Cellular fate and functions of glucosylceramide

Wat gebeurt er met glucosylceramide in de cel, en wat doet het?

(met een samenvatting in het Nederlands)

Proefschrift

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Jasja Wolthoorn

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Promotor: Prof. Dr. G.F.B.P. van Meer

Co-promotor: Dr. H. Sprong

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Contents

Abbreviations

AP	adaptor-protein complex
CGalT	UDP-Gal:ceramide galactosyltransferase
CGlcT	UDP-Glc:ceramide glucosyltransferase
CERT	ceramide transfer protein
ConcA	concanamycin A
CST	CMP-sialic acid transporter
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
FLIM	fluorescence lifetime imaging microscopy
Fuc	fucose
GalCer	galactosylceramide
GalT1	UDP-Gal:GlcCer galactosyltransferase 1 (LacCer synthase)
GlcCer	glucosylceramide
GlcSph	glucosylsphingosine
GLTP	glycolipid transfer protein
GM3	sialyllactosylceramide
GPI	glycosylphosphatidylinositol
GSL	glycosphingolipid
LacCer	lactosylceramide
Man	mannose
MVB	multivesicular body
PC	phosphatidylcholine
PI	phosphatidylinositol
PITP	phosphatidylinositol transfer protein
PNS	postnuclear supernatant
SG	steryl glucoside
SialT1	CMP-sialic acid:LacCer sialyltransferase 1 (GM3 synthase)
SM	sphingomyelin
SMS	sphingomyelin synthase
ST6GalI	CMP-sialic acid: α-2,6 sialyltransferase
TGN	*trans* Golgi network
UGT	human UDP-galactose transporter 1

Chapter 1

'The complex life of simple glycosphingolipids'

General introduction

Based on:

Degroote S, Wolthoorn J, van Meer G (2004) The cell biology of glycosphingolipids. *Semin Cell Dev Biol* 15:375-87.

Eukaryotes (and a few bacteria) contain a heterogeneous class of lipids called glycosphingolipids (GSLs): these lipids are composed of a ceramide backbone and a sugar headgroup. The hydrophobic ceramide part, consisting of a sphingoid base and a fatty acid, is inserted in a cellular membrane, whereas the sugar headgroup mostly faces the non-cytosolic space. Knockout studies in mice have started to reveal the importance of GSLs in organism development. Importantly, a knockout in the enzyme responsible for the synthesis of glucosylceramide (GlcCer), producing mice without glucose-based GSLs, was found to be embryonically lethal (Yamashita et al., 1999a). The removal of GSLs is also of physiological importance, as shown by the serious pathologies induced when the degradation of GSLs is deficient (Futerman and van Meer, 2004), highlighting again the importance of the balance between their synthesis and degradation. At the cellular level, GSLs exert important signaling functions possibly in the form of glycosignaling domains (Hakomori, 2002; 2004). In addition, cells use the capacity of GSLs to form ordered domains to create selectivity in membrane transport, important in the spatial organization of cells (Simons and van Meer, 1988). This review will focus on the synthesis of the main classes of mammalian GSLs, which are derived from the simple GSLs GlcCer and galactosylceramide (GalCer), and will discuss the sorting and transport of these GSLs, and their main functions in cells.

Diversity, synthesis and physical properties of glycosphingolipids

Heterogeneity of glycosphingolipids

GSLs exhibit a huge heterogeneity of structure, both in backbone and headgroup: more than 60 different sphingoid bases and more than 300 different oligosaccharide chains have been characterized so far, the combination of which creates thousands of different structures (Figure 1). The sphingoid bases can vary in length, saturation, hydroxylation, and branching (Karlsson, 1970). The main sphingoid base in mammals is sphingosine, or D-*erythro*-1,3-dihydroxy, 2-aminooctadec-4-ene or *trans*-4-sphingenine (d18:1). For example, in bovine kidney, sphingosine accounts for 80% of the total sphingoid bases (Karlsson et al., 1973). Sphinganine, which corresponds to sphingosine without the trans C4-C5 double bond, and phytosphingosine (C4-OH sphinganine), are also common sphingoid bases (Carter et al., 1954; Sastry and Kates, 1964). The fatty acid is amide-linked to the amino group of the sphingoid base. The fatty acid species are cell-type dependent, can vary in length, saturation and hydroxylation, but are mostly long (≥C16) and saturated in mammals (Rosenthal, 1987), sometimes they are unsaturated at C15 or hydroxylated at C2. GSLs can be divided into two main classes based on the first sugar linked to the ceramide backbone, glucose (Glcβ1-) or galactose (Galβ1-). In more specific cases, other sugars have also been found linked to ceramide: Fucα1–Cer in human colon cancer cells (Watanabe et al., 1976), GlcACer in *Flaviobacterium* (Yamamoto et al., 1978), Manβ1–Cer in several invertebrates (Hori et al., 1981). The major GSLs in

plants and fungi are based on inositolphosphoceramide, whereby mannose (α1-2, or α1-3) or glucuronic acid (α1-6) is transferred onto the inositol (Dickson and Lester, 1999). The common sphingoid base in these lipids is phytosphingosine, commonly C8 desaturated or, only in fungi, C9 methylated (Matsubara et al., 1987). The fatty acids are very long (C26) and often 2-hydroxylated.

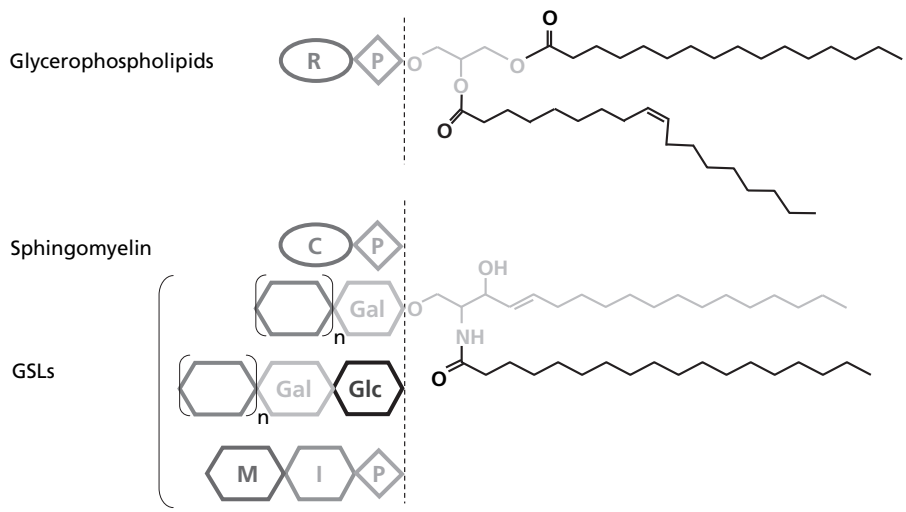

Figure 1. Structure of phospho- and glycolipids. Glycerophospholipids contain a phosphorylated glycerol backbone (light grey), substituted by two fatty acids, and the headgroups (R) choline, ethanolamine, inositol, or serine yielding PC (phosphatidylcholine), PE, PI and PS, respectively. Sphingolipids contain a sphingoid base like sphingosine (light grey) with an amide-linked fatty acid. The common mammalian phosphosphingolipid is the choline-containing sphingomyelin (SM). The glycosphingolipids (GSLs), carry sugars on the ceramide backbone. In mammals, the two main classes of GSLs contain either Gal or Glc as the first sugar. GalCer can then be galactosylated or sulfated. GlcCer can be further glycosylated to LacCer (Galβ1-4GlcCer), which serves as a precursor for different series of GSLs. Fungi and plants, besides GlcCer and complex GSLs produce the SM analog inositolphosphoceramide, which can be elongated by mannose and/or glucuronic acid.

Synthesis of glycosphingolipids

The first step in GSL biosynthesis is the condensation of serine and palmitoyl–CoA to 3-ketosphinganine, the precursor for all sphingoid bases. Subsequent acylation produces ceramide (Guillas et al., 2001; Schorling et al., 2001). In animals, the Δ4-*trans* unsaturation or C_4-hydroxylation of the sphingoid base most likely occur at this stage by a pair of newly identified desaturases DES1 and DES2 (Sperling et al., 2003) on the cytosolic side of the ER (Mandon et al., 1992; Hirschberg et al., 1993; Sperling et al., 2003). Ceramide can spontaneously cross the ER membrane and, in specialized cell types, be converted to GalCer. From the ER, ceramide can also follow the vesicular pathway to early Golgi compartments where it is converted to GlcCer. Alternatively, ceramide reaches the *trans* Golgi via CERT, a newly identified

ceramide transfer protein that is able to extract ceramide from the ER and deliver it to the Golgi after docking to phosphatidylinositol-4-phosphate (Hanada et al., 2003; Munro, 2003; Riezman and van Meer, 2004). This pathway may involve the membrane contacts between the ER and the *trans* Golgi (Ladinsky et al., 1999). Synthesis of the phosphosphingolipid sphingomyelin (SM) by the newly identified SM synthase in the *trans* Golgi (Huitema et al., 2004) depends on this pathway for ceramide supply (Hanada et al., 2003).

In mammals, GalCer derived GSLs are only present in specialized cells; they are the major lipids of the myelin sheath assembled around the axons of neuronal cells by oligodendrocytes and Schwann cells. In some animals, they are also present in epithelial cells of renal tubules and the gastrointestinal tract (Simons and van Meer, 1988). The transfer a Gal residue from UDP-Gal to ceramide occurs in the ER and is catalyzed by the UDP-Gal:ceramide galactosyltransferase or GalCer synthase CGalT (Schulte and Stoffel, 1993; Stahl et al., 1994; Schaeren-Wiemers et al., 1995; Kapitonov and Yu, 1997). CGalT is a class I integral membrane protein, belongs to the family of glucuronyltransferases, has an ER retention signal, and its active center is in the ER lumen (Sprong et al., 1998). It obtains its UDP-Gal substrate by retaining part of the Golgi UDP-Gal transporter in the ER (Sprong et al., 2003). On its way to the plasma membrane, GalCer passes the lumen of the Golgi apparatus. Here it can be sulfated to HO_3S-3GalCer (sulfatide). Especially in kidney epithelia, it can be galactosylated to Galα1-4GalCer. CGalT also produces animal Gal-diradylglycerol, which can be 3'-sulfated to seminolipid or, in plant chloroplasts, can be 6'-phosphorylated (Muller et al., 2000).

In contrast to GalCer, GlcCer is present in most eukaryotic cells and a few bacteria and serves as the major precursor for complex GSLs. It is synthesized by the UDP-Glc:ceramide glucosyltransferase or GlcCer synthase CGlcT, which has been cloned from many organisms (Leipelt et al., 2000; 2001). Sequence analysis suggests that CGlcT is a type III protein but, interestingly, it has no structure similarity with other proteins, including other glycosyltransferases. GlcCer synthesis occurs on the cytosolic side of Golgi membranes (Coste et al., 1986; Futerman and Pagano, 1991; Jeckel et al., 1992). CGlcT activity has been detected not only in the early Golgi compartments, but also in another compartment, possibly a pre-Golgi compartment (Futerman and Pagano, 1991) or the late Golgi (Jeckel et al., 1992). This observation could reflect the existence of two pools of GlcCer in cells, possibly having different functions.

Complex GSLs are made by the stepwise addition of individual sugars from their activated nucleotide precursors onto GlcCer. In mammals, the first reaction is the conversion of GlcCer to lactosylceramide (Galβ1-4GlcCer, or LacCer) by the LacCer synthase (Nomura et al., 1998; Takizawa et al., 1999). This enzyme, like all other glycosyl- and sufotransferases involved in the glycosylation of GSLs, acts in the Golgi lumen (Lannert et al., 1994; Kolter et al., 2002). Several galactosyl-, *N*-acetylgalactosaminyl-, *N*-acetylglucosaminyl -, sialyl-, and fucosyltransferases can

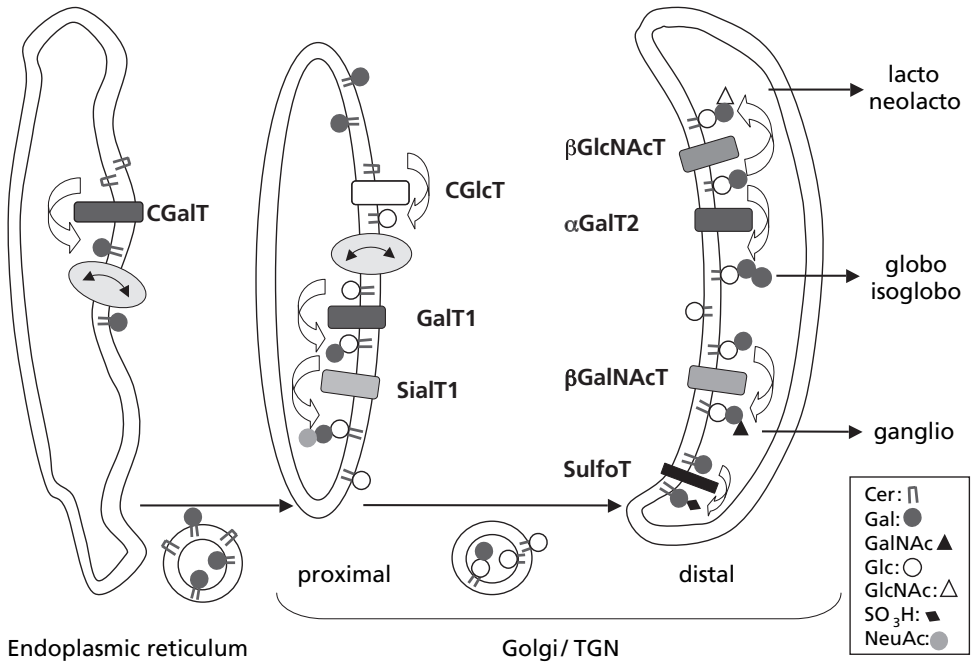

Figure 2. Topology of mammalian GSL synthesis. Ceramide is produced in the endoplasmic reticulum, where the sphingoid base can be desaturated or hydroxylated. In specialized cells, ceramide can be converted to GalCer in the ER lumen by the Gal transferase CGalT, also called GalT-1 (Basu et al., 1987). The glucosyltransferase CGlcT, active on the cytosolic side of the Golgi apparatus, synthesizes GlcCer which must be translocated to the Golgi lumen to be converted to LacCer by the β1,4-galactosyltransferase GalT1 (Giraudo and Maccioni, 2003) also called GalT-2 in a different nomenclature (Basu et al., 1987). LacCer is the precursor of different series of GSLs, mostly only one in any specific cell type. GalCer and LacCer can be sulfated in the late Golgi.

elongate the oligosaccharide chain of mammalian GSLs (Lloyd and Furukawa, 1998). Some of these enzymes are involved in the formation of the oligosaccharide backbone, the structure of which defines the different series of GSLs (see Figure 2, Table I). Other enzymes, like sialyltransferases and fucosyltransferases, are involved in the synthesis of the periphery of the oligosaccharide. Sialoglycosphingolipids are also called "gangliosides", and abbreviations have been assigned to them according to the number of sialic acids present and to their migration order in chromatography. The main abbreviations used are listed in Table II (Svennerholm system (Chester, 1998)). Except for ganglioside GM4, which corresponds to NeuAcα2-3GalCer, all gangliosides have LacCer as a precursor. Gangliosides are abundant in brain and nervous tissues. The carbohydrate chain of GSLs can also be sulfated by two different sulfotransferases: the Gal-3-*O*-sulfotransferase (Senn et al., 1990; Honke et al., 1997), in tissues that synthesize GalCer, mostly producing HSO_3-3GalCer but also HSO_3-3LacCer, a potential risk factor for type I diabetes mellitus, and a GlcA-

3-*O*-sulfotransferase, responsible for the synthesis of the HNK-1 epitope (HSO_3-3GlcAβ1-3Galβ1-3/4GlcNAcβ1-3Galβ1-4GlcCer) observed in developing central nerve and cauda equina in peripheral nerve (Bakker et al., 1997).

Table I. Root names and structures (recommendations for the nomenclature of glycolipids, http://www.chem.qmul.ac.uk/iupac/misc/glylp.html (Chester, 1998).

Root	Symbol	Structure
Ganglio	Gg	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer
Lacto	Lc	Galβ1-3GlcNAcβ1-4Galβ1-4Glcβ1-Cer
neolacto	nLc	Galβ1-4GlcNAcβ1-4Galβ1-4Glcβ1-Cer
Globo	Gb	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-Cer
Isoglobo	iGb	GalNAcβ1-3Galα1-3Galβ1-4Glcβ1-Cer
Mollu	Mu	GlcNAcβ1-2Manα1-3Manβ1-4Glcβ1-Cer
Arthro	At	GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ1-Cer

Table II. Some abbreviations for gangliosides in the Svennerholm system (Chester, 1998).

Abbreviation	Structure
GM4	NeuAcα2-3Galβ1-Cer
GM3	NeuAcα2-3Galβ1-4Glcβ1-Cer
GM2	GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-Cer
GM1a	Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-Cer
GM1b	NeuAcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-Cer
GD3	(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-Cer
GD2	GalNAcβ1-4 (NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-Cer
GD1a	NeuAcα2-3Galβ1- 3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-Cer
GD1b	Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-Cer
GT1a	NeuAcα2-8NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-Cer
GT1b	NeuAcα2-3Galβ1-3GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-Cer
GT1c	Galβ1- 3GalNAcβ1-4(NeuAcα2-8NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-Cer

Which sugars are present in the oligosaccharide backbone and periphery depends on the glycosyltransferases present and active in the Golgi apparatus of the cell, which may vary depending on the cell type, the developmental stage, or disease stage (Iwamori et al., 1984; Senn et al., 1990; Freischutz et al., 1994). For example, the presence of blood group ABH and of Lewis antigens will depend on the expression of certain glycosyltransferases (α1,3-*N*-acetylgalactosaminyltransferase, α1,3-galactosyltransferase, α1,2-fucosyltransferase for the ABH system, α1,2- and α1,3/4-fucosyltransferases for the Lewis system), which is genetically defined. The expression of β1,3-*N*-acetylglucosaminyltransferase, which is involved in the biosynthesis of glycolipids of the lacto- and neolacto-series, is highly regulated in mice and decreases after birth to undetectable levels in most cell types (Henion et al., 2001). Dramatic changes in glycolipid glycosylation and metabolism have been described in many cancer cells. Most of the time, these modifications in

glycosylation correspond to aberrant sialylation and/or fucosylation (Hakomori, 1986). The specific tumor associated antigens formed can be involved in adhesion events, or trigger intracellular signaling cascades involved in the development of the disease (Hakomori and Zhang, 1997).

Finally, in the yeast *Saccharomyces cerevisiae*, most of the genes involved in the biosynthesis of the inositolphosphosphingolipids have been identified (Beeler et al., 1997; Nagiec et al., 1997; Dickson and Lester, 1999; Lisman et al., 2004). Like inositolphosphoceramide synthesis, its mannosylation by the mannosyltransferases Csg1p and Csh1p occurs in the lumen of the medial Golgi (Dean et al., 1997; Lisman et al., 2004).

GSLs are degraded in the lysosomes, where they are sequentially hydrolyzed from the non-reducing end by specific hydrolases. Interestingly, GlcCer can be degraded not only by a glucocerebrosidase in lysosomes, but also in the cytosol by a non-lysosomal glucocerebrosidase (van Weely et al., 1993). Defects in the degradation of GSLs (either in glycosidases or their activator proteins) result in GSL storage in lysosomes, the species stored depending on which enzyme is deficient, and often cause very serious pathologies. Treatment of these diseases include replacement of the enzyme, gene therapy and inhibition of synthesis (Futerman and van Meer, 2004).

Physical properties of GSLs

Biophysical differences between GSLs and glycerolipids underlie the special behavior of GSLs in membranes. In GSLs, the region between the polar headgroup and the hydrophobic backbone contains chemical groups that can function both as hydrogen bond donor and hydrogen bond acceptor, in contrast to the glycerolipids, which only have hydrogen bond accepting properties in that part of the molecule (Pascher, 1976). Additional hydrogen bonding can occur between the sugar headgroups of GSLs. Another striking difference between GSLs and most glycerolipids is the fact that the lipid chains are saturated over at least the first 15 carbons of both chains (without disturbance by the double bond at the sphingosine C4 which is in the trans configuration). In combination with the higher hydrogen bonding, the saturated nature results in denser packing. This is measured as an increased melting temperature, or T_m, which corresponds to the temperature above which a bilayer of a single lipid switches from a frozen state, the gel or solid-ordered (s_o) phase to a fluid state, the liquid-crystalline or liquid-disordered (l_d) phase. GSLs have a much higher T_m than glycerolipids. The structural differences allow sphingolipids to self-associate amongst glycerophospholipids in the plane of the membrane as a flexible hydrogen-bounded network. In addition, cholesterol, a rigid and flat cylindrical lipid, also interacts preferentially with sphingolipids via van der Waals interactions (Boggs, 1987).

Studies in model membranes made of binary mixtures of lipids with different T_m have shown that phase separation can occur, resulting in the co-existence of a

s_o and a l_d phase. Nevertheless, this type of phase separation is probably not of physiological significance in mammalian cells, since membranes that contain high amounts of lipids of high T_m (like the plasma membrane) also contain high amounts of cholesterol, and cholesterol at high concentrations abolishes the s_o/l_d phase transition (Keough et al., 1989). Interestingly, in model membranes containing mixtures of a high T_m lipid and cholesterol, a fluid-fluid phase separation has been observed between the l_d and a liquid-ordered l_o phase (Recktenwald and McConnell, 1981). In addition, phase-behavior experiments using 3 different lipids of high T_m (di-saturated PC or a sphingolipid), low T_m (mono-unsaturated PC) and cholesterol (Silvius, 1992; Silvius et al., 1996; Ahmed et al., 1997; Xu and London, 2000) have shown that sterols have the ability to modulate the phase separation of lipids, depending on the concentration and the structure of the sterol used. Recently, de Almeida and co-workers have studied the co-existence of l_o, l_d and s_o phases in ternary mixtures of SM/mono-unsaturated PC and cholesterol. At a 1:1:1 ratio and 37˚C, it can be calculated that the l_o phase covered 2/3 of the surface, it was enriched in SM (1.5-fold) and cholesterol (4-fold) compared to the l_d phase, which in turn contained a 4 times higher PC concentration (de Almeida et al., 2003). Partitioning between such domains of a protein with a glycosylphosphatidylinositol (GPI) anchor was affected by the presence of 1 mol% of the ganglioside GM1 (Dietrich et al., 2001).

The relevant question for cell biology is: do l_d/l_o phase separations occur in biological membranes and are they of relevance for GSL distribution and function? The structural differences between GSLs and glycerophospholipids that promote their phase separation are conserved from vertebrates to yeast (Holthuis et al., 2001). Unfortunately, most evidence to date that domains enriched in GSLs exist in biological membranes is indirect (Harder, 2003). Most convincing are probably the microscopical observations that GSLs are clustered on erythrocytes (Thompson and Tillack, 1985), that the gangliosides GM1 and GM3 were concentrated in domains (Parton, 1994; Sorice et al., 1999), and that GM1 can be enriched in different domains than GM3 on the same cell (Gomez-Mouton et al., 2001). However, most indications for the lipid domain association have been obtained by using the detergent-resistance criterion. A number of membrane components that had been predicted to be in these “lipid rafts” from other approaches turned out to remain associated with membrane remnants after extraction with cold detergent (Brown and London, 2000). Although this technique has had great prospective value for how membrane signaling may work, especially at the immunological synapse (Harder, 2003), physical studies have suggested that there is no straightforward physical basis for why extraction at 4˚C would provide information concerning the situation at 37˚C (Heerklotz, 2002; de Almeida et al., 2003; Heerklotz et al., 2003): the results may be correct but by coincidence (de Almeida et al., 2003), which calls for caution in the interpretation of such data.

In order to avoid detergent extraction, pulse EPR (electron paramagnetic resonance) spin-labeling methods and single-molecule optical techniques have been

applied (Kenworthy et al., 2000; Dietrich et al., 2001; Subczynski and Kusumi, 2003) to monitor the entry and exit of probe molecules in domains in model membranes or in living cells. The results have provided evidence for small/unstable rafts in unstimulated cells and for larger stabilized rafts induced by oligomerization of GPI-anchored proteins or ligand binding (Harder, 2003), in which some proteins preferentially partition. Interestingly, the size of the confining domain for a GPI-anchored protein is reduced when cells are treated with inhibitors of GSL synthesis, suggesting that GSLs contribute to the physical properties of microdomains (Sheets et al., 1997). In conclusion, many studies suggest that GSL can be heterogeneously organized on living cells, although there is no strong consensus yet on the size, shape and dynamics of lipid rafts.

Traffic of glycosphingolipids

Subcellular distribution of glycosphingolipids

As would be expected from the location of many of their functions, GSLs have been found at the outside of the plasma membrane, although they usually constitute a minor fraction of the total plasma membrane lipids (van Meer and Holthuis, 2000). One exception is the apical plasma membrane of epithelial cells of the intestinal and urinary tracts, where GSLs are found at particularly high concentrations of 30-40 mol% of total lipid (Forstner et al., 1968; Kawai et al., 1974; Stubbs et al., 1979; Brasitus and Schachter, 1980). In comparison with the basolateral membrane of these cells, GSLs are almost 2-4 fold enriched in the apical membrane (Forstner et al., 1968; Douglas et al., 1972; Kawai et al., 1974; Stubbs et al., 1979; Brasitus and Schachter, 1980). Because the tight junction forms a lipid diffusion barrier between these two membrane domains, but exclusively in the non-cytosolic leaflet, that is where the differences in lipid composition must exist. If all GSLs were in the non-cytosolic leaflet and cholesterol would be distributed evenly over both leaflets, the apical surface of these cells would be completely covered by GSLs! High concentrations of galactosyl-GSLs also exist in myelin, while neuronal plasma membranes are rich in gangliosides. Intracellularly, GSLs are found in the vacuoles of the exocytic and endocytic pathways, with the exception of the ER that is low in GSL content (Matyas and Morre, 1987; van Genderen et al., 1991). As for mitochondria and peroxisomes, in general, no or very low levels of GSLs were found in these organelles. Indeed, Forssman glycolipid, a five-sugar GSL of the globo-series, was found at the plasma membrane, in endocytic compartments, to a low extent in the ER but excluded from mitochondria and peroxisomes (van Genderen et al., 1991). However, a highly increased content of the ganglioside GD1b was reported for mitochondria of malignant hepatoma (Dyatlovitskaya et al., 1976), and GD3 was found in mitochondria in ceramide-induced apoptotic cells (Malisan and Testi, 2002). Still, it has generally not been excluded that the GSLs actually resided in the mitochondria-

associated membrane, MAM, an ER subcompartment that closely associates and copurifies with mitochondria. The MAM has been reported to contain the enzymes of early GSL synthesis (Ardail et al., 2003). The various GSLs apparently display different distributions over the various intracellular membranes (Matyas and Morre, 1987; Muthing et al., 1998). Notably, while complex GSLs were found enriched on the plasma membrane, more of the GlcCer was localized to intracellular membranes (Warnock et al., 1993).

Glycosphingolipid transport and sorting

As most GSLs (and SM) are synthesized at the lumenal, non-cytoplasmic side of the Golgi, and reside on the plasma membrane surface and on the lumenal side of the endocytic organelles, the main transport mechanism must be the vesicular transport along the exocytic and endocytic recycling pathways (Figure 3). Indeed, it was shown for GM3 and for SM that they can only be transported from the Golgi to the plasma membrane via vesicular transport (Young et al., 1992; van Meer and Lisman, 2002). Also the recycling of GSLs and SM via the endocytic vesicular transport pathways has been described in great detail (Marks and Pagano, 2002). Since all vesicular transport pathways are directly or indirectly bidirectional, a major question has been how the cell maintains the different lipid compositions of its organelles. For example, how are newly synthesized GSLs preferentially transported to the plasma membrane and not to the ER, how are they preferentially incorporated into the apical pathway and into a specific endocytic pathway? Experiments on epithelial cells have led to one unifying hypothesis for the mechanism of GSL sorting: the lipid raft model (van Meer et al., 1987; Simons and van Meer, 1988). In this model which has been refined over the years by the contributions of many, sphingolipids in general and GSLs in particular form l_o domains in the non-cytosolic leaflets of the Golgi, plasma membrane and endosomes. Because phase boundaries are energetically unstable, rafts will have a tendency to minimize the length of the boundary by budding into a vesicle (Baumgart et al., 2003; Lipowsky, 2003) (Figure 4). These domains should incorporate proteins that can impose transport specificity, and, for a start, certain SNARE molecules involved in docking and fusion have now been reported to preferentially partition into lipid rafts (Lafont et al., 1999; Lang et al., 2001; Chamberlain et al., 2001; Chamberlain and Gould, 2002). It has been argued that the occurrence of a single type of lipid raft would be insufficient to explain the lipid sorting in the trans Golgi network of epithelial cells, where lipids to be transported back to the ER, unsaturated glycerophospholipids, would have to be segregated from basolateral lipids, SM and cholesterol, and from apical lipids, GSL and cholesterol (Young et al., 1992; van Meer and Lisman, 2002). The work on GSL sorting at the plasma membrane between pathways towards the Golgi and to the late endosomes clearly shows that GSL sorting can be regulated. The role of coat proteins like caveolin in the process is now being unraveled (Sharma et al., 2003; Singh et al., 2003).

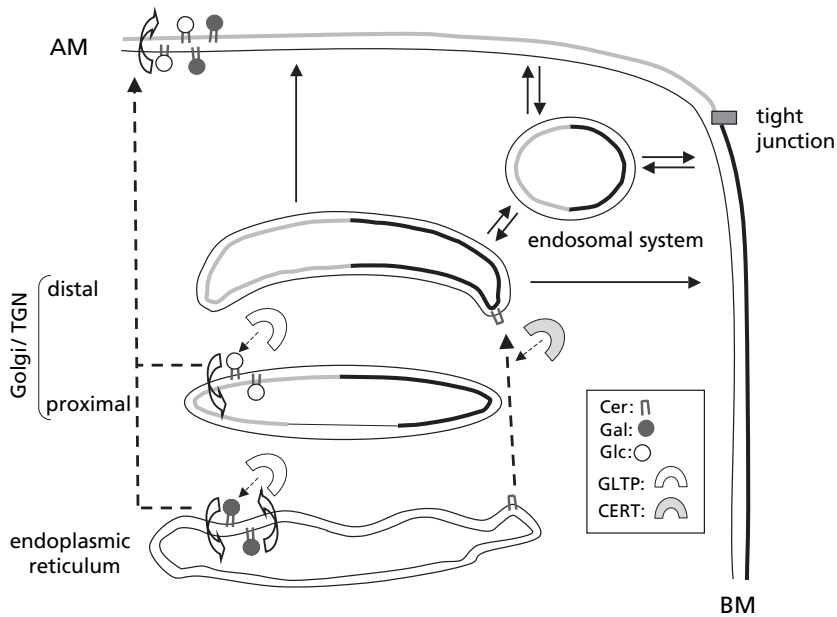

Figure 3. Intracellular transport of GSLs. Ceramide can be transported from the ER to the cis Golgi by vesicles. Alternatively, it is transported by the transfer protein CERT to the *trans* Golgi (Hanada et al., 2003), most likely via ER-Golgi contact sites (Munro, 2003). GalCer is able to translocate freely across the ER membrane (Burger et al., 1996), and is available for the glycolipid transfer protein GLTP, as is GlcCer on the cytosolic surface of the Golgi (Lin et al., 2000). GlcCer can flip across Golgi membranes spontaneously (Burger et al., 1996; Buton et al., 2002), or by the same ABC transporter as in the plasma membrane, most likely MDR1 (van Helvoort et al., 1996; Lala et al., 2000; De Rosa et al., 2003). In the lumenal leaflet of the Golgi lipids destined for the apical surface (light grey) are sorted laterally from basolateral lipids (fat and black) and from lipids in the retrograde direction (thin and black). AM: apical membrane; BM: basolateral membrane.

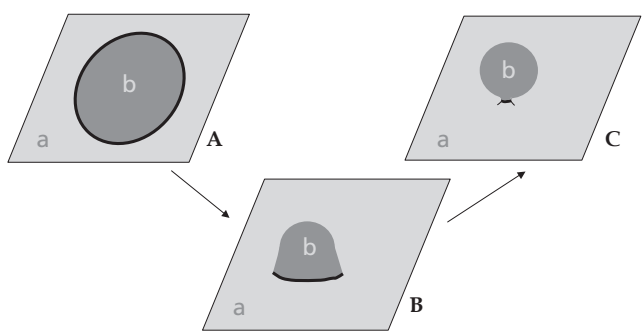

Figure 4. Behavior of co-existing fluid lipid phases. In a membrane containing co-existing fluid phases (a) and (b), with the (b) phase surrounded by the (a) phase (**panel A**), budding of the (b) domain will occur as soon as its size exceeds a certain threshold value (**panel B**), provided the tension within the membrane is sufficiently small. Very often, the final shape of (b) is a complete bud connected to (a) by a very thin neck (**panel C**). This model by Lipowski and co-workers (Lipowsky, 2003) was recently proven experimentally to be correct (Baumgart et al., 2003).

The fact that GlcCer is synthesized on the cytosolic surface of the Golgi implies that it can in principle follow three transport pathways (van Meer et al., 2003): (1) If it moves into budding transport vesicles it can reach the cytosolic surface of all membranes of the exocytic/endocytic membrane system. (2) It may be extracted from the membrane by a transfer protein and transferred to the cytosolic surface of other organelles, e.g. the mitochondria or peroxisomes. A glycolipid transfer protein has been identified (Lin et al., 2000), and cell fractionation has suggested that vesicle independent transport of GlcCer between Golgi and plasma membrane does indeed occur (Warnock et al., 1994). The function of this process is presently unclear. (3) GlcCer may flip to the lumenal leaflet of the Golgi membrane, where it becomes available for the LacCer synthase or where it can enter the lumenal aspect of transport vesicles. Evidence for fast, energy-independent GlcCer translocation across the Golgi membrane has been presented (Buton et al., 2002). In contrast, evidence has been presented to suggest that GlcCer is a substrate for the ABC-transporters MDR1 P-glycoprotein and MRP1 (Raggers et al., 2000). So, the possibility exists that GlcCer is removed from the cytosolic surface of the Golgi (Lala et al., 2000; De Rosa et al., 2003) or the plasma membrane (van Helvoort et al., 1996), and that its presence at the cytosolic surface reflects a hitherto unknown function. Actually, after synthesis in the ER lumen also GalCer is expected to have access to the cytosolic leaflet, and it may therefore have the same fate and function as GlcCer.

It is fully unclear how gangliosides like GD3 and Gd1b reach the mitochondrion (Malisan and Testi, 2002), because even if these lipids made it back to the ER lumen via the retrograde route, the experimental evidence suggests that complex GSLs are not able to translocate to the cytosolic surface (Buton et al., 2002). The fact that Forssman glycolipid was not found in mitochondria may suggest that there may be a selective transport process for the gangliosides.

Functions

Lessons from knockout mice

The importance of GlcCer-derived GSLs became obvious when the observation was made that embryos from a mouse with a knockout for this enzyme die at 7.5 days (Yamashita et al., 1999a), which shows that GSL synthesis is critical for embryonic development and for differentiation of certain tissues. Interestingly, a melanoma cell line GM95 that does not have the CGlcT lives (Ichikawa et al., 1994). Nevertheless, these cells have a defect in melanogenesis, linked to a sorting defect of melanosomal proteins (Sprong et al., 2001). This role of GSLs in protein sorting will be developed below. Knockouts for the ceramide glucosyltransferase have also been made in *P. pastoris* and *C. albicans*: they resulted in a complete loss of GlcCer, but this had no essential effect on cell growth (Leipelt et al., 2001).

Other mouse models lacking enzymes involved in the biosynthesis of GSLs have

been made. Mutant mice lacking the GM3 synthase (α2,3-sialyltransferase) appear rather normal but exhibit an enhanced insulin sensitivity, probably due to an increased phosphorylation of the insulin receptor (Yamashita et al., 2003). KO mice for the GM2/GD2 synthase (a β1,4-GalNAc transferase) are also viable, but exhibit defects in the nervous system, the maintenance and repair of nervous tissues, the differentiation of spermatocytes, and the regulation of the IL2 receptor complex (Takamiya et al., 1996; 1998, Sheikh et al., 1999; Furukawa and Takamiya, 2002). Double KO mice lacking the GM2/GD2 and GD3 synthases (β1,4-GalNAc transferase and α2,8-sialyltransferase, respectively) develop skin injury more rapidly, showing a role of higher GSLs in maintaining skin integrity (Inoue et al., 2002).

Mice with a knockout in the CGalT are alive and lack GalCer and sulfatide (Bosio et al., 1996; Coetzee et al., 1996). These mice display defects in the nervous system. The male were infertile because spermatogenesis was blocked before the first meiotic division; this was due to the absence of monogalactosylalkylacylglycerol and seminolipid (HO_3S-3Galβ1-alkylacylglycerol) in testis (Fujimoto et al., 2000). These data show a critical role for sulfoglycolipids in myelin function and spermatogenesis. Altogether, the observations made in mutant mice reflect the crucial role of GlcCer in embryonic development and differentiation, and the involvement of more complex GSLs in a number of important processes, especially in the nervous system.

Glycosphingolipids are part of ordered sphingolipid domains in cellular membranes and key molecules in recognition and signaling

As mentioned above, because of their tightly packed backbone GSLs have the property to interact preferentially with themselves and with SM and cholesterol in a phospholipid environment, therefore forming rafts (Simons and Ikonen, 1997; Simons and Toomre, 2000) (Figure 5). Raft formation is thought to be the basis for many of the properties of GSLs. They are thought to be enriched not only in specific proteins in their lumenal leaflet (Brown and Rose, 1992; Rodgers et al., 1994), but also in peripheral proteins carrying myristoyl and palmitoyl chains in their cytoplasmic leaflet, such as src-family kinases which are related to signal transduction (Hakomori et al., 1998; Hakomori and Handa, 2002). The notion of the glycosynapse has emerged, defined as a microdomain at the cell surface involved in glycosylation-dependent adhesion/recognition and signaling (Hakomori, 2003).

A number of studies have shown that GSLs are involved in many aspects of cell signaling. First, the carbohydrate part of GSLs itself is involved in a number a recognition processes: some saccharide determinants present in the carbohydrate chains of GSLs, generally at the periphery, can correspond to blood group antigens (ABH, P, Lewis, Ii systems), to development-associated antigens, or to abnormal disease-associated antigens. These epitopes are involved in several recognition processes, like cell-cell interaction, cell-substratum interaction, cell-pathogen interaction (Karlsson, 1986; Fantini et al., 2000). Interactions can occur between GSL and lectin-like proteins (Kopitz et al., 1998; Schnaar et al., 1998), or directly between

2 GSLs (Boggs et al., 2000; Wang et al., 2001a). Because they can interact with other proteins or sugars, GSLs are targets for many pathogens that use GSL-enriched domains as platforms to enter the cells: these pathogens can be viruses (for example, influenza virus (Suzuki, 1994), HIV (McAlarney et al., 1994), rotavirus (Delorme et al., 2001)), bacteria (*Helicobacter pylori* (Lingwood, 1999), *Neisseria gonorrhoeae* (Hugosson et al., 1998), *Escherichia coli* (Backhed et al., 2002)) and several toxins (Cholera toxin, Shiga toxin/verotoxin, *Clostridium botulinum* toxin) (Lencer et al., 1999; Inoue et al., 2001; Lingwood, 2003) can also directly bind GSLs. Interaction between GSLs and their interaction partner can subsequently trigger a variety of events, like entry of the pathogen in the cell, adhesion, growth, differentiation, migration, and apoptosis.

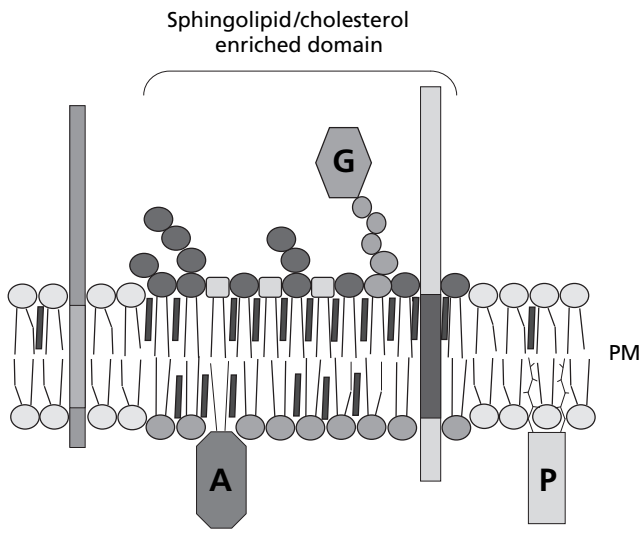

Figure 5. Schematic model of a sphingolipid/cholesterol raft. GSLs have a tendency to form a hydrogen-bonded network with SM and cholesterol in a phospholipid environment, forming rafts on the non-cytosolic side of the plasma membrane. GPI-anchored proteins (G) are enriched in these rafts. The composition of the inner leaflet of GSL enriched microdomains is unknown, but is probably special since only acylated proteins (A) are thought to associate with the cytosolic leaflet of rafts, whereas prenylated proteins (P) are excluded (Zacharias et al., 2002). Proteins that travel to the plasma membrane have in general longer transmembrane domains than Golgi resident proteins (Bretscher and Munro, 1993), and therefore may associate with GSL-enriched microdomains that are thicker than a typical phospholipid membrane (Kundu et al., 1996).

Second, it is thought that GSLs present in microdomains are involved in the modulation of signal transduction: they can interact with key transmembrane receptors that will trigger intracellular signaling cascades, resulting in regulation of cell proliferation and/or differentiation. Two major transmembrane signaling systems operating in the majority of eukaryotic cells, the growth factor receptor-associated protein kinases and PKC, have been shown to be modulated by gangliosides or their degradation products. For example, GM3 would be involved in the regulation of the epidermal growth factor receptor signal transduction, by preventing the dimerization of the receptor and therefore inhibiting the receptor kinase (Zurita et al., 2001). Similarly, tyrosine phosphorylation of the platelet-

derived growth factor receptor is inhibited by GM1 and other gangliosides (Bremer et al., 1984). PKC activity is inhibited by several glycolipids (Farooqui et al., 1988), including GD1b, GT1b, GM3. GSLs have also been shown to modulate integrin function (Wang et al., 2001b).

Finally, GSL synthesis intermediates or metabolites , i.e. ceramide, sphingosine, sphingosine-1P, lysogangliosides, can lead to inhibition or activation of many intracellular processes and are known as key mediators in apoptosis, proliferation, and stress response (Maceyka and Machamer, 1997; Liu et al., 1999).

Glycosphingolipids and protein sorting

GSL-enriched domains are thought to originate in the Golgi apparatus and it has been hypothesized that polarized epithelial cells use these domains as sorting platforms for the apical delivery of plasma membrane proteins (Simons and van Meer, 1988). Raft-association and subsequent apical sorting has been described for some transmembrane proteins and for GPI-anchored proteins (Simons and Ikonen, 1997). Later, it appeared that rafts also play a role in sorting processes not only in polarized epithelial cells, but also in many other cell types. Notably, in yeast *Saccharomyces cerevisiae*, lipid rafts are involved in the biosynthetic delivery of proteins to the plasma membrane (Bagnat et al., 2000; Bagnat and Simons, 2002; Watanabe et al., 2002). One idea is that sphingolipid/cholesterol rafts are thicker than a typical phospholipid/cholesterol membrane (Bretscher and Munro, 1993) and that plasma membrane proteins recognize these via their transmembrane domains which are longer than those of Golgi proteins.

How important are GSLs in raft function? It has been shown that GSL deficiency affects the formation of functional microdomains in lung carcinoma cells (Inokuchi et al., 2000). Moreover, the addition of GM1 to CHO-K1 cells modifies the distribution of GPI-anchored proteins in the plasma membrane, showing an effect in the organization of microdomains (Crespo et al., 2002), and the addition of gangliosides has been shown to displace GPI-anchored proteins from microdomains in MDCK cells (Simons et al., 1999). In agreement, the reduction of GSL levels in rafts affects the expression and function of GPI-anchored proteins, but does not impair signal transduction via the T cell receptor (Nagafuku et al., 2003). Nevertheless, it was also shown that GSLs are not essential for the formation of detergent-resistant domains and GPI-anchored protein sorting in melanoma cells, probably because the mutant cell line compensates the GSL defect by increasing SM synthesis (Ostermeyer et al., 1999). In yeast *Saccharomyces cerevisiae*, the absence of mannosylated GSLs does not modify protein sorting in the late Golgi (Lisman et al., 2004). These results suggest that GSLs are not essential for the formation of detergent-resistant domains, but could be involved in more specific functions fulfilled by these domains.

An essential function for glycosphingolipids in pigmentation

As mentioned above, mice with null-alleles for CGlcT were not viable (Yamashita et

al., 1999b; Sandhoff and Kolter, 2003), but a cell mutant unable to synthesize GlcCer by lack of CGlcT activity proliferates (Ichikawa et al., 1996). A clear phenotype in these cells was found much later, when Sprong et al. (2001) noted that the mutant melanoma cells were white while the parental cells were black. They showed that the first and rate-limiting enzyme in the pathway, tyrosinase, was present and capable of converting tyrosine to L-DOPA *in vitro*. In addition, the addition of exogenous L-DOPA resulted in pigment synthesis. Although all enzymes required for pigmentation were present in the mutant cells, they were incapable of forming melanin. Sprong et al. (2001) then observed that tyrosinase was mislocalized. Instead of being located in melanosomes, where pigment is normally synthesized, it was situated in the Golgi area. A second melanosomal enzyme, TRP-1, which escaped the Golgi complex of the cell mutant, was missorted via the plasma membrane. Sorting of these melanosomal enzymes and pigmentation could be restored when glycolipid-deficient cells were transfected with the glucosyltransferase, or incubated with glucosylsphingosine, which was converted to GlcCer by the cells. These results show that glycosphingolipids are required for melanosomal protein sorting and pigmentation. The molecular mechanism remains to be determined.

Scope of this thesis

It is clear that the simple glycosphingolipid glucosylceramide is of vital importance for multicellular organisms and required for a specialized process like pigmentation. The overall aim of the research described in this thesis was to investigate the role of glucosylceramide in the physiology of the cell. As the functionality of each lipid is determined by its local concentration in time, a first step was to obtain more insight in the spatial organization of sphingolipid metabolism. **Chapter 2** describes the ultra-structural localization of enzymes involved in sphingolipid synthesis in the Golgi of HeLa cells.

Glycosphingolipids are required for protein sorting to the melanosome for pigmentation. Because a functional adaptor protein, AP-3, is needed for pigmentation (Theos et al., 2005), in **Chapter 3** we investigated the transport of a different set of AP-3 dependent proteins, that are destined to the lysosome in melanoma cells. We report the unexpected finding that two melanosomal proteins contain dominant sorting information in their lumenal domain that requires glucosylceramide. In our search for lumenal determinants in the sorting of melanosomal proteins, we decided to test the lumenal pH (**Chapter 4**), which has been suggested to be an important parameter in pigmentation (Ancans et al., 2001; Brilliant, 2001). We learned that glycosphingolipids lower the lumenal pH in secretory organelles. They do so by activating the vacuolar proton pump possibly by binding directly to the proteolipid c-subunit, which forms the membranous rotor of the pump. **Chapter 5** discusses the implications of the findings presented in this thesis and proposes a model of

how sphingolipid metabolism may regulate the vacuolar pH in the secretory and endocytic pathway.

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Chapter 2

Ultra-structural organization of sphingolipid synthesis in the mammalian Golgi

Jasja Wolthoorn[1, §], Suzanne van Dijk[1, 2, §], Ann De Mazière[2], Hugo Maccioni[3], Judith Klumperman[2], Gerrit van Meer[1] and Hein Sprong[1]

[1] Membrane Enzymology, Bijvoet Center and Institute of Biomembranes, Utrecht University, [2] Cell Microscopy Center, Cell Biology, University Medical Center and Institute of Biomembranes, Utrecht University, The Netherlands. [3] Departamento de Química Biológica, Universidad Nacional de Córdoba, Ciudad Universitaria, Argentina

[§] Contributed equally

Summary

The functionality of each lipid is determined by its local concentration in time. In turn, this is determined by a balance between synthesis and hydrolysis and by transport of the lipid. In this study, we ectopically expressed the first enzymes involved in sphingolipid synthesis in the Golgi in HeLa cells, and determined their localization using both confocal laser scanning microscopy and quantitative immunoelectron microscopy. We found that GlcCer synthesis starts earlier in the Golgi than SM synthesis, but that the sub-Golgi localization of both synthases is overlapping in the late Golgi. SM and lactosylceramide synthesis takes place in the same late cisternae. We present a model how these enzymes play a role in the formation of sphingolipid/ cholesterol rafts in the late Golgi, but also in segregating different pools of the bioactive lipid ceramide.

Introduction

Sphingolipids comprise a small but vital fraction of membrane lipids in eukaryotes. Their basic building block is a long-chain sphingoid base, that can be anchored via an amide linkage to a saturated fatty acid, yielding ceramide. Sphingomyelin (SM) has in addition to its ceramide backbone, a phosphocholine headgroup, and occurs just like glucosylceramide (Glc β1-1Cer; GlcCer) in all mammalian cells (Fahy et al., 2005; Futerman and Riezman, 2005). GlcCer serves as the basis of the highly polymorphic class of complex glycosphingolipids (Degroote et al., 2004). Hundreds of different glycosphingolipids have been identified (Futerman and Riezman, 2005), implying that individual glycosphingolipids fulfill more roles in cell physiology than providing cellular membranes with high chemical and mechanical stability alone (Curatolo, 1987; Futerman and Hannun, 2004).

Specific glycosphingolipids serve as determinants of blood groups, whereas others take part in a variety of processes including differentiation (Watanabe et al., 1998; Yamashita et al., 1999), cell-cell interaction, and transmembrane signaling (Hakomori, 2002; 2004). Bacteria and viruses infect their host after binding certain glycosphingolipids via direct recognition (Smith et al., 2004; Olofsson and Bergstrom, 2005), or indirectly via rafts (Abraham et al., 2005). Although individual cells survive without glycolipids (Ichikawa et al., 1994), in melanocytes glycosphingolipids are required for the sorting of membrane proteins in the Golgi complex to melanosomes (Sprong et al., 2001). We found that glucosylceramide affects the pH in secretory organelles, most likely via activating the proton-ATPase (Chapter 4).

How glycosphingolipids carry out specialized functions at the molecular level is in many cases unclear. The functionality of each lipid is determined by its local concentration in time. This is determined by a balance between synthesis and hydrolysis, and by transport (van Meer, 2005). It is therefore important to know

exactly where lipids are synthesized and hydrolyzed, how these processes are controlled and, in addition, where and by what mechanisms they are transported. Since most enzymes of sphingolipid metabolism have been cloned (Hannun and Luberto, 2004), it is a challenge to find out how these enzymes are spatially organized and how their activity is controlled.

The localization of sphingolipid metabolizing enzymes has largely been addressed by measuring the activity of these enzymes in isolated subcellular fractions (see Table I). The usefulness of such approach, however, is limited, because of contamination. In addition, removal of contaminating membranes has the caveat of selecting a subfraction of the membrane of interest. Alternatively, enzymes associated with a given intracellular compartment may dissociate from accessory factors, resulting in reduced activity or specificity. Immunofluorescence and confocal laser scanning microscopy (CLSM) have been used to study the localization of these enzymes as well (Table I), but their resolution is limited to study sub-Golgi localization (e.g. this study). As a consequence, the localization and organization of sphingolipid-synthesizing enzymes is not resolved.

In this study, we ectopically express the first enzymes involved in sphingolipid synthesis in the Golgi in HeLa cells, and determined their localization using both CLSM and quantitative immunoelectron microscopy (IEM). We found that GlcCer synthesis starts earlier in the Golgi than SM synthesis, but that the sub-Golgi localization of both synthases is overlapping in the late Golgi. SM and lactosylceramide (LacCer) synthesis takes place in the same cisternae. The implications of these findings are discussed.

Results

Localization of GlcCer synthase and SM synthase

Ceramide is synthesized at the cytosolic leaflet of the endoplasmic reticulum (ER) (Venkataraman and Futerman, 2000) and transported to the Golgi compartment for conversion to SM and GlcCer. How ceramide is divided between these two processes is unclear, but may depend on the different modes of ceramide transport (Hanada et al., 2003) in combination with different localization of the GlcCer synthase (CGlcT) and Golgi-associated SM synthase (SMS1). Both enzymes localize to the Golgi, but their sub-Golgi localization has been controversial (Table I).

Our attempts to localize endogenous CGlcT and SMS1 with specific antibodies failed. As an alternative, we ectopically expressed epitope-tagged constructs in HeLa cells, and determined their cellular distribution using CLSM, and IEM. Both CGlcT and SMS1 had a nearly identical staining pattern with endogenous GM130, a *cis* Golgi marker (Nakamura et al., 1995) in transiently transfected HeLa cells (Figure 1). Neither SMS1 nor CGlcT were found in the nuclear envelope and ER (Kohyama-Koganeya et al., 2004).

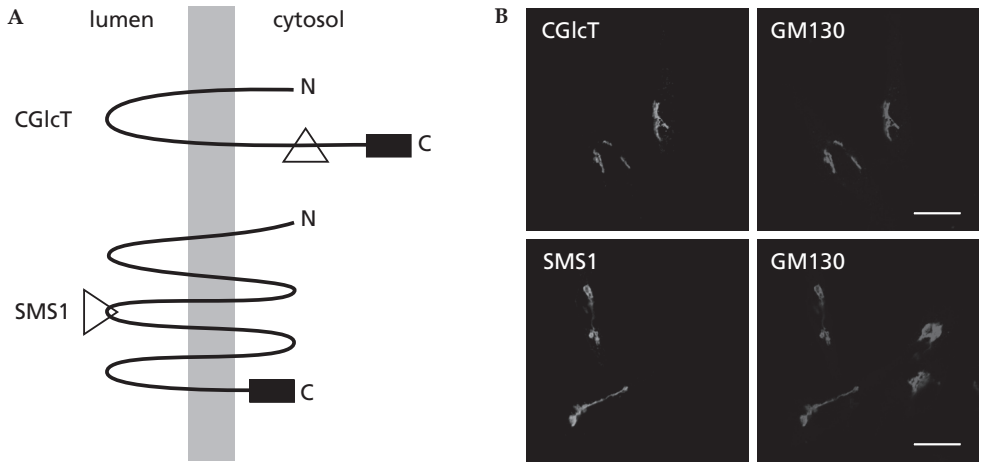

Figure 1. Localization of CGlcT and SMS1 in HeLa cells. (**A**) Topology of HA and V5 epitope tagged constructs of CGlcT and SMS1, respectively. CGlcT is a type III membrane protein and SMS1 is a multidomain spanning protein containing six predicted TM domains (Huitema et al., 2004). Tagged epitopes are indicated by black rectangles. The orientation of active center at the lumenal or cytosolic side is indicated by triangles. (**B**) HeLa cells were grown on glass coverslips and were transiently transfected with the constructs. One day after transfection, cells were fixed and double labeled with primary antibodies against HA or V5 epitopes and GM130, followed by labeling with secondary FITC conjugated antibodies and Texas-Red conjugated antibodies, respectively. Cells were visualized by confocal laser scanning microscopy. Scale bar, 10 μm.

To determine the sub-Golgi localization of CGlcT and SMS1, HeLa cell lines stably expressing these constructs were generated, and analyzed with IEM. Golgi stacks containing typically five cisternae were used for statistical further analysis, and endogenous GM130 was used as a *cis* Golgi reference for every labeling. Indeed, GM130 was restricted mostly to the first cisterna (81%) and only little label in the next cisterna (Figure 2 and 3). In contrast to GM130, labeling for CGlcT was spread over all five cisternae (Figure 2). CGlcT was not distributed equally, but was more concentrated in cisternae three to five (Figure 3). SMS1 was mostly present at the *trans*-side of the Golgi, and clearly peaked in the fourth cisterna (Figure 2). Quantification showed that CGlcT and SMS1 had a differential distribution: almost 50% of CGlcT localized to the first three cisternae, whereas only 33% of SMS1 was found there.

SMS1 and GM130 showed almost complete overlap by CLSM, but showed a completely different localization with IEM: GM130 at the *cis*-side and SMS1 at the *trans*-side of the Golgi. These results show that the resolution of CLSM is too low to study sub-Golgi localization.

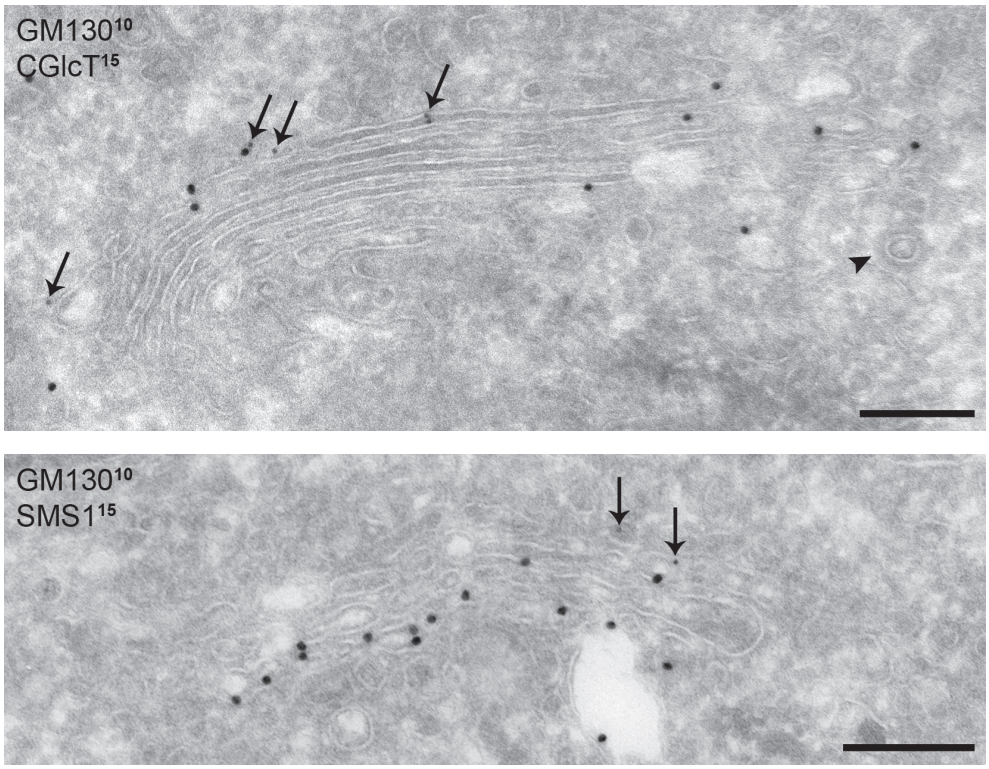

Figure 2. Sub-Golgi localization of GM130, CGlcT and SMS1 in HeLa cells. Cells stably transfected with the constructs were double labeled for GM130 as a *cis* Golgi reference (10 nm Protein A gold; indicated by arrows) and the specific enzyme (15 nm Protein A gold). Few labeling was present outside the Golgi. However, the antibodies were tested on unlabeled cells as well and gave a similar background labeling. Note that CGlcT is present in the outer areas of the cisternae. However, this was not confirmed by quantification. A clathrin coat, indicated by arrowheads, was often present on vesicles or tubules close to the TGN. Scale bar, 200 nm.

Localization of LacCer synthase and UDP-Gal transporter

GlcCer is synthesized at the cytosolic side of the Golgi (Coste et al., 1986; Futerman and Pagano, 1991; Jeckel et al., 1992), and is translocated to the Golgi lumen by an unidentified transporter or mechanism (Lannert et al., 1998). In the lumen, GlcCer is first converted to LacCer by the addition of a galactose moiety from UDP-galactose. LacCer synthesis not only depends on the localization of the LacCer synthase (GalT1) but also on the presence of the UDP-galactose transporter (UGT) (Sprong et al., 2003). Epitope-tagged constructs of GalT1 and UGT were transiently and separately expressed in HeLa cells and analyzed for their localization by CLSM (Figure 4). Both UGT and GalT1 had a typical Golgi distribution, corroborating previous findings (see Table 1).

Table 1. Subcellular localization of enzymes and transporters involved in sphingolipid synthesis in the Golgi of mammalian cell types. IEM: immunoelectron microscopy; IF: immunofluorescence microscopy; BFA: Brefeldin A.

Enzyme	Localization	Cell type	Technique	References
CGlcT (GlcCer synthase)	Golgi (*cis/ medial/ trans*)	HepG2 Rat liver	Activity assay: Localization C6-NBD-GlcCer by fractionation.	(Futerman and Pagano, 1991; Trinchera et al., 1991b; Jeckel et al., 1992)
	Golgi + ER + nuclear envelope	CHO	IF using epitope tagged CGlcT	(Kohyama-Koganeya et al., 2004)
SMS1 (Golgi associated SM synthase)	*cis* Golgi	HepG2 CHO	Activity assay: Localization C6-NBD-SM by fractionation.	(Jeckel et al., 1990; van Helvoort et al., 1997a; 1997b)
	TGN	Neurons HepG2	Activity assay: Metabolic labeling + BFA	(van Echten et al., 1990; van Meer and van 't Hof, 1993)
	Golgi	HeLa	IF using epitope tagged SMS1	(Huitema et al., 2004)
GalT1 (LacCer synthase)	Golgi	Rat liver	Metabolic labeling	(Trinchera et al., 1991a; 1991c)
	cis/ medial Golgi	Retina cells	Metabolic labeling + monensin	(Rosales Fritz et al., 1996)
	late Golgi	CHO-K1 Rat liver	Activity assay: Metabolic labeling + BFA	(Lannert et al., 1998; Giraudo and Maccioni, 2003)
SialT1 (GM3 synthase)	*cis* Golgi	Neurons Rat liver	Activity assay	(Trinchera and Ghidoni, 1989; van Echten et al., 1990)
	cis/ medial Golgi	Retina cells	Metabolic labeling + monensin	(Rosales Fritz et al., 1996)
	late Golgi	CHO-K1 Rat liver	Activity assays + BFA/monensin	(Lannert et al., 1998; Allende et al., 2000; Giraudo and Maccioni, 2003)
UGT (human UDP-galactose transporter)	Golgi	CHO	IF Activity assay	(Deutscher and Hirschberg, 1986; Sprong et al., 2003)
CST (CMP-sialic acid transporter)	Golgi	CHO	IF Activity assay	(Hirschberg and Snider, 1987; Eckhardt et al., 1996)

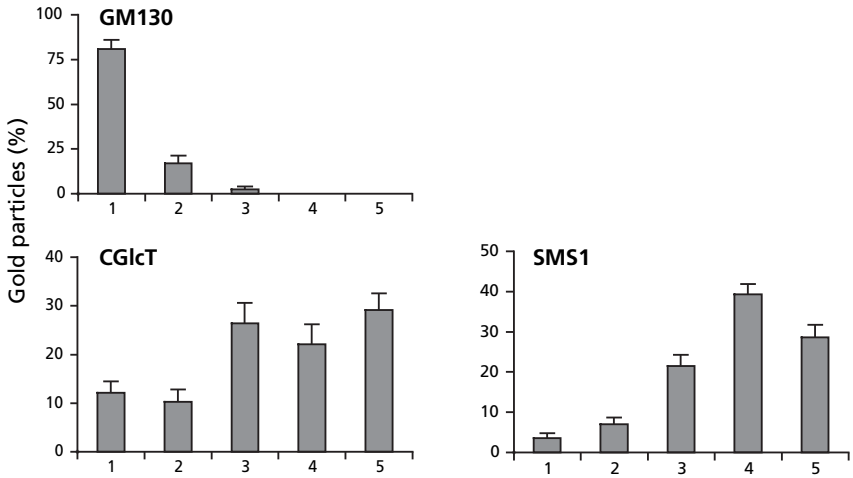

Figure 3. Quantitative distribution of GM130, CGlcT and SMS1. CGlcT and SMS1 were localized as described in Figure 2. For every cell line, 20 Golgi stacks containing five cisternae (numbered 1 to 5) were analyzed and labeling was quantified. Labeling for the reference GM130 was found almost exclusively in the *cis*-most cisterna of each Golgi and therefore this cisterna was denoted as cisterna 1. The number of gold particles per cisterna were expressed as a percentage of the total gold particles within that Golgi. The results for each enzyme are expressed as the average percentage ± SEM (n=20) of immunogold label for each cisterna. The total number of gold particles that were counted per protein were 117 for GM130, 146 for CGlcT and 211 for SMS1.

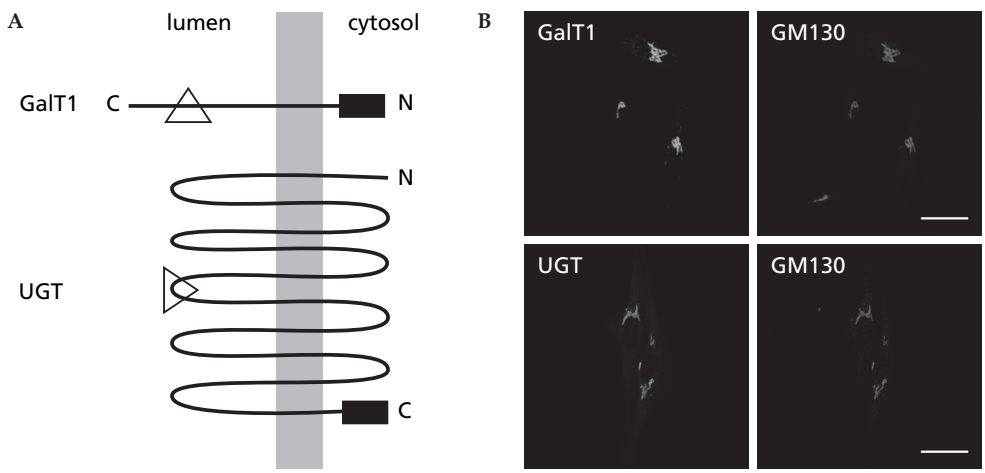

Figure 4. Localization of GalT1 and UGT in HeLa cells. (**A**) Topology of HA epitope tagged constructs of GalT1 and UGT. GalT1 is a type II membrane protein and UGT is a multidomain spanning protein containing ten predicted TM domains (Ishida and Kawakita, 2004). Tagged epitopes are indicated by black rectangles. The orientation of active centers are indicated by triangles. (**B**) HeLa cells were grown on glass coverslips and were transiently transfected with the constructs. One day after transfection, cells were fixed and double labeled with primary antibodies against HA epitopes and GM130, followed by labeling with secondary FITC conjugated antibodies or Texas-Red conjugated antibodies, respectively. Cells were visualized by confocal laser scanning microscopy. Scale bar, 10 μm.

The sub-Golgi localization of GalT1 and UGT was determined in HeLa cells stably expressing either construct by IEM. Labeling for LacCer synthase (GalT1) was found in all cisternae, but peaked in cisternae 4 and 5 (Figure 5 and 6). The Golgi distribution of UGT was more constricted than GalT1, and peaked in cisternae 3 and 4. Remarkably, UGT and GalT1 have overlapping but not an identical sub-Golgi distribution. Taken together, these data indicate that LacCer is synthesized at the *trans* Golgi at the same location as SM synthesis.

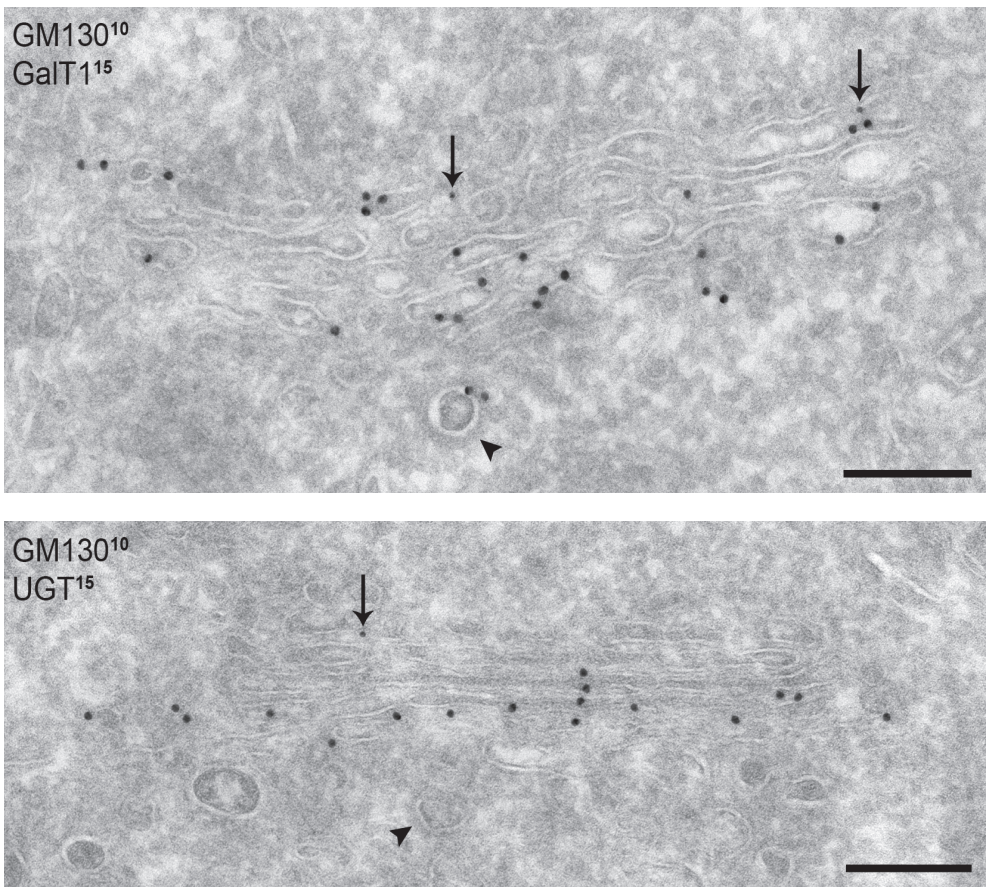

Figure 5. Sub-Golgi localization of GalT1 and UGT in HeLa cells. Thin frozen sections of HeLa cell lines stably transfected with the constructs were double labeled for GM130 as a *cis* Golgi reference (10 nm Protein A gold; indicated by arrows) and the specific enzyme (15 nm Protein A gold). Clathrin coat is indicated by arrowheads. Scale bar, 200 nm.

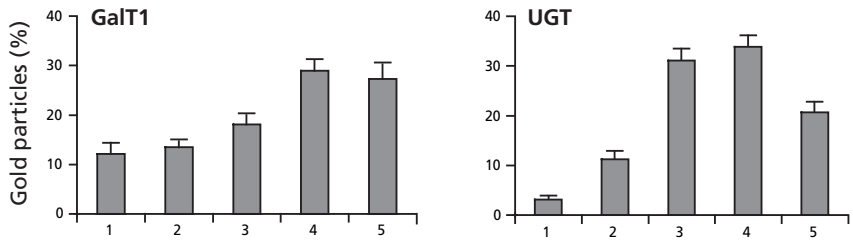

Figure 6. Quantitative distribution of GalT1 and UGT. The results for each enzyme are expressed as the average percentage ± SEM (n=20) of immunogold label for each cisterna. The total number of gold particles that were counted were 466 for GalT1 and 527 for UGT.

Discussion

A prerequisite for understanding how sphingolipid metabolism is organized and controlled is to know the localization of all the enzymes involved. CLSM is not suitable to study the sub-Golgi localization of Golgi enzymes, as even the *cis-trans* diameter of a Golgi is often smaller than the resolution of (conventional) laser scanning microscopes. For example, SMS1 and GM130 showed perfect overlap by CLSM, but with (IEM) GM130 was located in different Golgi cisternae than SMS1 (Figures 1-3). FRET measurements may indicate that two proteins are in the same cisternae, but does not necessarily reflect the localization of the majority of an enzyme (Giraudo et al., 2001; Giraudo and Maccioni, 2003).

Localizing endogenous CGlcT and SMS1 with specific antibodies failed. As an alternative, we expressed epitope-tagged version of proteins involved in sphingolipid synthesis. Although a 100-fold overexpression of a Golgi-resident protein had no effect on the distribution of this protein within the Golgi in HeLa cells (Rabouille et al., 1995), overexpression of a tagged version of a protein could lead to mislocalization. All our constructs distributed to the Golgi, without significant label outside the Golgi area. The Golgi localization of all our constructs is in agreement with previous findings on subcellular fractionation of endogenous enzymes (Table I).

Localization of GlcCer synthase and SMS1

CGlcT was found in the Golgi by both CLSM and IEM, but not in the ER. These findings are in line with subcellular fractionation studies (Futerman and Pagano, 1991; Trinchera et al., 1991b; Jeckel et al., 1992). We found that GlcCer synthesis starts earlier in the Golgi than SM synthesis, but that the sub-Golgi localization of both synthases is overlapping in the *trans* Golgi. What are the possible implications of the differential distribution of CGlcT and SMS1? Maybe ceramides transported from the ER to the *cis* Golgi via vesicular transport are being converted to GlcCer. CERT,

a cytoplasmic ceramide transporter, delivers ceramides to the *trans* Golgi for SM synthesis (Hanada et al., 2003). Knocking-down CERT resulted in decreased SM, but the effect on GlcCer synthesis was more complicated. GlcCer production from serine was reduced whereas the incorporation of sphingosine was unchanged. This may imply that exogenous sphingosine enters a spatially segregated ceramide pool that preferentially enters the *cis* Golgi by a CERT-independent pathway, presumably vesicular transport. A possible role for CGlcT in the *trans* Golgi might be to complete the transport block for ceramides: SMS1 on the lumenal side and CGlcT on the cytosolic side prevent mixing of the metabolic pool of ceramide with the signaling pool of ceramide at the cell surface.

Localization of LacCer synthase and UDP-Gal transporter

The localization of UGT was overlapping the GalT1 distribution in the Golgi, but it was also present in the medial Golgi cisterna. Because protein galactosyltransferases utilize UDP-galactose in the Golgi lumen (Furukawa et al., 2002), a widely spread availability of UDP-galactose in the Golgi cisternae might be necessary. Alternatively, UDP-galactose might be secreted if it is synthesized too much to the *trans*-side of the Golgi.

We found that LacCer synthesis located to the lumen of the *trans* Golgi cisternae, similar to SM synthesis. Sphingolipids and in particular glycosphingolipids have the propensity to cluster in an environment of other lipids (Brown and London, 2000). The formation of these lipid rafts are thought to be an essential feature of the sorting machinery of the Golgi (Simons and van Meer, 1988; Sharma et al., 2003). The localization of GalT1 and SMS1 may form the basis for raft biogenesis in the *trans* Golgi.

We have used a morphological approach to identify the organization of the first enzymes involved in sphingolipid synthesis in the Golgi in HeLa cells. The *in vivo* activity of these enzymes strongly depends on the local availability of their substrates, and on the presence of proteins that sense and control sphingolipid metabolism. Very little is known about these processes, and is subject of our future research.

Materials and Methods

Materials

The antibiotics geneticin G418 Sulphate and Hygromycin B were purchased from PAA Laboratories (Pasching, Austria) and Invitrogen Corporation (Carlsbad, CA) respectively. Lipofectamine 2000 reagent was from Invitrogen.

DNA constructs

CGlcT with a 3HA tag at the carboxy terminus was cloned in the following way.

cDNA of mouse CGlcT (Ichikawa et al., 1996) was amplified in a PCR reaction using the primers 5'-CGC AAG CTT ATG GCG CTG CTG GAC CTG GCC-3' and 5'-CGC TCT AGA CAC ATC CAG GAT CTC CTC-3'. The PCR product was cloned into pcDNA3.1-HA3 using the restriction sites HindIII and XbaI. A vector containing the cDNA of rat GalT1 (LacCer synthase; Giraudo and Maccioni, 2003) was used for amplification by PCR using the primers 5'-CGC AAG CTT CGG ATG TCT GCG CTC AAG CGG ATG-3' and 5'-CGC TCT AGA ATA GTC TTC AAC TGG AGC TAA CTC CG-3'. A 3HA tag was cloned at the amino terminus of GalT1 in a similar way as CGlcT-HA, but now using the plasmid pN3HA and the restriction sites HindIII and XbaI. The plasmid encoding human SMS1 tagged with the V5 epitope (Huitema et al., 2004) was kindly provided by K. Huitema. The plasmid encoding human UGT with an HA tag at the amino terminus has been described previously (Aoki et al., 1999). All constructs made by PCR were confirmed by sequencing both strands.

Antibodies

Polyclonal anti HA antibody (Y11) was from Molecular Probes, Eugene, OR. Mouse anti-V5 antibody was from Invitrogen. FITC- or Texas red-conjugated goat anti-rabbit and goat anti-mouse antibodies were from Jackson Laboratories, West Grove, PA. Additional antibodies used for electron microscopy included: mouse monoclonal 16B12 anti-HA (BAbCO, Richmond, CA; see (Pecot and Malhotra, 2004) and rabbit polyclonal anti-GM130 (gift from E. Sztul, University of Alabama, Birmingham, USA; described in Nelson et al., 1998).

Stable cell lines

HeLa cells from a low passage number (#4) were grown in DMEM, containing 10% FCS and were maintained at 37°C with 5% CO_2. Cells were transfected using lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. For transient transfection transcription was induced by butyrate (3 mM) 16 h before fixation. Stable transfections were performed on cells grown on a 3.5 cm dish. One day after transfection cells were trypsinized, diluted in selection medium (DMEM containing hygromycin B (200 U/ml) or G418 (0.8 mg/ml)) and divided over 4-6 24 wells plates. After approximately 3 weeks, individual or pooled colonies (maximum 3 colonies per 24 well; cells were induced by 3 mM butyrate 16 h before fixation) were selected for Golgi localization and a moderate expression level of the enzymes by immunofluorescence microscopy. Positive cells were grown on 6 cm dishes and induced by 3 mM butyrate 16 h before fixation for IEM.

Confocal laser scanning microscopy

Transfected cells grown on coverslips were fixed using 3% paraformaldehyde 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 goat 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). Images were obtained using a Nikon D-εclipse C1 confocal microscope.

Immunoelectron 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, subsequently 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 -120 ^{o}C, 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 and the *cis* Golgi marker GM130.

Quantification immunoelectron microscopy

For the sub-Golgi localization of the tagged enzymes, immunogold particles were quantified per cisterna. Therefore, 20 Golgi apparatuses were selected per enzyme on double labeled sections. The Golgi's were selected 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 reference. The GM130 marker was found almost exclusively in the *cis*-most cisterna of each Golgi and therefore this cisterna was denoted as cisterna 1. EM pictures of the selected Golgi's 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. The results for each enzyme are expressed as the average percentage ± SEM (n=20) of immunogold label for each cisterna. The reliability 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's. 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 value ≤ 0.05).

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Chapter 3

Lumenal domains of tyrosinase and TRP-1 contain glucosylceramide-dependent sorting information for melanosomes

Sophie Groux-Degroote[1,3], Suzanne M. van Dijk[1,2], Jasja Wolthoorn[1], Sylvia Neumann[1], Alexander C. Theos[4], Ann M. De Mazière[2], Judith Klumperman[2], Gerrit van Meer[1] and Hein Sprong[1]

[1]Membrane Enzymology, Bijvoet Center and Institute of Biomembranes, and [2]Cell Biology, Institute of Biomembranes, Utrecht University, Utrecht, The Netherlands. [3]Present address: Laboratoire de Chimie Biologique, Université des Sciences et Technologies de Lille I, Villeneuve d'Ascq, France. [4]Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

Summary

Fibroblasts have an intracellular pathway for transport of membrane proteins to lysosomes, which depends on the adaptor protein 3 (AP-3) complex. Melanocytes contain lysosomes as well as melanosomes. Transport of proteins to melanosomes also depends on AP-3, raising the question how sorting between melanosomal and lysosomal proteins occurs. We found that direct transport of the melanosomal protein TRP-1 to melanosomes was disturbed in the absence of GlcCer, while the AP-3 pathway was still functional. Remarkably, the lysosomal proteins LAMP-1 and -2 behaved opposite to TRP-1: they travelled via the cell surface in melanocytes, but took an intracellular pathway in the absence of glycosphingolipids. When the lumenal domains of TRP-1 and tyrosinase were replaced by those of marker proteins, the chimeras behaved like lysosomal proteins in all cell lines. We conclude that the lumenal domains of TRP-1 and tyrosinase contain sorting information that guides them to the melanosomes via an intracellular pathway which excludes lysosomal proteins and that is decoded only in the presence of glycosphingolipids.

Introduction

Melanin pigment is synthesized in melanosomes, specialized organelles in melanocytes that are situated along the secretory pathway but originate from a coated endosome. Melanosomes belong to the group of so-called secretory lysosomes, which are also found in platelets, cytotoxic T-cells and other cells of haematopoietic lineage (Blott and Griffiths, 2002). A common form of pigmentation-related disorders is oculocutaneous albinism (OCA), characterized by hypopigmentation. 40% of OCA is caused by defective functioning of the first enzyme in pigmentation, tyrosinase. Also mutations in the second enzyme, tyrosinase-related protein (TRP-1), accounts for a form of OCA (Newton et al., 2001). Other cases of oculocutaneous albinism display no defect in the enzymes themselves, but in their routing to the melanosomes. One example thereof is the subclass of Hermansky-Pudlak syndrome type II patients, who bear a mutation in the beta 3A subunit of the heterotetrameric AP-3 complex (Dell'Angelica et al., 1999). This adaptor complex binds to a dileucine signal in the cytosolic tail of melanosomal membrane proteins (Höning et al., 1998) and is required for their transport to the melanosomes (Boehm and Bonifacino, 2002). Notably, in non-melanosomal cells, AP-3 binds to the cytoplasmic signals of lysosomal membrane proteins (Höning et al., 1998), which in melanocytes are transported to lysosomes and not melanosomes (Raposo et al., 2001). In this paper we address how melanocytes differentially sort their lysosomal and melanosomal membrane proteins.

We have previously reported that the melanocyte cell line GM95 lacks pigmentation due to defects in transport of tyrosinase and TRP-1. Tyrosinase

accumulates in the Golgi area of GM95 cells, whereas TRP-1 still reaches melanosome-like structures, but indirectly via the plasma membrane. GM95 cells do not synthesize glycosphingolipids because they lack the glucosylceramide synthase (CGlcT). Protein sorting and melanin synthesis could be restored by transfection with CGlcT showing that glycosphingolipids are required for intracellular transport of melanosomal proteins (Sprong et al., 2001). Exactly which glycosphingolipids are involved in melanosomal protein sorting and at which step of the transport route has remained unclear.

The glycosphingolipid glucosylceramide (Glcβ1-1Cer; GlcCer) is synthesized by CGlcT in all mammalian cells, and serves as the basis for a highly polymorphic class of complex glycosphingolipids. Glycosphingolipids are of vital importance for mammals: mice with defects in CGlcT display are embryonically lethal (Yamashita et al., 1999). There is ample evidence to suggest that glycosphingolipids are not distributed randomly over the surface of membranes, but that they concentrate with cholesterol at specific locations into lipid rafts and that this forms the basis for their selective transport in the vacuolar membrane system of mammalian cells (Singh et al., 2003). Originally, this mechanism was proposed to drive the sorting of apical from basolateral membrane proteins in the secretory pathway of epithelial cells (Simons and van Meer, 1988). In neuroendocrine cells lipid rafts were reported to sort proteins at the Golgi into the secretory granules (Tooze et al., 2001). Independently, evidence has been provided for a role for lectins in sorting membrane proteins into the apical transport pathway (Rodriguez-Boulan et al., 2005). The missorting of melanosomal proteins in the absence of glycosphingolipids (Sprong et al., 2001) suggests that protein sorting towards melanosomes, secretory lysosomes (Blott and Griffiths, 2002), also may involve glycosphingolipid rafts or lectins.

In the present study, we addressed the role of glycosphingolipids in the formation of functional melanosomes. We found that GlcCer is required for the biogenesis of functional melanosomes, and for the intracellular sorting of melanosomal proteins. Furthermore, melanosomal proteins having their lumenal domain replaced are sorted to the lysosomes. We conclude that the discrimination between melanosomes and lysosomes depends both on lumenal sorting information in the cargo proteins and on the presence of GlcCer, and we discuss how the two may be linked.

Results

GM95 cells without glycosphingolipids do not possess melanosomes

To elucidate the role of glycosphingolipids in sorting melanosomal enzymes, we first set out for a detailed characterization of melanosome biogenesis and melanosomal protein distribution in glycosphingolipid-deficient GM95 cells in comparison with the parental MEB4 cells. As assessed by electron microscopy melanoma MEB4 cells display all stages of melanosome biogenesis (Seiji et al., 1963; Raposo and Marks,

2002), i.e. stage I, equivalent to an endosome with a bilayered coat, stage II, with a fibrillar content, stage III, with increasing melanin deposition, and the mature stage IV (Figure 1A). Immunogold labeling of ultrathin cryosections revealed that the melanosomal enzymes tyrosinase, TRP-1 and Pmel17 occur in the melanosomal stages I-IV and less extensively in early endosomes and multivesicular bodies (MVBs; Suppl. Table I), in line with previous observations (Raposo et al., 2001). By confocal microscopy, tyrosinase and TRP1 in MEB4 cells are largely separated from the lysosomal marker LAMP-1 (Figure 2A), corroborating that lysosomes and melanosomes are distinct organelles with different protein composition (Raposo et al., 2001). Glycosphingolipid-deficient GM95 cells do not synthesize melanin pigment. Electron microscopical analysis of these cells revealed that they were completely devoid of the characteristic stage II, III and IV melanosomes, although a subpopulation of MVB- and lysosomes contained a core with regular transverse and longitudinal striations, which were extremely rare in MEB4 cells (Figure 1B). These striations were different in appearance from the flexible fibrils in stage II melanosomes (compare Figures 1A,B). The MVBs containing striated bodies labeled positive for the lysosomal markers LAMP-1 and cathepsin D, as well as the melanosomal enzymes tyrosinase and TRP-1 (Suppl. Table I). The nature of the striated cores remained unclear, since they could not be labeled with HMB45 anti-Pmel17 antibodies that decorate the fibrils of normal melanosomes or with any of the antibodies against the above-mentioned other melanosomal proteins.

MVBs of GM95 cells contain tyrosinase and TRP-1 but no melanin

In GM95 cells tyrosinase transport is blocked in the Golgi area as seen by confocal fluorescence microscopy (Sprong et al., 2001). To define this block in more detail, we treated GM95 cells with microtubule depolymerizing nocodazole, leading to dispersion of the Golgi into numerous "ministacks" (Drecktrah and Brown, 1999). In ministacks, the colocalization of tyrosinase with the *trans* Golgi marker α-2,6 sialyltransferase was only partial (Figure 2A). Likewise, immuno-electron microscopy of GM95 cells showed tyrosinase both in the TGN and in MVBs, including those MVBs with striated material (Figure 2B, Suppl. Table I). TRP-1 mainly localized to melanosomes in MEB4 cells (Suppl. Table I), whereas the steady state distribution of TRP-1 in GM95 cells was comparable but not identical to that of LAMP-1 (Figure 2A, Suppl. Table I). The morphology of all other organelles as well as the intracellular distribution of the late endosome/lysosome markers LAMP-1 and cathepsin D, the early endosome markers Hrs and EEA1, and the 46 kD mannose-6-phosphate receptor CD-MPR, which cycles between TGN and endosomes, was comparable between GM95 and MEB4 cells (Suppl. Table I). These data show that in GM95 cells the compartments of the endocytic pathway are normally formed, and that glycosphingolipid synthesis is not required for proper sorting of endosome-lysosome associated proteins. Importantly, however, although tyrosinase and TRP-1 can reach endosomal compartments, melanin pigment was not synthesized.

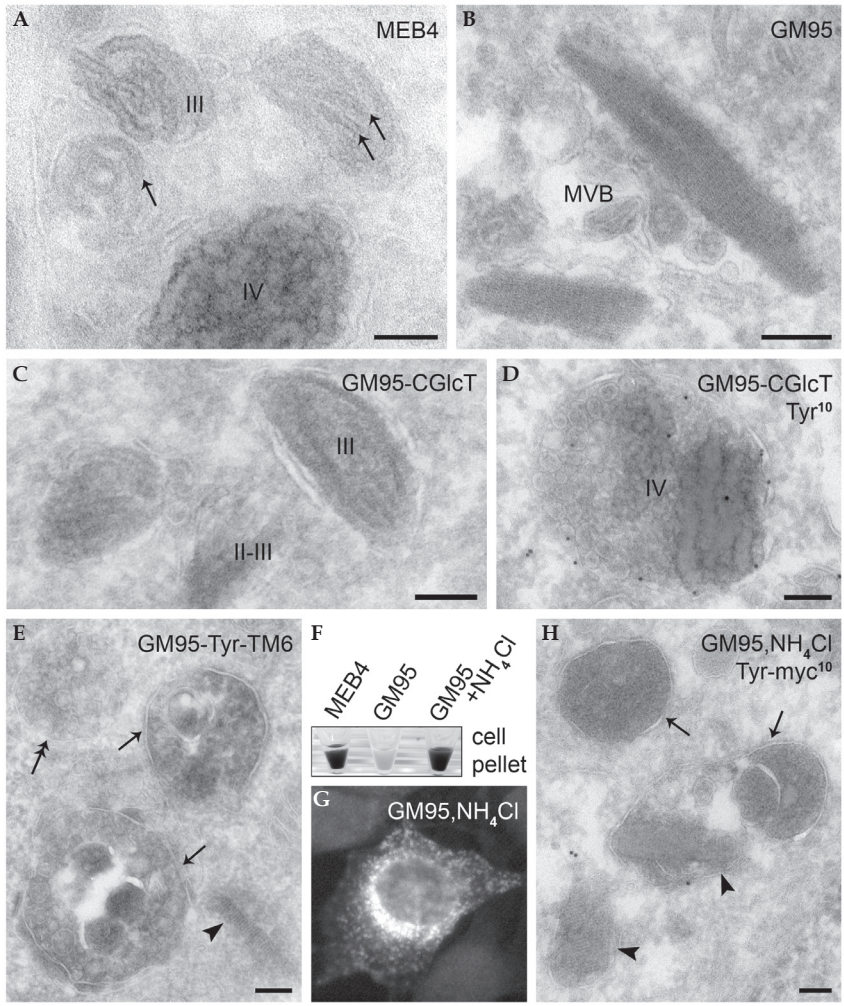

Figure 1. Melanosome formation in MEB4 but not in glycolipid-deficient GM95 cells. (A-E, H): Ultrathin cryosections. **(A)** MEB4 cells contain typical stage II melanosomes traversed by weakly banded fibrils of characteristic width (arrows), and stage III and IV melanosomes with increasing melanin deposition visible as increasing electron density. **(B)** GM95 cells, as well as GM95 cells transfected with tyrosinase-myc (shown here), have no characteristic stage II, III or IV melanosomes. Part of their MVBs contain rigid, longitudinally and transversely striated bodies. **(C, D)** GM95 cells transfected with CGlcT display true melanosomes with fibrils undergoing melanization. These melanosomes can be labelled with anti-tyrosinase antibodies and 10 nm gold particles (**D**). **(E)** In contrast, GM95 cells transfected with tyrosinase having an extended transmembrane domain (Tyr-TM6) lack melanosomes but have dark (arrows) as well as light (double arrow) MVBs and lysosomes occasionally containing a striated body (arrowhead). **(F-H)** GM95 cells were treated with 10 mM NH_4Cl for 1 h, and subsequently incubated overnight in normal medium. Cells were scraped, pelleted, and photographed (**F**), or tyrosinase was visualized by confocal immunofluorescence microscopy (**G**) and immuno-electron microscopy (**H**). They have no true melanosomes but electron-dense endosomal vacuoles (arrows), that contain tyrosinase (labeled by anti-myc antibody and 10 nm gold particles). Arrowheads, striated bodies. Scale bars, 100 nm.

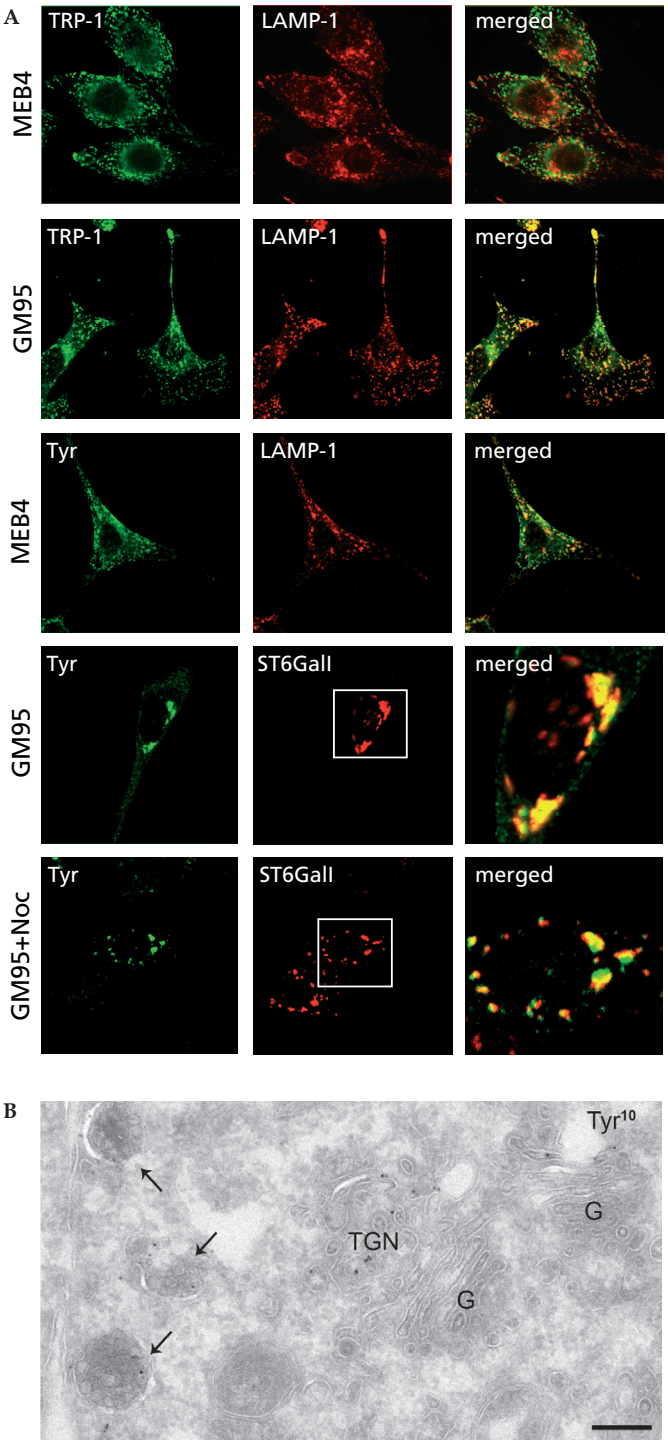

Figure 2. Localization of tyrosinase and TRP-1 in MEB4 and GM95 cells. (**A**) Cells (mock) transfected with the myc-tagged *trans* Golgi marker α-2,6 sialyltransferase were (mock) treated with 10 μM nocodazole for 3 h, fixed, labeled for TRP-1, LAMP-1, tyrosinase (Tyr) and sialyl-transferase (ST6GalI), and viewed by confocal fluorescence microscopy. Areas of overlapping distributions in the same optical section appear as yellow in the merged images. Compare the (strict) segregation of TRP-1 and LAMP-1 in MEB4 cells with the overlapping staining patterns in GM95 cells. In GM95 cells tyrosinase almost completely overlapped with the *trans* Golgi marker α-2,6 sialyltransferase. After fragmentation of Golgi membranes by nocodazole-induced microtubule disruption the overlap was reduced. (**B**) Ultra-thin cryosections of GM95 cells were labeled with anti-tyrosinase antibody and 10 nm gold particles. Tyrosinase is accumulated in the TGN, and present in nearby MVBs (arrows). G, Golgi apparatus. Scale bar, 200 nm.

Melanosome formation requires glycosphingolipids

To ensure that lack of melanosome formation in GM95 cells is indeed the result of lack of glycosphingolipids, we transfected GM95 cells with the missing CGlcT. This resulted in the recovery of organelles morphologically identical to stage II, and melanized stage III and IV melanosomes (Figure 1C) that could be labeled for tyrosinase (Figure 1D). Notably, two other treatments restored pigmentation, but did not lead to full recovery of the melanocyte phenotype: (i) A tyrosinase construct with an elongated transmembrane domain (Tyr-TM6) bypassed the transport block in the Golgi region (Sprong et al., 2001), but electron microscopy showed that GM95-Tyr-TM6 cells still lacked stage II, III and IV melanosomes. Instead, parts of the MVBs and lysosomes in these cells, including those containing the striated bodies, were highly electron-dense, suggesting melanin accumulation (Figure 1E). (ii) Treatment of mutant melanocytes with ammonium chloride or bafilomycin-A stimulates pigmentation (Ancans et al., 2001). When GM95 cells were treated for 8 h with 10 mM ammonium chloride or 10 μM bafilomycin-A (not shown) the cell pellet was black, and a fraction of tyrosinase located outside the perinuclear region (cf. Figures 1F and 1G). However, no melanosomes were observed. The black organelles resembled those in GM95-Tyr-TM6 cells (compare Figures 1E and 1H). Together, these results demonstrate that glycosphingolipids are required for melanosome biogenesis, and reinforce the notion that the presence of the tyrosinase and TRP-1 in endosomal structures alone is not sufficient for melanosome formation.

Need for GlcCer and not higher glycolipids in melanosome biogenesis

MEB4 cells express 3 major glycolipids: GlcCer, LacCer (Galβ1-4GlcCer), and GM3 (NeuAcα2-3Galβ1-4GlcCer). To address which glycosphingolipid is required for melanosome biogenesis, we reduced their levels using RNA interference. MEB4 cells were stably transfected with vectors that direct the synthesis of small interfering RNAs of ceramide transport protein (CERT) and LacCer synthase, and of lamin as a control. Knocking down CERT disrupted ceramide transport from the ER to Golgi, and consequently decreased the GlcCer and GM3 levels to < 30% of control values (Hanada et al., 2003). This reduction was sufficient for creating the pigmentation defect (Figure 3A), observed in the absence of GlcCer in GM95 cells (Sprong et al., 2001) and in an RNAi knock-out of MEB4 cells (Diallo et al., 2003). Knocking down LacCer synthase (GalT1) did not significantly change the level of GlcCer but reduced GM3 (and therefore LacCer) by a similar 70% as in the CERT knockdown. However, there was no effect on cell pellet color, showing that the synthesis of LacCer is not required for pigmentation.

The pigmentation defect in the absence of glycosphingolipids is accompanied by TRP-1 missorting (Sprong et al., 2001). Like tyrosinase, TRP-1 is normally transported from the Golgi to the melanosomes without passing over the cell surface. In GM95 cells, however, its presence at the cell surface is increased (Sprong et al., 2001). The uptake of anti-TRP-1 antibodies can serve as a read-out for the

amount of endocytosis from the plasma membrane and consequently indicates how efficient the glycosphingolipid-dependent sorting machinery transports TRP-1 to the melanosomes via the direct intracellular route. TRP-1 missorting in GM95 cells was indeed corrected by transfection of CGlcT (Sprong et al., 2001) and by exogenous glucosylsphingosine (Suppl. Figure 1). However, antibody uptake still occurred in GM95 cells in which pigmentation, but not melanosome biogenesis, was restored by transfection with elongated tyrosinase (Sprong et al., 2001) or treatment with NH_4Cl (Figure 3C). Knocking down CERT in MEB4 cells abolished pigmentation (Figure 3A), and induced anti-TRP-1 antibody uptake (Figure 3B). Addition of glucosylsphingosine to CERT-knockdown cells restored intracellular transport of TRP-1 (Figure 3B) and pigmentation (not shown). In contrast, knocking down LacCer synthase in MEB4 cells had no effect on pigmentation, nor did it induce antibody uptake (Figure 3B, lane 4). We conclude that intracellular transport of TRP-1 requires GlcCer, whereas LacCer or more complex glycosphingolipids are not needed.

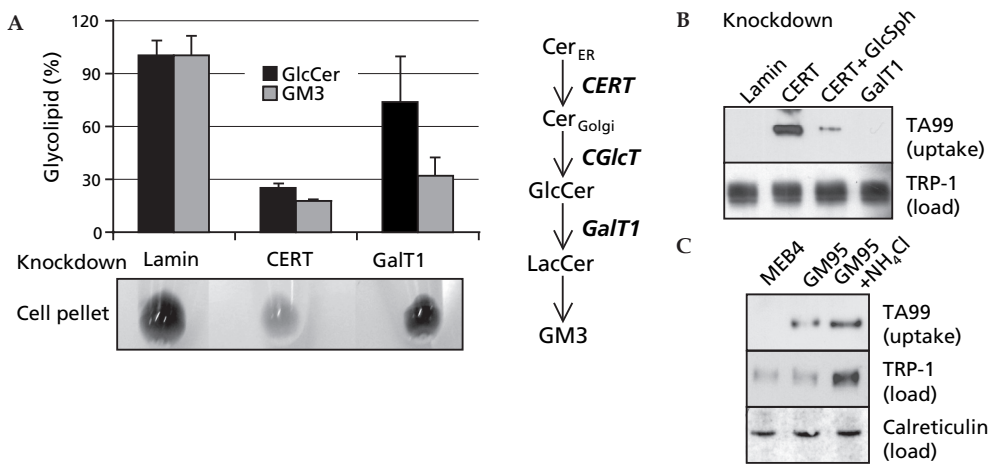

Figure 3. Role of GlcCer but not LacCer in pigmentation. (**A**) MEB4 cells stably expressing RNAi plasmids against the ceramide transfer protein CERT, LacCer synthase (GalT1), or lamin as a control were incubated overnight with [^{14}C]-serine. Lipids were extracted, separated by 2D-TLC, and radiolabeled lipids were quantitated using a phosphor-imager as described in Materials and Methods. Values are expressed as a percentage of GlcCer or GM3 in control cells (RNAi against lamin), and are the mean of 2 independent stable clones ± range. Parallel cell monolayers were scraped, pelleted, and photographed. (**B**) Independently, 3-cm dishes having the same number of RNAi expressing MEB4 cells were incubated with TA99 antibody against TRP-1 for 3 h at 37˚C. After washing, the internalized antibody was visualized by blotting using an HRP-conjugated secondary antibody. Total TRP-1 in the samples was detected by Western blotting using anti-pep1 (load). TRP-1 missorting induced by CERT knock-down was corrected by the continuous presence of glucosylsphingosine (GlcSph). (**C**) The same experiment was performed for GM95 cells incubated with 10 mM NH_4Cl for 8 h, a treatment that restored pigmentation (Figure 1F). These cells expressed more total TRP-1 (load) compared to the loading control calreticulin, probably due to reduced degradation in the lysosomes. However, NH_4Cl did not significantly correct antibody uptake.

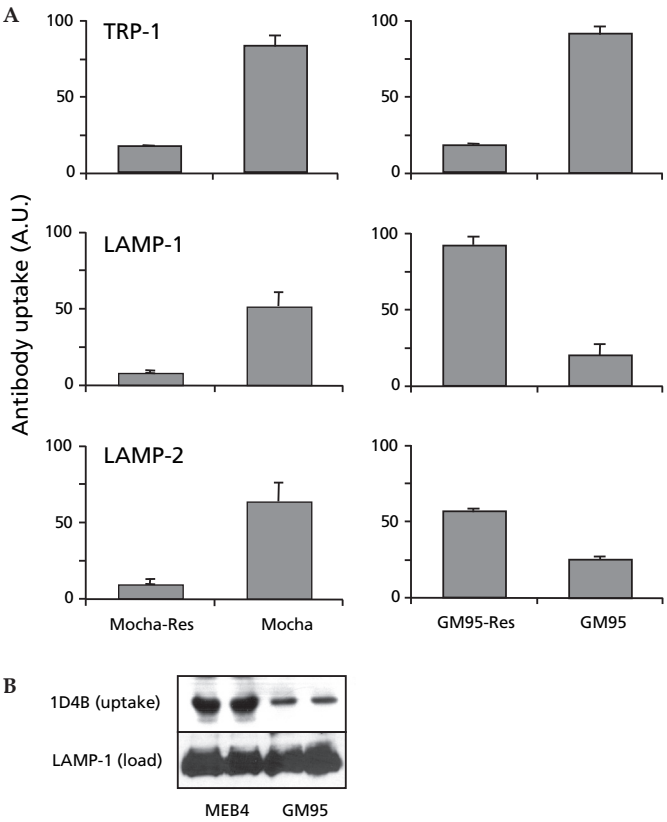

Figure 4. Transport of TRP-1 and LAMPs in δAP-3 or glycosphingolipid-deficient cells. 3-cm culture dishes of *mocha* fibroblasts transfected with TRP-1 (Mocha), *mocha* fibroblasts transfected with both TRP-1 and δAP-3 (Mocha-Res), GM95 and GM95-CGlcT (GM95-Res) cells were incubated with antibodies against the lumenal domain of TRP-1, LAMP-1, and LAMP-2. Internalized antibodies were visualized by Western blotting. To allow a quantitative comparison, different amounts of each sample were loaded on gel, and the total amount of LAMP-1 or TRP-1 in the samples was measured as an internal control. Signals were quantified using the Image Quant software. Data are means of at least 2 independent experiments. A.U., arbitrary units (see Materials and Methods).

Direct transport of melanosomal and indirect transport of lysosomal proteins in melanoma cells

The biogenesis of melanosomes requires a functional AP-3 pathway (Dell'Angelica et al., 1999). To test whether in fibroblasts the intracellular route of TRP-1 is AP-3 dependent, TRP-1 was transfected into *mocha* (deficient in AP-3 subunit δ) and *mocha*- rescued fibroblasts expressing the δ-subunit by transfection. We then compared by antibody-uptake the routing of TRP-1 to the lysosomes, its destination in these cells (Suppl. Figure 2), and found that TRP-1 was transported via the plasma membrane in *mocha* but not in *mocha*-rescued fibroblasts, demonstrating that direct transport of TRP-1 in fibroblasts depends on AP-3 (Figure 4A). Similarly, loss of AP-3 resulted in increased trafficking of LAMP-1 and LAMP-2 via the plasma membrane, corroborating previous findings in a number of cell types (Le Borgne et al., 1998; Dell'Angelica et al., 1999; Peden et al., 2004). As melanosomal and lysosomal proteins have different destinations in melanocytes (Figure 2), we then compared their routing in MEB4, GM95 and GM95-CGlcT melanocytes. We had already shown

that TRP-1 is transported via the intracellular pathway to melanosomes in MEB4 cells, but traveled over the cell surface in the glycosphingolipid-deficient GM95 cells (Sprong et al., 2001). Unexpectedly, we now found that lysosomal LAMP-1 and -2 displayed the opposite transport pattern than TRP-1: LAMP-1 and -2 traveled over the plasma membrane to reach the lysosomes in wild-type MEB4 melanocytes (Figure 4B). However, in the absence of glycosphingolipids, in GM95 cells, LAMP-1 and -2 did follow a direct pathway. Restoration of glycosphingolipid synthesis by CGlcT transfection increased the passaging of LAMP-1 and -2 via the plasma membrane. Thus, in MEB4, GM95 and GM95-CGlcT melanocytes LAMP-1 and -2 behave opposite to TRP-1 (Figure 4A, B).

The efficient direct transport of LAMP-1 and LAMP-2 to the lysosomes in GM95 cells indicated that the AP-3 machinery is present and active (Figure 4) and that the aberrant sorting of TRP-1 in GM95 cells is not due to a lack of AP-3. Indeed, the expression levels of the distinct AP-3 subunits as well as their recruitment to membranes were similar in MEB4 and GM95 cells (Figure 5A). Finally, the

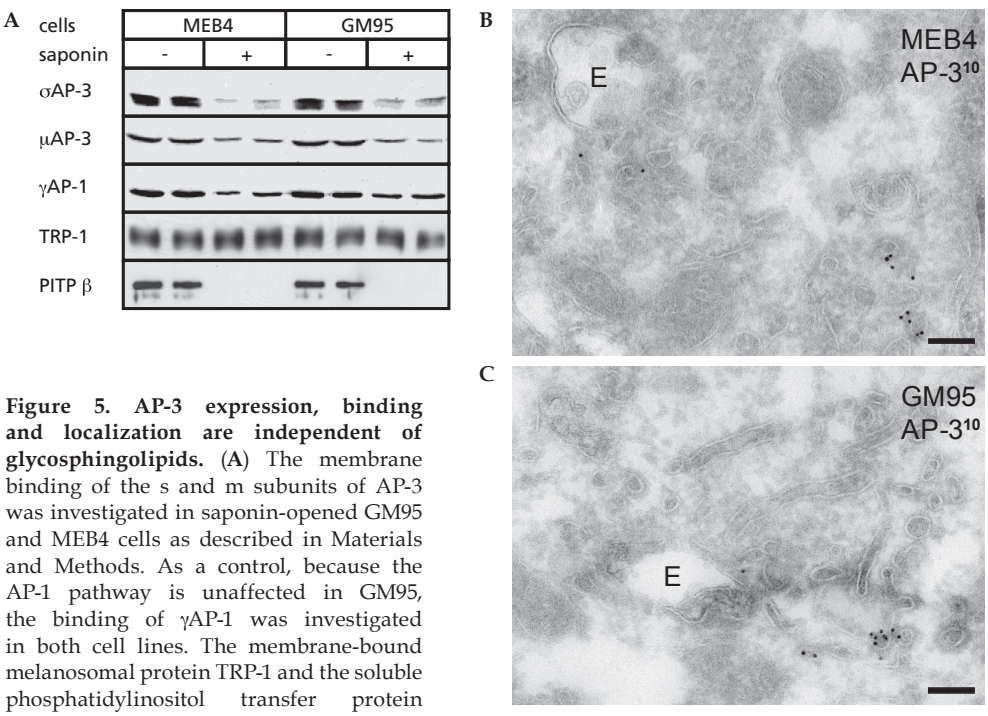

Figure 5. AP-3 expression, binding and localization are independent of glycosphingolipids. (**A**) The membrane binding of the s and m subunits of AP-3 was investigated in saponin-opened GM95 and MEB4 cells as described in Materials and Methods. As a control, because the AP-1 pathway is unaffected in GM95, the binding of γAP-1 was investigated in both cell lines. The membrane-bound melanosomal protein TRP-1 and the soluble phosphatidylinositol transfer protein PITPβ were used as controls for the opening procedure. The experiment was performed three times with comparable results. (B,C) AP-3 has a similar distribution in MEB4 and GM95 cells. Immunogold label (10 nm gold particles) for the δAP-3 subunit on ultrathin cryosections occurs both in MEB4 (**B**) and GM95 (**C**) cells on buds on endosome (E)-associated tubules that are often clathrin-coated. Scale bar, 100 nm.

ultrastructural localization of the AP-3 complex by immunogold labeling of ultrathin cryosections showed that in both cell types AP-3 associated with buds evolving from tubular endosomal compartments (Figure 5B), just as was previously shown in non-melanosomal cells (Peden et al., 2004).

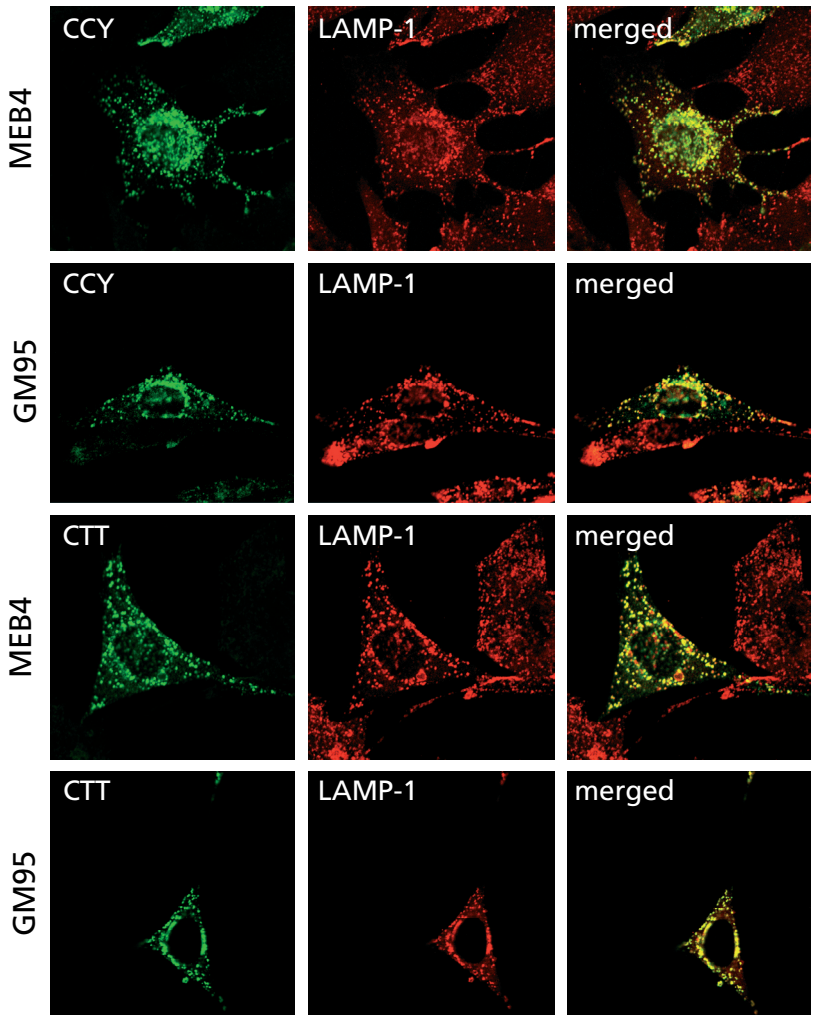

Figure 6. The lumenal domains of tyrosinase and TRP-1 determine their melanosomal localization. MEB4 and GM95 cells were (mock) transfected with CCY (lumenal and transmembrane domain CD25 and cytoplasmic tail tyrosinase) or with CTT (lumenal domain CD8, transmembrane and cytoplasmic tail TRP-1). Prior to fixation, cells were incubated with leupeptin for 3 h at 37˚C to prevent lysosomal degradation of the lumenal CD25- and CD8-domains. Cells were double labeled with antibodies against LAMP-1 and CD25 (CCY) and CD8 (CTT) and analyzed by confocal fluorescence microscopy. In contrast to tyrosinase (Figure 2) CCY is located to lysosomal structures in MEB4 as well as in GM95 cells. Also note the (strict) segregation between TRP-1 and LAMP-1 in MEB4 cells (in Figure 2), and the similar staining patterns of CTT and LAMP-1. The localization of CCT was similar to CTT (not shown).

Direct transport of melanosomal proteins depends on their lumenal domain

The different location of the melanosomal and lysosomal proteins in MEB4 melanocytes (Figure 2) and their opposite transport behavior (Figure 4) implied that next to the AP-3 motif in their cytoplasmic tail they must contain additional sorting information. To test whether melanosomal proteins contain dominant sorting information in their lumenal domains, we tested three chimeras that contained the cytoplasmic tail of melanosomal proteins and the lumenal domain of plasma membrane proteins: CTT, lumenal domain of CD8 and transmembrane and cytoplasmic tail of TRP-1; CCT, lumenal and transmembrane domain of CD8 and cytoplasmic tail of TRP-1; CCY, lumenal and transmembrane domain of CD25 and cytoplasmic tail of tyrosinase. First, we tested whether the lumenal domain determined the steady-state accumulation of tyrosinase and TRP-1 in the melanosomes. All chimeras fully colocalized with LAMP-1, both in MEB4 and GM95 cells (Figure 6). This implies that the lumenal/transmembrane domain of tyrosinase in MEB4 cells determines its localization in the melanosomes. Moreover, the results show that the information in the lumenal domain of TRP-1 determined its localization in melanosomes in MEB4 cells.

Next we studied whether the altered destination of the tyrosinase and TRP-1 chimeras correlated with a change in routing. In fibroblasts direct transport of all chimeras to the lysosomes was AP-3 dependent (Figure 7A), as was the transport of TRP-1 (Figure 4A). Remarkably, in MEB4 and GM95-CGlcT cells all chimeras were transported to the lysosomes via the cell surface. By contrast, in the absence of glycosphingolipids they were transported directly (Figure 7A). In addition, CTT transport over the cell surface was increased three-fold in GM95 incubated with glucosylsphingosine (Figure 7B), which restores all features of melanogenesis. Thus, removal of the lumenal domain of both TRP-1 and tyrosinase changed their trafficking pattern to that of lysosomal proteins. We infer that the lumenal domains of both tyrosinase and TRP-1 contain dominant information for following the direct transport pathway to melanosomes. The sorting information is functional only in the presence of glycosphingolipids in wild-type melanocytes.

Discussion

In melanocytes, melanosomal proteins are transported to melanosomes via a direct intracellular pathway that depends on AP-3 and glycosphingolipids. Unexpectedly, lysosomal membrane proteins which supposedly also follow an AP-3 mediated pathway appear to travel via the melanocyte cell surface. In the absence of glycosphingolipids this situation is reversed. The lumenal domain of the melanosomal proteins contains essential sorting information for the intracellular pathway to the melanosomes. This suggests that the critical role of glycosphingolipids

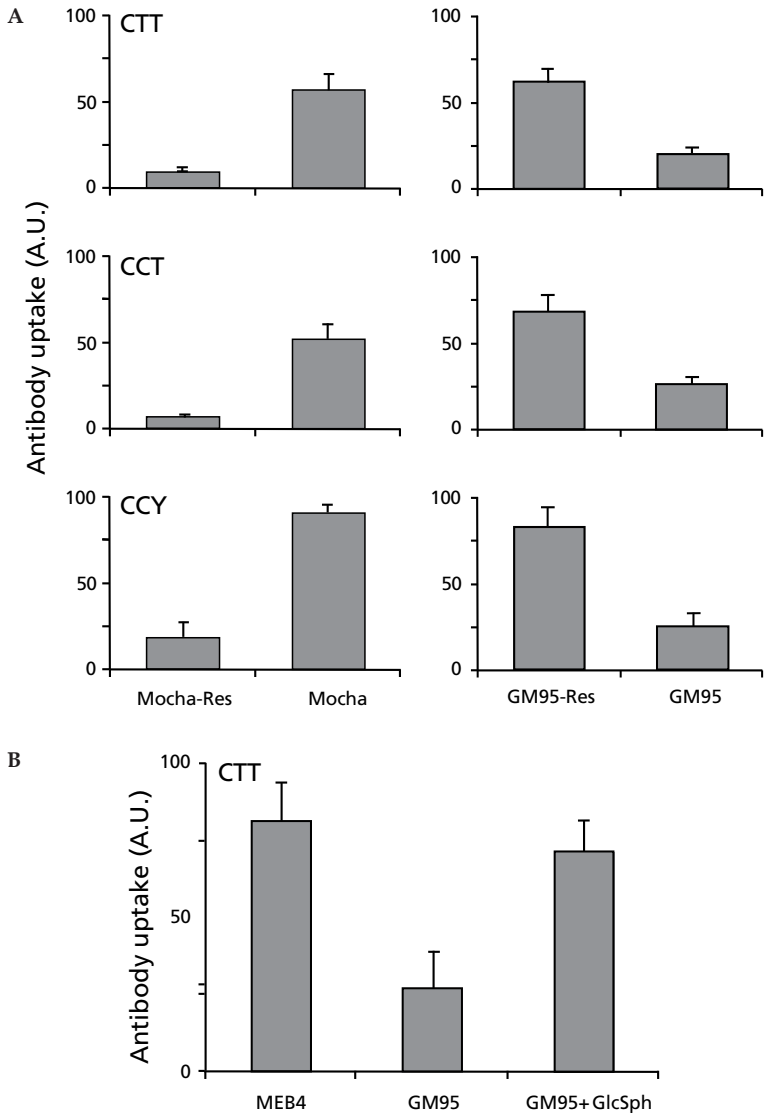

Figure 7. Lumenal domains of TRP-1 and tyrosinase contain glycolipid-dependent targeting information to the melanosomes. (**A**, **B**) 3-cm culture dishes of *mocha* (Mocha) and *mocha*-δAP-3 fibroblasts (Mocha-Res), and of MEB4, GM95 and GM95-CGlcT (GM95-Res) cells were transiently transfected with plasmids containing the CTT, CCT and CCY constructs: CTT, lumenal domain CD8 and transmembrane and cytoplasmic tail TRP-1; CCT, lumenal and transmembrane domain CD8 and cytoplasmic tail TRP-1; CCY, lumenal and transmembrane domain CD25 and cytoplasmic tail tyrosinase. After 1 d, the cells were incubated with anti-CD8 or anti-CD25 antibody for 3 h at 37°C. Internalized antibodies were visualized by Western blotting and quantified. Data are means of at least 2 independent experiments ($n \geq 2$). A.U., arbitrary units. In (**B**) GM95 cells transfected with CTT were grown in the continuous presence of glucosylsphingosine (GlcSph, 20 μM) starting 2 d before transfection.

in sorting lies on the lumenal surface, possibly via a lectin-based mechanism.

GM95 cells as a model to study glycosphingolipid function

GM95 cells are unable to synthesize glycosphingolipids and lack pigment and proper melanosomes (Figure 1). A mutant tyrosinase with a longer transmembrane domain overcame the transport block and restored pigmentation, but the mutant tyrosinase did not restore the formation of proper melanosomes (Figure 1). Also treatment with NH_4Cl restored pigmentation but not melanosome biogenesis or proper TRP-1 transport (Figures 1, 3), implying that it did not correct the basic defect in GM95 cells. Thus, formation of functional melanosomes requires intracellular sorting of TRP-1.

The NH_4Cl effect on pigmentation was probably provoked by its stimulating effect on tyrosinase stability and activity due to pH neutralization of endosomes that contained a low level of tyrosinase (Suppl. Table I; Ancans et al., 2001). In contrast, a knock-down of CERT and CGlcT in MEB4 cells (Figure 3; Diallo et al., 2003) mimicked the GM95 phenotype in depigmentation and in TRP-1 missorting (Figure 3). Furthermore, both CGlcT transfection of GM95 and exogenous glucosylsphingosine fully restored the melanocyte phenotype (Figure 1). This validates the GM95 cells as a system to address the molecular role of glycosphingolipids in pigmentation.

AP-3 mediated transport of melanosomal proteins

Melanocytes transport their melanosomal enzymes from the TGN to the melanosomes via an intracellular route that depends on the adaptor complex AP-3: the absence of functional AP-3 results in a pigmentation defect (Kantheti et al., 1998; Feng et al., 1999) and has been identified as the genetic defect in Hermansky-Pudlak syndrome type 2 patients (HPS2; Dell'Angelica et al., 1999; Huizing et al., 2002). Although both tyrosinase and TRP-1 contain the typical dileucine AP-3 recognition signal, Huizing et al. (2001) observed in HPS2 patient melanocytes that tyrosinase remained in the Golgi area while TRP-1 still occurred in peripheral structures. It was concluded that transport of tyrosinase but not that of TRP-1 is mediated by AP-3. However, the actual pathway of TRP-1 was not determined. We found in *mocha* fibroblasts with a defective AP-3 machinery that TRP-1 was still transported towards the endo-lysosomes, but it traveled via an indirect route over the cell surface (Figure 4). In fibroblasts in which a functional AP-3 complex was restored, TRP-1 traveled via a direct intracellular pathway. These data show that in fibroblasts TRP-1 is normally recognized by the AP-3 complex but in the absence of functional AP-3 shifts to an indirect pathway over the cell surface.

GM95 cells display a similar phenotype as HPS2 melanocytes (Huizing et al., 2001; Sprong et al., 2001), with perinuclear tyrosinase and more peripheral TRP-1 staining. Is should be noted that the block in tyrosinase transport is not complete as some tyrosinase in GM95 cells was found in endosomal structures (Suppl. Table I). In addition, TRP-1 followed the indirect pathway via the plasma membrane in GM95

cells as well as in the AP-3 deficient *mocha* cells (Figure 4). The altered trafficking of tyrosinase and TRP-1 suggested that the intracellular pathway for melanosomal proteins is hampered in GM95 cells. The quantity of AP-3, the fraction of AP-3 that was membrane-bound, and its intracellular localization as seen by electron microscopy were very similar between GM95 and MEB4 (Figure 5), suggesting that in these cells the AP-3 machinery is still functional. Together these data indicate that in melanocytes in addition to AP-3 an additional sorting mechanism is responsible for direct sorting of melanosomal proteins, which is absent from lysosomal proteins and only uncovered in the presence of glycolipids.

Sorting of melanosomal from lysosomal proteins

The lysosomal membrane proteins LAMP-1 and -2 contain a cytosolic AP-3 binding motif that directs them to the lysosomes in fibroblasts (Bonifacino and Traub, 2003). They follow the direct pathway in GM95 cells (Figure 4), but surprisingly in MEB4 melanocytes they followed an indirect pathway to the lysosomes, over the cell surface. This behavior was opposite to that of TRP-1 (Figure 4). This correlates with the fact that lysosomal and melanosomal proteins must contain different information to target them to the lysosomes and melanosomes, respectively. It turned out that the lumenal domain of the melanosomal proteins contains the specific targeting information, as replacement by the lumenal domain of non-specific plasma membrane proteins turned TRP-1 and tyrosinase into typical lysosomal proteins (Figure 7). So, how would this sorting system work?

So far, travel of lysosomal proteins (Le Borgne et al., 1998; Peden et al., 2004) and melanosomal proteins (Dell'Angelica et al., 1999) via the cell surface strictly correlates with the absence of a functional AP-3, and not any other adaptor complex. This suggests that in melanocytes melanosomal proteins bind AP-3 and travel to the melanosomes whereas the lysosomal proteins, LAMP-1 and -2, do not bind AP-3 and travel via the cell surface. A recent paper showed that the interaction of the AP-2 adaptor complex with its cargo depends on multimerization of the AP-2 binding site (Grass et al., 2004). So, possibly the lumenal domains of melanosomal proteins cause them to oligomerize in the relevant sorting compartment which then increases their affinity for AP-3. Lysosomal proteins would lose the competition for AP-3 binding from oligomerized melanosomal proteins and shift to the non-specific pathway to the cell surface (Le Borgne et al., 1998) (Figure 8).

In GM95 cells, which lack glycosphingolipids, the melanosomal protein TRP-1 traveled to the cell surface and was thus excluded from the direct intracellular pathway that was now followed by the lysosomal proteins (Figures 4). Glycosphingolipids are thus required for the proper functioning of the melanosomal targeting information in the lumenal domain of melanosomal proteins. Glycosphingolipids, more specifically glucosylceramide (Figure 3), may interact with melanosomal proteins via the formation of glycosphingolipid/cholesterol rafts. However, so far no difference in detergent-solubility of tyrosinase (one assay for raft association) was found

between MEB4 and GM95 cells (H. Sprong, unpublished data). Alternatively, an interaction may be mediated by a lectin-type interaction in the lumen of the sorting compartment (Schrag et al., 2003). In the model (Figure 8), the interaction with the glycosphingolipids would be required for the oligomerization of the melanosomal proteins. The lack of glycosphingolipids would result in the loss of clustering of the melanosomal proteins and a reduction in their affinity for AP-3. This would then result in binding of AP-3 to the lysosomal proteins by default, followed by the observed shift in routing. The findings in the present paper identify a sorting switch between melanosomal and lysosomal proteins. The properties of this switch and the direct or indirect role of AP-3 and glycosphingolipids are the topics of our present investigations.

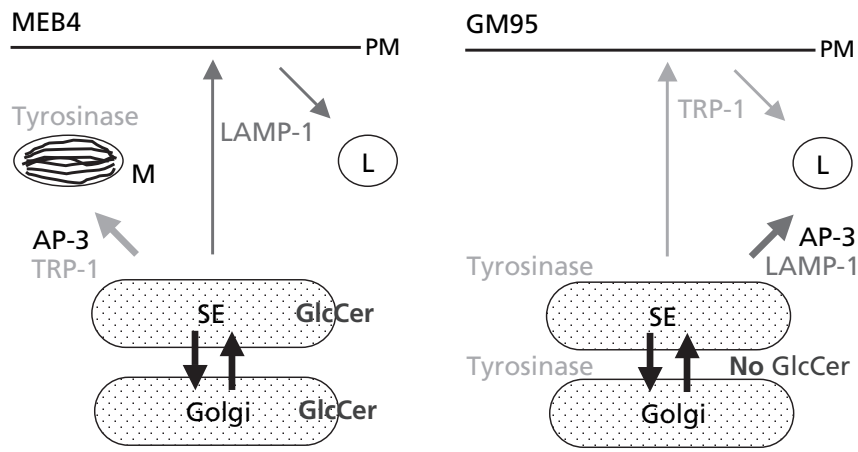

Figure 8. Model for the sorting of melanosomal and lysosomal proteins in melanocytes. In MEB4 cells, melanosomal proteins compete out the lysosomal proteins from the AP-3 pathway in a GlcCer dependent manner. Formation of AP-3 mediated melanosomal transport vesicles from a **S**orting station in the **E**ndocytic track (SE) results in melanosome formation. In GM95 cells, like in fibroblasts, LAMP-1 and LAMP-2 are transported directly to lysosomes. Tyrosinase is stuck in the Golgi/SE, TRP-1 is missorted, and mature melanosomes do not form.

Materials and Methods

Antibodies and reagents

Chemicals, if not indicated otherwise, were from Sigma (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 and reagents were from Invitrogen (Breda, The Netherlands). [^{14}C]-serine (5.59 Gbq/mmol) was from Amersham (Buckinghamshire, UK). Rabbit antisera against the cytoplasmic tails of mouse TRP-1 (anti-pep1) and mouse tyrosinase (anti-pep7)

were generated as described (Jimenez et al., 1988; 1991). Anti-pep7 was affinity-purified using the same peptide coupled to affigel-15 columns (Biorad, Hercules, CA). The rabbit anti-tyrosinase antibody used for electron microscopy was a kind gift of G. Raposo (Curie Institute, Paris, France). The rabbit antiserum against the lumenal domain of human CD25 was from M. Marks (University of Pennsylvania, Philadelphia, PA). The mouse antibodies against the different subunits of the adaptor protein complexes were from L. Traub (University of Pittsburgh, PA), except for the SA4 mouse anti-δAP-3 antibody, which was from A. Peden (Genentech Inc., San Francisco, CA). The rabbit antiserum against PITPβ was from G. Snoek (Utrecht University, The Netherlands). The mouse 9E10 antibody against the c-myc epitope, the mouse mAb 32M4 against the lumenal domain of human CD8, and the rat 1D4B antibody against the lumenal domain of mouse LAMP-1 were from Santa Cruz Biotechnology (Santa Cruz, CA), and the mouse mAb HMB45 against Pmel17 was from Lab Vision Corporation (Fremont, CA). The mAb ABL93 against the lumenal domain of LAMP-2 was from the Developmental Studies Hybridoma Bank (Iowa City, IA). The mAb TA99 against the lumenal domain of TRP-1 was from Abcam (Cambridge, UK). Rabbit polyclonal antibodies against Hrs, EEA-1, cathepsin D and CD-MPR were kind gifts of S. Urbé and M. Clague (University of Liverpool, UK), A. Hasilik (Phillips University, Marburg, Germany) and K. von Figura (University of Göttingen, Germany), respectively. FITC and Texas-red fluorescent secondary goat antibodies were obtained from Jackson (West Grove, PA). Rabbit anti-mouse immunoglobulin G, and HRP conjugated secondary goat antibodies were from DAKO (Glostrup, Denmark).

Plasmids

The chimera having the lumenal and transmembrane domains of CD25 and the cytoplasmic tail of tyrosinase (CCY-pCDM8.1) was from M. Marks (Calvo et al., 1999). TRP-1-pCMV6 and the chimeras of CD8 and TRP-1 (CCT- and CTT-pCDM8.1) were from A. Houghton (Vijayasaradhi et al., 1995). The construction of CGLCT-pCB7, myc-tagged Tyr, Tyr-TM6, and α2,6-sialyltransferase-pCB7 were described before (Sprong et al., 2001). Oligos used for RNA interference of CERT, LacCer synthase, and lamin (sequences available on request) were inserted between the BglII and HindIII sites of the RNAi plasmid pKoen (Deneka et al., 2003). All synthetic cDNAs were verified by restriction analysis and dye termination sequencing.

Cell culture and transfection

GlcCer synthase-deficient GM95 cells and their parental MEB4 cells from the RIKEN Cell Bank (Tsukuba, Japan) were grown in DME containing 4.5 g/l glucose and 10% fetal calf serum, at 37°C with 5% CO_2. Cells were stably transfected with CGlcT-pCB7 or myc-tagged α-2,6 sialyltransferase-pCB7, or transiently with CCY-, CTT- or CCT-pCDM8.1 using Lipofectamin 2000. Myc-transfectants were screened with immunofluorescence using the 9E10 antibody. Stable transfectants of CGlcT

(GM95-rescued) were screened by assaying the CGlcT as previously described (Sprong et al., 1998). MEB4 cells were stably transfected with RNAi plasmids (pKoen) against CERT, LacCer synthase, and lamin using Lipofectamin 2000. Stable transfectants were selected in the presence of hygromycin B (200 U/ml) or G418 (0.6 mg/ml) and individual colonies were obtained by limiting dilution subcloning. Stable transfectants carrying the pKoen constructs were initially selected for green fluorescent signal in the nucleus, and subsequently for their glycosphingolipid composition. For this, subconfluent cells on 3 cm dishes were incubated overnight in MEM alpha medium, 5% FCS and 15 kBq/ml [^{14}C]-serine. Cells were washed with PBS and lipids were extracted and analyzed as described (Sprong et al., 2003). The AP-3 δ-subunit–deficient *mocha* fibroblasts and *mocha* cells that were stably transfected with the δ-subunit (*mocha*-rescued) were from A. Peden, and were cultured as described (Peden et al., 2002; 2004). Cells were stably transfected with TRP-1-pCMV6 using Lipofectamin 2000. The stable transfectants of TRP-1 were selected in the presence of G418 (0.6 mg/ml) and hygromycin B (200 U/ml) and then screened and selected by Western Blotting using the anti-TRP-1 antiserum anti-pep1. Protein expression was induced by 5 mM sodium butyrate (Fluka, Buchs, Germany) 14-16 h prior to all experiments.

Immunofluorescence microscopy

Cells were grown on coverslips to 30-50% confluency. The cells were fixed with 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 goat antibodies in blocking buffer for 1 h. The coverslips were washed with blocking buffer, followed by two washes with PBS, rinsed briefly in deionized water and finally mounted in Mowiol 4-88 (Calbiochem, La Jolla, CA) containing 2.5% 1,4-dia zabicyclo[2.2.2]octane. The cells were examined with a Nikon D-eclipse C1 confocal microscope using separate filters for each fluorochrome viewed (FITC: L_{ex} = 488 nm and L_{em} = 500-530 nm; Texas red: L_{ex} = 543 nm and L_{em} = 570-600 nm). Examination of single-labeled cells with each primary/secondary antibody combination showed that no bleed-through occurred in the confocal conditions used.

Immuno-electron microscopy

Cells were fixed either overnight with 4% PFA, or for 2 h with 2% PFA + 0.2% glutaraldehyde or + 1% acrolein in 0.1 M phosphate buffer (pH 7.4) at room temperature. After fixation, the cells were rinsed with PBS and PBS containing 0.02 M glycine, scraped off the culture dishes and pelleted in 12% gelatin. Small blocks of the embedded cell pellets were infiltrated overnight with 2.3 M sucrose, mounted on aluminium pins and frozen in liquid nitrogen. Ultra-thin cryosections were cut at –120°C, 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

labeled with antibodies and protein A-gold as described earlier (Slot et al., 1991). The relative distribution of melanosomal and endosomal/lysosomal proteins was determined by random screening of single labeled sections from PFA-fixed cells. All gold particles on or up to 25 nm from a membrane-bound compartment were scored as label on that organelle type, and percentages of the total amount of gold particles counted were calculated for each compartment. Results are derived from total counts of 600 gold particles for tyrosinase and 200-300 for the other proteins.

Antibody internalization

Confluent cells on 3 cm dishes were incubated for 3 h at 37˚C with ~50 μg in 1 ml antibodies against the lumenal domain of LAMP-1, LAMP-2, TRP-1, CD8, and CD25 in medium containing 20 μg/ml leupeptin. Cells were washed 5 times with ice-cold PBS and lysed in reducing Laemli sample buffer. Equal amounts of protein were analyzed by SDS-PAGE, and the amount of internalized antibody was determined by Western blotting (Sprong et al., 1998). As an internal standard, LAMP-1 or TRP-1 was detected using the rat 1D4B antibody or the rabbit antiserum anti-pep1, respectively. Films were scanned on a Bio-Rad GS 700 imaging densitometer equipped with an integrating program. The maximum signal on a film that was still linear was determined by using a concentration series of blotted mouse IgGs, detected with anti-mouse peroxidase. It was set at 100 arbitrary units and the time of film exposure in each experiment was optimized to yield a value of roughly 100 A.U. for the spot with the highest amount of label.

Binding of adaptor proteins to membranes

Confluent cells on 3 cm dishes were washed 3 times with ice-cold PBS. To remove their cytosolic proteins, cells were then incubated with 1 ml PBS ± 0.1% ($^{w}/_{v}$) saponin for 20 min on ice. The incubation medium with cytosol was removed and the cells were washed with ice-cold PBS and lysed in reducing Laemmli sample buffer. Samples were analyzed by SDS-PAGE, followed by Western blotting. Contrary to the membrane protein TRP-1, the soluble protein PITPβ was almost completely removed by the saponin treatment.

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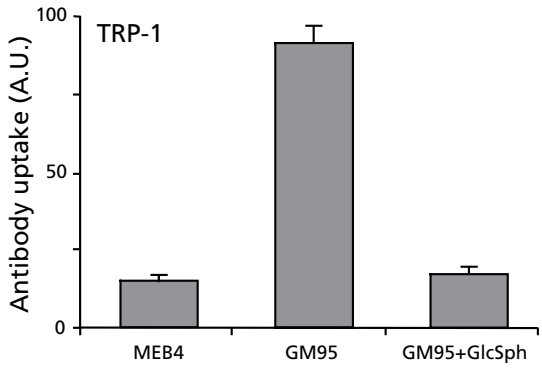
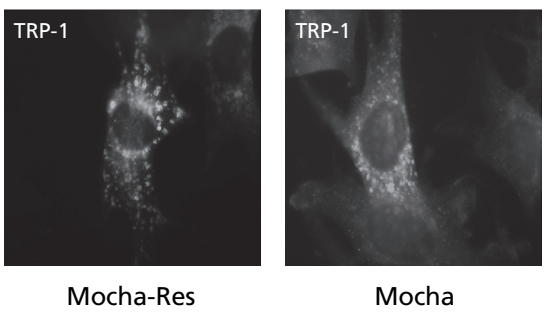


Supplementary Figure 1. Intracellular transport of TRP-1 is glycosphingolipid-dependent. MEB4 and GM95 cells were grown in the presence or absence of glucosylsphingosine (GlcSph, 20 μM) for 2 d. The cells were incubated with anti-TRP-1 (TA99) antibody for 3 h at 37°C. Internalized antibodies were visualized by Western blotting and quantified. Data are analyzed as described in the Methods section. A.U., arbitrary units.



Supplementary Figure 2. Cellular localization of TRP-1 in *Mocha* and *Mocha*-rescued cells. *Mocha* and *Mocha*-rescued (Mocha-Res) cells were transfected with TRP-1. Cells were labeled with antibodies against TRP-1, counterstained with FITC-labeled secondary antibodies, and analyzed by conventional fluorescence microscopy. The majority of TRP-1 localizes to punctate structures in the cytosol, resembling lysosomes. Note faint staining of the plasma membrane in *mocha* cells.

Supplementary Table I. Proteins in ultrathin cryosections were detected by specific antibodies followed by protein A-gold labeling as described in Materials and Methods. Randomly sampled gold particles were assigned to various organelles and their relative frequency in these compartments is expressed as '+++' for more than 25 %, '++' for 15-25 %, '+' for 5-15 %, '±' for 1-5 %, or '-' for less than 1 % of total label. Per cell line, 600 gold particles were scored for tyrosinase and 200-300 for the other proteins. [b] EE, early endosome; LE, late endosome; MVB, multivesicular body; Mel II, III, IV, melanosome stage II, III, IV; Tyr, tyrosinase; NA, not applicable. [c] MVBs with striated bodies occur very rarely in MEB4 cells.

Supplementary Table I. Subcellular distribution of endosomal/lysosomal markers in MEB4 and GM95 cells.

		Golgi stack	Vesicles/tubules			EE[b]	LE/MVB	lysosome	Mel II	Mel III	Mel IV	MVB + striated body[c]
			Golgi area	cytosol	endosome-associated							
Tyr[b]	MEB4	±	+	+++	±	+	+	±	±	+	++	-
	GM95	+	++	+++	+	+	++	±	NA	NA	NA	+
TRP-1	MEB4	±	±	+	-	++	+	±	+	++	++	±
	GM95	+	+	++	-	+++	++	-	NA	NA	NA	+
Pmel17	MEB4	-	±	±	-	++	±	-	+++	+++	+	-
	GM95	-	-	-	-	-	-	-	NA	NA	NA	-
LAMP-1	MEB4	-	±	+	-	+++	+	++	+	+	+	±
	GM95	-	±	+	-	+++	+	++	NA	NA	NA	++
cathepsin D	MEB4	-	-	-	-	+++	++	±	++	±	+	-
	GM95	-	-	-	-	+++	++	±	NA	NA	NA	++
Hrs	MEB4	+	+	+	±	+++	++	-	±	±	-	-
	GM95	±	+	±	-	+++	++	-	NA	NA	NA	±
EEA1	MEB4	±	+	+	+	+++	+	±	±	±	±	±
	GM95	±	+	+	+	+++	++	±	NA	NA	NA	+
CD-MPR	MEB4	-	+++	+++	++	±	+	-	-	-	-	-
	GM95	±	+++	+++	+	±	±	-	NA	NA	NA	±

Chapter 4

Glycosphingolipids lower the pH of the TGN and lysosomes in melanoma cells by activating the V-ATPase

Jasja Wolthoorn[1], Dave van den Heuvel[2], Maarten Egmond[1], Hans Gerritsen[2], Gerrit van Meer[1] and Hein Sprong[1]

[1] Membrane Enzymology, Bijvoet Center and Institute of Biomembranes, Utrecht University, [2] Molecular Biophysics, Debye Institute, Utrecht University, Utrecht, The Netherlands

Summary

The low pH in endocytotic compartments is an important physiological parameter that regulates numerous cellular processes. Using fluorescence lifetime imaging microscopy, the pH in the lumen of the *trans* Golgi network (TGN) and lysosomes of melanoma cells was found to be as low as 5 and 4, respectively. Melanoma mutant cells unable to synthesize glycosphingolipids had pH values >1 pH unit higher, similar to Hela cells and fibroblasts. The cytosolic pH was 7.5 in all cells. In an in vitro assay on isolated membranes the V-ATPase activity was two-fold higher in the wild-type than in the mutant melanoma cells. Exogenously added glucosylceramide, lactosylceramide and steryl glucoside, but not sphingomyelin or phosphatidylinositol, activated the V-ATPase in membranes from mutant but not from wild-type cells. The presence of glycolipids increased the K_i for the specific V-ATPase inhibitor concanamycin A 5-fold, suggesting competition for the same binding pocket on the membranous c subunit. A model is presented in which the concentration of the central sphingolipid metabolite ceramide regulates the pH in the secretory and endocytic transport pathway via glucosylceramide as the direct V-ATPase activator.

Introduction

The simple glycosphingolipid glucosylceramide (GlcCer) is synthesized by the ceramide glucosyltransferase (CGlcT) (Ichikawa et al., 1996) from UDP-glucose and ceramide. Thus, CGlcT is one of the enzymes that regulates the cellular pool of the pro-apoptotic ceramide (Futerman and Hannun, 2004). Remarkably, synthesis occurs on the cytosolic surface of the Golgi (Futerman and Pagano, 1991; Jeckel et al., 1992) and maybe ER (Kohyama-Koganeya et al., 2004), after which (some) GlcCer must flip to the Golgi lumen where it is converted to lactosylceramide (LacCer) the common precursor for the complex glycolipids. Complex glycolipids have functions on the cell surface, where they have been found to regulate the activity of many receptors, possibly in glycosignaling domains (Hakomori, 2002; 2004). In addition, glycosphingolipids are highly enriched on the apical surface of epithelial cells and microdomains of glycosphingolipids and cholesterol on the lumenal surface are thought to be an essential feature of the sorting machinery of Golgi and endosomes (Simons and van Meer, 1988; Sharma et al., 2003). Unrelated to GlcCer synthesis, specialized cells synthesize galactosylceramide (GalCer) by means of the ceramide galactosyltransferase on the lumenal aspect of the ER (Sprong et al., 2003). Newly synthesized GalCer is expected to equilibrate over both sides of the ER membrane (Burger et al., 1996) and to have access to the same sites as GlcCer. GalCer is partially converted to Galα1-4GalCer and sulfatide, HSO_3-3GalCer, in the Golgi lumen.

When searching for the cellular function of GlcCer and its derivatives, we unexpectedly noticed that a melanoma cell mutant, GM95, that was unable to synthesize glycosphingolipids by lack of CGlcT activity (Ichikawa et al., 1996), had lost the ability to synthesize pigment. This appeared to be due to a defect in transport of melanosomal proteins to the melanosomes (Sprong et al., 2001b). Subsequent experiments demonstrated that the block was situated in the TGN and recycling endosomes, and that the missorting was caused by information in the lumenal domain of the melanosomal proteins (Chapter 3). When we studied parameters determining the lumenal environment of the proteins, we measured a remarkable difference in pH between the TGN of the mutant and the parental cell line. When we subsequently addressed the question how the glycolipid would lower the pH in the organelles of the wild-type melanoma cells, we found a direct effect of the lipids on the activity of the V-ATPase in isolated membranes.

Results

MEB4 cells have a lower pH in the TGN and lysosomes than GM95 and HeLa cells

To measure the pH in the TGN we made use of the retrograde transport of the TGN-resident protein TGN38 to target fluorescein, a fluorescent pH-sensitive probe to the lumen of the TGN as described by Demaurex and co-workers (Demaurex et al., 1998). Cells were transfected with CD25-TGN38, a construct in which the lumenal domain of TGN38 was exchanged for the lumenal domain of the plasma membrane protein CD25. After the uptake of anti-CD25-FITC, the distribution of the antibody in wild-type MEB4 and mutant GM95 cells was similar to that of myc-tagged α-2,6 sialyltransferase, a *trans* Golgi marker (Figure 1A), but the two staining patterns did not perfectly overlap, as expected for a *trans* Golgi and a TGN protein. This was also the case in control HeLa cells, although some fluorescence was dispersed in punctate structures throughout the cell, as has been observed before (Demaurex et al., 1998).

The pH in the TGN was measured via the fluorescence lifetime of the endocytosed anti-CD25-FITC using fluorescence lifetime imaging microscopy (FLIM). We were able to measure selectively the lifetime of the fluorescence in the perinuclear area by using an intensity threshold (Materials and Methods and Supplementary Figure). When cells were incubated with the ionophore nigericin in K^+ buffer to equilibrate the intracellular and extracellular pH, the fluorescence lifetime of anti-CD25-FITC was sensitive to the pH of its environment, and a standard curve was obtained for calculating the pH in the TGN from the lifetime values (Figure 1B). Strikingly, in MEB4 cells the pH of the TGN was 5.1 ± 0.3, which was significantly lower than the pH in mutant GM95 cells (6.5 ± 0.3) (Figure 1C). When we used GM95 cells, in which the glucosyltransferase enzyme had been re-transfected (GM95-Res), the pH of the TGN (5.0 ± 0.3) was similar to the pH of wild type MEB4 cells, suggesting that

GlcCer synthesis is essential for the low pH in the TGN of MEB4 cells. The pH of the TGN in HeLa cells (6.6 ± 0.2) was similar to the pH in GM95 cells, showing that the TGN of MEB4 cells has an exceptionally low pH.

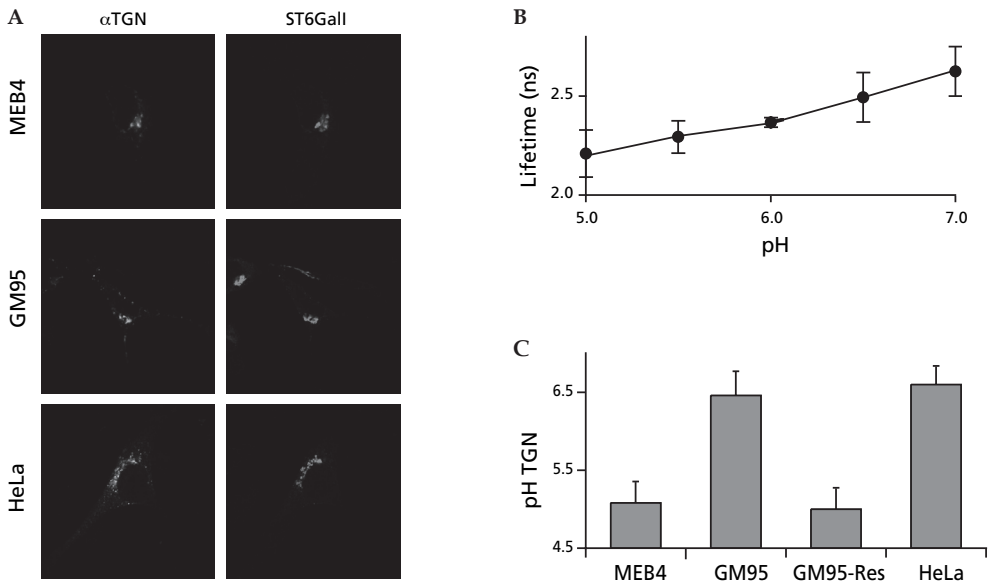


Figure 1. The pH in the TGN depends on glycosphingolipids. (**A**) Intracellular localization of endocytosed anti-CD25-FITC. MEB4, GM95, GM95-Res and HeLa cells expressing CD25-TGN38 were incubated with anti-CD25-FITC antibody (αTGN) overnight, fixed and permeabilized, and co-labeled with mouse anti-myc-α-2,6 sialyltransferase (ST6GalI). (**B**) pH calibration of anti-CD25-FITC in MEB4 cells using fluorescence lifetime imaging (FLIM). Calibration measurements on cells were carried out after 10 min incubation in the presence of nigericin (10 μg/ml) in 150 mM K^+ buffers varying in pH between 5.0 and 7.0 at 37°C. Curves measured in MEB4, GM95 and HeLa cells were overlapping (not shown). (**C**) pH in the TGN. Average lifetime values for anti-CD25-FITC in MEB4, GM95, GM95-Res and Hela cells, with n (#cells)= 23, n=23, n=13 and n=18, respectively, were correlated to the pH calibration curve in cells, yielding the average pH values of the TGN in these cells.

To see whether the effect of GlcCer was limited to the TGN, we measured the pH in lysosomes in MEB4, GM95, GM95-Res cells and in mouse fibroblasts as a control. Oregon Green (OG) was used as a fluorescent pH-sensitive probe coupled to anti-LAMP-1 antibody, which was taken up by endocytosis and ended up in the lysosomes as shown by perfect colocalization with the endogenous lysosomal protein LAMP-2 (Figure 2A). The fluorescence lifetime of anti-LAMP-1-OG was sensitive to the pH when bound and taken up by cells, at a pH range between 3.5 and 5.5. Like the pH difference in the TGN, the pH of lysosomes in MEB4 cells (4.1 ± 0.1) was significantly lower than the pH in GM95 cells (5.4 ± 0.2) (Figure 2C). GM95-Res cells had a lysosomal pH even lower (3.5 ± 0.1) than MEB4 cells, suggesting that

GlcCer synthesis is also essential for pH regulation in lysosomes. The lysosomal pH in mouse fibroblasts was similar to that in GM95 cells, showing that MEB4 cells have an exceptionally low pH in lysosomes.

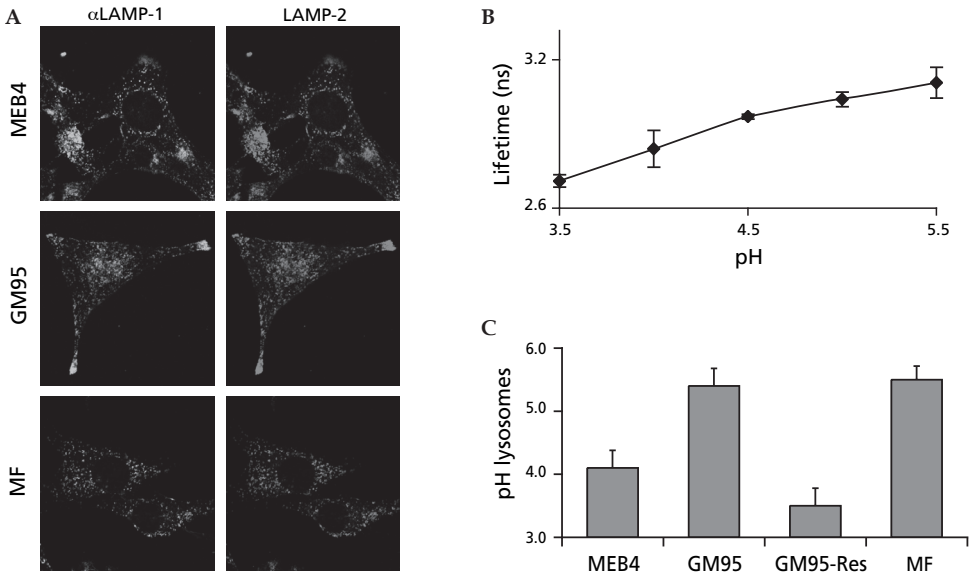


Figure 2. The pH in lysosomes depends on glycosphingolipids as well. (**A**) Intracellular localization of endocytosed Oregon Green (OG) labeled anti-LAMP-1. MEB4, GM95, GM95-Res cells and mouse fibroblasts (MF) were incubated with anti-LAMP-1-OG (α LAMP-1) for 5 h, fixed and permeabilized, and co-labeled with mouse anti-LAMP-2. (**B**) pH calibration of anti-LAMP-1-OG in MEB4 cells using FLIM. Calibration curves measured in MEB4, GM95 and MF cells were overlapping (not shown). (**C**) pH in lysosomes. Average lifetime values for anti-LAMP-1-OG in MEB4, GM95, GM95-Res and MF cells, with n (#cells)= 18, n=18, n=10 and n=6, respectively, were correlated to the pH calibration curve in cells, yielding the average pH values of lysosomes in these cells.

To see whether the lower lumenal pH in MEB4 cells was due to a lower cytosolic pH, we used carboxy SNAFL-diacetate, which becomes fluorescent after cleavage by cytosolic esterases, as a pH sensitive probe to measure the cytosolic pH by FLIM as described before (Sanders et al., 1995; Lin et al., 2003). In MEB4, GM95 and HeLa cells, SNAFL gave a cytosolic pattern and prominently stained the nucleus (Figure 3A). The fluorescence lifetime of the probe was sensitive to the pH although over a small lifetime range between pH 6.8 and 7.8. Also, calibration curves were slightly different for the different cell lines (Figure 3B). However, using the calibration curve for the corresponding cell line, no significant cytosolic pH difference was observed between MEB4 and GM95 cells, with a pH of 7.5 ± 0.0 (Figure 3C). Thus, GlcCer does not regulate the pH in the cytosol. HeLa cells had a similar cytosolic

pH (7.6 ± 0.0). Lifetime measurements in selected regions of interest yielded no significant difference in lifetime between the probe in the nucleus and the cytosol (not shown).

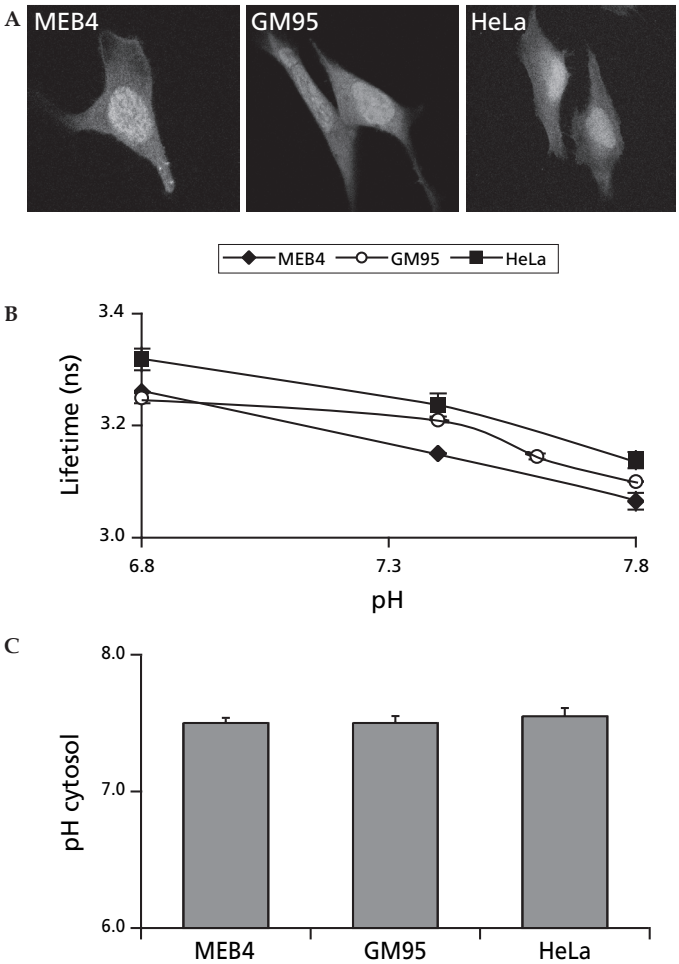


Figure 3. The cytosolic pH is independent of glycosphingolipids. (**A**) Intracellular localization of SNAFL after 10 min uptake in MEB4, GM95 and HeLa cells. (**B**) pH calibration of SNAFL in MEB4, GM95 and HeLa cells. The lines between the data points are intended 'to guide the eye'. For calculations a polynomial fit, degree =2 was used. (**C**) The pH in the cytosol. Average lifetime values for SNAFL in MEB4, GM95 and HeLa cells, with n (#cells)=7, n=4 and n=7, respectively, were correlated to the pH calibration curves in the corresponding cells, yielding the average pH values of the cytosol in these cells.

V-ATPase activity in total membranes

One of the major determinants of the intralumenal pH is the vacuolar or V-type ATPase, the proton pump that acidifies the lumen of organelles of both the secretory and endocytotic pathway. To test whether GlcCer or its metabolites stimulate the activity of the V-ATPase, we measured its activity in membranes isolated from the different cells using a colorimetric assay. We analyzed the free phosphate (P_i) produced by the ATPase in total membranes from MEB4, GM95 and GM95-Res cells in the presence of 5 mM Mg^{2+} and 2 mM ATP, concentrations that are 10-fold higher than the respective K_ms of the V-ATPase (David and Baron, 1994). To lower

the background in the assay we included inhibitors of mitochondrial and transport ATPases (2 mM azide and 0.1 mM vanadate). The V-ATPase activity was measured as the difference between the P_i production in the absence and presence of a specific inhibitor of the V-ATPase, concanamycin A (Wang et al., 2005). In line with the lower pH in the TGN and lysosomes in MEB4 cells, MEB4 cells displayed a two-fold higher V-ATPase activity than GM95 cells (Figure 4A). This activity was partially restored in GM95-Res cells, correlating GlcCer synthesis with V-ATPase activity. Expression levels of the B-subunit of the cytosolic V_1 domain of the V-ATPase, of the cytosolic protein GAPDH and of TRP-1, a membrane protein, were similar on Western blot (Figure 4B), showing that the 50% reduction in V-ATPase activity in GM95 cells was not due to a 2-fold lower expression level of the V-ATPase.

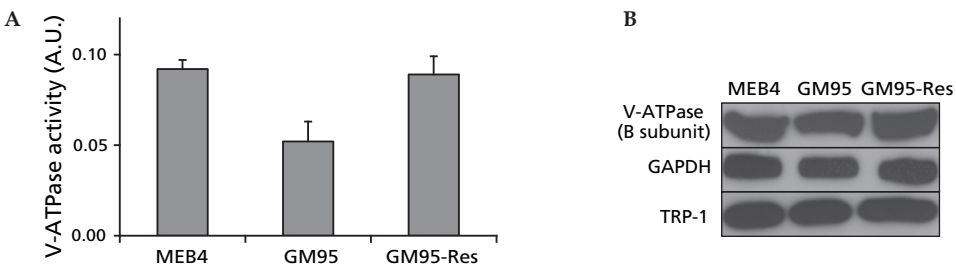


Figure 4. V-ATPase activity in total membranes of cells. (**A**) Total membranes isolated from postnuclear supernatants of MEB4, GM95 and GM95-Res cells were analyzed for V-ATPase activity. Bars represent the concanamycin-sensitive ATPase activities relative to the total activity in the absence of inhibitor, and is expressed in arbitrary units based upon the absorbance measured at 570 nm (in triplicate), per 0.01 mg protein per 30 minutes, as described in Materials and Methods. Activities were determined in at least two independent experiments with similar results. A representative experiment is shown. (**B**) Samples were analyzed for expression of the B-subunit of the V-ATPase (V_1 domain), GAPDH (a cytosolic protein) and TRP-1 (a membrane protein) by Western Blot.

V-ATPase activity in GM95 cell membranes is selectively restored by glycolipids

We next investigated whether GlcCer directly influences the activity of the V-ATPase. Because GlcCer is not water-soluble and does not efficiently insert into membranes when added as a monomer, we added liposomes containing 30 mol% GlcCer together with glycolipid transfer protein (GLTP; Mattjus et al., 1999) to isolated GM95 membranes and measured the V-ATPase activity. A maximal increase in activity of more than 50% was observed at the optimal GlcCer concentration (Figure 5). Also the glycolipids LacCer and steryl glucoside increased the V-ATPase activity of GM95 membranes. However, neither sphingomyelin (SM) nor phosphatidylinositol (PI) when added in the same concentration together with the PI/SM transfer protein PITPβ, stimulated the activity in GM95 membranes. These results on isolated membranes show that (simple) glycolipids stimulate the V-ATPase in melanoma cells specifically and directly, i.e. without need for transcription or vesicular transport.

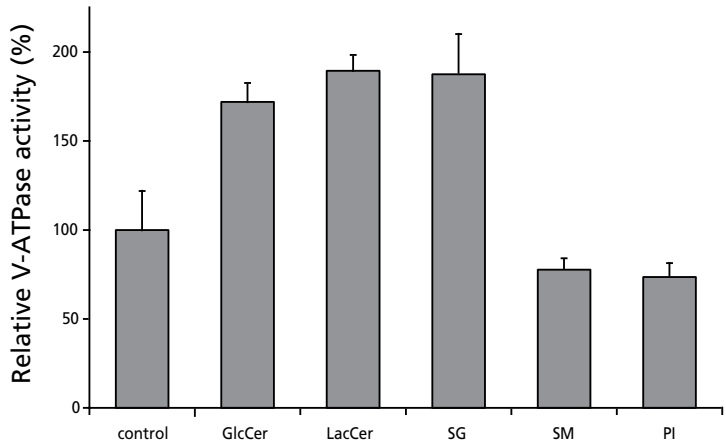
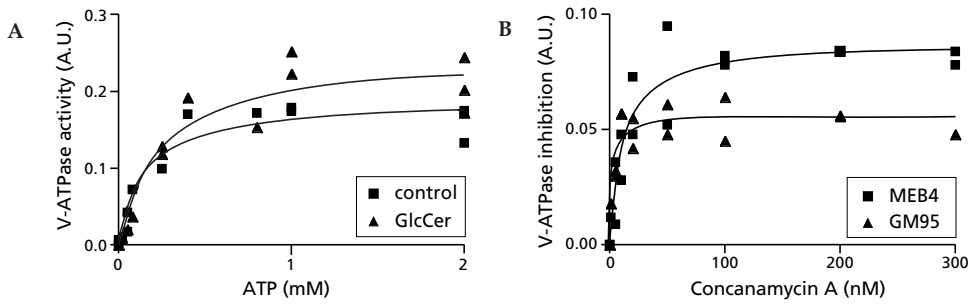


Figure 5. V-ATPase activity in total membranes of GM95 cells is restored by in vitro addition of GlcCer, LacCer or steryl glucoside, but not sphingomyelin or phosphatidylinositol. Total membrane fractions from GM95 cells were incubated with liposomes containing GlcCer, LacCer or steryl glucoside (SG) together with GLTP or sphingomyelin (SM) or phosphatidylinositol (PI) together with PITPβ for 30 min at 37°C, and analyzed for V-ATPase activity. Controls were incubated with liposomes containing only PC and cholesterol and GLTP or PITPβ. Bars represent the concanamycin-sensitive ATPase activities (Materials and Methods) and are expressed relative to the control. Activities were determined in at least two independent experiments with similar results. A representative experiment is shown.

Effect of glycosphingolipids on V-ATPase kinetics

Glycolipids increased the rate of ATP hydrolysis by the V-ATPase at steady state (Figures 4 and 5), which takes place at the A-subunits of the V_1 or soluble domain (Inoue et al., 2003). To investigate whether these lipids increase ATP binding to the V-ATPase, either directly or indirectly, we varied the ATP concentration in the ATPase assay and calculated the K_m (ATP) in GM95 membranes and GM95 membranes to which GlcCer had been added. GlcCer slightly increased the K_m (ATP) from 0.16 to 0.24 mM (Figure 6A), while the V_{max} increased from 0.19 to 0.25 due to addition of GlcCer (Figure 6A). As a result, higher V-ATPase activity is noted in the presence of GlcCer above 0.5 mM ATP (Figure 6).

The V-ATPase inhibitor concanamycin A binds the proteolipid c-subunits of the V_0 or membrane domain of the V-ATPase (Bowman et al., 2004; Whyteside et al., 2005). We tested different concentrations of concanamycin in the ATPase assay to calculate the K_i (concanamycin A). The K_i for concanamycin in MEB4 membranes (11.55 nM) is much higher than in GM95 membranes (2.24 nM), implying that in MEB4 much more concanamycin is needed to inhibit the V-ATPase. These results suggest the exciting possibility that glycolipids bind to the c-subunits of the V-ATPase or that they influence the conformation of the c-subunits more indirectly by changing their lipid environment.



condition	K_m (ATP) (mM)	sd	V_{max} (A.U.)	sd
control	0.16	0.05	0.19	0.01
GlcCer	0.24	0.08	0.25	0.02

cells	K_i (ConcA)(nM)	sd	V_{max} (A.U.)	sd
MEB4	11.55	3.54	0.09	0.01
GM95	2.24	0.91	0.06	0.00

Figure 6. V-ATPase kinetics. (**A**) Total membrane fractions from GM95 cells, incubated with and without GlcCer, were analyzed for V-ATPase activity. The activity was plotted against the ATP concentration to calculate the K_m (ATP) and V_{max} values, using the formula $V = \frac{Vmax \bullet S}{Km + S}$, where S represents the substrate concentration and V the velocity. Activities of three independent experiments were combined, values corresponding to conditions where no ATP was added were set to zero. K_m (ATP) is expressed in mM ATP. V_{max} is expressed in arbitrary units and represents the concanamycin-sensitive ATPase activity (Materials and Methods). (**B**) Total membrane fractions from MEB4 and GM95 cells were analyzed for inhibition of V-ATPase activity by concanamycin A. The activity was plotted against the concanamycin A concentration to calculate the K_i (concanamycin A) and V_{max} values. Activities of three independent experiments were combined, values corresponding to conditions where no concanamycin A was added were set to zero. K_i (concanamycin A) is expressed in nM. V_{max} is expressed in arbitrary units and represents the concanamycin-sensitive ATPase activity (Materials and Methods).

Discussion

In the present paper we report that the pH in the lumen of TGN and lysosomes of melanoma cells is much lower than in fibroblasts, and that this depends on the presence of glycosphingolipids. Various glycolipids activated the vacuolar proton ATPase in isolated membranes, and kinetic studies suggest that the glycosphingolipids compete for binding with the V-ATPase inhibitor concanamycin A. This implies that cells may use these glycosphingolipids to lower the pH of intracellular compartments via a direct binding to the V-ATPase.

Several years ago, we observed that mutant melanoma cells that were unable to synthesize glycosphingolipids no longer synthesized pigment. They did not assemble melanosomes and the transport of tyrosinase, the most important enzyme in melanin synthesis, was disrupted (Sprong et al., 2001a). In our more recent work (Chapter 3), we were able to assign the defect in transport to a factor on the lumenal side of the TGN or endosomal membrane, and in the present study we have identified one major lumenal parameter that is dramatically different between the cells with and without glycosphingolipids: the pH in the TGN/endosomes and lysosomes. How could a lower pH in the TGN or endosomes be involved in

protein trafficking and sorting? A gradient of pH exists along the organelles of the secretory and endocytotic pathway. While the lumenal pH is neutral in the ER, it gradually decreases towards the TGN, with a value of around 6, which is also the value generally reported for the early endosomes. The pH then further decreases to some 5.5 in late endosomes, and finally 5 or lower in the lysosomes. This gradient determines to a large degree how the cell manages the traffic, sorting and function of its organellar proteins along the vesicular transport routes (Weisz, 2003a), and a disturbed acidification may play a role in the pathology of several severe diseases (Weisz, 2003b). Especially the role of low pH in endocytosis and endocytotic recycling has been investigated in great detail, but also it is well understood that the low pH in the late exocytotic pathway is required for the proteolytic cleavage, aggregation and sorting of regulated secretory proteins (Tooze et al., 2001; Taupenot et al., 2005).

Proteolytic cleavages and aggregation also play a major role in melanosome biogenesis (Berson et al., 2003; Theos et al., 2005), and a role for a low pH has been proposed from studies on the p protein, a melanosomal transmembrane protein that is involved in the most common form of human oculo-cutaneous albinism (OCA2). From the observation that p protein-deficient melanosomes have an abnormal, non-acidic pH, it has been proposed that the p protein functions to regulate melanosomal pH by acting as a counterion transporter for the V-ATPase (Brilliant, 2001): anion cotransport allows the V-ATPase to generate a larger pH gradient and thus a lower lumenal pH. This is in agreement with our present observation that a low lumenal pH is required for melanogenesis. Exactly which steps in the processing and sorting of melanosomal proteins depend on the exceptionally low lumenal pH in the TGN and endosomes that we report here remains to be established, but proper transport of tyrosinase appears to be one of them (Sprong et al., 2001a; Toyofuku et al., 2002).

Contrary to the proposed role of p protein in lowering the lumenal pH, the defective pigmentation in p protein mutant cells can be corrected by compounds that raise organellar pH (Ni-Komatsu and Orlow, 2005), and it has been argued that p protein neutralizes melanosomes (Ancans et al., 2001). Similarly, we observed that neutralizing the pH by ammoniumchloride bypassed the requirement for glycosphingolipids and thus for a low pH (Chapter 3). However, whereas under the latter conditions melanin synthesis was restored, no proper melanosomes were assembled, suggesting that these experimental conditions are not of direct relevance for the biogenesis of melanosomes in vivo. Indeed, the observation that co-expression of p protein with tyrosinase in non-melanocytes partially corrected its mistrafficking (Ni-Komatsu and Orlow, 2005), fits better with lowering the lumenal pH during transport than with a role in melanin synthesis.

The pH gradient in the secretory pathway is regulated at multiple levels (Wu et al., 2001; Weisz, 2003a). The pH is determined by the balance between the pumping of protons, the conductance for counterions, and the leak of protons. Whereas general membrane properties like lipid composition play an important role, the

consensus is that the pH is mostly regulated by ion pumps and channels, the most important of which is the V-ATPase (Nishi and Forgac, 2002; Wagner et al., 2004). We now report that the V-ATPase can be activated in vitro by a number of glycolipids. It has been reported before that the activity of the purified tonoplast proton pump of plants is increased by steryl glucoside (Yamaguchi and Kasamo, 2001; 2002), and sphingolipids with a C26 acyl group turned out to be required for generating an active V-ATPase in yeast (Chung et al., 2003), which suggests that the activation of V-ATPase by specific lipids may be a general (eukaryotic) principle.

There are multiple ways how glyco(sphingo)lipids can activate the V-ATPase. Whereas the activation mechanism in yeast seemed to involve the processing of the soluble V_1 domain (Chung et al., 2003), the activation of in reconstituted membranes (Yamaguchi and Kasamo, 2001; 2002) or isolated cellular membranes (Figure 5) suggests a direct action of the lipid. The fact that we could activate the V-ATPase by the addition of lipids to isolated membranes argues against the possibility that the lipid would promote binding of the soluble V_1 domain to the membranous V_0 domain, since no free V_1 would be present in the isolated membranes. One possibility is that the lipid changes the immediate environment of the V-ATPase by creating a spingolipid/sterol rich environment, and indeed the V-ATPase has been found enriched in detergent-resistant membranes (Dermine et al., 2001; Yoshinaka et al., 2004) suggesting a preference for such a lipid composition. Another possibility is that the lipid directly binds to a defined binding pocket in the V-ATPase, and the concentration curve of inhibition by concanamycin (Figure 6) suggests just that. The K_i for concanamycin of 11 nM in wild-type membranes was reduced to 2 nM in the membranes of the mutant cells lacking glycosphingolipids (both K_i values are much higher than the value of 0.2-0.3 nM reported before, which is most likely due to the chemical instability of concanamycin, see (Whyteside et al., 2005)). This suggests the possibility that a glycosphingolipid competes with concanamycin for its binding site, which resides in the membranous c subunit (Bowman et al., 2004). This may also explain that the lack of glycosphingolipids also affected the pH of the lysosomes (Figure 2), where the V-ATPase has the same c subunits.

The concanamycin binding site resides on the cytosolic side of the c subunit (Bowman et al., 2004). Of the endogenous lipids that activated the V-ATPase, GlcCer and LacCer (Figure 5), only GlcCer has access to the cytosolic surface of the membrane (Lannert et al., 1994; Burger et al., 1996), making GlcCer the obvious candidate activator. Interestingly, GlcCer is synthesized on the cytosolic surface of the Golgi and is continuously removed from that surface by translocation across the membrane towards the lumenal leaflet where it is converted to lactosylceramide. This creates the remarkable possibility that the rate of glucosylceramide synthesis regulates the activity of the V-ATPase. The rate of synthesis in turn would depend on the activity of the glucosyltransferase and the ceramide concentration. Evidence has been provided that cells can regulate the activity of the glucosyltransferase, at the transcriptional level (Watanabe et al., 1998; Memon et al., 2001; Uchida et al.,

2004) or upon stimulation (Boldin and Futerman, 2000). Just as interesting, ceramide itself is a signaling lipid and a central metabolite in sphingolipid metabolism (Futerman and Hannun, 2004). Thus, cells may use the level of ceramide as a read-out for the status of their sphingolipid metabolism and use that, via the derived glucosylceramide as an input for regulating the lumenal pH, thereby coupling two important physiological parameters. All eukaryotic cells express the V-ATPase and most make glucosylceramide, which suggests that we may have stumbled onto an evolutionary conserved regulation mechanism.

Materials and Methods

DNA

The CD25-TGN38-pCDM8.1 construct, encoding the lumenal domain of human CD25 and the transmembrane and cytosolic domains of TGN38, was a gift from F. Maxfield (Cornell University, New York). Myc-tagged α-2,6 sialyltransferase-pCB7 and CGlcT-KKVK-pCB7 were made as previously described (Sprong et al., 2001a). GLTP-pQE-9 and PITPβ-pET15b were kind gifts from P. Mattjus (Åbo Akademi University, Turku, Finland) and M. Schenning (Utrecht University), respectively.

Antibodies and lipids

The rat conjugated anti-CD25-FITC antibody against human CD25 was from Serotec (Oxford, UK). The polyclonal rabbit anti human CD25 antiserum was a gift from M. Marks (University of Pennsylvania School of Medicine, Philadelphia, PA). Rabbit antiserum PEP1 against the cytoplasmic tail of TRP-1 was a gift from V. Hearing (NIH, Bethesda, MD). The monoclonal anti-c-myc 9E10 antibody and the rat 1D4B antibody against LAMP-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal anti-LAMP-2 antibody ABL-93 was from Developmental Studies Hybridoma Bank (Iowa City, IA). Oregon Green-514 carboxylic acid succinimidyl ester and 5-(and-6)-carboxy SNAFL-1 diacetate were from Molecular Probes (Eugene, OR). Oregon Green (OG) was coupled to anti-LAMP-1 antibody (1D4B) following instructions by Molecular Probes. Secondary antibodies were from Santa Cruz (Santa Cruz, CA). All lipids were obtained from Sigma (St. Louis, MO), except for phosphatidylinositol (Avanti Polar Lipids, Alabaster, AL) and steryl glucoside (Larodan Fine Chemicals, Malmö, Sweden).

Cell culture and transfection

CGlcT-deficient GM95 cells and their parental MEB4 cells were from RIKEN Cell Bank (Tsukuba, Japan) and were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37°C with 5% CO_2. HeLa cells (G. Warren, London) and mouse ear fibroblast WT1.2 cells (MF; J. Wijnholds, The Netherlands Cancer Institute) were grown under the same conditions as GM95

and MEB4 cells.

GM95 cells were stably transfected with CGlcT-KKVK-pCB7 using lipofectamine 2000 and selected in the presence of hygromycin B (200 U/ml) and individual colonies were obtained by limiting dilution subcloning. Stable transfectants were screened by assaying the CGlcT activity as previously described (Sprong et al., 1998). For pH measurements in the TGN, cells were grown on 3 cm glass bottom dishes (Mattek Corporation, Ashland, MA) and transiently transfected with CD25-TGN38-pCDM8.1 using lipofectamine 2000. After 5 h, transfection medium was exchanged for normal growth medium with anti-CD25-FITC (7.5 μl/ml) and the incubation continued for 16 h. For pH measurements in lysosomes, anti-LAMP-1-OG was added to untransfected cells and incubated for 5 h. For pH measurements in cytosol, cells were incubated for 20 min with 5 μM carboxy SNAFL-diacetate in HBSS, pH 7.4. For fluorescence lifetime imaging microscopy cells were incubated with DMEM/ Hepes (25 mM) pH 7.4 without phenol red containing 10% FCS at 37°C for measurements of the TGN and lysosomes, and in HBSS pH 7.4 for measurements of cytosolic pH.

pH calibrations

Calibration curves of the fluorescence lifetime of the various probes vs. the pH were generated using the high potassium/ nigericin method (Thomas et al., 1979). For measurements in the TGN, cells were washed twice in calibration buffer (100 mM KOAc, 50 mM KCl, 1 mM $MgCl_2$, 5 mM glucose) set at a pH value ranging from 5.0 to 7.0, followed by the addition of nigericin (10 μg/ml final concentration). After 5 min the fluorescence lifetime was measured in at least 3 different cells. Lysosomes were calibrated in the same calibration buffer with pH values ranging from 3.5 to 5.5. Calibration in the cytosol was done in phosphate buffer (100 mM K_2HPO_4/ KH_2PO_4, 50 mM KCl, 5 mM $MgCl_2$, 5 mM glucose) ranging from pH 6.8 to pH 7.8. Average lifetimes were plotted against the pH. Error bars represent the sample standard deviation (SD).

Fluorescence Lifetime Imaging Microscopy and statistical analysis

Lifetime measurements were performed using a confocal laser scanning microscope (CLSM, Nikon PCM2000). For excitation, 460 nm pulsed light was used. Therefore, the CLSM was equipped with a Tsunami Titanium:Sapphire laser (Spectra-Physics, Newport Corporation, Mountain View, CA) that produced 2 ps light pulses at 920 nm with a repetition rate of 82 MHz. For our purposes every 10th pulse was picked with a pulsepicker and the 920 nm was frequency-doubled to 460 nm using a LBO-crystal. The emitted fluorescence, selected with appropriate chromatic filters, was detected using a fast GaAsP photon-counting PMT (Hamamatsu, H7421-40, with a 450 ps transient time spread). The output pulses from the PMT were coupled to a four time-gated lifetime module, with four time-gates (de Grauw and Gerritsen, 2001). The four time-gate widths were set to 2 ns each without a delay between the time-gates. The start of the first time-gate was delayed until the intensity of a fast

decaying dye in the first time-gate was reduced to 10% of the maximal obtainable signal (Rose Bengal, $\tau = 90$ ps, Sigma, Zwijndrecht, The Netherlands (Rodgers, 1981)). In order to collect a stack of 4 time-gated intensity images of 160 × 160 pixels, cells were scanned at dwell times of 3 ms per pixel using the 50 μm pinhole of the CLSM and a 60× water immersion objective (Nikon, PlanApo, NA 1.2). The fluorescence lifetime (τ) per pixel was determined by fitting the 4 time-gated intensities (I(t)) per pixel, corrected for background, with a single-exponential decay function: $I(t) = I(o) \bullet e^{\frac{-t}{\tau}}$. These lifetimes were depicted in a lifetime image (Supplementary Figure). The regions with the highest fluorescence intensity were selected by an intensity-threshold and the average lifetime of the fluorescence in these pixels was determined from the lifetime histogram. No significant differences in average lifetimes were found when lifetimes of each pixel were weigthed or unweighted with their pixel-intensities, therefore we omitted this weighting factor from our calculations. For pH calculations, the pH calibration curve was used to determine the pH value corresponding to the average lifetime value. Variation in the pH was determined using the standard deviation of the average lifetime of the selected pixels of different cells. For each cell line lifetimes were measured on at least two independent experimental days. No statistically significant differences between days were found.

Membrane preparation for ATPase assays

Cells grown to confluency on 15 cm dishes were washed 3 times and scraped in homogenizing (HB) buffer (0.25 M sucrose, 3 mM imidazole, 1 mM EDTA). After pelleting, cells were frozen and thawed once and resuspended into 1 ml of HB + 25 mM KCl (HB/KCl buffer) and homogenized by passing them five times up and down through a 23 Gauge needle. A post nuclear supernatant (PNS) was obtained by centrifugation at 1000 g for 5 min. Membranes were separated from cytosol by ultracentrifugation for 1 h at 100,000 g in a Beckman tabletop centrifuge using a TLA55 rotor. The membrane pellets were resuspended into 200 μl HB/KCl buffer and further diluted to 0.5 mg total protein /ml using Bradford Reagent (Biorad, Hercules, CA) for protein determinations with bovine serum albumin as a standard.

Lipid addition from liposomes using transfer proteins

Lipids were dried and hydrated in HB/KCl buffer by incubation for 1 h at 60°C under adequate agitation. The resulting large multilamellar vesicles were disrupted by 4 freeze-thaw cycles followed by extrusion through a 0.4 μm filter. Unilamellar vesicles were obtained by extrusion through a 0.2 μm filter. For control liposomes, PC and cholesterol were mixed at a ratio of 66:34 mol%. PC/cholesterol/GlcCer liposomes were mixed at a ratio of 40:30:30 mol% respectively. Where indicated, GlcCer was exchanged for LacCer, steryl glucoside, SM or PI.

Purification of GLTP was based on (West et al., 2004). The protein was eluted in phosphate free buffer and the purity was confirmed by SDS-PAGE analysis. The

GLTP was concentrated to 5 mg/ml with a protein concentrator (Millipore, Billerica, MA). The transfer activity of purified GLTP was checked using pyrene-GlcCer as a donor system (Mattjus et al., 1999). PITPβ was purified as in (van Tiel et al., 2002). The PITPβ was concentrated to 5 mg/ml with a protein concentrator (Millipore). Transfer activity was checked using pyrene-PI as a donor system (Westerman et al., 1995).

A PNS from a confluent 15 cm dish was divided into 6 samples and pelletted by ultracentrifugation as described. Pellets were resuspended into 200 µl HB/KCl buffer at a concentration of ~0.5 mM total phospholipids (Rouser et al., 1970). PC/cholesterol liposomes or PC/cholesterol liposomes containing 30 mol% GlcCer, LacCer, steryl glucoside, SM or PI and GLTP or PITPβ at final concentrations of 0.15 mM and 0.25 mg/ml, respectively, were added and samples were incubated at 37°C. After 30 min samples were placed on ice and used in the ATPase assay at 0.5 mg total protein /ml.

ATPase assay

ATPase assays were performed on diluted membranes (0.01 mg protein) at 37°C in 25 µl buffer (0.25 M sucrose, 3 mM imidazole, 1 mM EDTA, 5 mM $MgCl_2$, 2 mM ATP, 2 mM sodium azide, 0.1 mM sodium vanadate with and without the specific V-ATPase inhibitor concanamycin A (200 nM) (Sigma)). Reactions were stopped after 30 min with 175 µl 40 mM H_2SO_4. Then, 50 µl dye solution (3 M H_2SO_4, 0.12% malachite green and 1.5 % ammonium molybdate) was added and after 20 min incubation at room temperature, the absorbance was measured at 570 nm using a Spectra Classic Microplate Reader (Tecan, Austria). For every experiment, the absorbance was measured in the same way, e.g. same volume per sample, same type of microtiter plates, same microplate reader. The specific V-ATPase activity was expressed as the difference between the absorbance of samples without and with concanamycin A, i.e. the concanamycin-sensitive ATPase activity, per 0.01 mg protein per 30 min.

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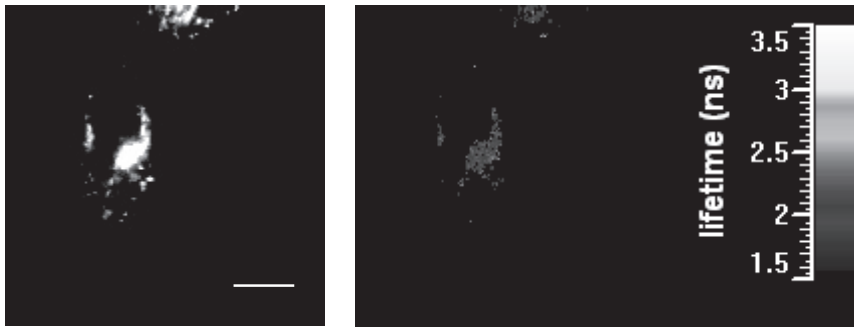

Supplementary Figure. Fluorescence lifetime imaging microscopy. An intensity threshold was applied to a confocal image (160×160 pixels) of a cell (left) to select the pixels with the highest intensity. For these pixels the average fluorescence lifetime is calculated and depicted in a lifetime image (right). In this case, cells were transfected with CD25-TGN38 and labeled with endocytosed anti-CD25-FITC. Scale bar, 5 μM. See the back of this thesis for a colored version of this figure.

Chapter 5

Summarizing discussion

The cellular lipidome of mammalian cells comprises over 1000 different lipids (van Meer, 2005), among which more than 500 different glycosphingolipids (GSLs) have been identified (Futerman and Hannun, 2004). Such complexity implies that individual glycolipids fulfill more roles in cell physiology than only providing cellular membranes with high chemical and mechanical stability (Curatolo, 1987). Knockout studies in mice have revealed the importance of these lipids in the development of multicellular organisms and in differentiation of tissues, particularly of the nervous system (**Chapter 1**). At the cellular level, GSLs serve functions in transmembrane signaling, possibly in the form of glycosignaling domains, and in cell-cell interactions (Hakomori, 2002; 2004). The fact that the GSL glucosylceramide (GlcCer), via protein sorting, is required for synthesis of the pigment melanin in melanoma cells, illustrates how a common and central glycolipid can affect a specific function. At the same time, our finding that glycolipids affect the pH in secretory organelles by activating the proton ATPase in these cells, opens the exciting possibility for a basic role for glycolipids in the physiology of all mammalian, and maybe even all eukaryotic cells.

Organization of sphingolipid synthesis

The functionality of each lipid is determined by its local concentration in time. In turn, this is determined by a balance between synthesis and hydrolysis and by transport of the lipid. It is therefore important to know exactly where lipids are synthesized and hydrolyzed, how these processes are controlled and, in addition, where and by what mechanisms they are transported. Since most enzymes of sphingolipid metabolism have been cloned, it is a challenge to find out how these enzymes are spatially organized and how their activity is controlled. Up till now, the localization of sphingolipid metabolizing enzymes has been largely addressed by measuring the activity in isolated subcellular fractions or by fluorescence microscopy. However, the resolution of such techniques is limited. As described in **Chapter 2**, we used a morphological approach to identify the localization of the first enzymes involved in sphingolipid synthesis in the Golgi in HeLa cells. Quantitative immunoelectron microscopy revealed that synthesis of GlcCer starts earlier in the Golgi than sphingomyelin (SM) synthesis, but that both synthases are present in the *trans* Golgi. What are the possible implications of the differential distribution of GlcCer synthase (CGlcT) and SM synthase (SMS1) in the Golgi? Maybe ceramides transported from the ER to the *cis* Golgi via vesicular transport are being converted to GlcCer. CERT, a cytoplasmic ceramide transporter, delivers ceramides to the *trans* Golgi for SM synthesis (Hanada et al., 2003). Knocking-down CERT resulted in decreased SM, but the effect on GlcCer synthesis was more complicated. GlcCer production from serine was reduced whereas the incorporation of sphingosine was unchanged. This may imply that exogenous sphingosine enters a spatially segregated ceramide pool that preferentially enters the *cis* Golgi by a CERT-independent pathway, presumably vesicular transport. A possible role for CGlcT in the *trans* Golgi might be to complete

the transport block for ceramides: SMS1 on the lumenal side and CGlcT on the cytosolic side prevent mixing of the metabolic pool of ceramide with the signaling pool of ceramide at the cell surface. We also found that LacCer synthesis located to the lumen of the *trans* Golgi cisternae, similar to SM synthesis. Sphingolipids and in particular glycosphingolipids have the propensity to cluster in an environment of other lipids (Brown and London, 2000). The formation of these lipid rafts is thought to be an essential feature of the sorting machinery of the Golgi (Simons and van Meer, 1988; Sharma et al., 2003). The localization of LacCer synthase and SMS1 may form the basis for raft biogenesis in the *trans* Golgi.

GM95 cells as a model to study glycosphingolipid function

Although glycosphingolipids are indispensable for development and survival of the organism, individual mouse melanoma cells without glycolipids by lack of CGlcT survive (Ichikawa et al., 1994). Our group did find a specific phenotype in these GM95 cells: GM95 cells lack melanosomal organelles containing melanin pigment, and display a transport block for the rate-limiting enzyme in pigmentation, tyrosinase, in the TGN (Sprong et al., 2001) and perinuclear endosomes (**Chapter 3**). Transfection with the CGlcT restored sorting of the enzymes and pigmentation. In order to find out which glycosphingolipid is involved, we used an RNAi approach. Wild type melanoma cells (MEB4 cells) with a knock-down of CGlcT or CERT were white due to missorting of melanosomal proteins, thereby mimicking GM95 cells. However, a knockdown of LacCer synthase had no effect on melanosomal protein sorting and pigmentation. This strongly indicates that GlcCer is the lipid required for protein transport to the melanosome and pigmentation. GlcCer was necessary and sufficient to fully restore melanosome biogenesis.

Sorting of melanosomal from lysosomal proteins

Melanocytes transport their melanosomal enzymes from the TGN to the melanosomes via an intracellular route that depends on the adaptor protein AP-3: the absence of functional AP-3 results in a pigmentation defect (Theos et al., 2005). Both tyrosinase and TRP-1 contain a dileucine-based motif in their cytosolic domain, which has been shown in vitro to be bound by AP-3 (Honing et al., 1998). In addition, in mocha fibroblasts defective in AP-3 but not in wild-type fibroblasts, ectopically expressed TRP-1 was missorted via the plasma membrane, indicating that its sorting depends on AP-3 (**Chapter 3**). This seems in contrast with the findings by (Huizing et al., 2001). However, these authors showed that TRP-1 still reaches peripheral organelles but did not study whether or not this transport was direct or via the cell surface. In order to nail down the molecular mechanism of the role of GlcCer in protein sorting to the melanosome, we investigated the role of AP-3 in this sorting process. Thereby, we set out to study the transport of a different set of AP-3 dependent proteins, that are destined to the lysosome in melanoma cells. In wild-type melanoma cells the lysosomal proteins LAMP-1 and -2 were misrouted via the

plasma membrane, in contrast to the melanosomal proteins TRP-1 and tyrosinase which followed an intracellular route. This situation was reversed in glycolipid-deficient GM95 cells, suggesting a competition for AP-3 between these two classes of proteins. Experiments using chimeras showed that TRP-1 and tyrosinase, in addition to their di-leucine AP-3 binding motif in the cytosolic tail, contain dominant sorting information for the melanosomes in their lumenal domain that requires GlcCer.

What is the underlying molecular mechanism sorting melanosomal from lysosomal proteins? In the end of chapter 3 we came up with a model in which AP-3 prefers binding melanosomal proteins over lysosomal proteins when GlcCer is present, whereas AP-3 prefers lysosomal proteins over melanosomal proteins in the absence of GlcCer. How could this work? One possibility is that binding of AP-3 to the cytosolic tails of these proteins depends on oligomerization of the proteins, in line with recent findings on the adaptor protein AP-2 (Grass et al., 2004). Such oligomerization may depend on glycosphingolipid-enriched microdomains or lectins in the Golgi lumen. Our later finding that GSLs affect the lumenal pH of the secretory and endocytotic organelles in melanoma cells, allows us to generate a more specific hypothesis (discussed below).

A role for glycosphingolipids in lumenal pH

In our search for the lumenal determinant in the sorting of the melanosomal proteins tyrosinase and TRP-1 that was dependent on GlcCer, we decided to test the lumenal pH (**Chapter 4**), which has been suggested to be an important parameter in melanogenesis (Ancans et al., 2001; Brilliant, 2001). Indeed, glycolipid-deficient melanoma cells had a different lumenal pH. The pH was more than one unit higher in the TGN and lysosomes compared to the parental cell line. In GM95 cells retransfected with CGlcT the organellar pH was similar to that in wild-type cells. This pH is regulated by a balance between the rates of intralumenal proton pumping, proton leak and counterion conductance (Weisz, 2003). The low pH is actively generated by the vacuolar proton pump, the V-ATPase (Schoonderwoert and Martens, 2001), and its activity may be regulated by lipids (Yamaguchi and Kasamo, 2001, 2002; Chung et al., 2003). However, the efflux of protons via leakage across the membrane can significantly contribute to the steady-state pH of the organelle. The extent of proton leakage is determined by membrane composition, e.g. the presence of cholesterol was shown to inhibit leakage by one third (Haines, 2001). Glycolipid-deficient GM95 cells might have enhanced proton leakage, as glycosphingolipids together with cholesterol increase packing of the membrane (Simons and van Meer, 1988). Finally, as protons are pumped inwards, an electrochemical gradient is built up that might limit acidification. However, anions can passively move in and cations can move out via channels. The melanocyte p protein, mutations in which are the most common cause of oculocutaneous albinism, has been proposed to be such an anion channel (Brilliant, 2001). This would be in line with a function of an unusually low pH in pigmentation, and GlcCer might modulate the presence or

activity of the p protein.

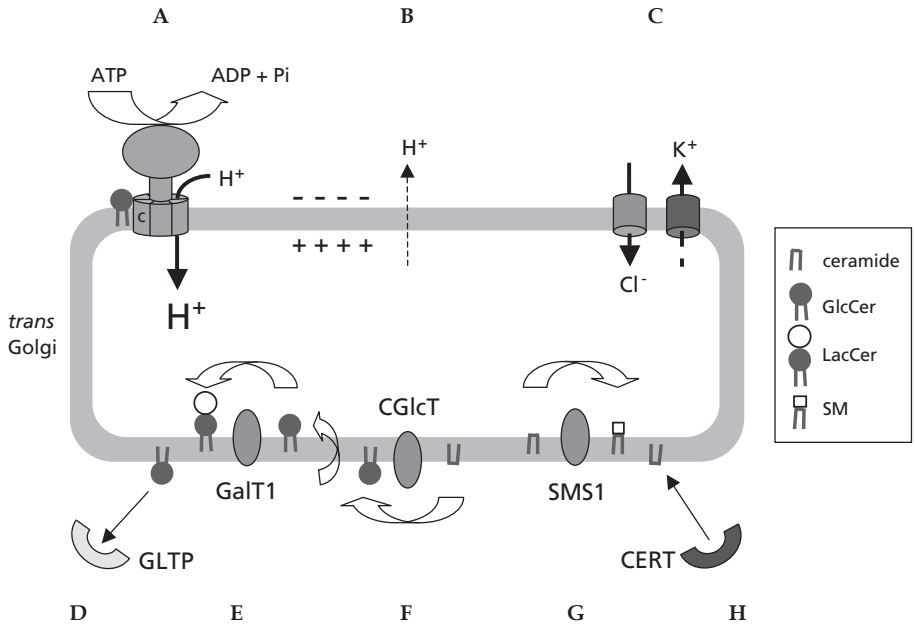

Figure 1. Model for regulation of the V-ATPase activity. The steady-state pH of the secretory organelle is maintained by active proton transport into the lumen (**A**), proton leak into the cytosol (**B**) and passive counterion conductance, mainly chloride and potassium ions, via channels (**C**). The low pH is actively generated by the V-ATPase. Upon ATP hydrolysis, protons are pumped into the lumen, thereby lowering the lumenal pH and generating a transmembrane potential, the magnitude of which is inversely proportional to the counterion conductance. The pump is activated by binding of glucosylceramide to the c-subunits of the V-ATPase at the cytosolic side of the membrane. Because of the continuous removal of the glycolipid from the cytosolic leaflet of the Golgi either by glycolipid transfer protein (GLTP; **D**) or by translocation across the membrane towards the lumenal leaflet where it is converted to lactosylceramide (**E**), regulation of the V-ATPase activity is achieved by the rate of glucosylceramide synthesis (**F**). This is determined by the relative activity of the glucosyltransferase (CGlcT) and sphingomyelin synthase (SMS1; **G**) and by the concentration of ceramide, delivered by CERT (**H**). In turn, the ceramide concentration is governed by the activities of the CGlcT and SMS1.

A role for glycosphingolipids in V-ATPase activity

Our in vitro activity assays immediately showed that whereas GM95 cells contained the same level of V-ATPase as MEB4 cells, its activity was only half (Chapter 4). The V-ATPase is composed of a peripheral V_1 domain, responsible for ATP hydrolysis, and the integral V_0 domain, mediating proton translocation across the bilayer. Their association is essential for proton translocation, and cycling between assembly and disassembly is an important regulatory mechanism. In yeast, pump assembly seems to require sphingolipids (Chung et al., 2003). Second, there are multiple isoforms of

some V-ATPase subunits, which are thought to play a crucial role in the targeting of the pump to distinct compartments. Third, also accessory proteins, like Ac45 (Supek et al., 1994) and AP2 (Myers and Forgac, 1993), have been shown to play a role in the targeting of the pump to a subset of compartments.

It is not likely that glycosphingolipids directly interfered with the assembly or targeting of the mouse V-ATPase. Isolated membranes of MEB4 and GM95 cells contained the same level of a subunit of the peripheral V_1 domain, which indicated proper assembly of the ATPase in the GM95 cells. A role for glycolipids in targeting the V-ATPase is not likely, first because the pH defect in GM95 cells was observed in both the TGN and the lysosomes. Second, because it is known that two different isoforms exist of the a-subunit of the V-ATPase in the Golgi and the vacuole in yeast (Manolson et al., 1992; 1994), the general pH defect suggests that activation of the V-ATPase by glycolipids is independent of the specific a-subunit present. Third, exogenously added glycolipids activated the V-ATPase in isolated membranes without cytosol, indicating that glycolipids do not activate the V-ATPase by transport of the pump to a specific organelle.

In **Chapter 4**, we discovered that glycolipids competed with the binding of a specific inhibitor, concanamycin A, to the V-ATPase. Concanamycin A binds to the c-subunits of the V-ATPase (Bowman et al., 2004; Whyteside et al., 2005), which are part of the V_0 domain. This suggests the exciting possibility that glycolipids (1) bind near or at these subunits as a co-factor or (2) influence the conformation of these subunits by changing the lipid environment as a solvent. As the c-subunits form part of the rotor of the pump, the glycolipid may facilitate the rotation. It has been shown that partitioning of the V-ATPase in microdomains increased the activity (Yoshinaka et al., 2004). Since the c-subunits are part of the integral membrane V_0 domain, glycolipids might stimulate the V-ATPase to partition into lipid microdomains, thereby increasing proton pumping. Otherwise, the glycolipid may change the lipid environment of the pump, e.g. via lateral pressure, thereby affecting its functionality. In the same way, different phospholipids altered the membrane environment of the plant plasma membrane proton pump 6-7 fold (Kasamo, 2003). The activity of the V-ATPase in isolated GM95 membranes was directly stimulated by exogenously added GlcCer, LacCer and steryl glucoside but not by sphingomyelin or phosphatidylinositol. This indicates that the glucose headgroup may be the important determinant. Glucose has many hydroxyl groups able to form hydrogen bonds with amino acids or phospholipids (Boggs, 1987). In order to get more insight in the molecular mechanism, the question is which natural glycosphingolipid affects the ATPase activity and at what side of the membrane?

How the Golgi ceramide pool may regulate lumenal pH using GlcCer as an activator

GlcCer, and not a complex glycosphingolipid, is required for melanosomal protein sorting and pigmentation (Chapter 3), suggesting that GlcCer itself stimulates the V-ATPase. We can presently not exclude that a metabolite of GlcCer, like

glucosylsphingosine (GlcSph), which lacks the fatty acyl chain and which is also a potential product of the glucosyltransferase, is a physiological activator of the V-ATPase. Exogenously added GlcSph restored melanosomal protein transport and pigmentation in the GM95 cells (Sprong et al., 2001), and stimulated the V-ATPase in our in vitro assay (not shown). A determination of the local concentrations of GlcCer and GlcSph and their K_m values for the activation is needed to resolve this issue. On the one hand, this will require high resolution analysis in terms of location and lipid composition (Wenk, 2005), on the other hand the enzymology will have to be performed in a chemically defined reconstituted model membrane system (Adachi et al., 1990).

Exogenously added GlcCer did not restore pigmentation, although it became complex glycosylated and thus was able to reach the lumen of the Golgi. In contrast, exogenous GlcSph which was partially acylated to GlcCer using acyl-CoA on the cytosolic surface (Farrer and Dawson, 1990) and yielded LacCer in the Golgi, did restore pigmentation. In addition, the activator lipid(s) competed with concanamycin A for the binding site on the proteolipid c-subunits (Chapter 4) on the cytoplasmic surface of the membrane (Bowman et al., 2004). GlcCer is synthesized on the cytosolic surface of the Golgi (Chapter 2). Therefore, the GlcCer concentration on the cytosolic surface of the Golgi (and recycling endosomes) appears to be the relevant parameter for the V-ATPase stimulation (and not the total GlcCer content of cells, which is very different for the various cell types). Interestingly, GlcCer is continuously removed from the cytosolic surface towards the lumenal leaflet of the Golgi where it is converted to LacCer, by a mechanism which is as yet unknown, but most likely involves a GlcCer translocator. In addition, GlcCer is expected to leave the cytosolic surface of the Golgi by means of the glycolipid transfer protein (Rao et al., 2004) to equilibrate with other cytosolic surfaces in the cell (Warnock et al., 1994). This removal turns GlcCer into a regulatory lipid, whereby its concentration at the V-ATPase binding site, and thereby the degree of activation of the ATPase, is determined by the balance of its synthesis and its removal (Figure 1).

It is presently unclear whether the removal of GlcCer from the cytosolic surface is regulated. In contrast, the rate of GlcCer synthesis depends on the activity of the glucosyltransferase (CGlcT) and on the concentration of its substrate ceramide, both of which are regulated: The activity of CGlcT is regulated, both at the transcriptional (Watanabe et al., 1998; Uchida et al., 2004) and post-translational level (Boldin and Futerman, 2000), on a time-scale of days down to minutes. In addition, cellular ceramide levels are highly regulated, and ceramide is a signaling molecule for apoptosis. Once again it is the ceramide concentration at the CGlcT that is important. Since the discovery of CERT (Hanada et al., 2003), a cytosolic protein that transfers ceramide from the ER to the Golgi, there is accumulating evidence that this protein is a critical determinant in regulation of local ceramide levels in time. Because all mammalian cells on the one hand synthesize GlcCer on the cytosolic surface of the Golgi and remove it by transmembrane translocation, and on the other hand

possess the V-ATPase, this mechanism of pH regulation is probably universal. Its importance may lie in the fact that in this way the concentration of a central lipid metabolite, ceramide, is coupled to a general physiological parameter, the lumenal pH in the secretory and endocytotic pathways.

A role for lumenal pH in protein sorting?

A controled lumenal pH is the basis of many cellular processes like protein sorting, proteolytic processing, protein glycosylation in the Golgi and hydrolysis in lysosomes. How could the lumenal pH in the TGN and endosomes be involved in sorting of tyrosinase and TRP-1 to the melanosome? Since there is ample evidence showing that a low lumenal pH is needed for oligomerization of prohormones and thereby partitioning into budding vesicles (Thiele and Huttner, 1998; Bell-Parikh et al., 2001), the low pH in the TGN of melanoma cells might induce clustering of specifically melanosomal proteins which may then result in the binding of AP-3. Although we have not been able to show a direct link between pH and oligomerization of melanosomal proteins, we now have indications that both protein glycosylation and the proteolytic processing of another melanosomal protein, Pmel17, are affected in the glycolipid-deficient GM95 cells. Clearly, the role of the lumenal pH in melanogenesis in health and in the various forms of oculocutaneous albinism is pleiotropic, and needs further investigation.

Concluding remarks

The organization of the synthesis of sphingomyelin and the simple glycosphingolipids in the Golgi appears to be highly important not only for creating sphingolipid/cholesterol rafts in the late Golgi but also for regulating numerous protein glycosylation, processing and sorting steps in the Golgi lumen via an intricate mechanism of pH regulation. The universal character of this regulatory system predicts that it represents one basic link between very different physiological parameters, sphingolipid homeostasis and pH regulation. This discovery may have an impact on developing cures for diseases of sphingolipid metabolism, like Gaucher and Farber disease, where the cellular accumulations of GlcCer and ceramide may actually induce pathogenesis via an effect on pH. On the other hand, the discovery of the role of pH in melanogenesis may contribute to a better understanding of various forms of oculocutaneous albinism for which the disease mechanism had remained unclear.

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Samenvatting

Promoveren

Aangezien de meeste mensen in mijn omgeving niet alleen willen weten wat ik onderzocht heb de afgelopen vierenhalf jaar, maar ook willen weten wat het nu is om onderzoek te doen en hoe ik dit heb ervaren, wil ik hier graag eerst wat over vertellen. Na mijn studie biologie ben ik, onder begeleiding van mijn promotor en daarbij halverwege mijn co-promotor, begonnen aan een promotieonderzoek uitgevoerd in de vakgroep "membraan enzymologie" aan de faculteit Scheikunde te Utrecht. Het onderzoek begon met de vraag hoe glycolipiden betrokken zijn bij pigmentvorming met als model de huidcellen van muizen: cellen met glycolipiden waren zwart en cellen zonder glycolipiden wit. Samen met een postdoc uit Frankrijk (een postdoc is iemand die na zijn of haar promotie nog weer een onderzoeksproject doet), hebben we ons twee jaar met deze vraag beziggehouden zonder direct het mechanisme op te kunnen helderen. Pas na die tijd kwam het onderzoek tot bloei. Door de zuurgraad (pH) in de cellen te meten, kwamen we erachter dat glycolipiden via de pH een rol spelen in pigmentatie. Hierbij wil ik aangeven dat de stappen in het onderzoek niet altijd logisch zijn. Je werkt volgens een model (hypothese), maar doordat vele brokjes informatie missen, je werkt als het ware met een “black box”, is dit model in de meeste gevallen misleidend. Bepaalde aannames blijken dan na verder onderzoek niet meer voor de hand te liggen. Aan de andere kant, door risico’s te nemen, zoals het meten van de pH, kun je zomaar stuiten op een nieuwe, mogelijk basale rol van glycolipiden in (pigment-) cellen.

Kortom, fundamenteel onderzoek doen kan soms erg frustrerend zijn, maar ook heel spannend en leuk. Ik vond het een mooie en leerzame ervaring. Hieronder volgt een samenvatting van het vakgebied celbiologie en van het onderzoek.

Celbiologie

Om meer inzicht te krijgen in hoe het fout kan gaan bij verschillende ziektes, is het belangrijk om het functioneren van cellen te begrijpen, de taak van celbiologen. Elk levend organisme bestaat uit tenminste één cel. Dierlijke cellen, inclusief die van de mens, hebben een grootte van ongeveer één honderdste van een millimeter. Ik vind het fascinerend dat elke cel een ander uiterlijk heeft, maar allemaal dezelfde genetische informatie (DNA) in zich draagt. Het DNA codeert voor eiwitten of enzymen die specifieke reacties in de cel uitvoeren, bijvoorbeeld het aanmaken van pigment, maar ook voor eiwitten die belangrijk zijn voor de structuur van de cel.

Door nu bepaalde eiwitten wel en andere eiwitten niet tot expressie te laten komen, kunnen cellen zich specialiseren, differentiatie genoemd, en samen een weefsel vormen.

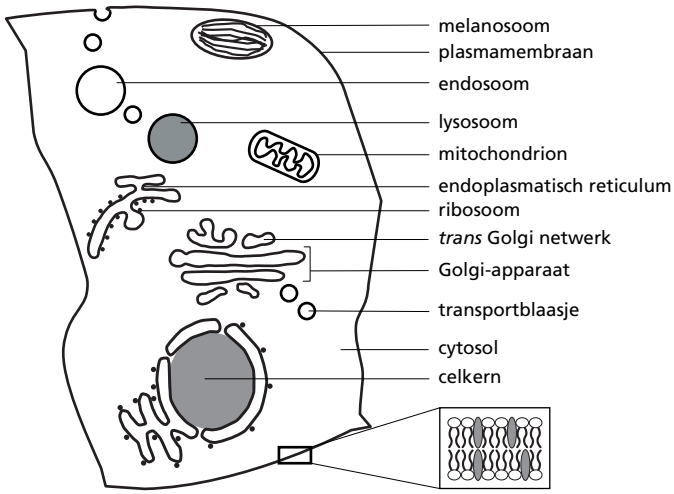
Cellen worden omgeven door een selectief doorlatende membraan van vetten (lipiden), de celmembraan. Deze is cruciaal voor het leven van de cel. De membraan omsluit de "celsoep", het cytosol genaamd, waarin allerlei organellen verankerd zijn. Elk van deze organellen, ook weer omgeven door een vetlaag, dient een specifieke functie in de cel. De belangrijkste organellen staan weergegeven in **Figuur 1**. Zo functioneert het lysosoom bijvoorbeeld als het afvalvat van de cel. Hierin worden vetten en eiwitten van de cel zelf of van buitenaf van bijvoorbeeld bacteriën afgebroken. Bij lysosomale stapelingsziekten vindt er een ophoping in dit organel plaats van bepaalde vetten die niet afgebroken kunnen worden, doordat lysosomale eiwitten betrokken bij deze afbraak defect zijn.

Eiwitten en lipiden functioneren alleen optimaal als ze op de goede plaats, zowel binnen als buiten de cel, terecht komen. Daarvoor is sortering en transport nodig. De meeste eiwitten hebben een bepaalde herkenningscode om op de goede plaats terecht te komen. Lipiden echter hebben deze code niet, maar kunnen wel met elkaar en met eiwitten een interactie aangaan. Omdat bepaalde lipiden, de sfingolipiden samen met cholesterol, kunnen clusteren in een omgeving van andere lipiden, wordt verondersteld dat dit cluster (raft) onderdeel uitmaakt van het sorteringsmechanisme voor lipiden en eiwitten. Echter, er is nog weinig bekend van het sorteringsmechanisme voor met name lipiden. Na sortering vindt er transport plaats, in de meeste gevallen via het afsnoeren van blaasjes van de membraan. Deze transportblaasjes worden vervolgens door sorteringseiwitten naar het juiste organel gestuurd.

Het onderzoek

In de meeste cellen bestaat slechts een klein gedeelte van het membraanoppervlak uit glycolipiden, lipiden met één of meerdere suikers, waar ze fungeren als versteviging en bescherming van buitenaf. Echter, dierlijke cellen bevatten wel 500 verschillende soorten glycolipiden. Dit betekent waarschijnlijk dat glycolipiden meer functies vervullen dan alleen maar als bouwsteen zijn van membranen. Van een aantal van deze lipiden is bekend dat ze bepalend zijn voor de verschillende bloedgroepen. Anderen worden herkend en gebonden door indringers als virussen en bacteriën.

Het doel van het onderzoek beschreven in dit proefschrift was om de functie van glycolipiden in cellen te bestuderen. In het algemeen geldt dat de functie van lipiden afhangt van de lokale concentratie van het lipide. Deze concentratie is weer afhankelijk van de activiteit van enzymen die de lipiden maken, afbreken en transporteren. In **hoofdstuk 2** hebben we gekeken naar de lokalizatie van enzymen betrokken bij de aanmaak van simpele glycolipiden en sfingolipiden, in HeLa cellen.



Figuur 1. Pigmentcel met organellen. De celkern is de opslagplaats van al het erfelijk DNA. Hier vindt de eerste stap voor de synthese van eiwitten plaats: de vertaling van DNA naar RNA. Het RNA wordt getransporteerd naar het cytosol, waar het vervolgens op ribosomen aan de buitenkant van het endoplasmatisch reticulum (ER) vertaald wordt in een slinger van aminozuren. De meeste van deze aminozuurketens of polypeptideketens worden in het ER gevouwen tot eiwitten. Hier worden ook de meeste lipiden aangemaakt. De eiwitten en lipiden kunnen in het Golgi-apparaat aangepast worden door het toevoegen van suikers. In het *trans* Golgi netwerk worden de eiwitten en lipiden van elkaar gescheiden, gesorteerd, en naar specifieke plaatsen gestuurd, bijvoorbeeld naar melanosomen in pigmentcellen. In dit organel vindt de aanmaak van pigment plaats. Endosomen en lysosomen zijn organellen die behoren tot de zogenaamde endocytische route verantwoordelijk voor opname van moleculen van buiten de cel of van de plasmamembraan. Mitochondriën zorgen voor de energiehuishouding van de cel en peroxisomen zorgen net als lysosomen voor de afbraak van moleculen.

We hebben gevonden dat de synthese van glucosylceramide eerder plaatsvindt in het Golgi dan de synthese van sfingomyeline, maar dat er overlap is aan de *trans* zijde van het Golgi. Dit zou mogelijk een rol kunnen spelen in het handhaven van twee verschillende pools van het substraat ceramide: een pool voor biosynthese in het Golgi en een pool voor signalering aan de plasmamembraan. Verder hebben we gevonden dat sfingomyeline en lactosylceramide synthese beide plaatsvinden aan de *trans* zijde van het Golgi. Dit zou de basis kunnen vormen voor het onstaan van rafts.

Een directe methode om de functie van glycolipiden te bestuderen is om knock-out muizen te "maken" zonder glycolipiden en te kijken naar het effect. Muizen zonder glycolipiden gaan dood als embryo na 7 dagen, wat laat zien dat glycolipiden van vitaal belang zijn voor de embryonale ontwikkeling. Echter, geïsoleerde huidcellen van deze muizen overleven, maar hebben een pigmentatiedefect door het missen van glycolipiden. In **hoofdstuk 3** hebben we gevonden dat het simpele glycolipide glucosylceramide voldoende en van essentieel belang is niet alleen voor pigmentie, maar ook voor het vormen van melanosomen, de organellen waar pigment gemaakt wordt.

Hoe is glucosylceramide nu betrokken bij pigmentvorming? Het was al bekend

dat glycolipiden nodig zijn voor de sortering en het transport van twee enzymen, tyrosinase en TRP-1, naar melanosomen, wat nodig is voor pigmentatie. Wij hebben gevonden dat het lumenale deel van deze eiwitten essentiële informatie bevat voor deze sortering (hoofdstuk 3). Toen we vervolgens op zoek gingen naar de link tussen glucosylceramide en sortering van de eiwitten via het lumenale deel, vonden we dat de zuurgraad of pH de bepalende factor was; cellen met en zonder glycolipiden hadden een verschillende pH in het *trans* Golgi netwerk en lysosomen (**hoofdstuk 4 en kader 1**). Ook ontdekten we verschillen in activiteit van een membraanpompje in deze organellen, de V-type ATPase, betrokkken bij de verzuring van organellen. We vonden dat glycolipiden de pomp konden activeren mogelijk door directe binding aan de rotor van de pomp. Het model aan het eind van het hoofdstuk beschrijft hoe sfingolipide metabolisme de pH in organellen van de cel zou kunnen reguleren.

Samenvattend kan gesteld worden dat het beschreven onderzoek heeft geleid tot vele nieuwe inzichten in de functies van glycolipiden en in het bijzonder glucosylceramide in de cel. Vooral de vinding dat glycolipiden de pH in secretie-organellen beïnvloeden door activatie van de protonenpomp in pigmentcellen, opent de mogelijkheid voor een basale rol van glycolipiden in de fysiologie van alle dierlijke cellen. Ongetwijfeld zullen deze en toekomstige vindingen bijdragen aan een beter begrip van pigmentziekten, maar ook van ziekten waarbij de pH in de cel verstoord is.

Kader 1. Hoe meet je de pH in cellen?

Het is mogelijk om de pH op specifieke plaatsen in de cel te meten. Een methode waar wij gebruik van gemaakt hebben is de combinatie van het ophopen van een fluorescente probe in de cel, waarvan de fluorescente levensduur afhankelijk is van de pH, samen met fluorescente levensduurmetingen. De probe is een eiwit of antilichaam tegen een eiwit met een fluorescent, pH gevoelig gedeelte . Doordat elk organel en het cytosol specifieke eiwitten hebben, kan in principe overal in de cel de probe opgehoopt worden. Om bijvoorbeeld de probe in lysosomen te krijgen, hebben we de probe gekoppeld aan een antilichaam tegen een lysosomaal eiwit. Doordat dit eiwit recycled tussen lysosomen en het celoppervlak, kan het eiwit de probe binden aan het oppervlak en vervolgens meenemen naar lysosomen. De volgende stap is om de fluorescente levensduur, wat hetzelfde is als de tijd van verval van fluorescentie, van de probe te meten. Hiervoor worden de cellen met de probe aangestraald met gepulsd laserlicht van een bepaalde golflengte in de orde van picoseconden, zodat de probe in een aangeslagen toestand komt. De tijd die de probe erover doet om weer in de grondtoestand te komen is afhankelijk van de pH. Door de metingen te ijken met verschillende pH buffers kan de levensduur gekoppeld worden aan de pH en kan uiteindelijk de pH bepaald worden.

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Jasja

Curriculum vitae

Jasja Wolthoorn werd geboren op 12 juli 1977 te Groningen. Na het behalen van haar Atheneum diploma in 1995 aan het Nienoord College in Leek, begon zij haar studie Biologie aan de Rijksuniversiteit Groningen. Zij deed een onderzoeksstage bij de onderzoeksgroep Moleculaire Genetica van Prof. Dr. O. Kuipers, onder begeleiding van Drs. J. Jongbloed. Verder deed ze stages bij DSM, Geleen, onder begeleiding van Dhr. T. Sonke, en in Philadelphia aan de University of Pennsylvania, Dept. of Pathology and Laboratory Medicine, onder begeleiding van Dr. M.S. Marks. Na het behalen van haar doctoraalexamen, begon ze in 2001 haar promotieonderzoek bij de vakgroep Membraan Enzymologie aan de Universiteit Utrecht onder begeleiding van Prof. Dr. G. van Meer en Dr. H. Sprong.