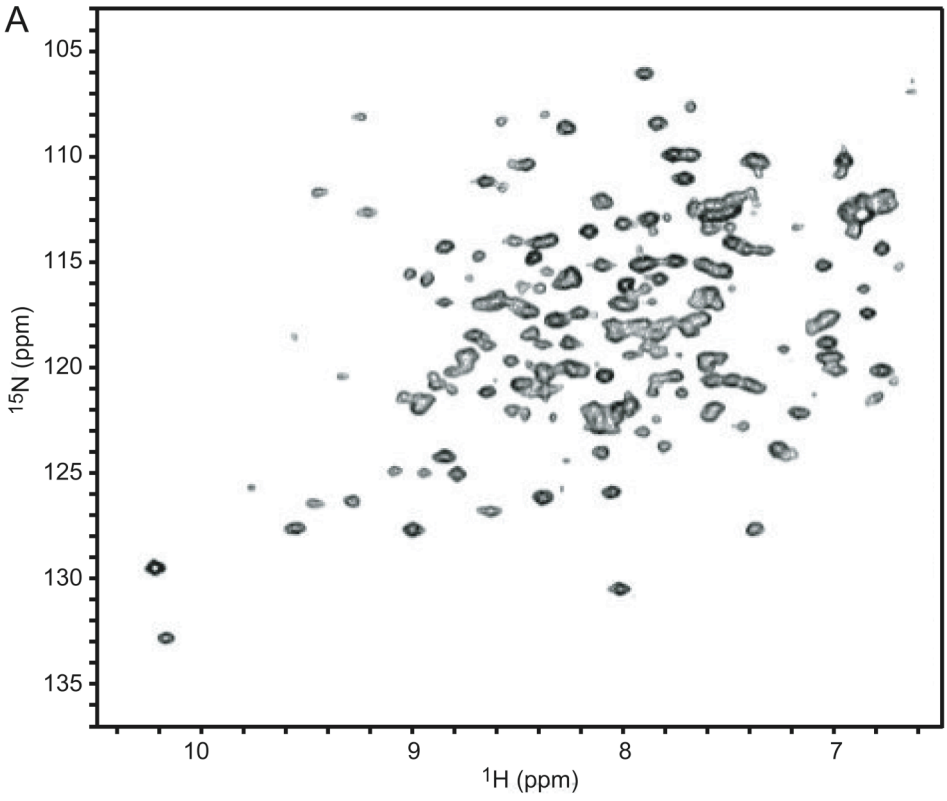
Structural consequences for GLTP upon uptake of glycolipid

Direct evidence for structural changes in GLTP resulting from incubations with vesicles containing 30 mol% GlcCer was obtained from 2D NMR measurements. HSQC spectra of apo-GLTP and an overlay of apo-GLTP with protein incubated with vesicles containing 30 mol% GlcCer were taken (Figure 6). Careful inspection of the data reveals shifted NH resonances after incubation of GLTP with these vesicles. Due to the limited solubility of the protein and protein denaturation no detailed information about the residues involved in binding of the glycolipid could be obtained, however.

Protein denaturation and the influence of bound GlcCer were studied further by CD experiments (Figure 7). For all GLTP species the loss of α-helical content with increasing temperatures was found to be independent of protein concentration. Lipid-free GLTP starts to denature at 43°C, while GLTP incubated with vesicles only containing PC and cholesterol even appears to be slightly less stable. In contrast, GLTP treated with vesicles containing 30 mol% GlcCer is much more stable than the other two species, as it starts to unfold at 53°C.

Discussion

Proteins interacting with poorly water-soluble lipids are generally faced with the problem of how to react specifically and rapidly with lipid molecules when these are present in aggregated form such as micelles or membrane vesicles. The kinetics of molecular processes at lipid-water interfaces require special treatment (Berg and Jain, 2002). For a protein at least two separate binding steps - and binding domains - are needed for proper interaction with aggregated lipids, one interfacial binding step and another step involving a single lipid molecule.



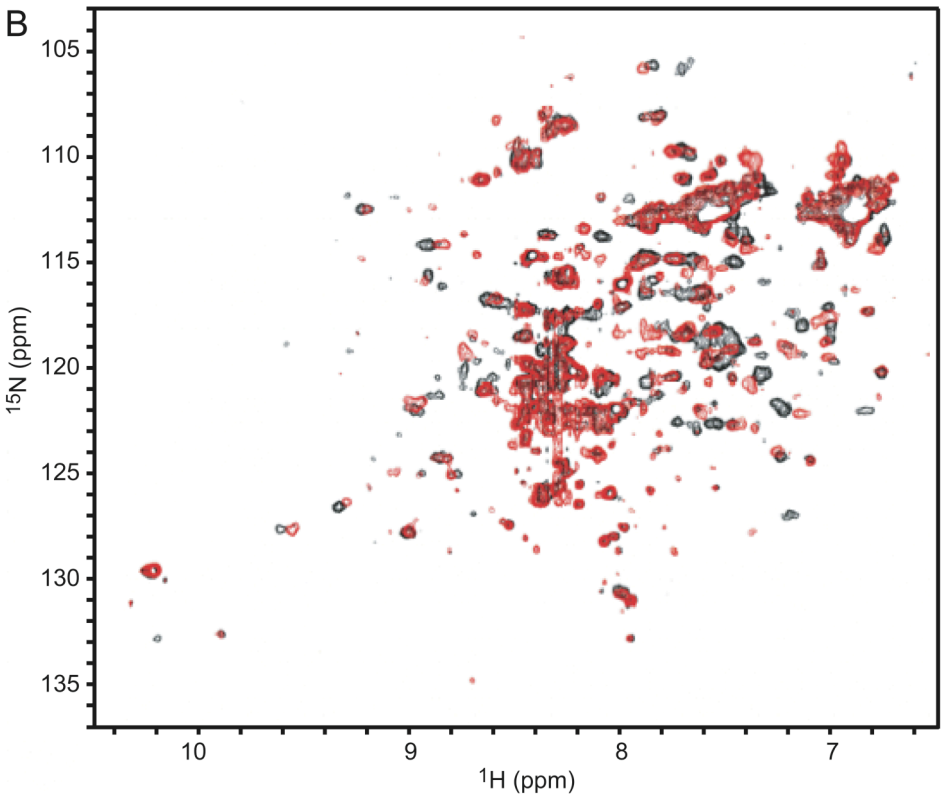


Figure 6: A: HSQC (heteronuclear single quantum correlation) spectrum of apo-GLTP **B:** Overlay of HSQC spectra of apo-GLTP and GLTP treated with vesicles containing 30 mol% GlcCer (red). Protein concentrations are 0.3 mM.

Using unilamellar vesicles containing 30 mol% GlcCer we find single turnover uptake of this lipid by GLTP to be a fast process. Furthermore, we obtained direct evidence that only half of the total amount of glycolipid in the vesicles is accessible to GLTP (Wong et al., 1984; Rao et al., 2004). This indicates that under these experimental conditions the vesicles stay intact even when all glycolipid has been extracted from the outer leaflet of the vesicular membrane. Titrations of fixed amounts of vesicles with GLTP yielded an affinity constant of GLTP for GlcCer uptake from these vesicles of approximately 25 nM. This value is close to the concentration of GLTP (10- 30 nM for small or large unilamellar vesicles) at which half maximal transfer activity was observed between donor and acceptor vesicles in previous work (Rao *et al.,* 2004). In our studies we observed signal changes due to glycolipid uptake from donor vesicles in the absence of acceptor vesicles when GLTP is present in µM amounts. Thus, for obtaining proper kinetic data in fluorescence based resonance energy transfer assays GLTP concentrations should be far below the total concentration of transferred glycolipid.

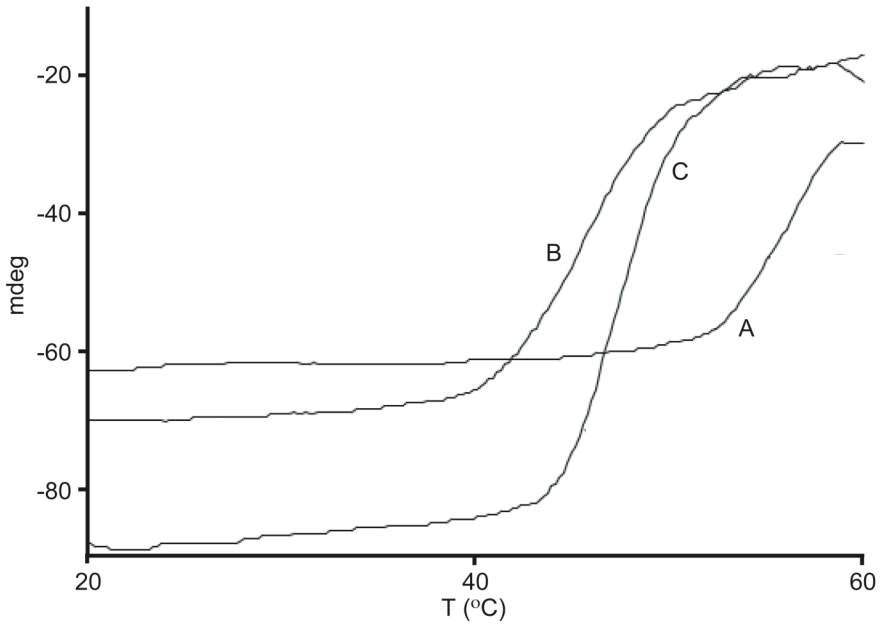


Figure 7: Changes in alpha helical content monitored at 220 nm for several GLTP species as a function of temperature. A: apo-GLTP, B: GLTP treated with vesicles consisting of 60 mol% egg PC and 40 mol% cholesterol, C: GLTP treated with vesicles containing 30 mol% GlcCer, 30 mol% egg PC and 40 mol% cholesterol. Protein concentrations were 10 μM for A, and 7 μM for B and C, respectively.

Attempts were made to study membrane binding in more detail by monitoring the intrinsic fluorescence of GLTP when titrated with vesicles containing cholesterol (40 mol%), egg-PC and GlcCer. The latter two lipids were varied such that the molar fraction of GlcCer varied between 0-30 mol%, while their total remained at 60 mol%. As described before (Li *et al.,* 2004) binding of glycolipid into the binding pocket of GLTP leads to a blue shift in the intrinsic fluorescence. In a recent study using tryptophan variants (West *et al.,* 2006) the importance of Trp96 for glycolipid transfer by GLTP was demonstrated. Therefore it is very likely that the blue shift in intrinsic fluorescence upon glycolipid uptake can be attributed to perturbation of Trp96 in GLTP. Using this intrinsic fluorescence we conclude from our titrations that GlcCer binding into the binding pocket is sensitive to the mole fraction of GlcCer present in the vesicles rather than its total concentration. In contrast to the high preference for GlcCer uptake no binding constant could be obtained for interaction of GLTP (at 0.5 μM) with the vesicles used up to 160 μM total lipid. Recently, direct evidence of the interaction of GLTP with vesicular membranes was obtained using labeled phospholipids (Rao *et al.,* 2005). From this work it was concluded that GLTP binds to vesicles also in the absence of glycolipid, but binding constants

are poor in the absence as well as the presence of glycolipid (around 200 µM total lipid). Given these binding constants it can be questioned whether vesicles stay intact when incubated with GLTP at protein concentrations around 200 µM. From our incubations of GLTP with radiolabeled lipids we concluded that the protein in the low µM ranges does not perturb the vesicular membranes. However, at high protein levels (*e.g.* 0.25 mM, as needed for NMR experiments), vesicular membranes were destroyed indeed leaving most of the lipid bound to the protein. This allowed us to identify amino acids at the surface of GLTP that are likely involved in membrane binding.

Using photo-CIDNP NMR an exposed and flexible Trp residue was clearly seen and identified as Trp142 based on its spectral properties (Mok and Hore, 2004), available structural data (Malinina *et al.,* 2004), solvent accessibility calculations (Kabsch and Sander, 1983) and point mutation of Trp142. The photo-CIDNP NMR signals were considerably reduced when GLTP was incubated with vesicles of various compositions. The strongest reduction was noted for 0.25 mM GLTP incubated with 2.5 mM vesicles containing 60 mol% of egg PC. We propose egg PC to cluster near Trp142 thereby suppressing the photo-CIDNP signals. This would indicate that Trp142 together with nearby solvent exposed hydrophobic residues (among which Ile143 and Ile147) form part of the interfacial binding domain of GLTP. We propose a model indicating a possible involvement of Trp142 in membrane binding and uptake of GlcCer (Figure 8). The protein does not need to penetrate deeply into the membrane for allowing transport of a glycolipid molecule to its binding pocket. When GLTP is incubated with vesicles containing 30 mol% GlcCer and 30 mol% egg PC (either lipid in three-fold molar excess over GLTP) the photo-CIDNP signals show higher intensities as found for vesicles not containing glycolipid. This can be explained when a substantial fraction of GlcCer is bound into the glycolipid binding pocket near Trp96 (Yamada et al., 1985; West et al., 2006), which is remote from the interfacial binding site around Trp142. Thus, in contrast with previous suggestions (Rao et al., 2005; West et al., 2006) we do not find evidence for a direct involvement of Trp96 in membrane binding. The Chemical Shift Pertubation data obtained upon treatment of GLTP with vesicles containing 30 mol% GlcCer clearly indicate that considerable conformational changes take place in the protein due to interaction with GlcCer. Close inspection of the crystal structure data for apo-GLTP and protein containing glycolipid (Malinina *et al.,* 2004), leads to the conclusion that about 20 backbone atoms of amino acid residues in close proximity to the glycolipid change their position in the structure. Since protein flexibility is likely needed for proper accommodation of GlcCer, the influence of GlcCer on protein stability was considered to be of interest. The effect of heat treatment on GLTP was investigated by CD measurements. While the α-helical content was found to drop rapidly at temperatures above 43°C, the presence of bound glycolipid improved the stability of GLTP by 12°C. In contrast, lipid vesicles

without glycolipid slightly destabilized the protein. Heat stability of GLTP has been studied before using glycolipid transfer rates as a measure of protein denaturation (Nylund and Mattjus, 2005), however, the stabilizing effect of bound glycolipid remained unnoticed.

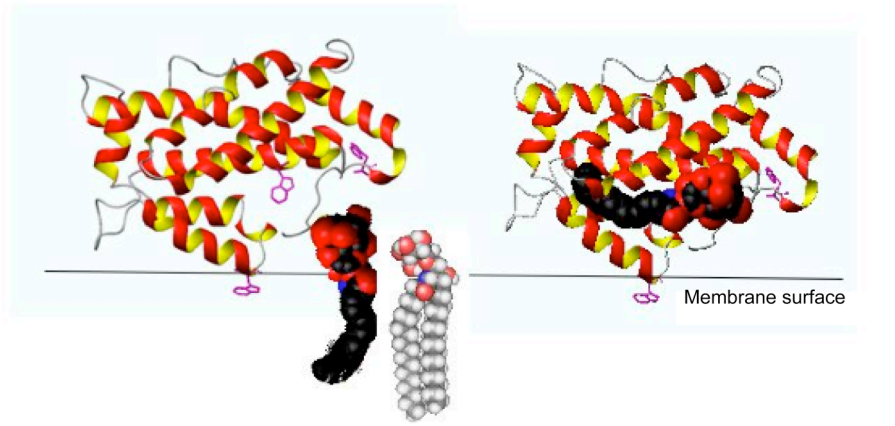


Figure 8: GLTP structures showing apo-GLTP (from 1swx.pdb, e.g. at http://www.ebi.ac.uk/msd) with the location of the three Trp residues present and GLTP containing bound LacCer (from 1sx6.pdb). The suggested role for Trp142 in interfacial binding is indicated. Trp96 becomes hidden by bound glycolipid after transfer to the binding pocket.

Consequences of high affinity for GlcCer for in vivo action of GLTP

Considering the high affinity of GLTP for GlcCer but the very low affinity for membranes one could assume that GLTP is required to keep GlcCer in a soluble form within the cytosol. As such GLTP could act as a kind of buffer for GlcCer and deliver it in situations it is needed or to special organelles in specialized cells. Compared to other lipid transfer proteins like FAPP2, which has a PH domain that targets the protein to the Golgi apparatus (Godi *et al.,* 2004) or CERT, which contains a PH domain for binding to the Golgi and a FFAT domain for binding to VAP proteins in the ER (Kawano *et al.,* 2006) it is difficult to identify membranes that GLTP would bind to. We and others (Rao *et al.,* 2005) find that the efficiency of uptake of glycolipid by GLTP depends on the mole fraction of the glycolipid. This characteristic of GLTP is likely important in defining donor or acceptor membranes *in vivo*. However, up to date there are no techniques available to measure lipid transfer *in vivo*, which would help to answer the questions concerning the identity of donor and acceptor membranes *in vivo*.

Summary

In conclusion we have found that the efficiency of uptake of glycolipid by GLTP depends on the mole fraction of the glycolipid in the outer leaflet of vesicular membranes. Binding of GlcCer to the protein already occurs at nM levels of GLTP. It is proposed that glycolipid transfer efficiency will be determined mainly by the rate of release of glycolipid from GLTP. For efficient transfer *in vivo* most likely additional factors are needed for proper GLTP action.

Acknowledgments

We thank Peter Mattjus for providing us with pQE9-GLTP, Gert Folkers for advising us in defining proper conditions for preparation of protein for HSCQ analysis and Hans Wienk for helping us with setting up the HSQC experiments and data processing. M.O. thanks the NUFFIC Huygens program for financial support.

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Pre- and Post-Golgi Translocation of Glucosylceramide in Glycosphingolipid Synthesis

Based on:

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Summary
Glycosphingolipids are controlled by the spatial organization of their metabolism and by transport specificity. Using immuno-EM we localize to the Golgi stack the glycosyltransferases that produce glucosylceramide (GlcCer), lactosylceramide and GM3. GlcCer is synthesized on the cytosolic side and must translocate across to the Golgi lumen for lactosylceramide synthesis. However, only very little natural GlcCer translocates across the Golgi *in vitro*. As GlcCer reaches the cell surface when Golgi vesicular trafficking is inhibited, it must translocate across a post-Golgi membrane. Concanamycin, a V-ATPase inhibitor, blocks translocation independently of multidrug transporters known to translocate short-chain GlcCer. Concanamycin did not reduce lactosylceramide and GM3 synthesis. Thus, GlcCer destined for glycolipid synthesis follows a different pathway and transports back into the ER via the late-Golgi protein FAPP2. FAPP2-knockdown strongly reduces GM3 synthesis. Overall we show that newly synthesized GlcCer enters two pathways, one towards the non-cytosolic surface of a post-Golgi membrane and one, via the ER, towards the Golgi lumen lactosylceramide synthase.

Introduction
Sphingolipids comprise a relatively small but vital fraction of the mammalian membrane lipids (Holthuis et al., 2001). Sphingomyelin (SM) carries a phosphocholine headgroup on a ceramide backbone and occurs in every mammalian cell, just like glucosylceramide (GlcCer). GlcCer serves as the basis for a highly polymorphic set of complex glycosphingolipids (GSLs). The unique physicochemical properties of sphingolipids allow different modes of interaction with their environment. Sphingolipids are concentrated at the cell surface and endocytotic membranes, where their bulk presence provides the membranes with chemical and mechanical stability. In addition, sphingolipids have the tendency to cluster with cholesterol in an environment of glycerolipids. The roles of sphingolipids in protein sorting, signaling and membrane deformation may therefore be explained by their ability to form lateral domains that specifically in- or exclude membrane proteins. In addition, the broad diversity in glycosidic structure allows individual GSLs to interact specifically with proteins, including viral and bacterial pathogens like Shiga-, and cholera-toxin. The mode of action of sphingolipids in cellular processes depends on their concentration in the various subcellular organelles and the transbilayer and lateral distribution in those membranes. How cells sense and control the sphingolipid concentration of their membranes is largely unknown, but the spatial organization of metabolism, the action of translocators and selectivity of transport are three important determinants, which are intrinsically linked. Many but not all enzymes of sphingolipid metabolism have been identified, some of them only very recently: the SM synthase family (Huitema et al., 2004; Yamaoka et al., 2004) and two non-lysosomal glucocerebrosidases (Yildiz et al., 2006; Boot et al., 2007; Hayashi et al., 2007).

One contemporary challenge is to unravel how sphingolipid metabolism is organized and controlled in the cellular context.

Ceramides are synthesized in the ER by various ceramide synthases (Pewzner-Jung et al., 2006). Some cell types express the galactosylceramide (GalCer) synthase, which acts on the lumenal side of the ER (Sprong et al., 1998; 2003). However, all other synthetic enzymes of ceramide-containing lipids are located in the Golgi, with the exception of the SM synthase 2 at the plasma membrane (Huitema et al., 2004). Newly synthesized ceramides are transported from the ER to the Golgi where they are converted by the GlcCer synthase (GCS) and the SM synthase 1 (SMS1). An important question is how cells regulate the ceramide supply to these enzymes. A first clue has come from the finding that the synthesis of SM, but not GlcCer, depended on ceramide transport to the trans-Golgi by CERT (Hanada et al., 2003), a pathway that is regulated via phosphoinositides, sterols and CERT phosphorylation (Perry and Ridgway, 2006). However, both SMS1 and GCS have been localized biochemically to the cis-medial Golgi, while GCS has also been assigned to pre-Golgi and trans-Golgi membranes (Futerman et al., 1990; Jeckel et al., 1990, 1992; Futerman and Pagano, 1991; Kohyama-Koganeya et al., 2004). The current agreement is that GlcCer is synthesized on a cytosolic surface and translocates across the Golgi membrane for higher GSL synthesis in the late Golgi (Lannert et al., 1994; 1998).

However, the consensus picture is based on evidence obtained before the recent identification of early enzymes in sphingolipid metabolism and transport. In addition, the bulk of the lipid data has been collected using short-chain sphingolipid analogs by the lack of tools to study natural sphingolipids. Such short-chain analogs have much higher off-rates from membranes and spontaneously exchange between membranes (Pagano et al., 1981). In addition, they are less well ordered in the membrane (Wang and Silvius, 2000). We have therefore addressed the following questions: (1) Where are the various enzymes situated along the Golgi stack? (2) Is natural GlcCer translocated across the Golgi membrane by multidrug transporters (van Meer et al., 2006)? (3) Is there a function for the glycolipid binding proteins GLTP in the cytosol (West et al., 2004) and FAPP2 on the trans-Golgi (Godi et al., 2004) in GSL metabolism and transport (Godi et al., 2004; Malinina et al., 2004; Vieira et al., 2005; 2006)? Using a novel assay for the transmembrane translocation of natural GSLs we report that GlcCer can reach the plasma membrane via non-vesicular transport and translocates to the cell surface. In contrast to the short-chain lipid analogs, natural GSLs are not translocated by the multidrug transporters but by a novel mechanism that, in turn, does not recognize short-chain lipids. In addition, we have uncovered a pathway used by most GlcCer to reach the Golgi lumen. It involves transport from the Golgi to the ER by the glycolipid binding protein FAPP2.

Results

Sphingolipid synthesis concentrates in the late Golgi and depends on the ceramide transport protein (CERT)

Because the intra-Golgi localization of the SMS1 and GCS is a matter of controversy (see Introduction), we first addressed their intracellular localization by immuno-fluorescence and -EM (IEM). Attempts to localize the endogenous enzymes of mammalian cells with specific antibodies have failed so far, most likely because of their low expression levels. As an alternative, we ectopically expressed epitope-tagged constructs in HeLa cells, and determined their cellular distribution using fluorescence microscopy (Fig. 1, Suppl. Fig. 1A) and EM (Fig. 2). In the confocal fluorescence microscope, both GCS and SMS1 displayed a virtually identical staining pattern with endogenous GM130, a cis Golgi marker (Nakamura et al., 1995), in transiently transfected HeLa cells (Fig. 1). Neither SMS1 nor GCS was found in the nuclear envelope and ER. To determine the intra-Golgi localization of GCS and SMS1, HeLa cell lines stably expressing these enzymes were analyzed by IEM. Golgi stacks containing five cisternae were used for statistical analysis, and endogenous GM130 was used as a cis-Golgi reference for every image. The intra-Golgi distribution of a protein was determined by counting all gold-particles over a Golgi-stack (n=20 per protein) after which the number of gold particles found over a specific cisterna was expressed as percentage of total gold (Fig. 2B). Indeed, GM130 was restricted mostly to the cis-most cisterna (81%), with only little label in the next cisterna (Fig. 2A,B). In contrast to GM130, labeling for GCS was found in all five cisternae but with higher concentrations in cisternae 3-5 (medial-trans). SMS1 was mostly present at the trans-side of the Golgi, and clearly peaked in the fourth cisterna. Quantitation showed that GCS and SMS1 had a significantly differential distribution: almost 50% of GCS localized to the first three cisternae, whereas only 33% of SMS1 was found there. Notably, SMS1 and GM130, which showed almost complete overlap by fluorescence microscopy, displayed different intra-Golgi distributions localization by IEM: GM130 was concentrated at the cis- and SMS1 at the trans-side of the Golgi.

LacCer synthesis not only depends on the presence of GlcCer, but also on the localization of the LacCer synthase (LCS) and the UDP-Gal transporter (UGT). LCS was found in all cisternae, but peaked in cisternae 4 and 5. The location of the UGT was more restricted to the medial-trans Golgi and peaked in cisternae 3 and 4. LacCer is further converted to GM3 by transfer of sialic acid from CMP-sialic acid. The GM3 synthase (GM3S) and the CMP-sialic acid transporter (CST) localized exclusively to the Golgi stack, predominantly to the trans side (Fig. 2). Although a 100-fold overexpression of a Golgi-resident protein had no effect on the distribution of this protein within the HeLa Golgi (Rabouille et al., 1995), overexpression of a tagged version of a protein could lead to mislocalization.

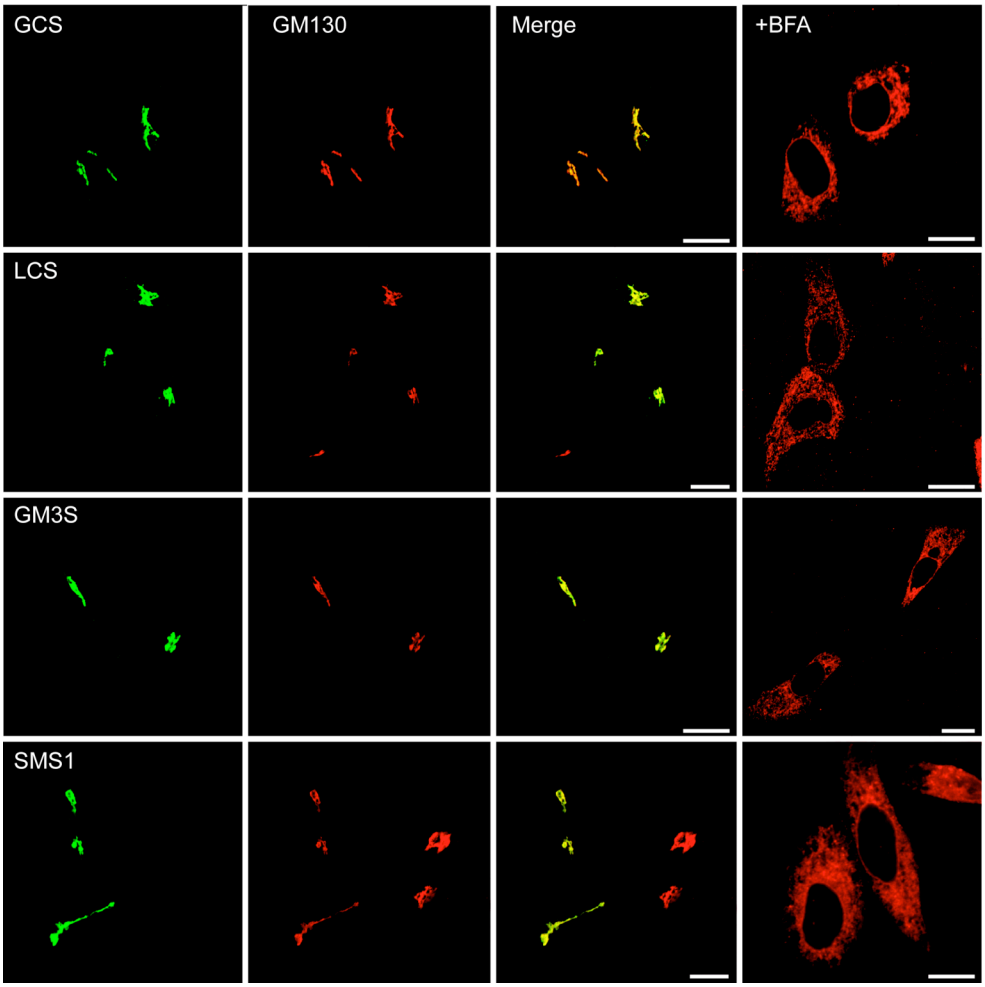


Figure 1. Cellular localization of enzymes in Golgi sphingolipid synthesis by confocal fluorescence microscopy. HeLa cells were transiently transfected with the epitope tagged enzymes (Table I). After 18 h cells were fixed, permeabilized, and labeled with rabbit antibodies against the HA or V5 epitopes and with mouse antibody against GM130, a cis-Golgi matrix protein. Cells were counterstained with FITC-labeled anti-rabbit (left column) and Texas red-labeled anti-mouse (GM130) antisera. Overlapping distributions appear as yellow in the merged images (Merge). After brefeldin A (BFA) treatment for 0.5 h, the glycosyltransferases were labeled with the specific antibodies followed by Texas-red labeled secondary antibodies. BFA fuses the Golgi stack to the ER. GCS, GlcCer synthase; LCS, LacCer synthase; GM3S, GM3 synthase; SMS1, SM synthase 1. For GCS, the transfected cells displayed 6.4x the activity of wild-type cells. Bars, 10 μm.

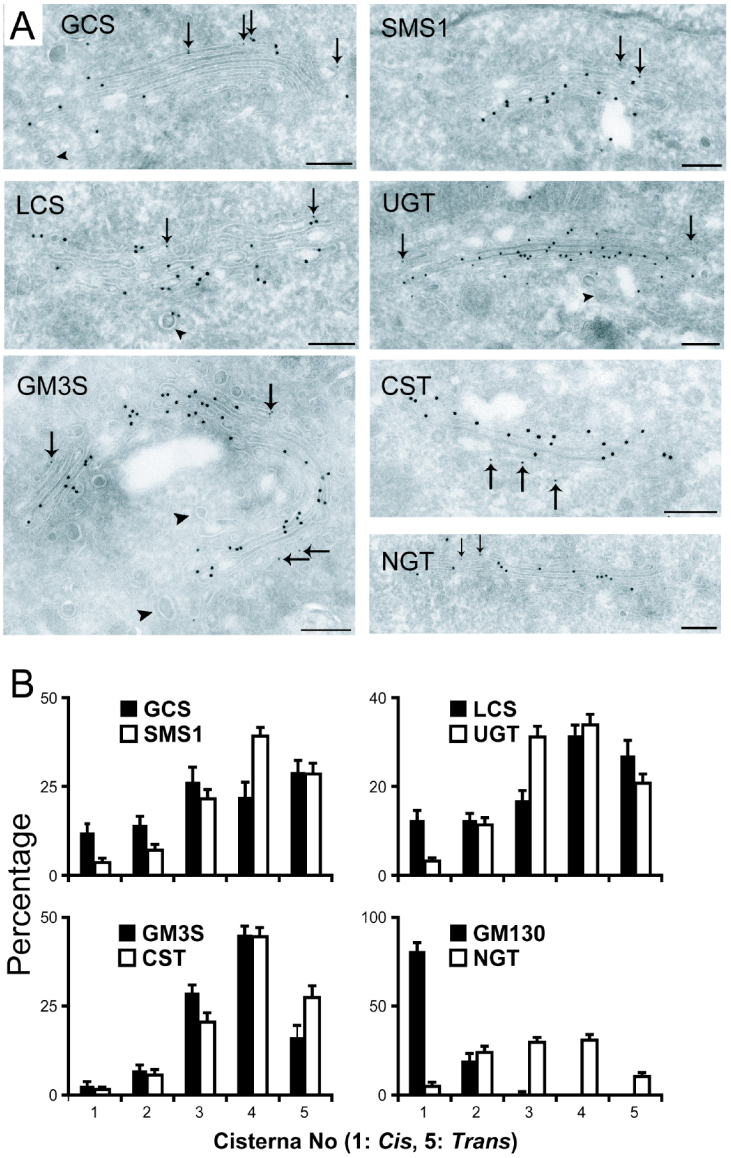


Figure 2. Cellular localization of enzymes in Golgi sphingolipid synthesis by IEM. (A) HeLa cells, stably transfected with the epitope tagged enzymes (Table I), were double-labeled for GM130 (10 nm protein A-gold; indicated by arrows) as a cis-Golgi marker and the specific enzyme (15 nm protein A-gold). Only little labeling was present outside the Golgi in all cases, and this was similar to the signal found on untransfected cells. Clathrin coats, indicated by arrowheads, were often present on vesicles or tubules close to the TGN. Abbreviations as in Fig.1; CST, CMP-sialic acid transporter (SLC35A1); NGT, UDP-N-acetylglucosamine transporter (SLC35A3) ; UGT, UDP-Gal transporter (SLC35A2). Bars: 200 nm. (B) For each enzyme, the gold-labeling was quantified in Golgi stacks that con-

tained five cisternae. The cisterna labeled with the cis-Golgi reference GM130 was denoted "1". The number of gold particles per cisterna was expressed as a percentage of the total gold particles within that Golgi stack: average % ± SEM (n=20).

As one internal control, we therefore determined the localization of the UDP-GlcNAc transporter (NGT), which provides the medial-Golgi GlcNAc transferase with its substrate. It localized to the 3 medial cisternae with only little labeling in the first and last Golgi cisternae (Fig. 2A,B). Taken together, the data indicate that the enzymes synthesizing LacCer and GM3 show a preferential medial-trans localization, like the SMS1, whereas the GCS is more evenly distributed over the Golgi stack.

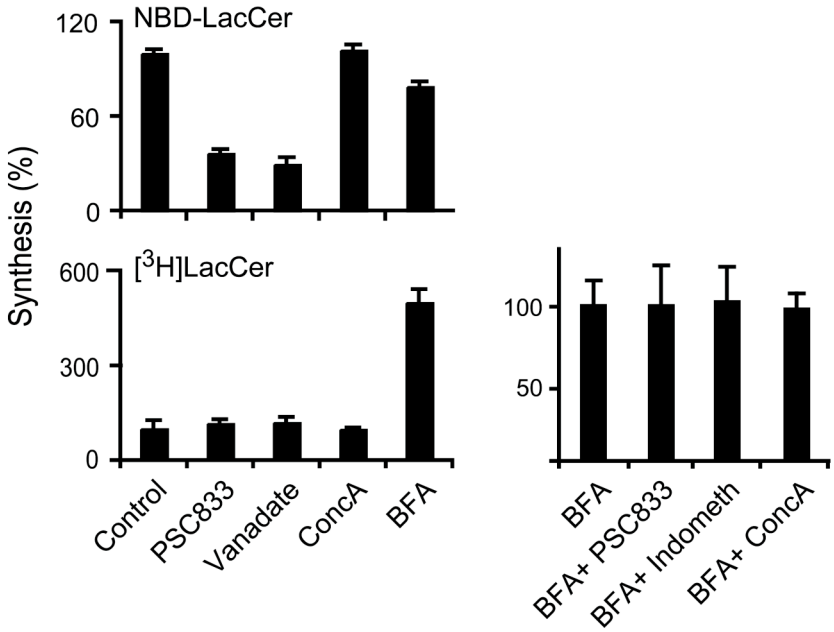


Figure 3. Natural GlcCer translocates less efficiently across the Golgi than across the ER membrane. Postnuclear supernatants (PNS) were prepared from HeLa cells that had been (mock) treated with 1μg/ml BFA. Equal volumes of PNS were incubated with or without 5 μM PSC833, 20 μM indomethacin (indometh), 1 mM vanadate or 5 nM concanamycin A (ConcA) for 10 min at 37°C, followed by a 1 h incubation with 2 mM UDP-Gal and 1 μM C_6-NBD-GlcCer complexed to BSA or 66 kBq /ml [^{3}H]GlcCer solubilized with recombinant glycolipid transfer protein, GLTP. Each panel represents an independent set of experiments (n=3). Lipids were extracted, analyzed and expressed as percentage of LacCer synthesized in the control.

Translocation of short-chain but not natural GlcCer across the Golgi membrane
Because GlcCer is synthesized at the cytosolic aspect of the Golgi (see Introduction), we next addressed the question whether GlcCer directly flips to the lumenal side where it can be utilized for complex GSL synthesis. Exogenously added C_6-NBD-GlcCer was readily converted to C_6-NBD-LacCer in a PNS of HeLa or CHO cells (Burger et al., 1996; Lannert et al., 1998; Sprong et al., 2003; Fig. 3). The translocation was 70% reduced by PSC833, an inhibitor of the ABC transporter ABCB1, a translocator for C_6-NBD-GlcCer (van Helvoort et al., 1996) and by vanadate, which blocks the nucleotide binding domain of ABC transporters. To test whether natural GlcCer translocates across the Golgi membrane, we monitored the conversion of exogenous [^{3}H]GlcCer to LacCer after insertion into the cytosolic leaflet of membranes in the HeLa PNS by the small cytosolic glycolipid transfer protein GLTP (Suppl. Fig. 2A,B). However, only little [^{3}H]LacCer synthesis was observed unless the Golgi had been fused to the ER by BFA prior to the experiment, in which case the synthesis of [^{3}H]LacCer, but not C_6-NBD-LacCer, went up 5-fold (Fig. 3). The translocation of [^{3}H]GlcCer was not inhibited by PSC833 or indomethacin, inhibitors of the ABC transporters ABCB1 and ABCC1, nor by concanamycin A, a vacuolar proton pump inhibitor. ABCB1 and -C1 translocate short-chain GlcCer (van Helvoort et al., 1996; van Meer et al., 2006) but the translocation of natural GlcCer across the ER-Golgi membrane was independent of these multidrug transporters.

Transport of simple but not complex glycosphingolipids continues with brefeldin A
In order to see whether GlcCer could translocate towards the outer leaflet of the plasma membrane, from where it can reach the site of LacCer synthesis (Trinchera et al., 1990; Sprong et al., 2001), we labeled the newly synthesized lipids with [^{14}C]palmitate, and after various times extracted the GSLs from the cell surface by GLTP. Based on previous experience, the assay was optimized starting from a 1,000-fold higher GLTP concentration than that used for the delivery of GlcCer to PNS membranes in Fig. 3, and the standard assay conditions were defined as 45 min at 37˚C with 1.5 mg/ml GLTP (Suppl. Fig. 2C,D). Addition of acceptor liposomes to the medium inhibited rather than stimulated GlcCer extraction by GLTP (Suppl. Fig. 2E), most likely because GLTP interacted with the liposomes even if they contained no GSLs, thereby lowering the free GLTP concentration. Under these conditions, GLTP extracted 30-50% of the radiolabeled GlcCer and 20-70% of the GM3 from various cell types in 45 min, but less than 0.5% of the SM and of the glycerophospholipids (Fig. 4A-C). To check whether the phospholipid distribution across the membrane was affected by the extraction of the GSLs, the exposure on the cell surface of inner leaflet phosphatidylserine was measured using FITC-labeled annexin V. No significant annexin binding was observed in the GLTP-treated cells as compared to a positive control (Fig. 4D). BFA induces fusion between the Golgi and the ER and blocks vesicular traffic from the merged ER-

Golgi to the plasma membrane. In MF cells BFA did not affect GM3 synthesis (Suppl. Fig. 4) but fully blocked its transport (Fig. 4A), in line with relocation of GM3S to the ER and the absence of a non-vesicular pathway for GM3 transport (Warnock et al., 1994). The same was observed for SGalCer in the oligodendrocytic D6P2T cells (Fig. 4C). In contrast, GlcCer transport persisted in the presence of BFA. GalCer (and galactosyldiacylglycerol), which are synthesized in the ER lumen of CHO cells transfected with the GalCS and of D6P2T cells, behaved identically to GlcCer.

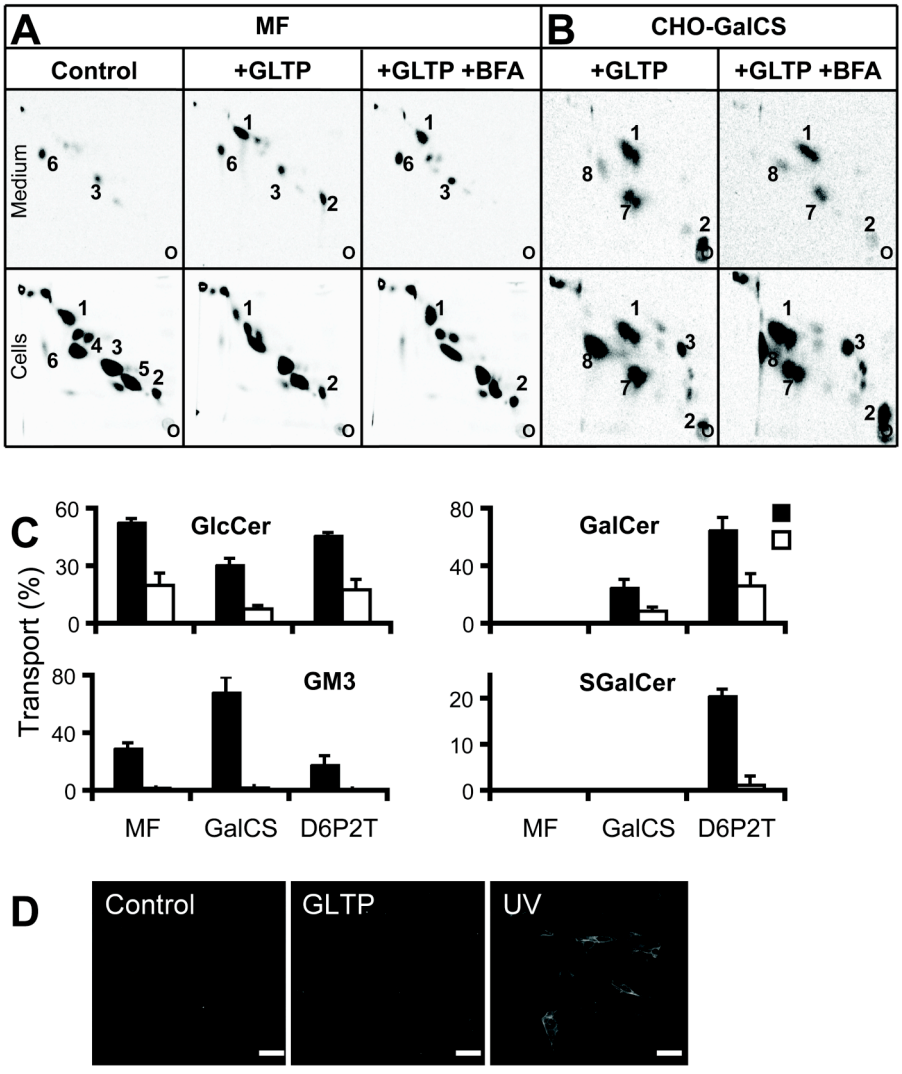


Figure 4. BFA completely blocks transport to the surface of complex glycolipids, but not of GlcCer and GalCer. (A) 2D-TLC radiogram of a typical experiment in which MF cells pretreated ± 1 μg/ml BFA and labeled with [^{14}C]palmitate for 1.5 h were incubated for 45 min at 37°C with 1.5 mg/ml GLTP. The lipids were extracted from cells and medium, separated by 2D-TLC and analyzed by phosphor-imaging (see Methods). The marked lipids are (1) GlcCer, (2) GM3, (3) PC, (4) phosphatidylethanolamine, (5) SM, (6) precursor [^{14}C]palmitate. The origin is marked by (o). 1st dimension: NH_4OH solvent. (B) CHO-GalCS cells were labeled with [^{14}C]galactose for 1.5 h +/– 1 μg/ml BFA, and transport of GlcCer, GalCer, and GM3 was assessed in a subsequent 45 min incubation with 1.5 mg/ml GLTP (medium), again +/– BFA. Lipid numbering as under (A), plus (7) GalCer and (8) galactosyldiacylglycerol. 1st dimension: NH_4OH solvent; borate plates. (C) MF, CHO-GalCS, and D6P2T cells were treated as under (A, B). Lipid transport as percent of that lipid found in the medium was calculated from at least 3 independent experiments for each lipid, each in triplicate. HeLa cells synthesized Gb3 instead of GM3, which could not be tested because its synthesis is interrupted by BFA (Suppl. Fig. 4). (D) MF cells were incubated with or without 1.5 mg/ml GLTP for 45 min at 37°C, fixed, stained with FITC-conjugated annexin-V, and analyzed by fluorescence microscopy. As a positive control, MF cells were forced into apoptosis by overnight growth in serum-free medium, exposure to 200 J/m^2 UV light and incubation in regular medium for 2 h at 37°C. The pictures were taken using identical settings. Bar: 10 μm.

Transfer proteins GLTP and FAPP2

The facts that GlcCer and GM3 were synthesized in the same compartment in the presence of BFA (Fig. 1), and that GlcCer but not GM3 reached the cell surface under these conditions implied that GlcCer had reached the plasma membrane in the absence of vesicular traffic. Because natural GlcCer, in contrast to short-chain C_6-NBD-GlcCer, does not readily exchange through the cytosol, we tested whether the transport in the presence of BFA was mediated by a glycolipid transfer protein. FAPP2, a protein that shares the glycolipid binding domain with GLTP, has been found ubiquitously expressed (Godi et al., 2004). However, the knockdown of FAPP2 in MEB4 cells had no effect on transport of newly synthesized GlcCer to the cell surface in the presence of BFA (Fig. 5A). Likewise, a knockdown of GLTP in D6P2T cells did not reduce transport of GlcCer (or GalCer, not shown) to the plasma membrane in the presence of BFA (Fig. 5B), indicating that the transport from the merged ER-Golgi to the plasma membrane in the presence of BFA was not mediated by these proteins, or that they are redundant. On the other hand, GLTP overexpression stimulated the transport of GlcCer (and GalCer) in the presence of BFA two-fold showing that GLTP *in vivo* is able to fulfill this function (Fig. 5C). Unexpectedly, FAPP2 knockdown in MEB4 cells reduced transport to the plasma membrane by 30% in the absence of BFA, and the knockdown of GLTP in D6P2T cells reduced transport of GlcCer (and also of GalCer, not shown) by 30-50%, implying that more than 30-50% of the GlcCer that reached the cell under normal conditions had been transported by FAPP2 or GLTP followed by translocation across a post-Golgi membrane. We did not succeed in making a double

knockdown for FAPP2 and GLTP, possibly because this is a lethal combination. Transport of GM3 in MEB4 cells was not reduced by FAPP2 knockdown. As before, it was effectively blocked by BFA (not shown).

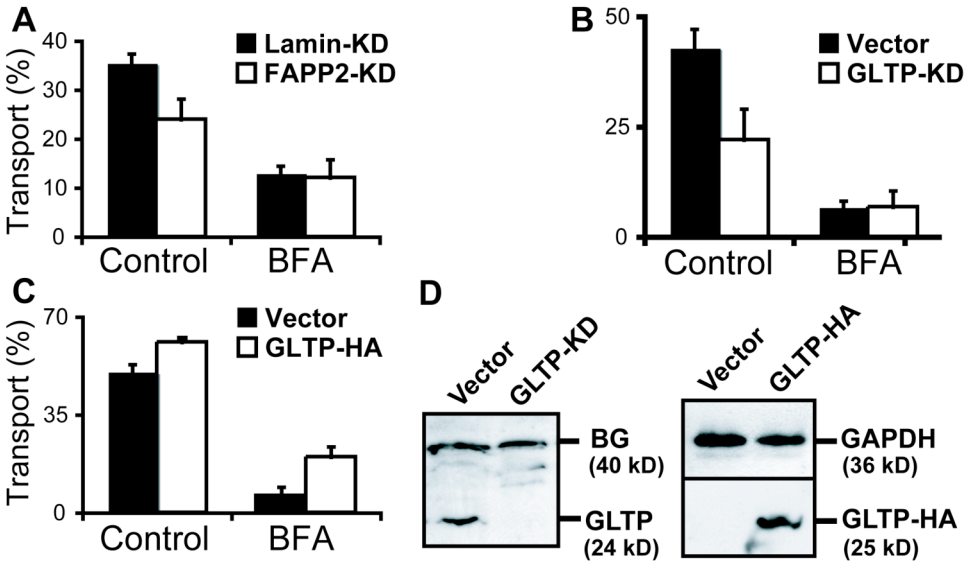


Figure 5. Effect of the expression levels of FAPP2 and GLTP on glycosphingolipid transport to the cell surface. (A) MEB4 cells stably expressing RNAi plasmids against FAPP2 or lamin were pre-incubated with or without 1μg/ml BFA for 0.5 h and labeled with [^{14}C]palmitic acid for 1.5 h. [^{14}C]GSLs were extracted from the cell surface during an additional 45 min-incubation with 1.5 mg/ml GLTP in the medium. The lipids in the cells and media combined with the washes were analyzed and quantified. Transport is expressed as the percentage of GlcCer recovered in the medium. (B, C) D6P2T cells stably expressing RNAi plasmids against GLTP or lamin (B) or stably transfected with GLTP (C) were treated with BFA and labeled with [^{14}C]galactose, and the transport of [^{14}C]GlcCer to the cell surface was measured as under (A). (D) Western blot analysis from D6P2T clones expressing either RNAi plasmids (B) against GLTP and lamin as control, or pCDNA3.1 plasmids with GLTP-HA (C) using a rabbit antiserum against mGLTP. A background band at ~40 kD (BG) or a blot against GAPDH were used as loading controls.

In contrast to C_6-NBD-GlcCer, natural GlcCer is not translocated by ABCB1 or C1 but by a novel mechanism sensitive to concanamycin A

We have previously reported that a variety of short-chain GlcCer analogs, including C_6-NBD-GlcCer, was transported to the cell surface by the multidrug transporters ABCB1 and ABCC1 (see van Meer et al., 2006). We therefore addressed whether the transport of natural GlcCer to the cell surface in the presence of BFA (Fig. 5) was mediated by these ABC transporters, by using a mouse fibroblast cell line derived from the Abcb1a/Abcb1b/Abcc1 triple-knockout (TKO) mouse (Wijnholds et

al., 2000), vs. TKO-cells stably transfected with human ABCB1 (MDR1). Transport of C_6-NBD-GlcCer was strongly reduced in TKO cells (down to zero with BFA) and was partially restored by transfection with ABCB1 (Fig. 6A), demonstrating that the translocation of C_6-NBD-GlcCer across the plasma membrane of MF cells was fully due to Abcb1 and Abcc1. In line with this, the C_6-NBD-GlcCer translocation in MF cells was reduced by specific inhibitors (PSC833 for Abcb1a/b) or general inhibitors (glibenclamide, vanadate) of ABC-transporters (Fig. 6B). In strong contrast, the knockout of the multidrug transporters had no effect on the transport of natural GlcCer neither in the absence, nor in the presence (not shown), of BFA. Transfection of the TKO cells with ABCB1 did not increase translocation of natural GlcCer, nor was there a significant effect of the various inhibitors, with one exception: concanamycin A, a specific inhibitor of the vacuolar proton ATPase (Bowman et al., 2006), essentially abolished the translocation of natural GlcCer without effect on C_6-NBD-GlcCer (Fig. 6B). Also in the absence of BFA, GlcCer transport to the cell surface was virtually abolished by concanamycin A (Fig. 6C), which cannot be explained by an inhibition of vesicular traffic as indicated by the mild reduction in GM3 transport. Concanamycin had no effect on the synthesis of GM3 from newly synthesized GlcCer (not shown).

GlcCer readily reaches the lumen of the ER in vivo

Because concanamycin did not affect the rate of GM3 synthesis, GlcCer destined for GM3 synthesis must have followed a different transport pathway, independent of the post-Golgi translocation that was inhibited by concanamycin. We therefore tested the possibility that this GlcCer reached the site of LacCer synthesis in the lumen of the Golgi via the ER. To test whether GlcCer is present in the ER lumen, we introduced an enzyme into the ER lumen, the SGalCer synthase, that transfers a sulfate from PAPS to GalCer but also GlcCer (Gasa et al., 1990) but that normally acts in the trans-Golgi in conjunction with the PAPS transporter (Farrer et al., 1995). A chimera (SGCS) was constructed of the SGalCer synthase to an HA-tagged PAPS transporter. In MEB4 cells SGCS showed a diffuse reticular staining and clear staining of the nuclear envelope. The patterns of SGCS and the cis-Golgi marker GM130 were mutually exclusive (Fig. 7A). In accordance with its location in the ER, SGCS remained sensitive to EndoH digestion (Fig. 7B) implying the presence of high mannose N-glycans. The molecular mechanism retaining the SGCS in the ER has remained unclear. It may contain a previously hidden ER retention signal or maybe the complex is partially unfolded. When SGCS-transfected MEB4 cells were labeled with [^{35}S]H_2SO_4, one major radioactive band appeared on TLC plates that ran faster than SGalCer (Fig. 7C). When GlcCer and [^{35}S]PAPS were added to a PNS of MEB4 cells transfected with the SGCS construct a lipid product was made that ran above SGalCer. Synthesis depended on GlcCer, on PAPS and on the transfection. A lipid with the same mobility on TLC was found in D6P2T cells. The band disappeared when the cells had been incubated with the GCS in-

hibitor *N*B-DNJ, identifying the unknown lipid as SGlcCer (Fig. 7D; Suppl. Fig. 3A). In addition, it was absent after a GCS-knockdown but not an LCS-knockdown (Suppl. Fig. 3B), which efficiently inhibited the synthesis of LacCer (not shown). Finally, similar amounts of the [^{35}S]lipid were synthesized in SGCS-transfected CHO and mutant CHO-lec8 cells (Suppl. Fig. 3C), which have three-fold reduced LacCer levels (Sprong et al., 2003). Thus, GlcCer reached the lumen of the ER where it was converted to SGlcCer by SGCS.

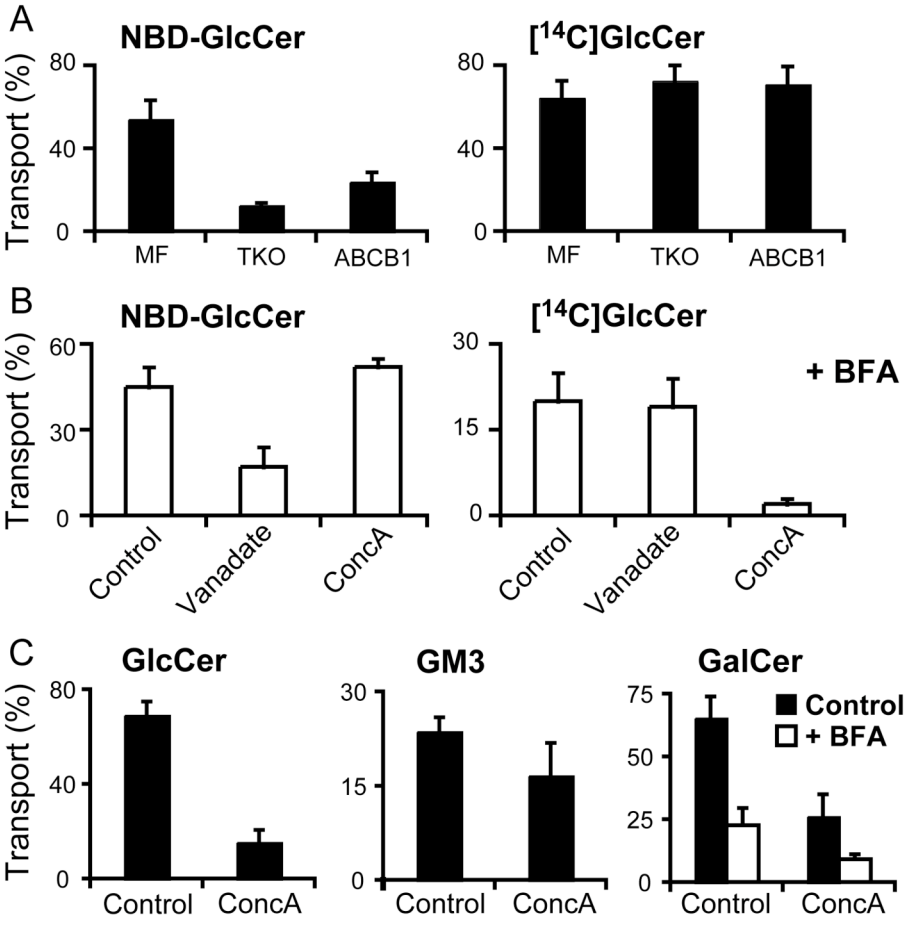


Figure 6. In contrast to C_6-NBD-GlcCer, endogenous GlcCer is translocated by a mechanism that does not depend on multidrug transporters but is blocked by concanamycin A.

(A) MF cells, TKO cells, a mouse fibroblast line with a triple knockout for the multidrug transporters Abcb1a, -b1b, and -c1, and TKO cells transfected with human ABCB1, were incubated with C_6-NBD-ceramide for 1 h or [^{14}C]palmitic acid for 1.5 h at 37˚C. Labeled GlcCer was extracted from the cell surface by BSA and GLTP, respectively, and quantitatively analyzed as described under Methods. The same experiment was carried out in the presence of

BFA (not shown). (B) MF cells were pre-incubated with 1μg/ml BFA, plus or minus 1 mM vanadate or 5 nM concanamycin A (ConcA) for 0.5 h at 37°C, and incubated with C_6-NBD-ceramide with BSA or [^{14}C]palmitate followed by GLTP, all in the presence of the inhibitors. 5 μM PSC833, 20 μM indomethacin, and 50 μM glibenclamide gave similar results to vanadate both, + and – BFA. (C) MF cells (GlcCer, GM3) or D6P2T cells (GalCer) were pre-incubated with or without 1 μg/ml BFA and 5 nM concanamycin A for 0.5 h at 37°C, followed by incubations with [^{14}C]palmitate and GLTP, all in the presence of the inhibitors. Labeled GlcCer was extracted from the cell surface, and quantified as under (A). Transport was calculated as % of the specific lipid that was recovered in the medium.

Role of FAPP2 in GlcCer transport to the ER

Selective GlcCer transport from the cytosolic surface of the Golgi back to the ER would be most easily explained by the activity of a cytosolic transfer protein, followed by a translocation across the ER membrane. We therefore tested the involvement of the glycolipid transfer protein FAPP2 in this pathway. When lamin- and FAPP2-knockdown cells were transfected with SGCS and labeled with [^{35}S]H_2SO_4, SGlcCer synthesis in the FAPP2-knockdown cells was only 30% of that of the control (Fig. 7D-F). In MEB4 cells FAPP2-knockdown resulted in a 2-fold decrease in the synthesis of GM3 with a concomitant accumulation of GlcCer (Fig. 7G). Similar results (Fig. 7H) were obtained in MDCKII cells where FAPP2 expression was reduced by retrovirus-mediated RNAi (Vieira et al., 2005). GalCer levels in these cells were normal, indicating that ceramide levels in the ER were unaffected. FAPP2 specifically affected the conversion of GlcCer to GM3 via LacCer. These experiments show that FAPP2, which binds to the Golgi (Suppl. Fig. 1A) via PI4P (Godi et al., 2004), plays an important role in the transport of GlcCer to the sulfation site in the ER and to the site of LacCer and GM3 synthesis in the lumen of the Golgi. Concanamycin A did not affect GlcCer translocation across the ER membrane (Fig. 3), nor did it affect GM3 synthesis from newly synthesized GlcCer (not shown) showing that ER translocation occurred by a different mechanism, and that the pathways of newly synthesized GlcCer to the Golgi lumen and to the plasma membrane are independent.

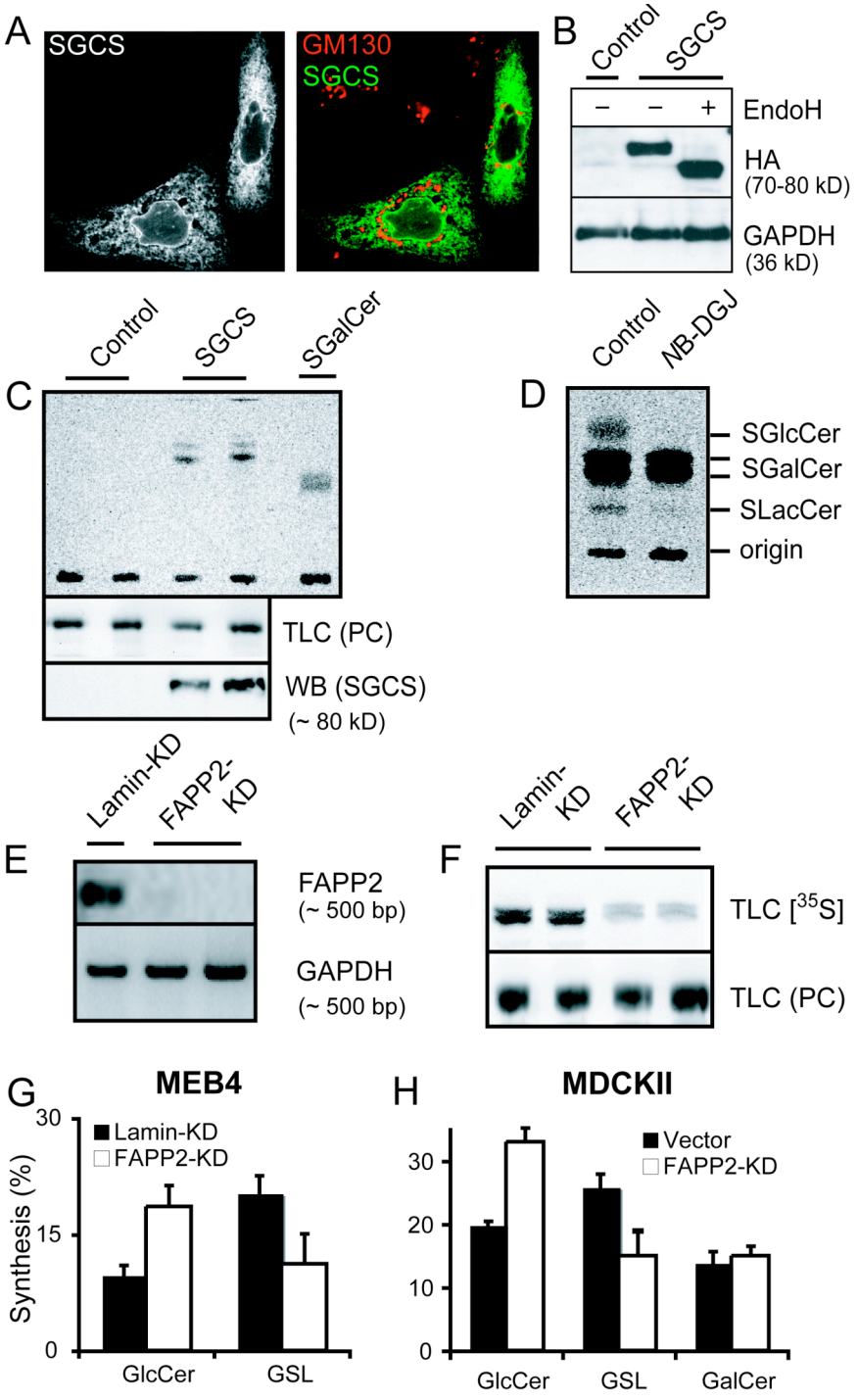

A
SGCS
GM130
SGCS
B
Control
SGCS
– – +
EndoH
HA
(70-80 kD)
GAPDH
(36 kD)
C
Control
SGCS
SGalCer
TLC (PC)
WB (SGCS)
(~ 80 kD)
D
Control
NB-DGJ
SGlcCer
SGalCer
SLacCer
origin
E
Lamin-KD
FAPP2-KD
FAPP2
(~ 500 bp)
GAPDH
(~ 500 bp)
F
Lamin-KD
FAPP2-KD
TLC [35S]
TLC (PC)
G
MEB4
Lamin-KD
FAPP2-KD
Synthesis (%)
30
15
0
GlcCer
GSL
H
MDCKII
Vector
FAPP2-KD
30
20
10
0
GlcCer
GSL
GalCer

Figure 7: Transport of newly synthesized GlcCer to the lumen of the ER and its conversion to GM3 are greatly reduced by knocking down FAPP2. (A) MEB4 cells were transiently transfected with a construct containing the sulfo-GalCer synthase and the PAPS transporter (SGCS). After 18 h the cells were fixed and labeled with rabbit antibodies against the HA tag of SGCS and with mouse antibodies against GM130, followed by FITC- (left) and Texas red-secondary antibodies (right). Bar: 10 µm. (B) Cells (mock-) transfected with SGCS were washed and lysed in 50 mM sodium citrate, pH 5.5, 2 mM EDTA, 1 mM 2-mercaptoethanol, 0.1% SDS, protease inhibitors. Half of the transfected sample received endoglycosidase H (EndoH), and all samples were incubated for 6 h at 30°C. Proteins were resolved on 7.5% SDS-PAGE followed by Western blot detection with rabbit anti-HA antibodies. The housekeeping enzyme GAPDH served as a loading control. (C) MEB4 cells (mock-) transfected with SGCS were incubated with 600 kBq [^{35}S]H_2SO_4/10^5 cells. After 24 h the lipids were extracted, separated by 1D-TLC, and stained with iodine to visualize the bulk lipid phosphatidylcholine (PC) as a loading control. [^{35}S]lipids were visualized by phosphor-imager screens. Western blot analysis with anti-HA antibodies was performed on parallel dishes. (D) D6P2T cells were labeled overnight with [^{35}S]H_2SO_4 in the presence or absence of 40 µM of the GCS inhibitor *N*-butyldeoxygalactonojirimycin (*N*B-DGJ), after which the lipids were analyzed by TLC and phosphor-imaging. The more hydrophilic spot was most likely SLacCer. (E) Semi-quantititative RT-PCR on RNAs from MEB4 clones stably expressing RNAi plasmids against PCR fragments of FAPP2 or lamin, as a control, to detect expression levels of FAPP2, and GAPDH as a control. (F) MEB4 cells stably expressing RNAi plasmids against FAPP2 or lamin were transiently transfected with SGCS. After 18 h, cells were labeled with 3,600 kBq/ml [^{35}S]H_2SO_4 for 8 h. The lipids were analyzed as under (C). Parallel dishes had equal expression of SGCS as checked by Western blotting against HA (not shown). (G, H) MEB4 cells stably expressing RNAi plasmids against FAPP2 or lamin, and MDCKII cells infected with empty virus (control) or with retrovirus-mediated RNAi against FAPP2 were labeled overnight with 72 kBq/ml [^{14}C]galactose. Lipids were extracted, separated by 2D-TLC, and radiolabeled lipids were quantified using a phosphor-imager as described in Materials and Methods. GSL: LacCer plus GM3 (MEB4) and a mix of complex GSLs (MDCKII). Values are expressed as a percentage of total [^{14}C]lipids, and are the mean of 3 experiments in triplicate.

Discussion

Until recently, sphingolipid assembly in the Golgi was thought to be organized according to the same simple linear model as glycoprotein processing (Fig. 8A). Ceramide synthesized in the ER would, because it is not water-soluble, be transported by the secretory pathway to the cis-Golgi where it would be converted to SM by SMS (Futerman et al., 1990; Jeckel et al., 1990) and to GlcCer by GCS (Futerman and Pagano, 1991; Jeckel et al., 1992). Subsequently, GlcCer would encounter the enzymes of complex GSL synthesis in an ordered array along the Golgi stack (Trinchera et al., 1990a). The finding that GlcCer is synthesized on the cytosolic surface but converted to LacCer in the lumen forced the question whether and how GlcCer crosses the Golgi membrane. This seemed solved by (a) the finding that short-chain GlcCer analogs were able to cross the Golgi membrane (Lannert et al., 1994; 1998; Burger et al., 1996), (b) the identification of the multidrug transporters

ABCB1 and ABCC1 as floppases for these molecules (see van Meer et al., 2006), and (c) a correlation between ABCB1 activity and complex GSL synthesis in living cells (see de Rosa et al., 2004). However, the mechanism of SM synthesis is much more intricate, in that SMS receives its substrate via the cytosolic transfer protein CERT, that binds to ER and trans-Golgi and is regulated at various levels via phosphorylation (Perry and Ridgway, 2006; Kumagai et al., 2007; Fugmann et al., 2007).

Also the synthesis and processing of GlcCer are more sophisticated than suspected (Fig. 8B): (a) The GCS is concentrated in the trans-Golgi, not in the cis-Golgi, and its activity was inhibited over 2-fold upon CERT knockdown. (b) In contrast to short-chain GlcCer, natural GlcCer did not flop efficiently across the Golgi membrane and was not a substrate for the multidrug transporter ABCB1. (c) Instead, newly synthesized GlcCer reached the outside of the plasma membrane by a non-vesicular transport pathway and was translocated via a mechanism that was inhibited by concanamycin A, an inhibitor of the vacuolar ATPase, suggesting the involvement of a proton gradient. (d) Finally, most GlcCer reached the LCS in the Golgi lumen via the ER, with a role for the trans-Golgi glycolipid-binding protein FAPP2 in shuttling GlcCer to the ER. This suggests that GlcCer may play a role in transport and sorting events at the ER. In addition, FAPP2 appears to regulate complex GSL synthesis.The finding that multiple pathways remove GlcCer from the cytosolic surface of the trans-Golgi suggests the possibility that GlcCer exerts a physiological function at that location.

Intra Golgi organization of sphingolipid metabolism

The finding that SM synthesis depends for its ceramide supply on the ER/trans-Golgi protein CERT suggested that the Golgi SMS is located in the trans Golgi (Munro, 2003). Indeed, in Hela cells transfected SMS1 peaked in the 4th cisterna out of 5 (Fig. 2). Like Hanada et al. (2003) we observed that CERT knockdown also affected GCS synthesis from [^{14}C]serine, suggesting that [^{14}C]serine derived ceramide is transported by CERT to the GCS in the trans-Golgi. However, these authors reported that GlcCer synthesis from [^{3}H]sphingosine was independent of CERT, suggesting the existence of different ceramide pools and preferential delivery of [^{3}H]sphingosine-derived ceramide by vesicular transport to cis-medial Golgi GCS. A bimodal distribution of GCS, and not SMS, had been observed before by cell fractionation (Futerman and Pagano, 1991; Jeckel et al., 1992), and is now corroborated by IEM of the transfected GCS (Fig. 2). In contrast to the Drosophila GCS (Kohyama-Koganeya et al., 2004), mammalian GCS was not in the ER. In the Golgi lumen, GlcCer is converted to LacCer, which in most cells is efficiently sialylated to GM3. The responsible enzymes, LCS and GM3S, have been assigned to the Golgi stack from the fact that they were still able to utilize newly synthesized GlcCer and LacCer after BFA treatment (see van Meer, 1993). Cell fractionation

assigned LCS to the trans-Golgi (Lannert et al., 1998), and GM3S to the cis-Golgi (Trinchera et al., 1990a) but GM3 synthesis was found highest in the trans-Golgi (Lannert et al., 1998; Allende et al., 2000). IEM located both enzymes and their nucleotide sugar transporters to the trans-Golgi, peaking in cisterna 4 (Fig. 2), whereas NGT, the transporter for UDP-GlcNAc into the medial-Golgi, was more evenly distributed. Biochemical experiments have indicated that LCS and GM3S form a molecular complex (Giraudo and Maccioni, 2003), explaining the efficient utilization of LacCer by GM3S.

Translocation of GlcCer towards the non-cytosolic surface

After synthesis, GlcCer must cross a membrane to reach the LCS in the Golgi lumen. When exogenous GlcCer analogs with shortened fatty tails were added to the Golgi they were converted to LacCer (Lannert et al., 1994; 1998; Burger et al., 1996), a process sensitive to the ABCB1 inhibitor PSC833 (Fig. 3). This suggests translocation by Abcb1, which also translocates newly synthesized C_6-NBD-GlcCer across plasma membranes (van Helvoort et al., 1996; Fig. 6A, B). In contrast, LacCer synthesis from natural GlcCer and translocation of newly synthesized natural GlcCer across the plasma membrane were insensitive to PSC833 (Fig. 6B). The latter translocation was sensitive to concanamycin, which suggests the involvement of a pH gradient. Because endosomal recycling still occurs under BFA (Lippincott-Schwartz et al., 1991), the translocation most likely occurred in the endosomes. How the low pH affects transmembrane translocation of GlcCer is presently under investigation. Although concanamycin has some inhibitory effect on vesicular traffic (Fig. 6C; Weisz, 2003), this does not explain the abolition of cell surface appearance under non-BFA conditions. One other potential translocator for GlcCer, ABCA12, identified in skin (Lefevre et al., 2003), is not expressed in the cell lines used in this study.

The synthesis of LacCer from exogenous GlcCer in a PNS was greatly stimulated by BFA (Fig. 3). This suggests that GlcCer translocation across the trans-Golgi membrane was limiting, and that GlcCer efficiently flopped across the membrane of the ER-Golgi compartment which for 75% consists of ER membrane (but is not the native ER membrane). Also GalCer (and galactosyldiacylglycerol) appears to flip across the ER-Golgi, but in the opposite direction: after synthesis on the lumenal side of the ER-Golgi in the presence of BFA, GalCer still reached the cell surface (Fig. 4B,C). In the absence of vesicular traffic from this organelle to the plasma membrane (as evidenced by the lack of transport of GM3, SGalCer, and proteins: Suppl. Fig. 5), this transport must have involved translocation towards the cytosolic surface of the ER membrane, monomeric transport through the cytosol to the cytoplasmic side of the plasma membrane and outward translocation across the plasma membrane. There are a number of studies in the literature that have addressed lipid flipping in the ER and every lipid tested so far readily moves across

the ER membrane in both directions by an energy-independent mechanism (reviewed in Papadopulos et al., 2007), and we now find that this event is insensitive to inhibition of the multidrug transporters (Fig. 3B).

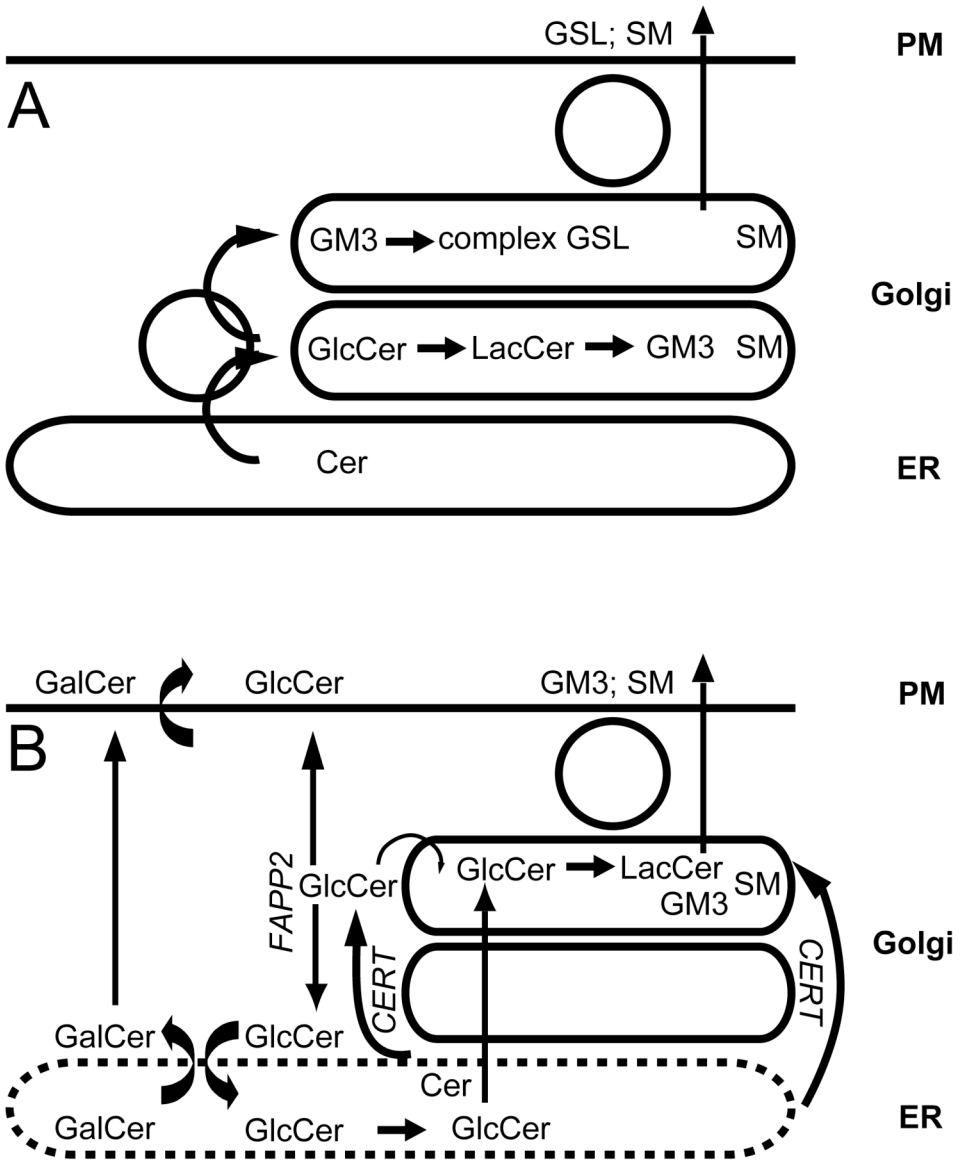


Figure 8: Synthesis and transport of glycosphingolipids. (A) In the traditional model, ceramide moves to the cis-Golgi via vesicular transport, and is converted to SM and GlcCer. GlcCer is further processed by transferases in the trans-Golgi and TGN. (B) In the updated model most ceramide is transported to the trans-Golgi by CERT, SM is produced in the lumen and GlcCer on the cytosolic surface. Most GlcCer is transported back to the ER via FAPP2, while another fraction reaches the cytosolic surface of the plasma membrane (or

endosome) where it is translocated. The ER GlcCer (our assays do not strictly exclude the possibility that this is actually the cis-Golgi) enters the secretory pathway and is converted to LacCer and GM3 in the trans-Golgi, followed by further modification in the TGN, dependent on cell type. It remains unclear whether any GlcCer translocates directly across the Golgi membrane.

Cytosolic GlcCer transport and ER lipid rafts

Under BFA when vesicular transport, measured as appearance of GM3 and SGalCer, was inhibited, natural GlcCer still reached the cell surface (Fig. 4C). This shows that GlcCer synthesized in the ER-Golgi reached the cytosolic surface of the plasma membrane or an endosomal compartment (cf. Warnock et al., 1994). Surprisingly, neither a knockdown of FAPP2 nor of GLTP, the two known cytosolic proteins with a glycolipid binding domain, had an inhibitory effect, suggesting the involvement of a third protein or an independent mechanism like membrane contact sites (Levine and Loewen, 2006). The knockdowns of these proteins did have an inhibitory effect in the absence of BFA, where virtually all GlcCer arriving at the cell surface was translocated by the concanamycin-sensitive non-ER mechanism. This suggests that under normal conditions GlcCer destined for the cell surface is transported by FAPP2 or GLTP from the cytosolic surface of the Golgi to the post-Golgi membrane where subsequent translocation occurs. The observation that the GlcCer transport remaining under BFA conditions was insensitive to FAPP2 or GLTP knockdown suggests that either there is a redundancy between these proteins, or that, possibly, ultrastructural connections between the Golgi and post-Golgi membranes are involved, that are broken by BFA (Lippincott-Schwartz et al., 1991). After translocation of GlcCer to the non-cytoplasmic leaflet of the post-Golgi membrane, part of this GlcCer might recycle to the Golgi where it would be converted to LacCer (Trinchera et al., 1990; Sprong et al., 2001), but this pathway may not be very active considering the lack of effect of concanamycin on GM3 synthesis (not shown).

The finding that GlcCer reached the ER lumen as measured via an assay involving enzymatic modification suggested the involvement of one of the two known cytosolic GlcCer binding proteins. Because MEB4 cells have low levels of GLTP (not shown), we knocked-down FAPP2, a protein with a GLTP domain that is recruited to the trans-Golgi and TGN by PI4P (Godi et al., 2004; Suppl. Fig. 1A). The FAPP2 knockdown dramatically reduced GlcCer transport to the ER lumen (Fig. 7F). FAPP2 may transport GlcCer as a monomer via its GlcCer binding domain, analogous to ceramide transfer by CERT. We can presently not exclude the possibility that FAPP2 is involved in the generation of retrograde vesicles that on their cytosolic surface are enriched in GlcCer. FAPP2 knockdown inhibited GM3 synthesis by 50% (Fig. 7G, H), suggesting that under normal conditions at least half of the GlcCer used for GM3 synthesis passes through the ER. It would be delivered to the

cytosolic ER surface by FAPP2, flip across the ER, and be concentrated into ER-Golgi transport vesicles. This lateral concentration in the ER membrane may be very similar to the proposed formation of lipid rafts in the Golgi (van Meer, 1993). The localization of the Drosophila GCS to the ER (Kohyama-Koganeya et al., 2004) has the same implications. Also GalCer, which is synthesized on the lumenal surface of the ER of some cell types, may contribute to such a mechanism. The occurrence of lipid rafts in the ER (defined by detergent-insolubility) has been reported for yeast (Bagnat et al., 2000) and for mammalian cells, where they were GlcCer dependent (Smith et al., 2006). They were suggested to play a role in protein sorting and toxin retro-translocation across the ER membrane, respectively. The effects of FAPP2 knockdown on sphingolipid-mediated events at the TGN and apical plasma membrane of MDCK cells (Vieira et al., 2005; 2006) may be due to the inhibition by FAPP2 of the GlcCer flux through the ER or to its inhibitory effect on complex GSL synthesis (Fig. 7H).

Nearly all GlcCer that reached the cell surface followed the concanamycin-sensitive pathway (Fig. 6C). Concanamycin did not inhibit transport across the ER-Golgi (Fig. 3), which suggests that the GlcCer that enters the Golgi via the ER is converted to higher glycosphingolipids and does not reach the cell surface as GlcCer. Thus, no GlcCer would pass through the trans Golgi lumen to the plasma membrane, in contrast to GalCer which is not further glycosylated. This may explain why oligodendrocytes synthesize very little SGlcCer as compared to SGalCer (Fig. 7D).

Physiological functions of GlcCer

Apart from being the precursor for the complex GSLs and playing a role in membrane organization as part of lipid rafts on the lumenal surface of cellular membranes, GlcCer may exert specific functions at one other location that is unusual for GSLs, the cytosolic surface of the Golgi and the ER membrane. GlcCer is synthesized on the cytosolic surface of the Golgi, from where it does not simply disappear by translocation across the Golgi membrane. From independent work we have suggested a function for GlcCer on the cytosolic surface of the Golgi in sorting membrane proteins to the melanosomes (Sprong et al., 2001), which we have now identified as a stimulation of the vacuolar proton pump (Wolthoorn et al., man. in prep.). Relevant issues are the sidedness of GlcCer in the ER and the location and mechanism by which it flops to the lumenal side of the membrane. Also the function of the recently discovered GlcCer degrading ER and cytosolic b-glucosidases (Yildiz et al., 2006; Boot et al., 2007; Hayashi et al., 2007), the absence of which results in raised GlcCer levels, remain to be resolved. The fact that the GCS occurs in most eukaryotes, even in 50% of all fungi (Saito et al., 2006), suggests that eukaryotes use GlcCer for basic functions that we are about to unravel, but it is likely that evolution has overlaid the basic principles by many levels of regulation which

may explain why these basic principles of GlcCer action have remained obscure to date.

Materials and Methods

Materials

BSA, fraction V, and other chemicals, if not indicated otherwise, were from Sigma-Aldrich (St. Louis, MO) and used in the highest purity available. Silica TLC plates were from Merck (Darmstadt, Germany), organic solvents were from Riedel de Haën (Darmstadt, Germany), and cell culture media, reagents and FCS were from PAA (Pasching, Austria). Cell culture plastics were from Costar (Cambridge, MA). Tran[^{35}S]label (>37 TBq/mmol), [^{35}S]H_2SO_4 (74 MBq/mmol), D-[1-^{14}C]galactose (1.8 GBq/mmol), [U-^{14}C]palmitic acid (18 GBq/mmol), [9,10(n)-^{3}H]palmitic acid (1.5 TBq/mmol) and L-[3-^{14}C]serine (1.9 GBq/mmol) were from GE Healthcare (Diegem, Belgium) and MP Biomedicals (Amsterdam, Netherlands). Lipids and lipid standards were from Avanti (Alabaster, AL). C_6-NBD-fatty acid was from Molecular Probes (Eugene, OR). C_6-NBD- and [^{3}H]palmitoyl-GlcCer were synthesized as described in Suppl. Material. EST clones were from RZPD (Berlin, Germany). Rabbit antisera against the V5 and HA epitope (Y11) were from Sigma-Aldrich and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. The rabbit peptide-antibody against mGLTP was a kind gift of Kyoko Aikawa-Kojima (Tokyo, Japan). The rabbit serum against GM130 was a gift from Elizabeth Sztul (Birmingham, AL). Mouse anti-V5 antibody was from Invitrogen. Mouse mAb 16B12 anti-HA from BabCO (Richmond, CA). Mouse-anti GM130 antibodies were from BD Biosciences (Erembodegem, Belgium). Mouse anti GAPDH was from Applied Biosystems (Nieuwerkerk a/d lJssel, Netherlands). Fluorescent secondary goat antibodies were obtained from Jackson Immuno-Research Laboratories, Inc. (West Grove, PA). Horseradish peroxidase-conjugated secondary goat anti-rabbit lgG was from DAKO (Glostrup, Denmark). Recombinant GLTP was produced as described under Suppl. Methods.

DNA constructs

Plasmids are described in Table I. ORFs were amplified by PCR using cDNA clones as template. PCR products were gel-purified and cloned into mammalian expression vectors containing a sequence encoding for a triple HA tag (3*YPYDVPDYA) at the 5'- or 3'-end or a single HA or V5 tag (GKPIPNPLLGLDST) at the 3'-end. SGCS is a chimeric construct containing a triple HA tag, the rat 3'-phosphoadenosine 5'-phosphosulfate (PAPS) transporter, a double myc tag as spacer (2*EQKLISEEDL) and the mouse SGalCer synthase. Primers containing RNAi sequences of FAPP2 and lamin were inserted between the BglII and HindIII sides of the RNAi plasmid pKoen (Deneka et al., 2003). All synthetic constructs were verified by restriction analysis and dye termination sequencing of both strands.

Cell culture, transfection and RNAi

HeLa, CHO, CHO-lec8 cells were from ATCC (Rockville, MD). D6P2T cells were a kind gift from Steven Pfeiffer, Farmington, CT (Bansal and Pfeiffer, 1987). CHO-GalCS cells have been described (as CHO-CGalT: van der Bijl et al., 1996). MEB4 cells were from RIKEN Cell Bank (Saitama, Japan). MEB4-pKoen-GCS, and MEB4-pKoen-LCS will be described elsewhere (Wolthoorn *et al., in preparation*). The mouse fibroblast MF and TKO lines (Wijnholds et al., 2000; de Rosa et al., 2004) were from Piet Borst (Amsterdam, Netherlands). MDCK strain II cells infected with recombinant retroviruses carrying short hairpin RNA against FAPP2 have been described (Vieira et al., 2005). All cells were grown in DMEM, stable glutamine, 4.5 g/l glucose, 10% FCS at 37°C with 5% CO_2. Stable transfectants were grown in the presence of 200 U/ml hygromycin B or 0.6 mg/ml geneticin (G418). Cells were transfected using ~1 μl Lipofectamine 2000 and ~0.3 μg DNA per cm^2 cells. For transient protein expression, the cells were used one day after transfection. For the generation of stable transfectants, the cells were trypsinized 24 h after transfection, and divided over 4x 15 cm dishes in culture medium containing hygromycin B or G418. Medium was refreshed weekly, and after approximately 3 weeks individual colonies were selected for green fluorescence in the nucleus (Deneka et al., 2003) and trypsinized using a metal cylinder.

Quantitative immuno-EM (IEM) and confocal laser scanning microscopy

Stably transfected HeLa cells were fixed with 2% PFA + 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and incubated for 4 h at room temperature followed by an overnight incubation at 4°C. After fixation, cells were rinsed with PBS and with PBS containing 0.02 M glycine, scraped and pelleted in 12% gelatin. Small blocks of the embedded cell pellets were infiltrated overnight with 2.3 M sucrose, mounted on aluminum pins and frozen in liquid nitrogen. Ultrathin cryosections were cut at –120ºC, picked up with 1% methylcellulose, 1.2 M sucrose, thawed and collected on grids. After washing with PBS containing 0.02 M glycine, sections were double-labeled, as described earlier (Slot et al., 1991), with antibodies against the tags (mouse) and the *cis* Golgi marker GM130 (rabbit). For the sub-Golgi localization of the tagged enzymes, immunogold particles were quantified per cisterna. For each enzyme, 20 Golgi stacks were selected on double-labeled sections based on the following criteria: clear visibility of 5 cisternae per Golgi, which we denoted cisterna 1 to 5 (Martinez-Menarguez et al., 2001), the presence of at least 5 gold particles for the specific tag and the presence of GM130 label as a cis-Golgi reference. The cisterna containing the GM130 marker was located at one end of each Golgi stack and was denoted cisterna 1. EM pictures of the selected Golgi stacks were made at a magnification of 30,000x or 40,000x. The number of gold particles for the specific tag was counted per cisterna and expressed as a percentage of the total gold particles within that Golgi stack. The results for each enzyme are expressed as the average percentage ± SEM (n=20) of immunogold label for each cisterna. The reli-

ability of the sample size was determined by accumulating data (for a given enzyme) until average percentages remained stable within 10% of the end value, for the last added 5 Golgi stacks. 200-500 gold particles were localized for each protein. The significance of the peak value of the gold particle percentage in a given cisterna was determined by performing paired t-tests between cisternae with the lowest and highest percentage of gold label ($p \leq 0.05$). Confocal laser scanning microscopy was performed as described under Suppl. Methods.

Transport incubations on intact cells

24-well dishes with ~2*10^5 MF, CHO-GalCS, or D6P2T cells were pre-incubated with various drugs (1 μg/ml brefeldin A; BFA, 5 μM PSC833, 50 μM glibenclamide, 1 mM vanadate) for 0.5 h, and metabolically labeled in the presence of inhibitors with radioactive precursors or C_6-NBD-ceramide at 37˚C. For natural lipids and proteins, cells were labeled with 24 kBq [1-^{14}C]palmitate/ml, 15 kBq/ml [3-^{14}C]serine, 3,600 kBq/ml [^{35}S]H_2SO_4, or 72 kBq/ml [^{14}C]galactose in culture medium for 1.5 h at 37°C and 5% CO_2. Serine labeling was carried out in MEMa, because of its lower level of serine. The medium was replaced by HBSS' containing purified GLTP to extract GSLs from the cell surface at 37°C. After varying times and GLTP concentrations (see Results: typically, 45 min with 1.5 mg/ml GLTP ± BFA), the medium was removed and the cells were washed twice with 0.5 ml HBSS'. Wash buffers and medium were pooled and centrifuged for 5 min at 800*g* to remove detached cells. The lipids were extracted from medium and cells as described below. Transport is calculated as [^{14}C]- or [^{35}S]lipid in the medium as % of the total amount of that lipid in medium plus cells. Effects of drugs or RNAi treatments on synthesis were determined by measuring the sphingolipid of interest against an internal standard like [^{14}C]phosphatidylserine, in triplicate. For C_6-NBD-GlcCer labeling, cells were incubated for 1 h with 1 μM C_6-NBD-ceramide in HBSS' + 1% BSA (w/v) to back-exchange newly synthesized C_6-NBD-lipids appearing at the surface at 37°C. The buffer was replaced by HBSS' + 1% BSA and incubated for 0.5 h on ice. The lipids were extracted from the combined label- and washing-buffers and from the cells as described under lipid analysis (see below). Transport is expressed as C_6-NBD-lipid in the medium as % of total C_6-NBD-lipid. Measurements were performed in triplicate. For protein transport analysis, cells were incubated with 800 kBq/ml [^{35}S]amino acids in culture medium for 135 min. Cells and media were analyzed as described under SDS-PAGE and Western blotting (below). Transport was calculated as % of control.

Golgi translocation assay measuring [^{3}H]LacCer synthesis

15 cm^2 dishes with (~1.5*10^7) HeLa cells were pre-incubated in the presence or absence of 1 μg/ml BFA in culture medium for 0.5 h at 37°C in 5% CO_2. Cells were then washed 2x with PBS and scraped in homogenizing buffer (120 mM K^+-glutamate, 15 mM KCl, 5 mM NaCl, 0.8 mM $CaCl_2$, 5 mM $MnCl_2$, 2 mM $MgCl_2$, 1.6

mM EGTA, 20 mM HEPES/KOH, pH 7.2, 1 µg/ml apoprotein, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 5 µg/ml antipain and 1 mM benzamidine). Cells were centrifuged at 300*g* for 5 min at 4°C, taken up in 800 µl homogenizing buffer and broken up by passing 10x through an EMBL homogenizer (0.016 ball size). Nuclei and unbroken cells were removed from PNS by spinning for 10 min at 300g at 4°C. Soluble GLTP/[^{3}H]GlcCer complexes were prepared as follows: 0.4 MBq [^{3}H]GlcCer in 10 µl ethanol were injected into 1,200 µl homogenizing buffer containing 12 µg GLTP. The mix was incubated for 20 min at 37°C and then spun down for 5 min at 20,000g to remove aggregates. 100 µl PNS (0.25 mg/ml) were incubated for 1 h at 37°C in the presence or absence of BFA, with 100 µl GLTP/[^{3}H]GlcCer complexes, 2 mM UDP-Gal and 2 mM ATP in a volume of 500 µl. Lipids were extracted as described under lipid analysis and LacCer synthesis was measured as % of control.

Lipid analysis

Lipids were extracted and applied to TLC plates, which, when used to separate GalCer from GlcCer, had been dipped in 2.5% w/v boric acid in MeOH and dried, all as before (van der Bijl et al., 1996). Lipids were generally separated by 2D-TLC using in the first dimension either $CHCl_3$/MeOH/25% v/v NH_4OH/water (65:35:4:4, v/v) or $CHCl_3$/MeOH/0.2% aqueous $CaCl_2$ (55:45:10, v/v), with for the second dimension the acidic solvent, $CHCl_3$/MeOH/acetone/HAc/water (50:20:10:10:5, v/v). C_6-NBD-lipids were analyzed by 1D-TLC in the acidic running solvent. 1D-TLC plates of [^{35}S]H_2SO_4 labeled cells and of PNS were developed in the $CaCl_2$ mixture. Radiolabeled spots were detected by exposure of phosphor-imaging screens and read-out on a Personal FX phosphor-imager. TLC plates with fluorescent lipids were directly developed using a STORM 860 phosphor-imager (Molecular Dynamics, Sunnyvale, CA). Spots were identified by comparison to standards and quantified using the Quantity One software (Biorad, Hercules, CA).

SDS-PAGE and Western blotting

Cells were washed 3 times with PBS and were resuspended in protein sample buffer (200 mM Tris-HCl pH 6.8, 3% w/v SDS, 12% v/v glycerol, 1 mM EDTA, 0.003% w/v bromophenol blue, 50 mM dithiothreitol). Media were centrifuged at 3,000*g* to remove cell debris, and 1/3 volume of 4x protein sample buffer was added. Samples containing SGCS were incubated 10 min at room temperature and 0.5 h at 50°C. All other samples were heated for 5 min at 95°C and resolved by SDS-PAGE on 7.5% minigels. Radioactive gels were dried and analyzed by fluorography or a Personal FX phosphor-imager using Quantity One software. For Western blotting, nitrocellulose transfers were blocked for 1.5 h in PBS, 5% Protifar (Nutricia, Zoetermeer, Netherlands), 0.2% Tween 20 (blotto). Primary antibody incubations were for 1 h in blotto. Detection was with horseradish peroxidase-conjugated secondary antibodies using enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK).

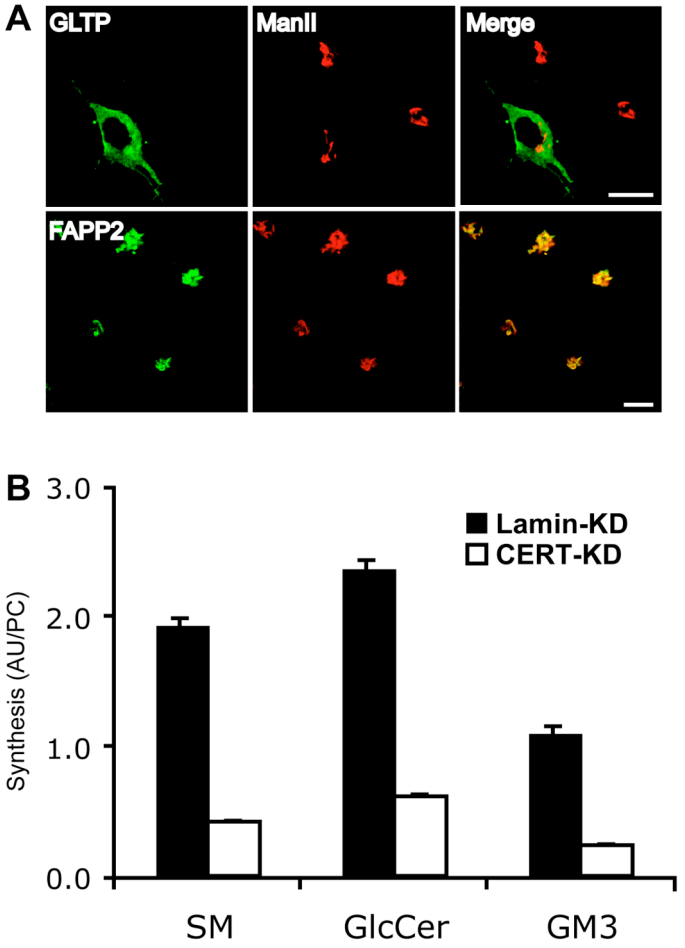
Supplemental information.

Suppl. fig. 1 shows that FAPP2 is localized in the perinuclear region of Hela cells where it partially colocalizes with the medial Golgi marker mannosidase II, while GLTP distributes all over the cytosol except for the nucleus. Knocking down CERT resulted in a 80% reduction in the synthesis of SM, and 70% reduced synthesis of GlcCer and GM3, in line with a trans Golgi location of a large fraction of GCS. Suppl. fig. 2 shows the purified recombinant GLTP as a single band in a Coomassie stained gel. The protein extracted C10-pyrene-GlcCer, but not SM from membranes. Extraction of radiolabeled GlcCer from fibroblasts was concentration- and time-dependent, while exogenous liposomes inhibited the extraction. Suppl. Fig. 3 shows that the product of the ER sulfotransferase construct SGCS was not SLacCer. Product synthesis was inhibited by the GCS inhibitor *N*B-DGJ and by GCS knockdown, but not by knocking down LCS. In addition, there was no reduced synthesis of the compound in Lec8 cells which synthesize far less LacCer because they lack the galactose importer in their ER and Golgi. Suppl. fig. 4 shows that BFA does not inhibit GM3 synthesis but does inhibit the synthesis of Gb3 in HeLa cells. Finally, Suppl. Fig. 5 shows that BFA fully inhibited protein secretion in melanocytes, but that FAPP2 knockdown had no effect on protein secretion.

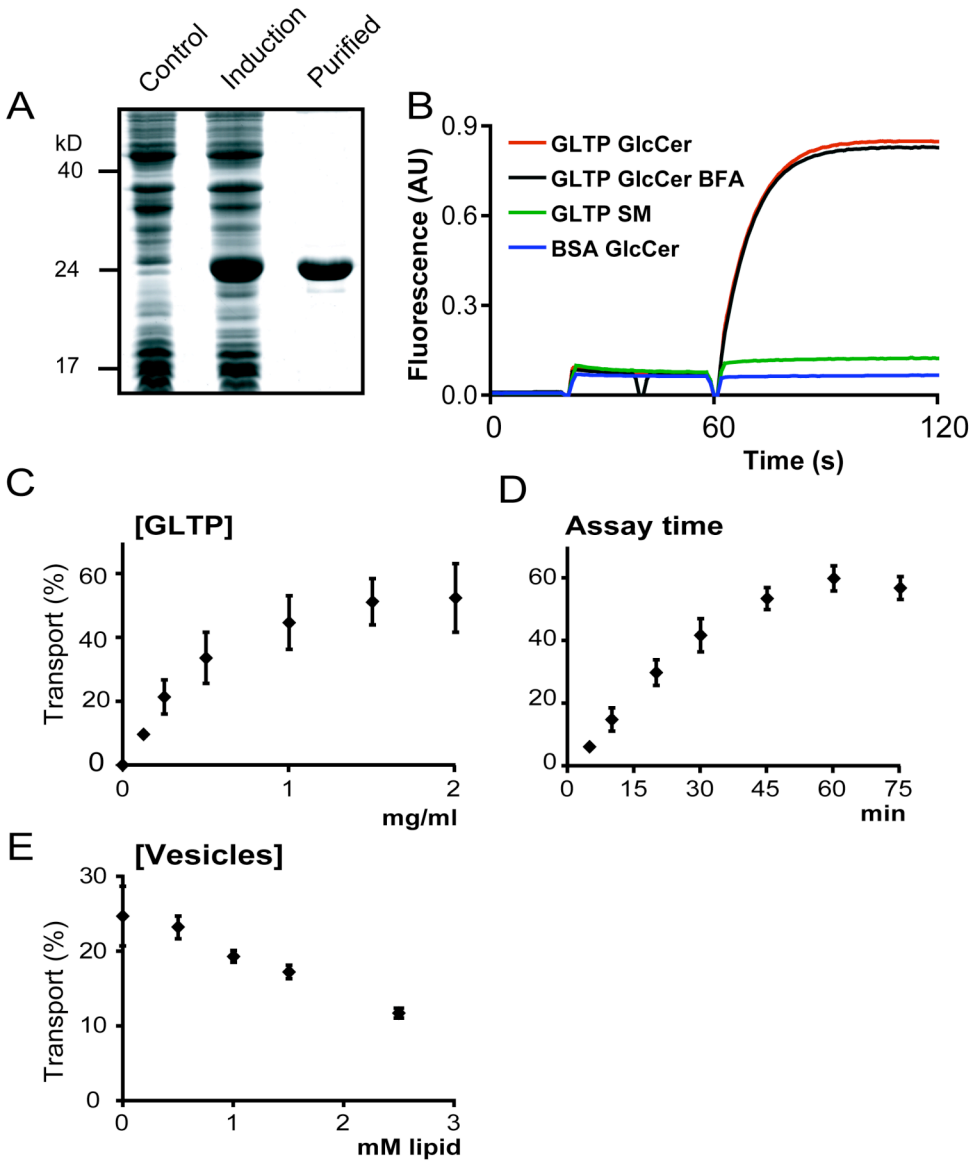
Table I
cDNAs and constructs used

Gene	Accession Number	Plasmid	Species	Tag (N/C)	Origin
CST	NM006416	pCDNA3.1h	human	HA (C)	Ashikov et al., 2005
FAPP2	NM032639	pCDNA3.1h	human	HA (C)	RZPD, Berlin, Germany
GCS	NM003358	pCDNA3.1h	human	HA3[2] (C)	Sprong et al., 1998
GLTP[1]	NM175799	pCDNA3.1h	bovine	HA (C)	West et al., 2004
GLTP[1]	NM175799	pQE9	bovine	HIS6 (N)	West et al., 2004
GM3S	AF119416	pNHA3	mouse	HA3 (N)	Giraudo and Maccioni, 2003
LCS	AF097158	pNHA3	mouse	HA3 (N)	Giraudo and Maccioni, 2003
NGT	NM012243	pCDNA3.1	human	V5 (C)	Ashikov et al., 2005
SGCS[3]	NM199111 + NM016922	pNHA3	rat + mouse	HA3 (N)	RZPD, Berlin, Germany
SMS1[1]	NM147156	pCDNA3.1	human	V5 (C)	Huitema et al., 2004
UGT[1]	NM005660	pMKIT-neo	human	HA (C)	Miura et al., 1996
MDR1[1]	NM000927	pRc/RSV	human	-	van Helvoort et al., 1996
CERT	NM023420	pKoen	mouse	RNAi	Synthetic
FAPP2	BC052360	pKoen	mouse	RNAi	Synthetic
GCS	NM011673	pKoen	mouse	RNAi	Synthetic
Lamin	NM001002011	pKoen	mouse	RNAi	Synthetic
LCS	AF097158	pKoen	mouse	RNAi	Synthetic

[1] The construction of this plasmid has been described (see Origin).
[2] HA3: triple HA-tag.
[3] Chimeric construct, see Materials and Methods.

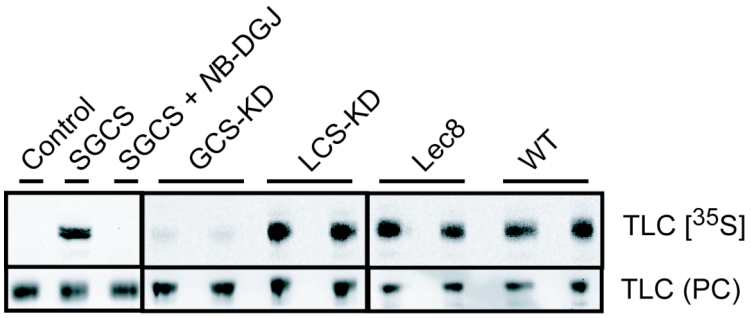


Suppl. figure 1. Proteins involved in Golgi sphingolipid transport. (A) Intracellular localization of GLTP and FAPP2. HeLa cells were transiently transfected with GLTP-HA or FAPP2-HA. After 18 h cells were fixed, permeabilized, and labeled with rabbit antibodies against HA and with mouse antibody against mannosidase II (ManII), a medial Golgi marker. Cells were counterstained with FITC-labeled anti-rabbit (left column) and Texas red-labeled anti-mouse (middle column) antisera. Overlapping distributions appear as yellow in the merged images (right column). (B) GlcCer synthesis depends on CERT. MEB4 cells stably expressing RNAi plasmids against CERT or lamin were incubated overnight with [^{14}C]serine. Cells were washed with PBS and lipids were extracted and analyzed as described under Methods.



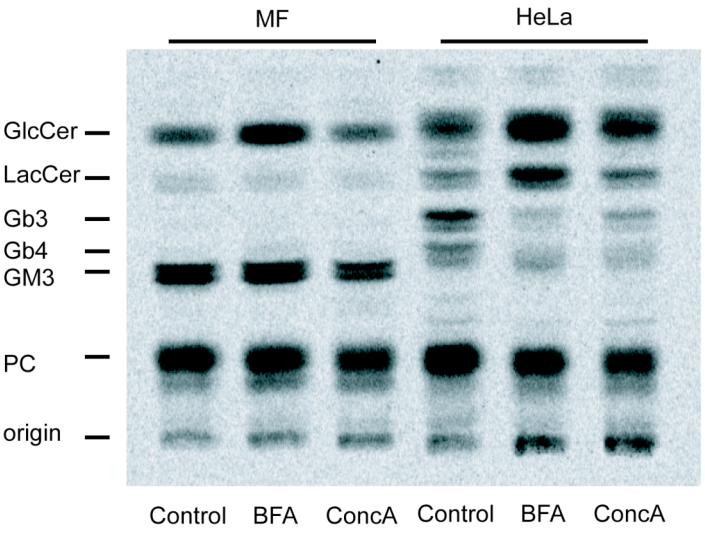
Suppl. figure 2. Optimized GLTP assay for the cell surface appearance of newly synthesized radiolabeled glycosphingolipids. (A) Expression of bovine GLTP (23-24 kD) in *E.coli* (Control) was induced for 2 h at 37°C (Induction). Cells were lysed and GLTP was affinity-purified by its his-tag in the absence of detergents (Purified). Samples were analyzed by SDS-PAGE, and proteins were visualized with Coomassie brilliant blue, all as described under Supplementary Methods. (B) Dequenching of C_{10}-pyrene-GlcCer, when extracted as a monomer from a selfquenching environment by GLTP. At t = 20 s, 1 µM C_{10}-pyrene-GlcCer or -SM was injected into HBSS' from ethanolic solution. At t = 60 s, 2.5 µg GLTP or BSA was injected per ml. GlcCer but not SM was extracted. BFA had no effect on GlcCer extraction. A typical experiment is shown. (C, D) Dishes with MF cells were labeled with [^{14}C]palmitate for

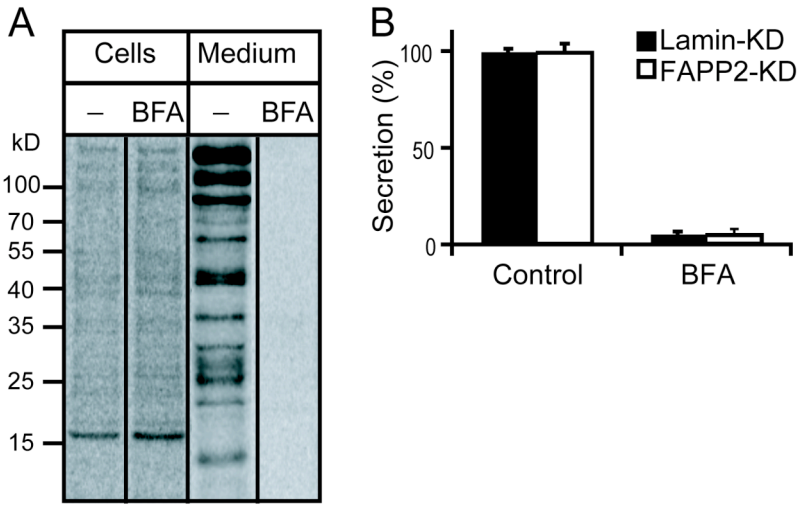
1.5 h at 37°C. The medium was replaced by HBSS' containing varying concentrations of purified GLTP for 1 h at 37°C (C), 1.5 mg/ml GLTP during varying incubation times (D), or 1.5 mg/ml GLTP for 45 min at 37°C (E) to extract GSLs arriving at the cell surface. At lower temperatures GSL extraction was less efficient. The medium in (E) contained varying concentrations of sonicated egg PC/cholesterol liposomes. Radioactive lipid in the medium was expressed as percent of total. Each point represents triplicate measurements ± S.D. Based on these data, the standard incubation conditions for the assay for cell surface GlcCer were defined as 1.5 mg/ml GLTP, 45 min at 37˚C in the absence of liposomes.



Suppl. figure 3. The product of SGCS is not SLacCer. Cells were transfected with SGCS and after 18 h the cells were incubated with were incubated with 600 kBq [^{35}S]H_2SO_4/10^5 cells. 24 h later the lipids were extracted, separated by 1-D acidic TLC, and stained with iodine to visualize the bulk lipid PC as a loading control. [^{35}S]lipids were visualized by phosphor-imager screens. (A) MEB4 cells plus/minus 40 µM *N*-butyldeoxygalactonojirimycin (*N*B-DGJ), an inhibitor of GlcCer synthesis. (B) MEB4 cells stably expressing RNAi plasmids against GCS (KD-GCS), and against LCS (KD-LCS). (C) CHO cells, and CHO cells lacking the UDP-Gal transporter UGT (CHO-lec8). The findings that the sulfated lipid was formed in the LacCer synthase knockdown and in the absence of a functional UDP-Gal transporter (which is required for LacCer synthesis) exclude the possibility that the sulfated lipid was SLacCer. A low level of SLacCer was found in D6P2T cells (Fig. 4D). SLacCer was identified according to 3 criteria. (1) It is a sulfated lipid made by intact cells that synthesize GalCer, GlcCer and LacCer. (2) The lipid is more polar that SGalCer, and much more polar than SGlcCer. (3) The lipid is derived from GlcCer because labeling no longer occurs in the presence of an inhibitor of GlcCer synthesis. The fact that MEB4 and CHO cells do not synthesize GalCer rules out the possibility that the sulfated lipid was SGalCer.

Suppl. figure 4. Glycosphingolipid synthesis in MF and HeLa cells is differentially affected by BFA and concanamycin A. MF and HeLa cells were incubated with [^{14}C]palmitate for 24 h, after which the radiolabeled lipids were separated by $CaCl_2$ TLC as described under Methods. BFA boosted GlcCer synthesis. While BFA had no effect on the conversion of GlcCer to LacCer and the complex GSL GM3 in MF cells, BFA strongly reduced the synthesis of the complex GSLs Gb3 and Gb4 from LacCer in HeLa cells, as reported by others (Sherwood and Holmes, 1992). Concanamycin A had a small effect on the synthesis of GM3, Gb3 and Gb4. PE: phosphatidylethanolamine.





Suppl. figure 5. BFA blocks protein secretion from the ER. To measure protein transport, MEB4 cells were (mock-) treated with BFA, and labeled with 1,800 kBq/ml [^{35}S]amino acids for 4h. Proteins in cells (0.03%) and medium (3%) were analyzed by SDS-PAGE, and protein secretion was expressed as % of the control.

Supplemental Materials and Methods

C_{10}-pyrene fatty acid was a gift from Pentti Somerharju (Helsinki, Finland). C_{10}-pyrene-SM and C_{10}-pyrene-, C_6-NBD-, and [^{3}H]palmitoyl-GlcCer were synthesized as described (Kishimoto, 1975). Briefly, 25 µmol of the 1-β-D-glucosylsphingosine or sphingosylphosphocholine, 25 µmol C_{10}-pyrene- or C_6-NBD- or 36 MBq [^{3}H]palmitic acid, 50 µmol triphenylphosphine and 50 µmol 2,2-dithiodipyridine were dissolved in 450 µl dimethyl formamide. The mixture was shaken vigorously in the dark at RT overnight. After drying under N_2, the products were dissolved in $CHCl_3$/MeOH (2:1, v/v) and applied to a silica-60 column to remove labeled fatty acids from the labeled sphingolipid. The purity and identity of the product were confirmed by TLC using standards. The concentration was determined spectrophotometrically or by liquid scintillation counting.

Synthesis and purification of GLTP

pQE9-GLTP was transformed into *E.coli* BL21 and selected for ampicillin resistance. A culture was grown in LB medium at 37°C until OD600 was 0.6. Expression of GLTP was induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h. The bacteria were harvested and resuspended in lysis buffer (300 mM NaCl, 10 mM imidazole, 50 mM NaH_2PO_4, pH 8, 1 µg/ml apoprotein, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 5 µg/ml antipain and 1 mM benzamidine). Cells were sonicated with a Branson Sonifier 450 (Danbury, CT). Debris was centrifuged for 0.5 h at 15,000*g* at 4°C. The His6 tagged protein was purified on a Ni-NTA agarose column and concentrated and the buffer exchanged to HBSS' using Amicon centrifugation tubes (Millipore, Billerica, MA). The protein (7.5 mg/ml) was stored in HBSS' with 10% glycerol at -80°C. The activity of purified GLTP was measured as the fluorescence dequenching that occurs when C_{10}-pyrene-GlcCer from a self-quenching vesicle is bound as a monomer to the hydrophobic binding side of GLTP (West et al., 2004). An ethanolic solution of C_{10}-pyrene-GlcCer or -SM was injected into 1 ml HBSS' at room temperature to yield self-quenching vesicles at a concentration of 1 µM (CMC <1 nM), 1% ethanol. After 40 s 2.5 µg GLTP or BSA was added, and the fluorescence was measured at λ_{ex}=343 nm, λ_{em}=378 nm. Maximum fluorescence was determined by addition of TX100, 1% v/v.

Confocal laser scanning microscopy

Transiently transfected cells grown on coverslips were fixed using 3% PFA in PBS for 20 min, blocked in PBS containing 0.5% BSA and 0.1% saponin (blocking buffer) for 1 h and labeled with primary antibodies in blocking buffer. The coverslips were washed and counterstained with fluorescently labeled secondary antibodies in blocking buffer for 1 h. The coverslips were washed with blocking buffer, PBS and water and mounted in Mowiol 4-88 (Calbiochem, La Jolla, CA) containing 2.5% 1,4-diazabicyclo [2.2.2] octane at 20˚C. MF cells on glass coverslips were washed twi-

twice with HBSS', fixed with 3% PFA, stained with FITC-conjugated annexin-V for 45 min, and analyzed. Pictures of the different samples were taken using the same settings. Images were obtained through a Nikon Plan Apo 60x A/1.40 lense on a Nikon D-εclipse C1 confocal microscope using the filter settings: FITC: λ_{ex} = 488 nm and λ_{em} = 515 LP; Texas red: λ_{ex} = 568 nm and λ_{em} = 585 LP). Single-labeled cells with each primary/secondary antibody combination showed that no bleed-through occurred.

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Abbreviations

BFA, brefeldin A; C_6-NBD-, *N*-6-NBD-aminohexanoyl-; GalCer, galactosyl β1-1 ceramide; GalCS, GalCer synthase; GCS, GlcCer synthase; GlcCer, glucosyl β1-1 ceramide; GLTP, glycolipid transfer protein; GM3, sialyllactosylceramide; GM3S, GM3 synthase; GSL, glycosphingolipid; HBSS', HBSS with 20 mM Hepes pH 7.4; LacCer, galactosyl β1-4 GlcCer; LCS, LacCer synthase; PAPST, 3'-phosphoadenosine 5'-phosphosulfate transporter (SLC35B2); PC, phosphatidylcholine; PNS, postnuclear supernatant; SGalCer, galactosylceramide sulfate; SGCS, chimeric protein consisting of an HA-tagged PAPST and SGalCer synthase; SM, sphingomyelin; SMS1, SM synthase 1.

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Hitch-hiking between cells on lipoprotein particles

Based on

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Summary
Cell surface proteins that are covalently linked with lipids associate with specialized membrane domains. Morphogens like Hedgehog and Wnt use their lipid-anchors to bind to lipoprotein particles and employ lipoproteins to travel through tissues. Removal of their lipid-anchors or decreasing lipoprotein levels give rise to adverse Hedgehog and Wnt signaling. Some parasites can also transfer their glycosylphosphatidylinostol-anchored surface proteins to host lipoprotein particles. These antigen-loaded lipoproteins spread throughout the circulation, are endocytosed by different cell surface receptors, and probably hamper an adequate immune response. Together, these findings imply a widespread role for lipoproteins in intercellular transfer of all types of lipidated proteins, and may have various functional consequences. We discuss how lipid-modified proteins may be transferred to and from lipoproteins at the molecular level.

Introduction
Proteins that are modified with lipids often tightly associate with cellular membranes (Bijlmakers and Marsh, 2003). Furthermore, the physical properties of lipid-anchors are likely to control the localization of their proteins to particular membrane domains, called rafts. Rafts can laterally include or exclude distinct lipids and proteins within a membrane and play pivotal roles in protein sorting and signaling (Simons and van Meer, 1988; Simons and Ikonen, 1997). Acylated and prenylated proteins occupy different areas on the cytosolic surface of the plasma membrane (Zacharias et al., 2002), whereas glycosylphosphatidylinositol (GPI) anchored proteins are enriched in rafts at the cell surface in a debatable manner (Brugger et al., 2004; Glebov and Nichols, 2004; Sharma et al., 2004; Schuck and Simons, 2006). Recent findings point to another, inventive role for lipid anchors of surface proteins in trafficking.

Hedgehog, Wnt and EGFR-ligands are families of secretory ligands that travel long distances -over 30 cell diameters- through the aqueous extracellular space, and signal in a concentration dependent fashion (Neumann and Cohen, 1997; Zeng et al., 2001). Their signal transduction pathways controls patterning and proliferation during development and are often involved in the maintenance of the morphology of adult tissues. Members of these families have been shown to be palmitoylated (Mann and Beachy, 2004; Miura et al., 2006), and Hedgehog proteins contain an additional cholesterol modification at their C- termini as well. Hedgehog and Wnt proteins were shown to participate in DRM-factions, suggesting the presence in rafts (Rietveld et al., 1999; Karpen et al., 2001; Zhai et al., 2004). Their lipid-anchors are essential for intercellular trafficking and proper signaling (Miura et al., 2006; Miura and Treisman, 2006). Smith-Lemli-Opitz syndrome, desmosterolosis and lathosterolosis are diseases caused by defects in the final stages of cholesterol synthesis. Many of the developmental malformations in these syndromes have

been ascribed to defective Hedgehog signaling (Cooper et al., 2003; Gofflot et al., 2003).

Like morphogens, GPI-anchored proteins can undergo intercellular transfer (Kooyman et al., 1995; McCurry et al., 1995; Anderson et al., 1996; Ilangumaran et al., 1996; Liu et al., 2002; Onfelt et al., 2004). Paroxysmal nocturnal hemoglobinuria (PNH) is a chronic disease with severe hemolytic anemia, and is caused by a defect in the biosynthesis of GPI-anchors (Rosse, 2001; Almeida et al., 2006). Erythrocytes from PNH-patients lack two GPI-anchored proteins, CD55 and CD59, which inhibit the formation of the membrane attack complex of complement, and are extremely sensitive to complement-mediated lysis. Transfer of GPI-anchored proteins to deficient cells has been demonstrated in cell culture and animal models. Transfer of CD55 and CD59 from microvesicles to PNH-erythrocytes reduced their susceptibility to complement-mediated lysis (Sloand et al., 1998). Ectopically expressed CD59 was also transferred from erythrocytes to the vascular endothelial cells, and from seminal fluid to prostasomes (Kooyman et al., 1995; Rooney et al., 1996). Furthermore, cell-to-cell transfer of another GPI-anchored protein, Thy-1, occurred in chimeric murine embryoid bodies composed of normal and PNH-cells (Dunn et al., 1996). In parasitemic patients the variant surface glycoprotein (VSG) was transferred from trypanosomal membranes to erythrocytes (Rifkin and Landsberger, 1990), possibly leading to one of the pathological features associated with trypanosome infection: anemia. Conversely, some parasites have been shown to acquire host CD55 to protect themselves against complement lysis (Pearce et al., 1990).

How proteins with such strong membrane affinity move from producing cells to surrounding cells is puzzling, and more than one mechanism has been proposed to explain how lipid-modified proteins move through tissues (Ilangumaran et al., 1996; Miller, 1998; Mann and Culi, 2005; Eaton, 2006). Shedding of GPI-anchored proteins can be mediated by proteases or phospholipases, leaving the lipid anchor in the donor membrane. Proteins can also be released from cells together with their lipid modification (Walter et al., 1992; Rooney et al., 1996; Cavallone et al., 2001; Schwartz et al., 2005). One of the simplest models is the formation of micelle-like multimers (Rooney et al., 1996; Zeng et al., 2001), in which the hydrophobic groups of e.g. Wnt or Hedgehog are arranged in such a way that the protein complex becomes soluble in a polar medium. Alternatively, lipid-anchored proteins can be released on membranous exovesicles containing a complete membrane bilayer, like exosomes (de Gassart et al., 2003; Fevrier et al., 2004), surfactant-like particles (Eliakim et al., 1989) or nodal vesicular parcels (Tanaka et al., 2005). Such particles could be generated by vesiculation of plasma membrane protrusions (Eliakim et al., 1989)], or by an exosome-related mechanism (Rabesandratana et al., 1998; Denzer et al., 2000). Another possibility is that cells pass on Wnt proteins via cell-

cell contacts or through long cellular extensions called cytonemes (Ramirez-Weber and Kornberg, 1999; Hsiung et al., 2005). Membranous transporters are not unique for lipid-modified proteins: They can also transfer transmembrane and cytosolic proteins between cells (Thery et al., 2001; Wubbolts et al., 2003).

Lipoprotein mediated transport of lipid-modified proteins

Two recent studies found that lipoprotein particles could act as vehicles for the intercellular movement of lipid-modified proteins. Lipoprotein particles are large, globular complexes composed of a central core of hydrophobic lipids that are surrounded by a monolayer of membrane lipids. They are hold together by one or more members of a family of apolipoproteins, of which some are covalently linked to palmitate as well (Zhao et al., 2000). Lipoprotein particles allow intercellular transport of water-insoluble lipids, fat and signaling metabolites throughout the aqueous circulation of multi-cellular organisms. In theory, the lipid-modification of proteins can anchor the protein in the exoplasmic leaflet of cell membranes as well as in the outer phospholipid layer of lipoproteins (Vakeva et al., 1994). In this way, lipid-modified proteins get solubilized and will be transported together with lipoproteins.

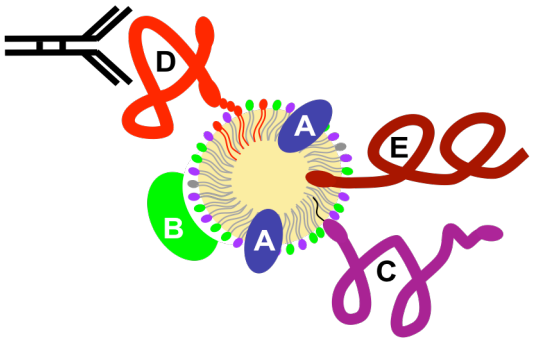

Figure 1: Association of lipid-modified proteins with lipoproteins

The hydrophobic core of a lipoprotein particle is surrounded by a monolayer of membrane lipids. Integral apolipoproteins (A), like apoAI and ApoB100 form belt-like structures around the particle, and provide structural stability. Peripheral apolipoproteins (B) , such as GPI-PLD, interact with surface lipids via amphipatic helices. The covalent lipid-modification of surface proteins (C) equally well fit in the outer leaflet of the plasma membrane as in the monolayer around lipoprotein particles. Also parasite lipid-modified proteins (D) can associate lipoproteins. Parasitic GPI-anchors are often structurally different from mammalian ones, making them resistant for GPI-specific phospholipases. In theory, membrane proteins that span a membrane only partially, or that are cleaved intramembranously, could associate with lipoproteins as well (Haas et al., 1997).

Transport of proteins associated with lipoproteins affect lipoprotein transport and vice versa. For example, LDL-receptors bind apoB, and co-endocytose LDL together with parasite GPI-

anchored proteins (Sprong et al., 2006). Neutrophils that recognize and endocytose parasite GPI-anchored proteins, also take up LDL via the Fc-receptor (Sprong et al., 2006).

In the first study Drosophila Wnt and Hedgehog proteins were found to co-purify with lipoproteins from tissue homogenates, and to co-localize with lipoprotein particles in the developing wing epithelium. Furthermore, reduction of lipoprotein levels showed that these particles are required for long-range, but not short-range signaling (Panakova et al., 2005). Consistent with this idea, multiple Drosophila GPI-proteins were associated with the lipoprotein fraction in a GPI-specific phospholipase (PLC) sensitive way (Panakova et al., 2005).
Despite the many possibilities suggested by this model, direct evidence that lipoproteins function as intercellular carriers of lipidated morphogens is lacking. Considering that lipoproteins are indispensable for systemic lipid transport and metabolism, it will be very difficult to design an indisputable *in vivo* experiment (Mann and Culi, 2005).

Recently, we discovered that GPI-anchored coat proteins of two parasites, schistosomes and trypanosomes, were physically associated with the host lipoprotein particles in the blood stream of infected patients (Sprong et al., 2006). As a consequence, cells expressing LDL-receptors endocytosed parasite GPI-anchored proteins together with low-density lipoprotein (LDL) particles, which resulted in a lysosomal accumulation of parasite proteins, and an abnormal lysosomal morphology. Upon infection with one of these parasites, the host immune system promptly generates antibodies towards the highly antigenic coat proteins, but only inadequately attacks the parasite. In serum from patients suffering from chronic schistosomiasis, we found that antibodies were bound to the patients' own lipoprotein particles, probably via the parasitic antigens. Therefore, LDL particles were co-endocytosed with antibodies by neutrophils via their Fc receptor. The lipoprotein accumulation in neutrophils was associated with apoptosis and reduced neutrophil viability. These findings could explain the reduced lipoprotein content and the neutropenia found in patients with chronic schistosomiasis, and provide new clues to how schistosomes might trick the immune system.

The finding that lipid-modified proteins can insert into lipoproteins raises many more questions: How do lipid-modified proteins get inserted into lipoproteins? Mammalian plasma lipoproteins have been classified into five major groups based on their size and density, but lipoproteins are also found in cerebrospinal fluid, the interstitial space of the brain, and in other body fluids. On what kind of lipoproteins are lipid-modified proteins secreted? Lipoproteins consist of different apolipoproteins and some of them are exchangeable. Are there apolipoproteins that specifically bind certain lipid-modified proteins? Contradictory to Wnt and Hedgehog secretion palmitoylation of the drosophila EGF Receptor Ligand Spitz (Spi) anchors

the protein more tightly to the plasma membrane of producing cells and thereby increases its signaling (Miura et al., 2006). Thus, can all lipid-modified proteins be secreted by lipoproteins or is a specific machinery required to facilitate the uptake of particular lipidated proteins? How does the interaction with receiving cells take place? Can lipidated proteins insert back into membranes? The lipidation of Wnt and Hedgehog and their presence at lipoproteins as well as GPI-anchored proteins are new and unexpected findings. Therefore the most important question is what are the (patho)physiological implications of this resourceful mechanism?

Incorporation into lipoproteins

Lipidated proteins that hitchhike from cell to cell on lipoproteins might exploit similar strategies and mechanisms that are utilized for intercellular lipid transport (Rodenburg and Van der Horst, 2005; Krimbou et al., 2006). Their association and dissociation with lipoproteins might take place on different sites: In the secretory route where lipoproteins such as LDL and chylomicrons are assembled, at the cell surface and during endocytosis and recycling where for instance HDL is (de)lipidated. Cell surface proteins acquire their lipid-modification post-translationally in the endoplasmic reticulum (Chamoun et al., 2001; Chatterjee and Mayor, 2001; Micchelli et al., 2002), the organelle where VLDL, LDL, and chylomicrons are assembled as well. It is likely that GPI-anchored proteins and morphogens can be incorporated in these lipoprotein particles during assembly or transport to the cell surface. Remarkably, inadequate lipoprotein assembly results in impaired embryonic neurodevelopment (Huang et al., 1995; Raabe et al., 1998), a phenotype comparable to defective Hedgehog signaling. Furthermore, combining fat metabolism with morphogenic signals allows tissues to modify their organization in response to altered nutrient uptake, and may give novel clues about the role of dietary fat in carcinogenesis (Weisburger, 1997). Nascent HDL particles are secreted from liver and intestinal cells in an almost lipid-free form, which would require a direct protein-protein interaction between the apolipoprotein and the lipid-modified protein for co-secretion.

GPI-anchored proteins, and most likely also lipid-linked morphogens, are enriched at the cell surface. They are endocytosed and recycled back to the cell surface via different mechanisms (Mayor and Riezman, 2004). Only a few cell types synthesize lipoproteins themselves: Most cells interact with lipoprotein particles present in their external environment either on the cell surface or in the endocytic route. Association of lipid-modified proteins to lipoproteins could happen at the cell surface or during endocytosis and recycling of HDL particles (Schmitz et al., 1985; Sun et al., 2006). In mammals, LDL is normally endocytosed and degraded in lysosomes, but some cells might be able to recycle LDL back to the cell surface (Dehouck et al., 1997; Van Hoof et al., 2005). An endosomal pathway exists in trypanosomes that specifically endocytose and recycle surface GPI-anchored proteins together with

LDL (Pal et al., 2002). This route might be required for the release of VSG on host lipoprotein particles into the circulation (Sprong et al., 2006). Remarkably, secretion of Wnt in C. elegans requires the retromer, a multiprotein complex involved in intracellular membrane trafficking (Coudreuse et al., 2006). Transfer of Wnt to lipoproteins could take place in endosomal organelles, and the retromer could be required for recycling the complex back to the cell surface (Seaman, 2005; Prasad and Clark, 2006).

Lipoprotein receptors

Each type of lipoprotein has a distinct apolipoprotein (that defines the specific physiological functions) that functions to mediate binding of the lipoprotein to cell surface receptors. Lipoprotein receptors are indispensable for efficient lipid-transfer between cells and lipoproteins, although they are not involved in the actual transfer itself. These receptors bring lipoproteins in close proximity to the membrane where the transfer can take place. Maybe, lipoprotein receptors could be involved in transfer of lipid-modified proteins in a similar fashion. Two large families of cell surface receptors mediate binding to lipoproteins: The scavenger-receptors (Murphy et al., 2005) and the LDL-receptor family (Herz and Bock, 2002).

Scavenger receptors are defined as cell surface membrane proteins that bind chemically modified lipoproteins such as acetylated low-density lipoprotein and oxidized LDL (Brown et al., 1979; Goldstein et al., 1980). They often bind a variety of ligands. The prototypic scavenger receptor SR-BI is an HDL receptor that mediates the cellular uptake of cholesterol esters from HDL (Rigotti et al., 2003; Connelly and Williams, 2004). *In vitro* studies have shown that CD59 can bind to HDL, (Vakeva et al., 1994), but SR-BI knockout mice have no symptoms similar to PNH. So far, a role for scavenger receptors in Hedgehog or Wnt signaling has not been found.

The LDL receptor family consists of seven structurally related transmembrane proteins. While the LDL receptor plays an essential role in cholesterol homeostasis, the other family members fulfill a variety of biological functions, many of which are not directly related to lipid metabolism. They bind and endocytose a multitude of extracellular ligands and also directly participate in signal transduction processes. Several members of the LDL-receptor family (LRPs) have been shown to be involved in Wnt and Hedgehog signaling (Spoelgen et al., 2005). LRP-2 has been shown to endocytose Hedgehog (McCarthy et al., 2002), and the LRP2 knockout mouse displays defects that are consistent with loss of Hedgehog signaling (Willnow et al., 1996). LRP5 and LRP6, and maybe also LRP-1 are required for Wnt signaling (Wehrli et al., 2000; Zilberberg et al., 2004). Whether these receptors directly interact with Wnts and Hedgehogs alone or whether the interaction is specific for lipoprotein-associated morphogens is unknown (Wu and Nusse, 2002).

Furthermore, it will be interesting to investigate whether these receptors are also involved in the secretion and recycling of morphogens (Marois et al., 2006).

Heparan sulfate proteoglycans

Interestingly, trafficking of both lipid-modified morphogens and lipoproteins also involves another family of cell surface co-receptors. Heparan sulfate proteoglycans (HSPGs) are large molecules composed of repeated sulfated disaccharides covalently attached to core proteins. HSPGs are abundantly expressed by virtually all mammalian cells and are found on cell surfaces and in the extracellular matrix. The negatively charged sulfate and carboxyl groups of the heparan sulfate in HSPGs interact with positively charged residues on lipoproteins and many other secretory proteins with low affinities. HSPGs of the extracellular matrix play important roles in lipoprotein retention (Pillarisetti et al., 1997), and cell surface HSPGs allow cell adhesion and uptake - even in the absence of lipoprotein receptors- by enhancing the accessibility of lipoproteins to lipoprotein receptors (Kolset and Salmivirta, 1999; Llorente-Cortes et al., 2002). Interestingly, heparan sulfate is a basolateral sorting determinant, that may influence the polarized secretion of lipoproteins after synthesis or during transcytosis (Mertens et al., 1996; Obunike et al., 2001).

HSPGs affect trafficking of lipid-modified morphogens via several different mechanisms (Lin, 2004; Hacker et al., 2005; Eaton, 2006). Mutations that block the synthesis of heparan sulfates impair intracellular accumulation, gradient formation and long-range signaling in *Drosophila* of Wnt and Hedgehog, but also of Decapentaplegic, a non lipid-modified morphogen (Bornemann et al., 2004; Han et al., 2004; Takei et al., 2004). HSPGs at the cell surface, like drosophila dlp (Eaton, 2006) and from the ECM (drosophila trol (Park et al., 2003)) might restrict the diffusion of morphogen throughout the epithelium, effectively increasing the local morphogen concentration. Remarkably, diffusion of Hedgehog without a lipid-modification is independent of HSPGs (Lin, 2004). Maybe HSPGs restrict morphogen diffusion indirectly by inhibiting the movement of their lipoprotein carrier. Alternatively, HSPGs like drosophila dally, could affect intracellular trafficking of morphogens. HSPGs might direct apical/basolateral sorting in morphogen-producing cells whereas HSPGs in receiving cells might affect lysosomal degradation of morphogens together with their carriers and stimulate their recycling (Eaton, 2006; Marois et al., 2006).

Transporters and translocators

The mechanisms underlying the biogenesis, maturation and disassembly of lipoprotein particles at the molecular level are complex and not well understood (Shelness and Ledford, 2005; Krimbou et al., 2006). The two extremes, monomeric lipid transfer and fusion with membranes, require both protein machinery. ABC-transporters transfer lipids and maybe also lipid-modified proteins to secretory lipo-

protein particles (van Meer et al., 2006). Transfer of lipidated proteins could occur passively and reversibly, maybe enhanced by this binding of lipoprotein to surface receptors, or by unknown machinery. Remarkably, Dispatched - a member of the sterol-sensing receptor family - is essential for the release of Hedgehog from cells, but is not required for the release of a non-cholesterol-modified form, suggesting that Hedgehog requires a transporter-like function of Dispatched (Burke et al., 1999; Ma et al., 2002). Another member of this family, Patched binds Hedgehog in receiving cells, and is involved in signaling. However, *C. elegans* has nearly 30 genes related to Patched and Dispatched, but has no Hedgehog (Incardona, 2005). A recent study showed that Patched is involved in secretion of the 3β-hydroxysteroid (pro-)vitamin D3 (Bijlsma et al., 2006). Confusingly, two other members of this family, NPC1 and NPC1L1, have been implicated in the inward transport of cholesterol to cytosolic leaflets of cell membranes (Chang et al., 2006). Ultimately, functional reconstitution of purified members of this family in model membranes is a necessary step to test their ability to move sterol across the membrane bilayer.

Intercellular movement

Solubilization of anchor-intact lipid-modified proteins by incorporation into lipoproteins enables them to get transported through aqueous environments. But why are GPI-anchored proteins not abundantly present on lipoproteins in the circulation? GPI-anchors can be cleaved by specific phospholipases and the only mammalian member cloned to date is GPI-specific phospholipase D (GPI-PLD). GPI-PLD is relatively abundant in serum (~10 µg/ml) and has a well-characterized biochemistry, but its physiological role is completely unknown (Raikwar et al., 2005). GPI-PLD is a HDL-associated protein, and is able to exchange between different lipoprotein classes (Hoener and Brodbeck, 1992; Deeg et al., 2001). Although GPI-PLD specifically cleaves GPIs *in vitro*, GPI-PLD appears to cleave GPI-anchored proteins from the cell surface only if the membrane is perturbed with detergents, raising the possibility that GPI-PLD is catalytically inactive in serum or requires a change in the membrane environment of the substrate to allow cleavage (Low and Huang, 1991; Bergman and Carlsson, 1994; Deng et al., 1996; Deeg et al., 2001). Possibly, its activity is directed towards GPI-proteins released onto lipoproteins. GPI-PLD might prevent systemic spread of GPI-proteins released on lipoproteins, and could very well be involved intercellular in gradient formation of GPI-anchored proteins.

Concluding remarks

Lipoprotein carriers may determine the intercellular distribution and activity of lipidated proteins. Conversely, the fate and function of lipoproteins might very well be determined by their cargo. Indeed, the importance of HDL-associated proteins in lipid metabolism and atherosclerosis has been increasingly appreciated (Navab et

al., 1998; Shih et al., 1998). Maybe, many more lipidated proteins utilize this system to communicate to neighboring cells (Knoll and Drescher, 2002). A new and exciting study revealed the presence of GPI-anchored Prion on LDL (Safar et al., 2006). Finally, it seems that parasites utilize this trick as well. Survival of parasites depends on their ability to escape the host innate immune system. Unraveling how lipidated proteins associate with lipoproteins can provide new approaches to tackle infectious diseases, Creutzfeld-Jacob-disease and morphogen-related cancers.

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Role of lipoproteins and their receptors in the release of mammalian Wnt

Based on:
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Abstract
Very little is known about the release and transport of Wnt proteins from mammalian cells. Lipoproteins may act as carriers for the intercellular movement and gradient formation of the lipid-linked morphogens Wingless and Hedgehog in *Drosophila*. To investigate whether such a mechanism can occur in mammals, we have studied Wnt release in cultured mammalian cells. Wnt3a associated with lipoproteins in the culture medium and not with extracellular vesicles or exosomes. Although Wnt3a was associated with both high- (HDL) and low-density lipoproteins (LDL), only HDL allowed Wnt3a release from mouse fibroblasts. The release of Wnt3a was inhibited by lipid-free apoAI, suggesting a role for the HDL-receptor scavenger receptor class B type I/II (SR-BI/II). Cells that lack functional lipoprotein receptors were unable to release Wnt3a, but ectopic expression of the scavenger receptor class B isoforms I and II restored Wnt release onto lipoproteins. Remarkably, Wnt3a lacking its palmitate moiety was released in a lipoprotein-independent manner, demonstrating the dual role of palmitoylation in membrane and lipoprotein binding. We additionally found that Wnt3a can be released from enterocyte cell lines on endogenously expressed lipoproteins. We further discuss the physiological implications of our findings.

Introduction
Wnt proteins constitute a family of signaling molecules that control growth and patterning during animal tissue development (Logan and Nusse, 2004) as well as the maintenance and the regeneration of adult tissues (de Lau et al., 2007; Stoick-Cooper et al., 2007). Disturbances in Wnt signaling result in both degenerative diseases and cancer (Sancho et al., 2004). In mice about 20 different Wnt proteins have been identified serving different fuctions (for more information visit the Wnt homepage: www.stanford.edu/~rnusse/wntwindow.html). Wnt proteins act as morphogens: They are expressed locally, released into the extracellular space where they establish a concentration gradient eliciting distinct, concentration-dependent responses in the neighboring cells. On the other hand, Wnt proteins tightly bind to membranes, most likely via covalent lipid modifications. Several family members have been shown to be modified by a palmitate moiety at a conserved cysteine residue and by a palmitoelate moiety at a conserved serine residue (Willert et al., 2003; Zhai et al., 2004; Takada et al., 2006; Galli et al., 2007; Kurayoshi et al., 2007). Whereas the first lipid modification is important in Wnt signaling activity (Willert et al., 2003; Komekado et al., 2007; Kurayoshi et al., 2007) the second modification is involved in proper intracellular sorting (Tanaka et al., 2000; Zhai et al., 2004; Takada et al., 2006; Galli et al., 2007). Another important morphogen is Hedgehog, which is also lipid-modified (Porter et al., 1996). How molecules with strong membrane affinity can spread in an aqueous environment and how their concentration gradient is formed remains elusive (Mann and Culi,

2005). Several mechanisms have been proposed to explain how the lipid-modified morphogens Hedgehog and Wnt move through tissues. One of the simplest models is the formation of micelle-like multimers (Zeng et al., 2001), in which the hydrophobic groups of Wnt or Hedgehog are arranged in such a way that the protein complex becomes soluble in a polar medium. Another possibility is that cells pass on Wnt proteins via cell-cell contact sites or through long cellular extensions called cytonemes (Ramirez-Weber and Kornberg, 1999; Hsiung et al., 2005). Alternatively, Wnt proteins may assemble onto membrane vesicles that are released from cells, such as exosomes (Greco et al., 2001; Fevrier and Raposo, 2004), surfactant-like particles (Eliakim et al., 1989) or nodal vesicular parcels (Tanaka et al., 2005).

Recently, lipoprotein particles were proposed to act as vehicles for the intercellular movement of lipid-modified proteins (Panakova et al., 2005). Lipoprotein particles are large, globular complexes composed of a central core of hydrophobic lipids that are associated with apolipoproteins and surrounded by a monolayer of membrane phospholipids. Lipoproteins allow intercellular transport of water-insoluble lipids and fat throughout the circulation of multi-cellular organisms. In theory, the lipid-modification of Wnt can anchor the protein in the exoplasmic leaflet of cell membranes as well as in the outer phospholipid layer of lipoproteins. *Drosophila* Wingless/Wnt was found to co-purify with lipoproteins from tissue homogenates and with lipoprotein particles in the developing wing epithelium. Furthermore, reduction of lipoprotein levels narrowed the range of Wingless signaling (Panakova et al., 2005). However, direct evidence that lipoproteins function as intercellular carriers for lipid-modified morphogens is lacking. Furthermore, it is unclear whether this mechanism could operate in organisms other than *Drosophila.* For the transport of lipid-modified proteins between cells, they first have to be extracted from the membrane of the producing cells, then they have to be transported and bind to the receiving cells. In this study, we have investigated whether lipoproteins are involved in the release of Wnt3a from mammalian cells.

Studying the role of lipoproteins as protein carriers is hampered by the complexity of multicellular organisms, especially in mammals (Mann and Culi, 2005). More than ten different apolipoproteins are expressed, which form or associate with different classes of lipoproteins. Furthermore, the formation of lipoproteins depends on the developmental stage and is tissue specific. Additionally, a number of lipoprotein receptors is expressed that bind more than one kind of lipoprotein and can act redundantly. The circulation and the extracellular space contain a large amount of lipoproteins, whereas the action of lipid-modified proteins is restricted in space. Another experimental complexity is that interference with lipoprotein metabolism in living animals gives rise to disturbances in nutrient supply, complicating the inter-

pretation of phenotypes. For these reasons, we studied the release of Wnt from cultured mammalian cells.

Results

Active Wnt co-fractionates with lipoproteins

Mouse fibroblast L-cells stably transfected with Wnt3a (L-Wnt3a) release active Wnt3a in the medium (Willert et al., 2003). To determine if the protein is present in the medium either as a soluble protein, associated with exosomes or with lipoprotein particles, we fractionated conditioned medium by differential centrifugation and analyzed all fractions by Western blotting. To mark exosomes, we additionally transfected L-Wnt3a cells with CD63-GFP. CD63 is a tetraspanin that localizes to internal vesicles of multi-vesicular bodies, and is released on exosomes (Escola et al., 1998; Wubbolts et al., 2003). Exovesicles were pelleted by ultracentrifugation and in contrast to CD63, which was pelleted completely, Wnt3a remained in the supernatant, as did lipoproteins (Figure 1A). To distinguish between freely soluble Wnt3a and Wnt3a with lipoproteins, the supernatant was subjected to isopycnic density centrifugation. During this procedure lipoproteins float to the top of a self-forming density gradient, whereas soluble proteins migrate to the bottom fractions of the gradient. Wnt3a (Figure 1B) as well as Wnt3a-myc (Figure 3A) co-fractionated with lipoproteins in the top low-density fraction, while soluble proteins, such as IgGs, are present in bottom, higher-density fractions (Figure 1B). This suggests that Wnt3a was associated with lipoproteins in the medium. Because of its lipid modifications Wnt proteins are hydrophobic, which could target them to cellular membranes. Site-directed and naturally occurring mutations of the palmitate-modified cysteine (C77 of mouse Wnt3a) gave rise to normally released but inactive Wnt, indicating that the lipid moiety is important for signaling (Willert et al., 2003; Takada et al., 2006; Kurayoshi et al., 2007). Removal of the palmitate moiety by site-directed mutagenesis resulted in efficient release of Wnt3a-C77S-myc from L-cells (Figure 1B). Wnt3a-C77S-myc was present as a soluble protein in the medium since it migrated to the bottom fractions together with soluble serum proteins, but no longer co-fractionated with lipoproteins on isopycnic density gradients (Figure 1B). Taken together these results suggest that Wnt associated with lipoprotein particles in the medium, depending on the presence of palmitic anchor. Most likely one lipid modification is not sufficient for binding of Wnt to membranous structures, as it is known for small GTPases (Behnia and Munro, 2005).

Mammals have 5 major classes of plasma lipoproteins that can be distinguished on the basis of their physical density using fractionation techniques. To investigate on which class of lipoproteins Wnt3a is present, we separated conditioned medium from L-Wnt3a cells by discontinuous density centrifugation (Redgrave et al., 1975). Wnt3a was found in the LDL as well as in the HDL density fractions (Figure 1C).

The physical interaction of Wnt3a with lipoproteins in the medium was directly assessed by co-immunoprecipitation. L-Wnt3a cells were incubated in the presence of human or bovine serum and Wnt3a was precipitated with anti-Wnt3a antibodies.

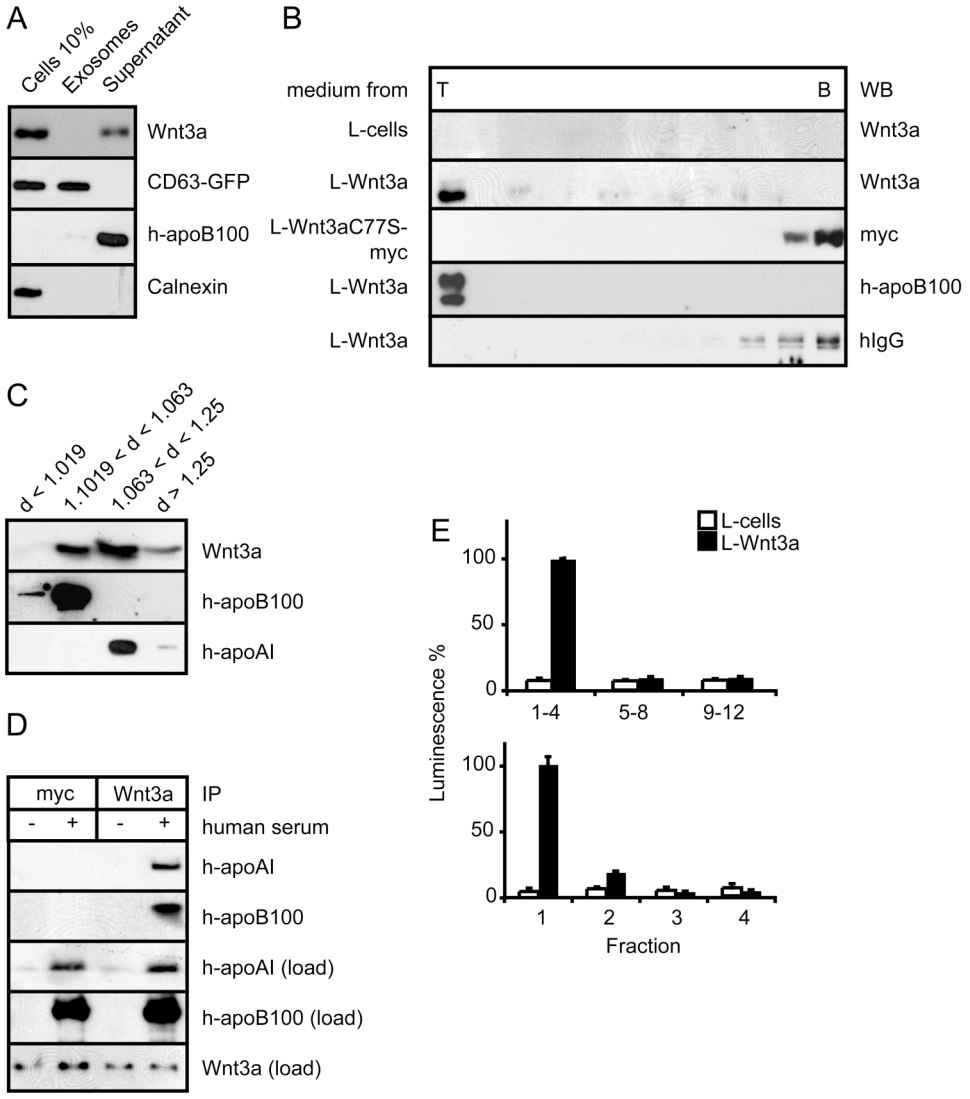

Figure 1: Association of Wnt3a with lipoprotein particles

A: L-cells stably expressing Wnt3a (L-Wnt3a) were transiently transfected with CD63-GFP and incubated with culture medium containing 10% FCS and 0.5% human serum for 3 days. Cells were scraped, and pooled with the 10,000*g* pellet from the medium (Cells). The supernatant was centrifuged for 1h at 120,000*g,* and separated into pellet (Exosomes) and supernatant (S120). **B:** Twelve fractions were taken from top (T) to bottom (B) of KBr isopycnic density gradients of the S120 from media of L-cells stably or transiently (mock-) expressing

Wnt3a or mutant Wnt3a-C77S-myc (L-Wnt3a-myc) and analyzed by Western blotting for the migration of Wnt proteins and compared them to lipoprotein associated proteins (h-apoB100) or soluble proteins (hIgGs). **C:** S120 from media of L-Wnt3a cells were analyzed by discontinuous KBr density fractionation to separate different classes of lipoproteins and analyzed for the presence of Wnt3a and apolipoproteins (h-apoAI, h-apoB100) by Western blotting. **D:** L-Wnt3a cells were incubated with culture medium containing either 10% FCS (-) or 5% FCS and 5% human serum (+) for 3 days. Wnt3a from the medium was adsorbed to protein A-sepharose with rabbit antisera against Wnt3a or myc as a control (IP). Immunoprecipitates were subjected to SDS-PAGE and Western blotting. Co-precipitated human lipoproteins were detected using antisera against h-apoA1 and h-apoB100, respectively. The relative amounts of lipoproteins and Wnt3a in the media prior immunoprecipitation (load) were determined on a fraction of the media by Western blotting. Co-precipitation was specific, since immunoprecipitation with a control antibody (myc) did not pull down either h-apoAI or h-apoB100. **E:** To measure which fraction of the medium contained active Wnt3a, conditioned medium of L-cells or L-Wnt3a cells was subjected to a KBr gradient (as in Figure 1B) and the fractions were exchanged to normal medium. L-cells stably expressing the superTopFlash system (L-STF) were incubated with fractions overnight and luciferase activity was determined.

To detect lipoproteins we used antibodies that recognize human but not bovine apolipoprotein AI and B100, the major protein constituents of HDL and LDL, respectively. To determine whether Wnt3a is active under these conditions, we subjected conditioned medium of L-Wnt3a cells to isopycnic density fractionation and divided the gradient in three or twelve fractions, exchanged the KBr solution to normal medium and performed a luciferase based activity assay, using the super-Topflash system (STF, Mikels and Nusse, 2006). In this system luciferase is expressed as a reporter gene, responding to Wnt signaling activity. When fractions were incubated with L-cells stably expressing STF for 24h only the lipoprotein-containing fraction induced luciferase activity (Figure 1E).

Wnt is released on HDL

From the preceding data, we concluded that Wnt3a in the medium is present on both HDL and LDL particles. We wondered how Wnt3a is transferred from cells to lipoprotein particles in the medium. Secreted proteins having hydrophobic properties might generally stick to membranous serum components like lipoproteins after they are released from cells. Alternatively, Wnt3a may be selectively delivered to lipoproteins. When L-Wnt3a cells were incubated with increasing amounts of delipidated FCS (DL-FCS), Wnt3a wasn't found in the medium, showing that the presence of lipoproteins is required for its release (Figure 2A). In contrast, mutant Wnt3a lacking its palmitic anchor was released into the medium also in the absence of lipoproteins (not shown). The addition of increasing amounts of LDL did not lead to release of Wnt3a from cells either. However, Wnt3a release into the medium was only restored by the addition of HDL (Figure 2A). To confirm that lipo-

proteins are required for the release of functional Wnt3a, conditioned medium from L-Wnt3a cells was produced with and without 10% FCS. Only medium containing lipoproteins exhibited activity, whereas medium without lipoproteins only showed minor activity (Figure 2B). To exclude that structures or particles that generally have the capability to sequester hydrophobic molecules lead to the extraction of Wnt3a from plasma membranes, we tested whether small unilamellar vesicles (SUVs) were capable to solubilize Wnt3a. However, release of Wnt3a after incubation with vesicles was not observed (Figure 2C).

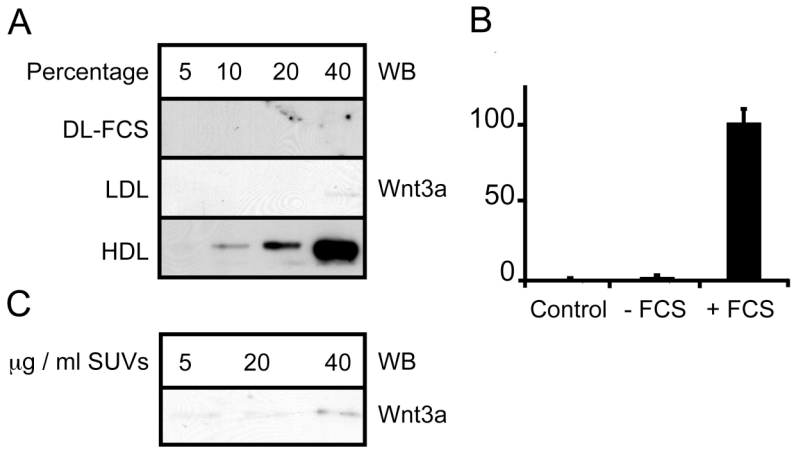

Figure 2: Release of Wnt depends on HDL particles
A: L-Wnt3a cells were incubated for 3 days with culture medium containing the indicated concentrations of delipidated FCS (DL-FCS) or 10% DL-FCS plus increasing concentration of purified LDL or HDL particles. The soluble fraction of the medium was subjected to KBr isopycnic density centrifugation. The two top fractions were pooled and analyzed by Western blotting against Wnt3a. **B:** Conditioned medium of L-cells grown with 10% FCS (Control) or L-Wnt3a cells grown with or without 10% FCS was incubated overnight on L-STF cells. and luciferase activity was measured to determine Wnt activity in the medium. To exclude that cells grown without FCS are less viable or produce less Wnt3a, we determined the ability to activate the STF system in a co-culture assay after the collection of conditioned medium. No differences between both conditions were observed (not shown). **C:** L-Wnt3a cells were incubated for 3 days with culture medium containing the indicated concentrations of small unilamellar PC/cholesterol/PS vesicles. The soluble fraction of the medium was subjected to KBr isopycnic density centrifugation and the two top fractions were pooled and analyzed by Western blotting against Wnt3a.

Release of Wnt3a is facilitated by the SR-BI/II receptor

Our results argue against an unspecific release of Wnt3a on lipoproteins or other serum components, but indicate the specific use of HDL particles. This prompted us to investigate their role in Wnt3a release in more detail. Cells have two principal

mechanisms to exchange lipids with HDL, utilizing distinct cell surface proteins and HDLs as key components: (i) the scavenger receptor class B type I or II (SR-BI/II), which is a receptor that binds lipid-rich HDL with high affinity and mediates both selective lipid uptake from and release to HDL and (ii) the ATP-binding cassette transporter A1 (ABCA1), which mediates cholesterol efflux to lipid-poor pre-HDL and to lipid-free apoAI (Wang et al., 2000; Van Eck et al., 2004). To test whether SR-BI or other lipoprotein receptors are involved in Wnt3a release, we transfected Wnt3a into LDL receptor-deficient CHO-*ldl*A-7 cells (ldlA, Sege et al., 1984), which express only low amounts of HDL binding/selective uptake activity (Acton et al., 1994). Whereas Wnt3a was barely released from these cells into the medium, transfection of SR-BI or its isoform SR-BII resulted in a significant increase in Wnt3a release (Figure 3A). Wnt3a was not released when *ldl*A cells were co-transfected with either the LDL receptor (Figure 3A), ABCA1 or ABCG1, another ABC-transporter that facilitates the efflux of cholesterol to HDL (Figure 3B). This shows that SR-BI/II specifically facilitated Wnt3a release onto HDL. Mutant Wnt3a lacking the palmitic anchor was released normally from *ldl*A cells (not shown), suggesting that the mutant protein cannot be anchored sufficiently to cellular membranes. To test whether non-lipidated apoAI allows the release of Wnt3a, we incubated L-Wnt3a cells with increasing concentrations of purified apoAI, but no release could be observed (Figure 3C). Moreover, in the presence of serum lipoproteins the addition of lipid-free apoAI inhibited Wnt3a release from L-Wnt3a cells in a dose-dependent manner. Since lipid-free apolipoproteins can also bind to SR-BI and compete with binding of lipid-rich HDL (Xu et al., 1997; Liadaki et al., 2000), apoAI possibly blocked the release of Wnt3a by interfering with SR-BI function, explaining the inhibitory effect of apoAI on Wnt3a release.

Release of Wnt via endogenous lipoproteins

L-cells do not synthesize lipoproteins themselves. Instead, these cells released Wnt on exogenous HDL, which required the HDL receptor SRBI/II. However, knockout mice lacking SRBI/II or mice with defective HDL particles have no reported defects in Wnt signaling (Rigotti et al., 1997). This suggests that alternative or related mechanisms exist. For example, cells that synthesize and assemble lipoproteins themselves might secrete Wnt directly on these lipoproteins (Neumann et al., 2007). Therefore we studied whether the human enterocyte cell line Caco-2 releases Wnt3a on endogenously produced lipoproteins. Caco-2 cells are polarized epithelial cells from the intestine and express Wnt proteins (Holcombe et al., 2002; Gregorieff et al., 2005) as well as lipoproteins, mostly the LDL related chylomicrons (Levy et al., 1999).

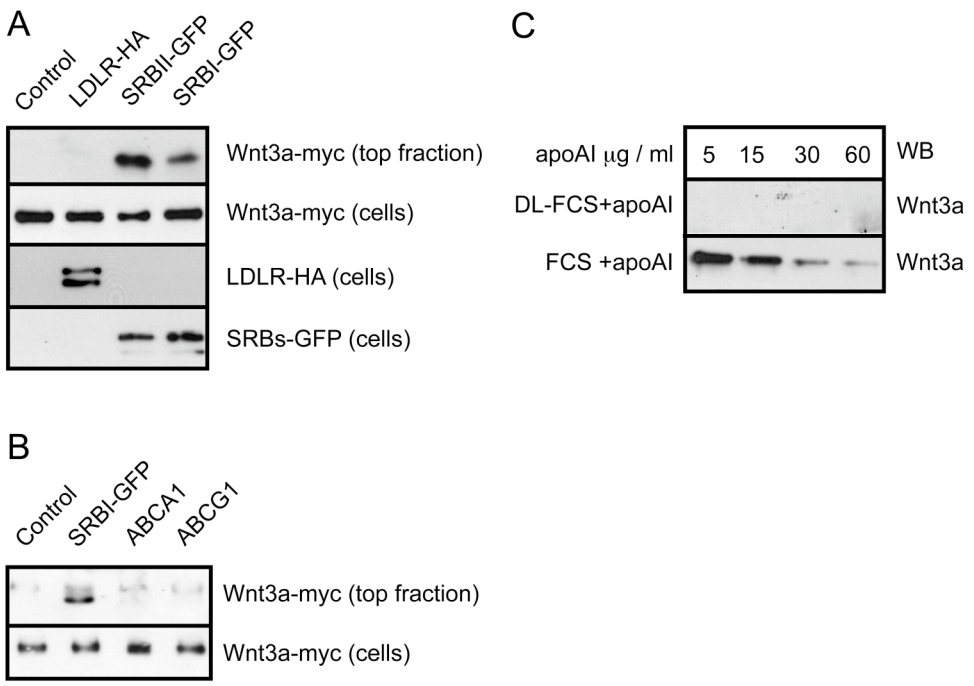

Figure 3: Role of SR-BI/II in Wnt3a release
A: To investigate if HDL receptors are involved in Wnt3a release, we made use of a cell line defective in lipoprotein receptors *ldl*A (Kingsley and Krieger, 1984). *ldl*A cells transiently (mock) expressing HA-tagged LDL-R, and GFP-tagged SR-BI or SR-BII were co-transfected with Wnt3a-myc and incubated in culture medium with 20% FCS for 3 days. The relative amount of Wnt3a in the lipoprotein fraction of the media was analyzed by western blotting (top fraction). The cellular expression levels of the lipoprotein receptors and Wnt3a were determined (cells). **B:** Cells can bind HDL via different cell surface receptors. To test if other receptors than SR-BI/II are involved in Wnt3a release *ldl*A cells transiently (mock) expressing GFP-tagged SR-BI, ABCA1-V5 or ABCG1-GFP were co-transfected with Wnt3a-myc and incubated in culture medium with 10% FCS for 3 days. Media and cells were analyzed as above. **C:** SR-BI/II can bind to lipid poor apoAI or lipidated HDL. To study which particle can facilitate Wnt3a release, L-Wnt3a cells were incubated with 10% DL-FCS (panel 1) or 10% FCS (panel 2) and increasing amounts of purified apoAI for 3 days. The soluble fraction of the medium was subjected to isopycnic density centrifugation and the first two fractions were pooled and analyzed by Western blotting for the presence of Wnt3a.

PD-7 cells, a subclone of Caco-2, produce only low amounts of mature lipoproteins compared to other Caco-2 clones (Salvini et al., 2002) and are therefore a good model to distinguish between endogenously produced and exogenously added lipoproteins. Secreted lipoproteins from PD7 cells fractionated in the high-density bottom fraction of the density gradient (Figure 4A) and were absent from the top fraction, indicating that they are poorly lipidated. Although exogenous lipoproteins

were present, Wnt3a-myc secreted from PD7 cells co-fractionated with apoB100 in the high-density fractions of the gradient, also when cells were grown on Transwell™ inserts (not shown). Thus, Wnt3a could be released as a soluble protein or be associated to the poorly lipidated apoB100. To test the interaction between Wnt3a-myc and lipoproteins under these conditions we tested whether apoB100 can be co-precipitated with Wnt3a-myc. We observed that apoB100 is not pulled down in the absence of Wnt3-myc or with a control antibody but specifically co-precipitated with anti-myc antibody, indicating a direct interaction between Wnt3a-myc and apoB100 (Figure 4B). In contrast, the parental Caco-2 cells secrete lipoproteins that are fully lipidated and fractionate in the top fraction of a density gradient. In Caco-2 cells stably expressing Wnt3a-myc (Caco-Wnt3a) Wnt3a-myc co-fractionates with lipoproteins and is absent from the high-density bottom fraction (Figure 4C). These experiments show that Wnt3a associated with endogenously produced lipoproteins, which may have taken place in the secretory pathway or at the cell surface.

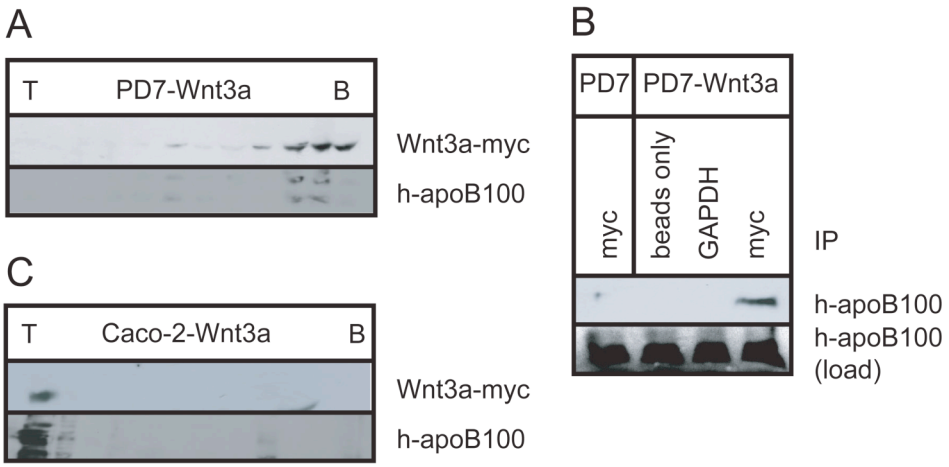

Figure 4: Wnt3a can be secreted through endogenous lipoprotein particles
A: PD7 cells stably expressing Wnt3a-myc (PD7-Wnt3a) were incubated with medium containing 10% FCS for 3 days. The conditioned medium was subjected to a KBr isopycnic gradient and fractions were analyzed by Western blotting for Wnt3a-myc and newly synthesized h-apoB100. Both proteins co-fractionate in the bottom fraction of the gradient. **B:** To examine if both proteins associate, conditioned medium of PD7 cells or PD7-Wnt3a cells was produced as above and probed by co-immunoprecipitation for interaction of Wnt3a with lipid poor apolipoproteins. Conditioned medium of PD7 cells or PD7-Wnt3a cells was divided into 3 parts and Wnt3a-myc was adsorbed to protein G-sepharose without antibody or mouse anti-GAPDH antibody as controls (IP) or with mouse anti-myc antibody. Immunoprecipitates were subjected to SDS-PAGE and Western blotting against h-apoB100. The relative amount of h-apoB100 in media prior to immunoprecipitation (load) was determined on a fraction of

the media by Western blotting. **C:** Compared to PD7 cells, wild type Caco-2 cells secrete more lipoproteins into the culture medium (Salvini et al., 2002). Caco-2 cells stably expressing Wnt3a-myc (Caco2-Wnt3a) were incubated with medium containing 10% FCS for 3 days. The conditioned medium was subjected to a KBr isopycnic gradient and fractions were analyzed by Western blotting for Wnt3a-myc and h-apoB100.

Discussion

Although many components of the Wnt signaling cascade have been identified and characterized only little is known about the release of Wnt into the extracellular space and its trafficking to neighboring cells, particularly in mammals. Several mechanisms have been proposed for the transport of lipid-modified proteins through the aqueous environment of cells and tissues. Recently it was suggested that Wingless, a Wnt homologue in *Drosophila*, is released and transported via lipoproteins (Panakova et al., 2005). In this study, we observed that Wnt3a is released from mammalian cells by lipoproteins present in the culture medium and that the protein was active under these conditions. The release of Wnt3a occurs specifically to HDL particles in a SR-BI/II dependent manner. Furthermore Wnt3a was released on newly synthesized lipoprotein particles from enterocyte cell lines.

Lipid modifications are required for the association with lipoproteins

Members of the Wnt family have been shown to be lipid-modified by palmitic acid (16:0) at a conserved cysteine residue (C77 in Wnt3a, Willert et al., 2003) and by palmitoleic acid (16:1) at a conserved serine residue (S209 in Wnt3a, Takada et al., 2006). Whether all members of this family carry a lipid anchor is not clear, however the critical residues for lipid modifications are highly conserved between family members and species. Nonetheless, only a number of Wnt proteins have been characterized in respect to the lipid anchor so far (Willert et al., 2003; Zhai et al., 2004; Takada et al., 2006; Galli et al., 2007; Kurayoshi et al., 2007). As proteins with different lipid anchors associated with lipoproteins in *Drosophila*, we expect the association of Wnt proteins with lipoproteins also to occur with Wnt proteins other than Wnt3a. However, very recently it was shown that WntD, a *Drosophila* Wnt protein that does not have a mammalian homologue, is not lipid modified (Ching et al., 2008). Removal of the palmitate moiety allows normal release of Wnt to the cell culture medium but impairs its function, whereas removal of the palmitoleate moiety impedes transport of Wnt from the ER to the plasma membrane (Tanaka et al., 2000; Willert et al., 2003; Zhai et al., 2004; Takada et al., 2006; Galli et al., 2007; Komekado et al., 2007; Kurayoshi et al., 2007), indicating several distinct roles of the lipid modifications. Despite the normal release from cells of mutant WntC77S, which lacks the palmitate moiety, into the medium we find that mutant WntC77S was present as a soluble protein, in contrast to wild type Wnt3a, which was associated with lipoproteins. This indicates that the palmitoleate moiety on Ser209 by itself is not able to anchor Wnt3a to lipoproteins. Probably a single lipid

modification is not sufficient to efficiently target proteins to membranous structures as is the case for cytosolic small GTPases (Behnia and Munro, 2005). On the other hand it cannot be excluded that the removal of the lipid anchor leads to a structural rearrangement of Wnt, which impairs signaling activity and lipoprotein binding.

Lipoproteins and lipoprotein receptors

In theory, all classes of lipoproteins have the capability to carry lipid-modified proteins in the phospholipid monolayer surrounding the fat core. A possible role for lipoprotein receptors is to bring lipoprotein particles in close vicinity to the exoplasmic leaflet of cell membranes, which contain Wnt. Then, Wnt can be transferred to the lipoprotein particles, either by passive diffusion or by an active, but yet unidentified, protein machinery. In our experiments Wnt is released from cells only to HDL, but not to LDL. However, once associated to lipoproteins Wnt could freely exchange between lipoprotein particles, similar to other proteins, like the small apolipoproteins apoE and apoC (Havel et al., 1973; Mahley, 1988; Shearer et al., 2004). The release of Wnt was dependent on HDL and the SR-BI/II receptor, but not on LDL and the LDL receptor. What determined this specificity? One possibility could be sorting in the endocytic system, which was found to be essential for Wnt release (Banziger et al., 2006; Bartscherer et al., 2006; Coudreuse et al., 2006; Franch-Marro et al., 2008; Yang et al., 2008). Whereas LDL particles and their associated proteins are taken up via the LDL receptor and targeted for degradation to the lysosome, HDL can be recycled by SR-BI/II or ABCA1 (Eckhardt et al., 2004; Neufeld et al., 2004; Hassan et al., 2008). Thus if Wnt would have been released onto LDL, both proteins would have been targeted to lysosomal degradation. In addition we found a more efficient release of Wnt from *ldl*A-cells transfected with SR-BII compared to SR-BI. SR-BII preferentially localized to endocytic compartments and the plasma membrane (Eckhardt et al., 2004), suggesting a role in HDL recycling. Possibly Wnt can also be released on recycling lipoprotein particles other than HDL (Fazio et al., 2000; Roosendaal et al., 2008).

Intercellular transport of Wnt in vivo

Depending on the specific Wnt protein affected, the loss of Wnt signaling is lethal or leads to phenotypes that have defects in limb outgrowth, bone development or adipocyte maturation in adult mice (Parr and McMahon, 1995; Bennett et al., 2005). The SR-BI/II knock-out mouse does not show phenotypes related to impaired Wnt signaling (Rigotti et al., 1997) contrasting our findings in mammalian cell culture systems. This could be due to redundancy of receptors from the large family of scavenger receptors, which bind a broad range of hydrophobic molecules. On the other hand, the large family of low density lipoprotein receptor related proteins (LRPs) could be involved in the recycling of lipoproteins (Farkas et al., 2004), maybe contributing to Wnt release. Furthermore during development different lipo-

proteins and lipoprotein receptors than in adult tissues can be involved. It is difficult to obtain experimental evidence for lipoproteins as morphogen transporters in living animals, because interference with lipid metabolism alters physiological processes. Perhaps the use of inducible reporter genes for Wnt signaling in combination with inducible, tissue specific knockdowns of lipoproteins and their receptors will give more insight in Wnt transport *in vivo*.

Different from the *Drosophila* model, where lipoproteins are synthesized in the fat body and Wnt is produced in other cell types, some mammalian cell types producing Wnt are also able to synthesize lipoproteins (Holcombe et al., 2002; Gregorieff et al., 2005). We find first evidence that in these cells Wnt is present on endogenously produced lipoproteins. In contrast to the fibroblast system, Wnt3a co-fractionated together with poorly lipidated lipoproteins in conditioned medium from PD7 cells, instead of being released onto exogenous lipoproteins. Most likely this occurs because the SR-BI/II receptor is localized apically (Cai et al., 2004), whereas lipoproteins are secreted basolaterally (van Greevenbroek et al., 1995) together with Wnt3a (not shown). Whether in these cells the association of Wnt3a with lipoprotein particles occurs in the secretory pathway or as a consequence of extraction from the plasma membrane via secreted lipoproteins has to be further investigated. Nevertheless, lipid modification of Wnt and lipidation of lipoproteins both take place in the ER. Therefore it is likely that association of both proteins occurs in the same compartment. This pathway would give cells the opportunity to flexibly react to changes in nutrition. In mammals the lipid-modified hormone Ghrelin was found to be associated to VLDL and HDL particles in serum (De Vriese et al., 2007). Ghrelin is a small peptide hormone carrying a octanoate moiety (Kaiya et al., 2001) and is secreted from the stomach and the intestine upon starvation to induce food uptake. This is another example of regulating fat metabolism by the action of lipid-modified proteins.

Other lipid-modified proteins

A rising number of extracellular proteins have been identified as containing a lipid anchor. For all of these proteins it is unclear how they are transported through an aqueous environment. Lipoproteins have the potential to be a vehicle for a variety of lipid-modified proteins. In *Drosophila* the lipid-modified morphogens Hedgehog and Wingless as well as GPI-anchored proteins were found to be associated with lipophorin (Panakova et al., 2005), indicating that the incorporation of lipid-modified proteins into the phospholipid monolayer of a lipoprotein is rather unspecific. In contrast our findings suggest that the transfer of the lipid-modified protein Wnt3a occurs specifically to HDL. The fact that not all lipoprotein receptors facilitate the release of Wnt implies that, in addition to recruiting the lipoprotein to the membrane, a specific interaction between either the lipid-modified protein and the lipoprotein or the lipoprotein receptor could be involved. Theoretically lipid-modified

proteins and lipoprotein receptors could also be laterally restricted to distinct membrane domains, depending on the physical properties of the lipid-modification and the transmembrane domains of the receptor. Thus the interaction between lipoproteins and lipid-modified proteins may be rather unspecific, although in the context of lateral organization of membranes combined with individual protein-protein interactions, specificity in uptake can be obtained. Whether specificity is only relevant for efficient release or also serves additional functions remains to be determined. As many different proteins are associated to lipoprotein particles (Panakova et al., 2005; Sprong et al., 2006; O'Brien, 2007; Vaisar et al., 2007) the role of lipoproteins and their associated proteins should be reconsidered.

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Materials and Methods

Materials
Chemicals, if not indicated otherwise, were from Sigma (St. Louis, MO) and used in the highest purity available. Organic solvents were from Riedel de Haën (Darmstadt, Germany), and cell culture media and reagents were from Invitrogen (Breda, The Netherlands). Cell culture plastics were from Costar (Cambridge, MA). Mouse anti-myc 9E10, rabbit anti-myc A14 and rabbit anti-HA Y11 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-human apoliprotein AI was from Calbiochem (La Jolla, CA) and antisera against apolipoprotein B100 were from Calbiochem (La Jolla, CA) or from Santa Cruz Biotechnology (C1.4, Santa Cruz, CA). The mouse antibody against green fluorescent protein JL8 was from Clontech (Mountain View, CA). Rabbit anti-calnexin antiserum (Zhang *et al.*, 1997) was a kind gift of Ineke Braakman (Utrecht University, Netherlands). Rabbit anti-Wnt3a antiserum was a kind gift from Roel Nusse (Stanford University, Stanford, CA). Mouse anti-GAPDH was from Ambion (Applied Biosystems, Nethelands). Anti-hIgG was a kind gift of Jeanette Leusen (University Medical Center Utrecht, Netherlands). Horseradish peroxidase conjugated secondary goat antibodies were from Pierce (Perbio, Netherlands) Myc-peptide was synthesized using an Applied Biosystems 431A peptide synthesizer.

Sera and lipoprotein preparation

Fetal calf serum (Hyclone, Logan, UT) was delipidated (DL-FCS) by solvent extraction. Therefore serum was mixed with an equal volume of a 2:1 mixture of diisopropyl ether: n-butanol, stirred at room temperature for 30 min, and phases were separated by centrifugation at 5,000g for 30 min. The aqueous phase was re-extracted with an equal volume of diisopropyl ether, and subsequently dialyzed against PBS. This treatment resulted in the total loss of intact lipoprotein particles, as judged by the loss of proteins from the top fraction of a KBr gradient centrifugation that was analyzed by SDS-PAGE and coomassie brilliant blue staining. The cholesterol content was reduced to less than 5% of untreated serum, as determined using the Amplex Red Cholesterol assay kit (Molecular Probes, Eugene, OR). Human serum was obtained from healthy fasting human donors, and HDL and LDL were prepared by discontinuous density centrifugation as previously described (Redgrave et al., 1975), dialyzed against PBS, and stored under nitrogen at 4°C. The protein contents of the preparations were determined using the BCA assay (Pierce Chemical Co.). Small unilamellar vesicles (SUVs) were generated by sonication on ice (Smith et al., 1994) of egg PC:cholesterol:egg PS (50: 50:1, mol/mol).

Plasmids

pcDNA3-LDLR-HA was kindly provided by Jürgen Gent (Utrecht University, The Netherlands). The construction of pEGFP-SRBI and pEGFP-SRBII has been described (Eckhardt et al., 2004). pcDNA3.1/V5-His-TOPO-ABCA1 and pEGFP-ABCG1 were from Gerd Schmitz (Regensburg, Germany). pEGFP-CD63 was from Gillian Griffiths (Oxford, United Kingdom). pcDNA3-Wnt3a was from Damien Coudreuse (Rockefeller University, New York). A myc-tag was appended at the carboxy-terminus of Wnt3a by PCR and ligated into pcDNA3 (pcDNA3-Wnt3a-myc). Cysteine 77 of Wnt3a was mutated into a serine by PCR-mutagenesis, yielding pcDNA3-Wnt3a-C77S-myc. All synthetic cDNAs were verified by restriction analysis and dye termination sequencing.

Cell culture and transfection

Mutant Chinese hamster ovary *ldl*A (clone 7) cells that are LDL receptor-deficient, and express very little SR-BI or HDL binding/selective uptake activity (Acton et al., 1994; Acton et al., 1996) were kindly provided by Monty Krieger (MIT Department of Biology, Cambridge) and Jürgen Gent (Utrecht University, The Netherlands). *ldl*A and mouse L-cells (ATCC, Rockville, MD) were grown in DMEM containing 4 mM L-glutamine, 4.5 g/L glucose, 10% FCS (culture medium) and were maintained at 37°C with 5% CO_2. Control L-cells were stably transfected with pcDNA3 and maintained in culture medium containing 0.4 mg/ml geneticin. L-Wnt3a cells (ATCC, Rockville, MD) were cultured in culture medium with 0.4 mg/ml geneticin.

For Wnt activity measurements L-Wnt3a cells were kindly provided by Daniele Tauriello (Utrecht University, The Netherlands) and cells were cultured in medium containing 50 µg/ml zeocin (Invitrogen). L-cells stably transfected with the superTOP-flash system (L-STF) were kindly provided by Roel Nusse (Stanford University, CA) and cultured in medium containing 0.8 mg/ml geneticin.

Wnt conditioned medium

Unless indicated otherwise, in experiments detecting human lipoproteins by Western blotting, human sera were used. Cells on 3 cm dishes were transiently (mock) transfected using Lipofectamine 2000 (Invitrogen), and grown in Optimem (Invitrogen) with DL-FCS, FCS, purified HDL or LDL or lipid-free apoA1 at 37°C with 5% CO_2 for 3 days. Cell debris was removed by centrifugation at 10,000*g* and the supernatant centrifuged for 1-3 hours at 39,000 rpm at 4°C in a SW41Ti rotor (Beckman). Lipoprotein particles were separated from soluble proteins by isopycnic density centrifugation. In short, KBr was added to the supernatant to a final concentration of 0.33g/ml, and the sample was spun for 2 days at 37,000 rpm at 4°C in a SW41Ti rotor. Twelve fractions were taken from the top. The proteins were precipitated with trichloroacetic acid. In some cases, the proteins from the supernatant were directly precipitated with trichloroacetic acid.

Wnt activity measurements:

L-cells or L-cells stably transfected with Wnt3a were grown on 15 cm dishes. Protein expression was induced with 2 mM butyrate for 3-5 days, when cells where 60% confluent. Conditioned medium (20 ml) was collected and cell debris was removed by centrifugation. KBr gradients were performed and divided in three (pooled) or twelve fractions. Fractions were exchanged to normal medium using an Amicon column (Millipore) to normal medium without FCS. L-cells stably transfected with Super Top-Flash system were grown on a 24 well and incubated overnight with a 1:1 dilution of a fraction or with normal medium in a final volume of 300 μl. Cells were lysed and luciferase activity was measured using the BrightGlo system (Promega).

Immunoprecipitation

Conditionened medium of cells was subjected to ultracentrifugation at 120,000*g* in a SW41Ti rotor (Beckman) and the supernatant was precleared during a 2-hour incubation with 0.25 volumes of sepharose CL4B beads. A fraction of each supernatant was used to determine relative amounts of apoA1 and apoB by Western blotting. The remainder was incubated with anti-Wnt3a, anti-myc or anti-GAPDH

adsorbed to protein A- or protein G-sepharose (GE Healthcare). Immunoprecipitates were washed at least 10 times with 5 volumes PBS (without Ca^{2+} and Mg^{2+}), 0.5% bovine serum albumin, and once with PBS (without Ca^{2+} and Mg^{2+}). Proteins were eluted from beads using either 150 mM NaCl, 2 mM EDTA, 100 mM Tris-Cl pH 8.3, 0.5% Nonidet -P40, 0.5% sodiumdeoxycholate, and 0.1% SDS or Laemmli sample buffer containing 5% ß-mercaptoethanol or myc-peptide.

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GPI-specific phospholipase D releases GPI-anchored proteins from lipoprotein particles

Summary
Glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) is an abundant protein in serum. However, its physiological role is unclear. Here, we demonstrate that GPI-PLD, which is inactive to GPI-anchored proteins at cell surfaces, can cleave GPI-anchored proteins that were transferred from cultured cells to lipoprotein particles. Conversely, when the activity of GPI-PLD was inhibited chemically, by bacterial lipids or heat-inactivated bacteria, GPI-anchored proteins were found on lipoproteins. Thus, GPI-PLD may be involved in the removal of GPI-anchored proteins from circulating lipoproteins and LPS may interfere with this process. This may have consequences for systemic infections.

Introduction
The glycosylphosphatidylinositol (GPI) anchor is a posttranslational modification on many eukaryotic proteins that attaches them to the outer leaflet of cellular membranes. GPI-specific phospholipases cleave the GPI-anchor. They release a soluble protein, leaving the lipid anchor in the membrane. The only GPI-specific mammalian phospholipase known is GPI-phospholipase D (GPI-PLD). The protein is relatively abundant in serum (Davitz et al., 1987) but whether it is involved in the release of GPI-anchored proteins from cells is not known. GPI-PLD is secreted by hepatocytes (Raymond et al., 1994) and to a lesser extent by macrophages (Xie and Low, 1994). In addition, expression of GPI-PLD has been detected in many other tissues (LeBoeuf et al., 1998).

GPI-PLD possibly functions intracellularly (Brunner et al., 1994; Hari et al., 1996; Mann et al., 2004), but whether this role involves the cleavage of GPI-anchored proteins during their transport to the cell surface, the release of proteins from the plasma membrane, or the breakdown of exogenous GPI-anchored substrates taken up by the cell is unknown. Various (patho)physiological conditions have been associated with either an increase or a decrease in serum GPI-PLD activity (Raymond et al., 1994; Rhode et al., 1999; Gray et al., 2008), but the physiological significance of these changes is unclear. *In vitro,* GPI-PLD specifically hydrolyses GPI-anchored proteins, but serum GPI-PLD can cleave GPI-anchored proteins from the cell surface only if the membrane is perturbed with detergents. One possibility is that an inhibitor of GPI-PLD is present in serum. Another possibility is, that a change in membrane environment is required for cleavage (Low and Huang, 1991).

Most GPI-PLD in serum is associated with HDL and *in vitro* HDL increased cleavage of GPI-anchored proteins by GPI-PLD (Hoener and Brodbeck, 1992; Deeg et al., 2001). In *Drosophila*, which lacks GPI-PLD, GPI-anchored proteins were found associated with lipoproteins (Panakova et al., 2005). On the other hand, in mam-

mals, with abundant GPI-PLD, GPI-anchored proteins were hardly found on lipoproteins (Vakeva et al., 1994; Gao et al., 2006; Safar et al., 2006; Sprong et al., 2006). Generally, lipoproteins can act as carriers for lipid-modified proteins (Panakova et al., 2005; Sprong et al., 2006; Neumann et al., 2007). Therefore we investigated whether GPI-PLD can release GPI-anchored proteins from lipoprotein particles.

Material and Methods

Materials

Chemicals and cell culture materials were from the usual sources (Panakova et al., 2005). Mouse anti-GFP was from Roche (Basel, Switzerland). Horseradish peroxidase-conjugated secondary goat antibodies were from DAKO (Glostrup, Denmark). Phosphatidylinositol-specific phospholipase C (PI-PLC) was from Invitrogen (Breda, Netherlands). GPI-PLD was purified as before (Civenni et al., 1999).

Release of GPI-GFP from cells

Her14 cells stably expressing GFP-GPI (GFP-GPI cells), a kind gift from Erik Hoffman (Utrecht University, Netherlands), were cultured in DMEM, 4.5 g/l glucose, 10% FCS (Bader et al., 2007). GFP-GPI cells were incubated with phenol red-free DMEM and (mock) treated with 300 mU PI-PLC or 300 mU GPI-PLD (4h, room temperature). GFP fluorescence in the medium was measured using a PTI Quantamaster (PhotoMed GmbH, Germany) at λex = 473 nm and λem = 590 nm.

Media

Media contained 10% of the following FCS: (1) Untreated. (2) GPI-PLD was inactivated by raising the pH of FCS to pH 11 for 30 min. Before incubations with cells the pH was neutralized. (3) 30 ml of *Neisseria meningitidis* wild type or LpxA$^-$ were grown to an OD600 of 0.7, heat-inactivated (65°C, 1h), pelleted and mixed with 4 ml FCS. (4) LPS was mixed with FCS (50 µg/ml).

Cell incubations and lipoprotein separation

15 cm^2 dishes of GFP-GPI cells were incubated with 20 ml of medium containing 10% of the FCS treated as described. After 2–3d 12 ml medium was collected, centrifuged at 4,000*g* for 15 min and subsequently at 100,000*g* for 1h. Media were (mock) incubated with 300 mU PI-PLC or 300 mU GPI-PLD (5h, 37°C). Lipoprotein particles were separated from soluble proteins by isopycnic density centrifugation (Sprong et al., 2006). Proteins were precipitated from the top 1 ml with trichloroacetic acid and analyzed by Western blotting against GFP.

Results and Discussion

GPI-PLD cleaved GFP-GPI from lipoproteins, not from cells

The GPI-anchor associates proteins to the cell surface, but many of these proteins also exist in a soluble form in the circulation (Ferguson, 1992; Labeta et al., 1993). These could be generated through cleavage by proteases or phospholipases. In *Drosophila* GPI-anchored proteins were found associated with lipoproteins, like other lipid-modified proteins (Panakova et al., 2005; Eugster et al., 2007). This organism lacks GPI-PLD. Because GPI-PLD is inactive on plasma membranes (Low and Huang, 1991), we wanted to test whether GPI-PLD would cleave GPI-anchored proteins from lipoproteins. GFP-GPI cells were (mock) incubated with PI-PLC or GPI-PLD. Without phospholipases present, some GFP-GPI was released, possibly by proteolytic cleavage. PI-PLC released GFP from the membrane, whereas GPI-PLD did not (Figure 1A), corroborating earlier findings (Low and Huang, 1991). To investigate whether GFP-GPI was associated to lipoproteins, conditioned medium of GFP-GPI cells was subjected to isopycnic density centrifugation to separate lipoproteins from other serum proteins (Panakova et al., 2005; Sprong et al., 2006). When cells were incubated with FCS, GFP-GPI did not co-fractionate with lipoproteins (Figure 1B). GPI-PLD can be inhibited by treatment of serum at pH 11. Under these conditions GFP-GPI fractionated with lipoproteins, indicating association. To show that this effect was due to GPI-PLD inactivation, we added back the purified enzyme to the pH-treated conditioned medium of GFP-GPI cells. This released GFP-GPI from lipoproteins (Figure 1B). Therefore, we concluded that GPI-PLD cleaved GFP-GPI from lipoproteins in culture medium but not from cell surfaces.

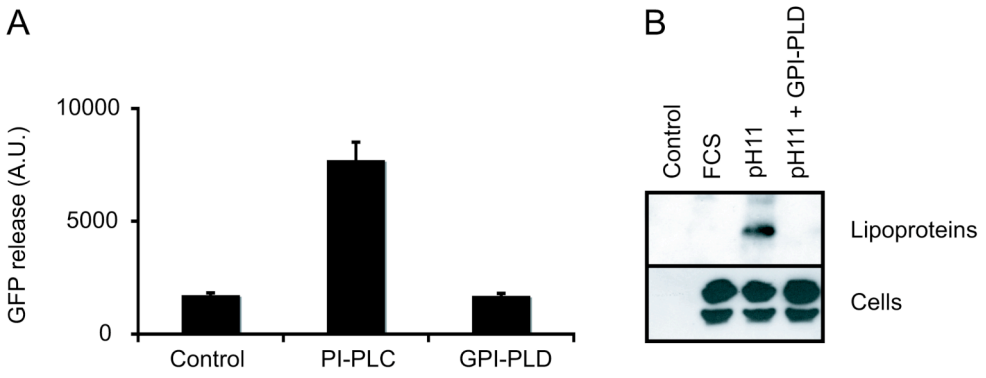

Figure 1: GFP-GPI is cleaved from lipoproteins by GPI-PLD

A: Her14 cells overexpressing GFP-GPI were incubated for 4h at room temperature with or without purified PI-PLC (300 mU) or GPI-PLD (300 mU) and released GFP was measured in a fluorimeter. Some release was observed without phospholipases present (Control). GFP release from cells was increased by PI-PLC but not by GPI-PLD. **B:** GPI-PLD was inhibited

by raising the pH of FCS to pH 11 for 30 min. Before incubation on cells the pH was neutralized. Her14 cells overexpressing GFP-GPI were incubated with or without pH-treated FCS. Medium was collected after 2-3 days and subjected to an isopycnic KBr gradient to separate lipoprotein particles. Proteins from the top fraction of the gradient were precipitated with trichloroacetic acid and subjected to Western blot against GFP (Lipoproteins). Equal GFP-GPI expression under the different conditions was confirmed by Western blot analysis (Cells). In samples of untransfected cells (Control) no signal for GFP was detected. Only when GPI-PLD was inhibited by pH treatment GFP-GPI co-fractionated with lipoproteins. It was released from lipoproteins when the collected medium was treated with purified GPI-PLD before centrifugation.

GFP-GPI is on lipoproteins in the presence of bacterial lipids

In vitro, GPI-PLD can be inhibited by lipids, amongst them bacterial LPS (Low and Huang, 1993). We wanted to test whether this is also relevant for the cleavage of GPI-anchored proteins from lipoproteins. GFP-GPI cells were incubated with purified bacterial lipids or heat-inactivated bacteria. When cells were incubated with purified LPS from *E. coli*, GFP-GPI was present on lipoproteins (Figure 2A). It could be released by PI-PLC, showing that the GPI-anchor was responsible for anchoring the protein to lipoproteins. Also when incubated with heat-inactivated *Neisseria meningitidis* GFP-GPI was present on lipoprotein particles in a PI-PLC dependent manner. The mutant LpxA$^-$, which lacks the first enzyme of LPS synthesis (Steeghs et al., 1998), did not show this effect (Figure 2B).

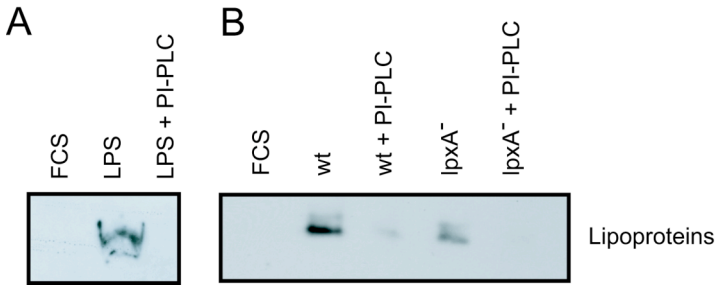

Figure 2: GPI-anchored proteins co-fractionate with lipoproteins when bacterial lipids are present

GPI-PLD can be inhibited by bacterial lipids (Low and Huang, 1993). Therefore we wanted to test, if under these conditions GFP-GPI is found on lipoproteins. **A:** Medium of Her14 cells overexpressing GFP-GPI with or without 5 µg/ml purified LPS was collected after 2-3 days and subjected to isopycnic density centrifugation. GFP in the top fraction of the gradient was analyzed as above. When LPS was present, GFP-GPI co-fractionated with lipoproteins in a PI-PLC dependent manner. **B:** GFP-GPI cells where incubated with medium containing heat-inactivated wild type (wt) *Neisseria meningitidis* or a mutant that cannot express LPS (LpxA$^-$) and media were analyzed as above. Only after incubation with wild type *Neisseria meningitidis* GFP-GPI was found on lipoprotein particles, whereas the incubation with the

mutant form had only a slight effect. GFP-GPI could be released from lipoproteins with PI-PLC.

Although the protein activity of GPI-anchored proteins is often well-characterized, the functional contribution of the GPI-anchor is not known. Therefore it is difficult to predict what would be the consequences of their presence on lipoproteins. Because GPI-PLD is inhibited by LPS (Low and Huang, 1993) and GPI-PLD activity is strongly reduced in systemic infections (Rhode et al., 1999), one possibility is that GPI-anchored proteins involved in the innate immune response may be affected by GPI-PLD activity. Interestingly, the LPS receptor CD14, which is directly involved in first responses to bacterial infections, is a GPI-anchored protein and its protein moiety was released from mononuclear cells (Labeta et al., 1993). Both anchored and non-anchored CD14 elicit immune responses. Further investigation whether there are differences in activity between CD14 present on lipoproteins and soluble CD14 are needed. It has to be noted, that many bacterial pathogens express PI-PLC, which is also capable of cleaving GPI-anchored proteins. PI-PLC may counteracts the inhibition of GPI-PLD and thereby influences the outcome of systemic infections.

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Nederlandse Samenvatting

Introductie

Cellen zijn de kleinste zelfstandige eenheid van het leven. Ze zijn omgeven door een dun laagje lipiden, membraan genoemd, die hen van de buitenwereld afgrenst en beschermt. Eenvoudige levensvormen, met name bacteriën, zijn ééncellige organismen met een eenvoudig bouwplan. Meer complexe levensvormen, zoals gist of zoogdieren, bestaan uit één of meerdere cellen, die in compartimenten onderverdeeld zijn, bijvoorbeeld de celkern. Deze zogenoemde organellen zijn ook begrensd door een membraan.

Membranen bestaan uit lipiden. Dat zijn moleculen met een hydrofobe (niet van water houdend) staart en een hydrofiele (van water houdende) kopgroep, die spontaan een laag kunnen vormen in het water en zo een reactieruimte kunnen creëren, waar biochemische reacties plaats kunnen vinden, zoals in een reageerbuisje. Echter, lipiden zijn niet alleen zoals het glas van het reageerbuisje, maar ze participeren ook direct in reacties of zijn betrokken bij hun regulering. Dus lipiden hebben vele belangrijke functies in een cel. In een cel vind men heel veel verschillende lipiden, waarschijnlijk om verschillende functies uit te voeren. Toch zijn de functies van lipiden niet goed bekend.

Lipiden zijn niet slechts membraancomponenten, maar kunnen ook direct aan eiwitten gebonden zijn. Deze eiwitten zijn dan verankerd in een membraan, want door het lipide zijn ze hydrofoob. Vele verschillende eiwitten zijn lipide-geankerd, wat een mechanisme is om hun lokalisatie in een cel te bepalen en daardoor hun functie. Ook eiwitten buiten de cel kunnen lipide-geankerd zijn.

Het onderzoek

In mijn onderzoek heb ik de transport van lipiden en lipide-geankerde eiwitten bestudeerd. Het transport van moleculen in een cel of tussen cellen is heel belangrijk, zoals onze maatschappij ook niet zonder fietsen, auto's, treinen, vliegtuigen en schepen kan bestaan. De bestudering van het transport van een molecuul geeft ook informatie over de functie van dit molecuul. Omdat lipiden en lipide-geankerde eiwitten hydrofoob zijn, hebben ze een transport vehikel, een soort ferryboot nodig. Eén manier van reizen voor lipiden is via membraanblaasjes, dit zijn kleine kogelachtige structuren, die door een membraan omgeven zijn. De andere mogelijkheid is een transporteiwit dat het lipide uit de membraan extraheert en dan specifiek ergens anders heen brengt. Ook lipide-geankerde eiwitten die buiten de cel aanwezig zijn, moeten getransporteerd worden. Ze kunnen wederom verschillende transportmechanismen gebruiken. Bijvoorbeeld, extracellulaire membraanblaasjes, exosomen genoemd, of transporteiwitten.

Hoe werkt het glycolipide transfer protein - GLTP?
Glycolipiden horen bij de klasse van sfingolipiden en ze tonen de hoogste diversiteit onder de lipiden, want ze kunnen vele combinaties van suikermoleculen als kopgroep hebben. Al zesentwintig jaar geleden hebben wetenschappers een eiwit geïsoleerd, dat glycolipiden van de ene membraan naar de andere membraan kan verplaatsen. Ondanks veel onderzoek weet men in essentie niet wat voor functie dit eiwit in de cel heeft. Opvallend is dat men GLTP in cellen nauwelijks aan membranen gebonden kan vinden. Daarom hebben wij onderzocht hoe GLTP aan modelmembranen bindt. Wij hebben een aminozuur van GLTP geïdentificeerd, dat een interactie met membranen aangaat. Verder kan dit eiwit ook detecteren, hoeveel glycolipiden er in een membraan zitten.

Hoe kan glucosylceramide flip-floppen?
Glucosylceramide is het eenvoudigste van de glycolipiden met slechts een glucose als kopgroep. Dit lipide wordt op de buitenkant van de Golgi-membraan gemaakt, die naar het cytosol wijst. Maar alle andere glycolipiden, die op glucosylceramide gebaseerd zijn, worden aan de andere kant van de membraan gemaakt. Dus moet glucosylceramide de membraan oversteken, een proces dat flip-flop genoemd wordt. Waar dat precies gebeurt in de cel is niet helemaal duidelijk. Wij hebben gevonden dat een grote hoeveelheid van glucosylceramide in het ER naar de andere kant flipt, maar dat dat ook kan gebeuren in de plasmamembraan of in lysosomen. Bovendien moet glucosylceramide dan nog van het Golgi naar het ER getransporteerd worden. Wij hebben gevonden dat daarvoor het eiwit FAPP2 gebruikt wordt. FAPP2 is ook een glycolipide transfer eiwit, dat heel erg op GLTP lijkt. Daardoor konden wij een volledig nieuwe transportroute voor glucosylceramide beschrijven.

Hoe wordt het lipide-geankerde proteïne Wnt tussen cellen getransporteerd?
Wnt is een eiwit dat heel belangrijk is in de vormgeving van het organisme. Samen met andere eiwitten is het belangrijk voor het bouwplan van de lichaam, dus: waar moet het hoofd, een arm of een been groeien. Wnt en ook andere soortgelijke eiwitten worden in bepaalde cellen gesynthetiseerd, maar dan worden ze naar andere cellen getransporteerd, om deze cellen mee te delen, bij welk lichaamsdeel ze horen. Ondanks dit feit is Wnt eigenlijk niet goed oplosbaar in de vloeistof die de cellen in ons lichaam omgeeft, want het heeft twee lipide-ankers. In de fruitvlieg *Drosophila* heeft men gevonden dat lipoproteïnen een soort ferryboot kunnen zijn voor Wnt. Lipoproteïnen zijn eiwitten die vet transporteren in de bloedbaan. Ze zijn gespecialiseerd in het transporteren van in water onoplosbare moleculen. Omdat Wnt twee lipide-ankers heeft geven lipoproteïnen het een lift. Wij hebben gevonden dat dit mechanisme ook in zoogdiercellen kan werken. Het transport van Wnt moet strak gereguleerd worden, aangezien anders de lichaamsdelen niet goed terecht komen. Daarom mag Wnt niet zomaar meeliften. Wij hebben gevonden, dat be-

paalde receptoren op het celoppervlak het vervoer van Wnt op lipoproteïnen controleren.

Wat is de functie van GPI-specifiek fosfolipase D (GPI-PLD)?

Een andere klasse van lipide-geankerde eiwitten zijn GPI-geankerde proteïnen. GPI is een bepaald soort lipide-anker. Ook deze eiwitten zijn op het oppervlak van cellen aanwezig, maar soms worden ze ook in de bloedstroom gevonden of op andere cellen waar ze niet gemaakt worden. Dus ook deze eiwitten worden getransporteerd. Wij vinden dat ook deze eiwitten op lipoproteïnen terecht kunnen komen. In het bloed is een enzym aanwezig, dat het GPI-anker van eiwitten af kan knippen: GPI-PLD. Wij hebben gevonden dat alleen wanneer de activiteit van dat eiwit onderdrukt is, GPI-geankerde eiwitten op lipoproteïnen kunnen meeliften. Wat zou hiervan de functie zijn? Dit is moeilijk te voorspellen, want de functie van vele GPI-geankerde eiwitten is niet bekend. Één manier om GPI-PLD te onderdrukken is de aanwezigheid van bacteriële lipiden. Daarom denken wij, dat dit mechanisme een functie heeft in het afweerstelsel. Sommige proteïnen die betrokken zijn bij het bestrijden van infecties zijn GPI-geankerd. Misschien verandert hun activiteit, als ze op lipoproteïnen terechtkomen, en zijn ze op deze manier beter in staat bacteriën te bestrijden.

Deutsche Zusammenfassung

Einleitung

Eine Zelle ist die kleinste selbstständige Einheit des Lebens. Sie wird umgeben von einer dünnen Schicht aus Lipiden, die man Zellmembran nennt. Membranen beschützen die Zelle vor Einflüssen aus der Umgebung und grenzen sie nach außen hin ab. Lipide sind Moleküle mit einer hydrophilen (wasserliebend) Kopfgruppe und einem hydrophoben (wasserabweisend) Schwanz. Zellen höher entwickelter Lebensformen, unter anderem Bäckerhefe, oder mehrzelliger Lebensformen wie Pflanzen oder Säugetiere, besitzen auch innere Membranen, welche die so genannten Organellen umgeben. Organellen sind Reaktionsräume, in denen spezifische, chemische Bedingungen herrschen um zelluläre Aufgaben zu erfüllen. Somit können durch Membranen Reaktionsräume geschaffen werden, wie in einem Reagenzglas. Hierbei spielen die Lipide nicht nur eine strukturelle Rolle, sondern nehmen auch direkt an biochemischen Reaktionen teil oder regulieren Proteine, die biochemische Reaktionen ausführen. Da viele biochemische Prozesse an Membranen stattfinden, ist deren Beschaffenheit, also ihre jeweilige Lipidzusammensetzung sehr wichtig. Darum ist es unabdingbar zu erforschen, wie bestimmte Lipidzusammensetzung generiert und aufrechterhalten werden. Lipide sind nicht nur Bestandteile von Membranen, sondern können auch kovalent an Proteine gebunden sein. Diese Proteine besitzen also einen Lipidanker, der sie wegen seiner Hydrophobizität an Membranen bindet (verankert). Dies ist wichtig um sie an einem bestimmten Ort innerhalb oder an der Außenseite der Zelle zu lokalisieren und somit ihre Funktion zu regulieren.

Die Fragestellungen

Während meiner Doktorarbeit habe ich den Transport von Lipiden und lipidmodifizierten Proteinen studiert. Der Transport von Molekülen innerhalb und zwischen Zellen ist sehr wichtig für die Funktion von biologischen Systemen, so wie unsere Gesellschaft auch nicht ohne Verkehr und Verkehrsmittel funktioniert. Weiterhin erhält man durch Informationen über den Transport eines Moleküls auch Hinweise über die eigentliche Funktion des Moleküls. Weil Lipide und lipidmodifizierte Proteine hydrophob sind, und somit unlöslich in der Flüssigkeit in und um Zellen, benötigen sie ein Vehikel für ihren Transport, sozusagen eine Fähre. Eine Möglichkeit für den Transport von Membrankomponenten sind Vesikel, das sind kleine, kugelartige Strukturen, die von Membranen umgeben sind. Das hydrophobe Molekül ist dann in die Membran eingebettet. Eine andere Möglichkeit sind Transportproteine, die zum Beispiel das Lipid aus der Membran extrahieren, um es dann an seinen Bestimmungsort zu transportieren. Lipid-modifizierte Proteine, die unter anderem an der Außenseite von Zellen zu finden sind, werden durch ähnliche Mechanismen transportiert.

Wie funktioniert das Glykolipid Transfer Protein - GLTP?

Glykolipide gehören zu der Klasse der Sphingolipide und weisen die höchste Variation unter den Lipiden auf. Dies macht sie sehr interessant, weil sie dadurch wahrscheinlich viele verschiedene Funktionen ausüben können. Schon vor 26 Jahren haben Wissenschaftler ein Protein isoliert, das Glykolipide von einer Membran zu einer anderen transportieren konnte. Obwohl dieses Protein seitdem weitreichend erforscht wurde, ist es immer noch unklar, welche Funktion es in Zellen hat. Es ist auffallend, dass GLTP innerhalb von Zellen eigentlich nicht an Membranen gebunden vorliegt. Darum haben wir untersucht, wie GLTP eigentlich an Membranen bindet. Dabei konnten wir eine Aminosäure identifizieren, welche verantwortlich ist für die Wechselwirkungen von GLTP und Membranen. Außerdem kann GLTP auch detektieren, wie viel Glykolipid in einer Membran vorhanden ist, was vielleicht bedeutet dass es eigentlich ein Sensor ist anstatt eines Transport Proteins.

Was ist die zelluläre Route von Glukosylceramide?

Glukosylceramid, das einfachste der Glykolipide, wird auf der cytosolischen Seite am Golgi Apparat synthetisiert. Jedoch werden alle komplexen Glykolipide, die auf Glukosylceramid basiert sind, im Lumen des Golgi synthetisiert. Das bedeutet, dass Glukosylceramid die Membran überqueren muss, ein Prozess, der Flip-Flop genannt wird. Wir haben gefunden, dass Glukosylceramid vom Golgi zum ER transportiert wird, wo es ins Lumen flippen kann und dann via Vesikel zum Golgi zurück transportiert wird, wo es zu komplexen Glykolipiden umgesetzt wird. Der Transport vom Golgi zum ER findet durch das Glykolipid Transfer Protein FAPP2 statt. FAPP2 hat eine ähnliche Struktur wie GLTP, kann aber zusätzlich auch an den Golgi binden. Weiterhin kann Glukosylceramid auch an der Plasmamembran oder an endocytischen Organellen flip-floppen.

Wie wird das lipid-modifizierte Protein Wnt zwischen Zellen transportiert?

Wnt ist ein Protein, das zu den Morphogenen gehört. Morphogene sind für die Formgebung eines Organismus verantwortlich. Vereinfacht gesagt bestimmen sie wo ein Kopf wächst, ein Bein und ein Arm an einem Körper. Dies geschieht dadurch dass Morphogene den Zellen Signale übermitteln, wo sie sich im Körper befinden und wie sie sich entwickeln müssen. Wenn etwas in diesen Signalkaskaden außer Balance ist, kann sich kein vollständiger Organismus entwickeln oder es entstehen Krankheiten wie Krebs. Morphogene werden typischerweise von bestimmten Zelltypen ausgeschüttet und lösen ein Signal in „empfangenden“ Zellen aus. Deshalb müssen sie zwischen Zellen transportiert werden. Manche Morphogene, so wie Wnt, sind aber fest verankert in Membranen durch ihre Lipidanker. In der Fruchtfliege *Drosophila* sind Lipoproteine mögliche Transporter für lipidmodifizierte Morphogene. Wir haben herausgefunden, dass auch in Säugetierzellen Wnt von Lipoprotein extrahiert werden kann und dass dafür ein bestimmter Lipoprotein Rezeptor benötigt wird.

Binden GPI-verankerte Proteine an Lipoproteine?

Eine andere Art lipid-modifizierter Proteine sind GPI-verankerte Proteine, wobei GPI für eine bestimmte Art Lipidanker steht. In *Drosophila* findet man diese Proteine auch an Lipoproteine gebunden, in Säugetieren jedoch kaum. Was kann der Grund dafür sein? Im Prinzip können Proteine mit Lipidanker, unabhängig davon, welcher Lipidanker vorhanden ist, an Lipoproteine binden. Im Blut von Säugetieren ist ein Protein vorhanden, dass GPI-Anker abschneiden kann: GPI-spezifische Phospholipase D (GPI-PLD). Da GPI-verankerte Proteine auch auf Zelloberflächen zu finden sind, könnte GPI-PLD diese Proteine entweder von der Zelloberfläche abschneiden, oder vielleicht von Lipoproteinen. Wir und andere haben herausgefunden, dass GPI-PLD nicht an der Zelloberfläche schneiden kann. Stattdessen zeigen unsere Experimente, dass wenn man GPI-PLD inhibiert, GPI-verankerte Proteine an Lipoproteine gebunden sind. Im Organismus wird GPI-PLD vielleicht von bakteriellen Lipiden inhibiert, somit könnten einige Proteine, die an der Immunantwort beteiligt sind und gleichzeitig einen GPI-Anker besitzen, reguliert werden.

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Curriculum Vitae

Sylvia Neumann was born 30. July 1979 in Eisenhüttenstadt (Germany). After grammar school at the Gymnasium for Natural Science “Carl Friedrich Gauss” in Frankfurt/Oder (Germany) she started to study Biochemistry at the “Ruhr Universität” Bochum (Germany). Her studies were finished with her Diploma thesis about the “Transcriptional Regulation of the MMP2 Promoter” in the Lab of Bertram Opalka and Hans Christoph Kirch at the University Medical Center, Essen (Germany). For her doctoral studies she moved to the lab of Gerrit van Meer and Hein Sprong at the University Utrecht (The Netherlands) to study “Proteins mediating intra- and intercellular transport of lipids and lipid-modified proteins”. After her PhD Sylvia plans to start her postdoctoral studies in the Lab of Sandy Schmid at the The Scripps Research Institute, San Diego (USA).

Sylvia Neumann was participating in dancing groups and dancing theatres since her school time. Together with her friends from “Fire and Flame” (Eisenhüttenstadt), the dancing theatre “macasju” (Bochum) and “Dansimprovisatie Utrecht” (Utrecht) she enjoyed many years of training, workshops and especially performances.