THE ROYAL SOCIETY

Published online 17 March 2003

The fate and function of glycosphingolipid glucosylceramide

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In higher eukaryotes, glucosylceramide is the simplest member and precursor of a fascinating class of membrane lipids, the glycosphingolipids. These lipids display an astounding variation in their carbohydrate head groups, suggesting that glycosphingolipids serve specialized functions in recognition processes. It is now realized that they are organized in signalling domains on the cell surface. They are of vital importance as, in their absence, embryonal development is inhibited at an early stage. Remarkably, individual cells can live without glycolipids, perhaps because their survival does not depend on glycosphingolipid-mediated signalling mechanisms. Still, these cells suffer from defects in intracellular membrane transport. Various membrane proteins do not reach their intracellular destination, and, indeed, some intracellular organelles do not properly differentiate to their mature stage. The fact that glycosphingolipids are required for cellular differentiation suggests that there are human diseases resulting from defects in glycosphingolipid synthesis. In addition, the same cellular differentiation processes may be affected by defects in the degradation of glycosphingolipids. At the cellular level, the pathology of glycosphingolipid storage diseases is not completely understood. Cell biological studies on the intracellular fate and function of glycosphingolipids may open new ways to understand and defeat not only lipid storage diseases, but perhaps other diseases that have not been connected to glycosphingolipids so far.

Keywords: glycolipids; sphingolipids; lipid rafts; translocators; flippases

1. GLYCOSPHINGOLIPIDS: A SPECIAL CLASS OF CELL MEMBRANE LIPIDS

Higher eukaryotes contain a class of lipids in their cellular membranes that differs from the regular membrane phospholipids in the remarkable variation in its head group structures. These are the glycosphingolipids. They are anchored to the membrane by a two-tailed lipid anchor, ceramide, to which is attached a mono-, di- or polysaccharide. Most glycosphingolipids contain lactosylceramide, Gal(β1-4)Glc(β1-1)ceramide, as their basic disaccharide (Sandhoff & Kolter 2003). A number of other carbohydrates can be attached to the galactose by various different glycosidic linkages. Some specialized membrane surfaces are completely covered by glycosphingolipids. This has been reported for the outer, non-cytoplasmic surface of the apical plasma membrane domain of epithelial cells in the intestine and in the urinary bladder (see Simons & Van Meer 1988). Myelin, the specialized plasma membrane of oligodendrocytes and Schwann cells, contains a high concentration of galactosylceramide and its sulphated form, sulphatide (Horrocks 1967). Other membranes contain more modest concentrations of glycosphingolipids. They appear to be concentrated in the plasma membrane and in membranes of the endocytotic recycling system (Van Genderen *et al.* 1991), and are generally thought to be limited to the non-cytoplasmic leaflet of the lipid bilayer. The enormous range of head group structures and their occurrence on the cell surface already suggested that they function in specific recognition between cells and in subsequent signalling towards the cell interior (Hakomori 2002). A breakthrough in our understanding of the molecular mechanism behind this signalling came when it was realized that the ceramide lipid backbone provides these lipids with special physical properties. In the presence of cholesterol they can segregate from the bulk of the membrane phospholipids, which are based on a diacylglycerol backbone (Simons & Van Meer 1988). The glycosphingolipids aggregate into a more ordered, but still fluid 'liquid-ordered' domain (Brown & London 2000; Harder 2003). With the glycosphingolipids, also the ceramide-based phospholipid sphingomyelin partitions into these domains. They can cover a large fraction of the cell surface. By mechanisms that are largely unknown, membrane proteins involved in signalling collect in the domains (or 'rafts') and, because this partitioning depends on the signalling state of the protein, rafts are essential in bringing signalling proteins together (e.g. Pierce 2002). An exciting finding is that a number of different types of lipid raft may exist within a single membrane (Schnitzer *et al.* 1995; Gómez-Moutón *et al.* 2001). One of the greatest unknowns concerning these rafts is the lipid and protein organization on the cytosolic surface (Van Meer 2002). In model membranes sphingolipid domains in one leaflet are able to recognize domains in the opposed bilayer leaflet (Dietrich *et al.* 2001). Although caveolae are believed to consist of a glycolipid/cholesterol-enriched domain in the

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One contribution of 17 to a Discussion Meeting Issue 'Glycolipids in cell biology and medicine'.

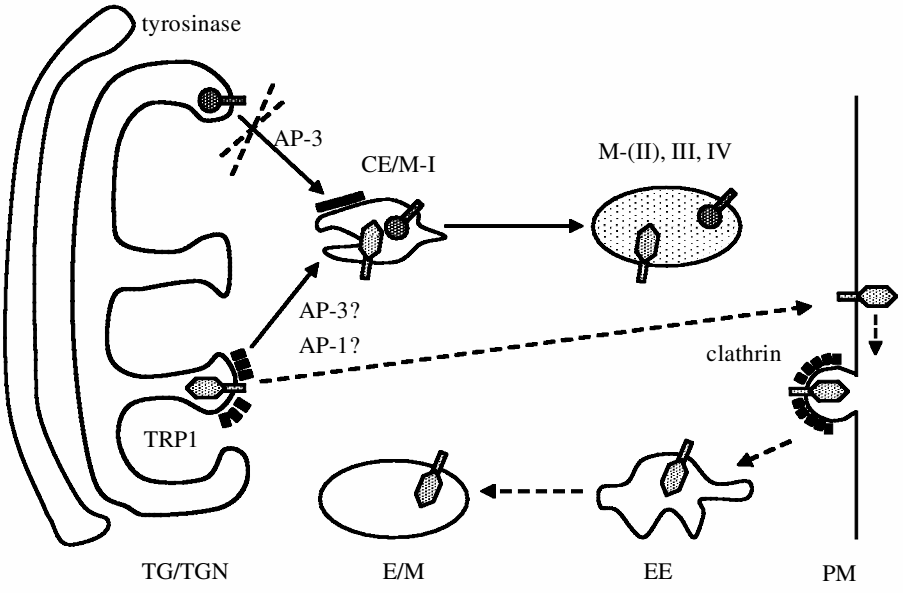

Figure 1. Trafficking defects of melanosomal proteins in glycolipid-deficient melanocytes. In normal melanocytes, tyrosinase and TRP1 are thought to be delivered directly from the TGN to preformed melanosomes via a direct pathway (black arrows) (see review by Raposo & Marks (2002)). Only a small proportion of these proteins travels via the cell surface. The adaptor protein complex AP-3 seems to be involved in tyrosinase sorting. The adaptor protein complexes AP-1 or AP-3 may be involved in TRP1 sorting. In glycolipid-deficient cells, tyrosinase does not reach the melanosomes, and is retained in the Golgi area (dashed cross). TRP1 still reaches the melanosomes, but travels via the cell surface (dashed arrows; Sprong *et al.* (2001)). The difference in the length of the transmembrane domain of tyrosinase and TRP1 could explain the different pathways followed by the two proteins. Abbreviations: AP, adaptor protein complex; CE, coated endosome; EE, early endosome; E/M, endosome/melanosome; M-I, -II, III, IV, stage I, II, III, IV melanosome.

external leaflet of the plasma membrane that is coupled to another cholesterol-enriched lipid domain on the cytosolic side, this has not been demonstrated directly (see Van Meer 2002).

In a recent paper (Sprong *et al.* 2001), the groups of P. van der Sluijs and ourselves demonstrated that glycosphingolipids are required in pigment cells for the transportation of newly synthesized enzymes from the Golgi complex to the melanosomes, where they are required for the synthesis of the melanin pigment. Studies on the intracellular sorting of glycosphingolipids had initially suggested that the sorting of membrane proteins to the apical membrane of epithelial cells was mediated by their partitioning into glycolipid microdomains on the lumenal aspect of the Golgi membrane (Simons & Van Meer 1988). However, the glycosphingolipid-dependent sorting mechanism in pigment cells does not seem to involve lumenal glycosphingolipid rafts. By contrast, they suggest the possibility that the simple glycosphingolipid GlcCer functions on the cytosolic surface of, most likely, the Golgi complex in the budding of the transport vesicles that are destined for the melanosomes.

All complex glycosphingolipids are assembled by the stepwise addition of sugars to GlcCer in the lumen of the Golgi (Sandhoff & Kolter 2003). However, biochemical studies on the synthesis of GlcCer (Coste *et al.* 1986; Futerman & Pagano 1991; Trinchera *et al.* 1991; Jeckel *et al.* 1992) and, later, the cloning of the glucosyltransferase (Ichikawa *et al.* 1996) demonstrated that this glycolipid is synthesized on the cytosolic surface of the Golgi. Thus, GlcCer must translocate across the Golgi membrane to gain access to the lactosylceramide synthase (Lannert *et al.* 1994). Generally, it has been observed that this is mediated by an energy-independent translocase. However, we have found that GlcCer (analogues) can be translocated across the plasma membrane by one or more ABC transporters (Van Helvoort *et al.* 1996; Raggers *et al.* 1999), which suggests that this lipid may serve a function on the cytosolic surface of the organelles between the sites of synthesis (Golgi) and translocation (plasma membrane).

2. AN ESSENTIAL FUNCTION FOR GLYCOSPHINGOLIPIDS IN PIGMENTATION

Mice with null-alleles for the glucosyltransferase were not viable (Yamashita *et al.* 1999; Sandhoff & Kolter 2003), but a cell mutant unable to synthesize GlcCer by lack of glucosyltransferase 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 the melanosomes, it was situated in the Golgi area. A second melanosomal enzyme, TRP1, which escaped the Golgi complex of the cell mutant, travelled to the plasma membrane, and reached endosomes by subsequent endocytosis (figure 1). From the notion that the plasma membrane proteins tend to have longer

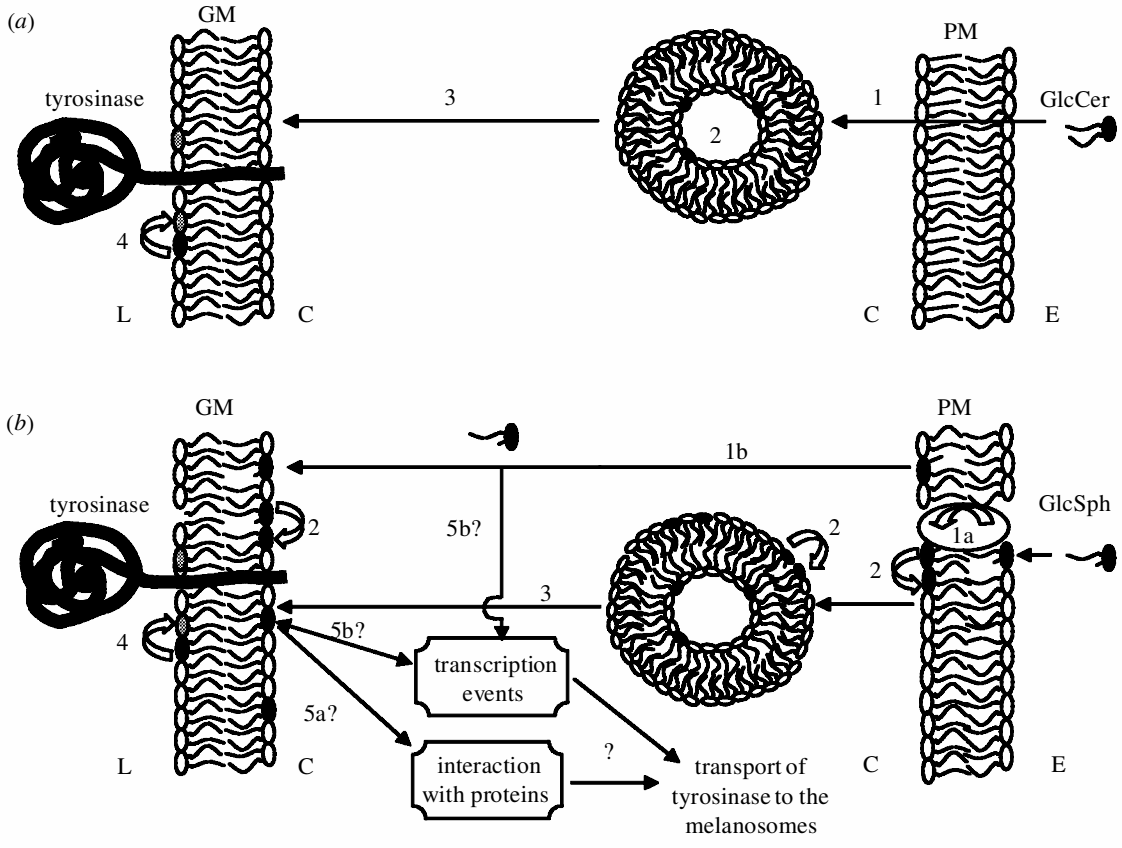

Figure 2. Exogenous GlcSph, but not GlcCer, restores tyrosinase sorting to the melanosomes. (*a*) When GlcCer is added to the medium of GM95, the synthesis of higher glycolipids is observed (4), showing that part of this lipid reaches the lumen of the Golgi, probably via endocytosis (1), with the bulk of GlcCer being in the lumen of the vesicles (2), and then fusion with the Golgi membrane (3). Tyrosinase sorting is not restored. (*b*) When GlcSph is added from the outside, the same amount of higher glycolipids is synthesized (4) but tyrosinase sorting is now restored. The conversion of GlcSph to GlcCer (2), a reaction that takes place in the cytosol, suggests that part of the GlcSph can reach the cytosolic leaflets of membranes, by translocation across the plasma membrane and monomeric transportation through the cytosol (1a and 1b). Vesicles can be formed at the plasma membrane, potentially containing GlcCer and GlcSph on both leaflets, which can fuse with the Golgi membrane. Part of the GlcCer ends up in the lumen of the Golgi, where conversion to higher glycolipids occurs (4), but part of GlcCer/GlcSph can still be present in the cytosolic leaflet of the Golgi, where it could restore tyrosinase sorting either directly by interacting with proteins involved in budding/sorting of tyrosinase, or indirectly by inducing transcription of genes that encode proteins involved in these events (5a and b). Abbreviations: C, cytosolic leaflet; E, exoplasmic leaflet; GM, Golgi membrane; L, lumenal leaflet; PM, plasma membrane.

hydrophobic transmembrane domains than Golgi-resident proteins (Bretscher & Munro 1993), it was then noted that TRP1 contained a longer stretch of hydrophobic amino acids than tyrosinase. When a tyrosinase construct was made with a transmembrane domain, which was six amino acids longer, it escaped the Golgi, and the mutant cells made pigment. This demonstrated that glycosphingolipids were exclusively needed for transporting tyrosinase out of the Golgi area, and not for pigment synthesis itself. Transfection of mutant cells with the glucosyltransferase restored tyrosinase transportation. However, the exogenous addition of the enzyme's product, GlcCer, did not. Transportation was restored when not GlcCer but GlcSph, which lacks one fatty tail, was fed to the mutant cells. GlcSph was converted by the cells to GlcCer, and both lipids, when added exogenously, reached the lumen of the Golgi where they were converted to lactosylceramide and its sialylated form GM3 (figure 1). Apparently, this was irrelevant for restoring pigmentation. As GlcSph is converted to GlcCer on the cytosolic surface (Farrer & Dawson 1990) from acyl-CoA, one possibility is that the GlcCer concentration on the cytosolic surface is the relevant parameter for restoring tyrosinase transportation (figure 2). However, it cannot be excluded that GlcSph by itself is the active species. Because this lipid is present in very small amounts, it is unlikely that it would act as a cofactor for the protein machinery needed for vesicle budding. However, it could be directly involved in signalling events (Hannun & Bell 1987).

If GlcCer would 'act' on the cytosolic surface, two scenarios can be envisioned. GlcCer could form a domain on the cytosolic surface that would most likely also be enriched in cholesterol. Such a domain could then recruit specific proteins (Van Meer 2002) needed for creating the coat that is required for the budding of the transport vesicle destined for the melanosome. Alternatively, GlcCer on the cytosolic surface might be recognized by the glycolipid transfer protein, a cytosolic protein with the capability of binding glycosphingolipids (Lin *et al.* 2000), which might then recruit other proteins. A number of indications predict that the adaptor complex AP-3 is involved in this process (Dell'Angelica *et al.* 1997, 1999; Höning

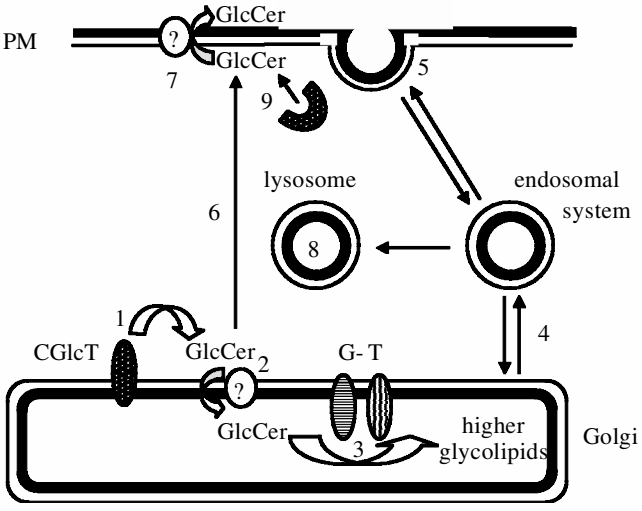

Figure 3. Synthesis, transportation and degradation of GlcCer. GlcCer is synthesized on the cytoplasmic leaflet of the Golgi apparatus (1), and can translocate across the Golgi membrane to the lumen (2), where it is a substrate for glycosyltransferases involved in the biosynthesis of higher glycolipids (3). Transport vesicles can be formed at the Golgi and reach the plasma membrane (4). GlcCer can be present in the lumenal leaflet of the transport vesicles, and after fusion with the plasma membrane (5), ends up at the cell surface. GlcCer can also reach the cytosolic leaflet of the plasma membrane, either by monomeric transportation, or via the cytosolic leaflet of the transport vesicles (6). GlcCer could then be translocated at the cell surface by the action of MDR1 PgP (7; R. J. Raggers *et al.*, unpublished data). There are bi-directional vesicular pathways between the plasma membrane, the Golgi apparatus and the endosomes. GlcCer is finally degraded either in lysosomes by the lysosomal glucocerebrosidase (8), or by the non-lysosomal glucocerebrosidase (9; Van Weely *et al.* 1993). Abbreviations: CGlcT, ceramide glucosyltransferase; G-T, glycosyl transferase; PM, plasma membrane.

et al. 1998; Kantheti *et al.* 1998). An initial indication for the theory that GlcCer may act as a cofactor for vesicle budding on the cytosolic surface of the Golgi complex comes from the finding that plasma membranes contain proteins that are capable of removing GlcCer from the cytosolic surface by translocating the lipid to the outer leaflet of the plasma membrane lipid bilayer. So far, it has only been demonstrated that GlcCer analogues carrying a short acyl chain can be translocated across plasma membranes by the ABC transporters MDR1 P-glycoprotein and MRP-1 (Van Helvoort *et al.* 1996; Raggers *et al.* 1999). It is a challenge to find out whether natural GlcCer is a substrate for these transporters. In addition to an assay for the sidedness of GlcCer based on chemical oxidation (Sillence *et al.* 2000), an assay has now been developed making use of GlcCer hydrolysis by the lysosomal glucocerebrosidase and the activator protein saposin C (R. J. Raggers *et al.*, unpublished data). These assays should be of help in determining whether or not natural GlcCer is translocated across the plasma membrane and whether indeed similar activities exist in intracellular membranes (Lala *et al.* 2000) (figure 3).

3. THE MODEL

Glycosphingolipids, most likely GlcCer, are required for the vesicular pathway from the Golgi complex to the melanosome. As this pathway involves the budding of carrier vesicles from the trans-Golgi network or from an organelle intermediate between the trans-Golgi network and the melanosome, the simplest mechanism would be that GlcCer is required for the formation of a functional protein coat on the cytosolic surface of these membranes. After budding and transportation to the melanosome the active lipid GlcCer might be removed from the cytosolic surface by a translocator (Borst & Oude Elferink 2002), which would make the process unidirectional. A defect in this machinery may lie at the basis of some forms of the Hermansky–Pudlak syndrome, a group of related recessively inherited human pigmentation disorders (Feng *et al.* 2002), as has already been demonstrated for the AP-3 complex. Finally, melanosomes are part of the endosomal membrane system (Raposo & Marks 2002). It will be interesting to see the consequences of the absence of glycosphingolipids on the structure and function of endosomes in regular cells that do not make pigment.

The authors thank their fellow scientists in the glycosphingolipid community that have propelled the field forward over the past decades. It is more alive than ever! They also want to thank them for their help in supplying them with their valuable cell lines, antibodies, constructs and inhibitors. For their contributions to the actual experiments and for provoking ideas, the authors thank their coworkers through the years. This work was financially supported throughout the years by the Mizutani Foundation for Glycoscience, the Netherlands Foundations for Chemical Research (CW), Life Sciences (ALW) and Medical Sciences (ZonMW), INSERM and SENTER, the Dutch Ministry of Economic Affairs.

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GLOSSARY

ABC: ATP-binding cassette
GlcCer: glucosylceramide
GlcSph: glucosylsphingosine
TGN: trans Golgi network