Principles of Lysosomal Membrane Digestion: Stimulation of Sphingolipid Degradation by Sphingolipid Activator Proteins and Anionic Lysosomal Lipids

Thomas Kolter and Konrad Sandhoff

Kekulé-Institut für Organische Chemie und Biochemie der Universität, 53121 Bonn, Germany; email: tkolter@uni-bonn.de; sandhoff@uni-bonn.de

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glycosphingolipids, GM2-activator, lysosomes, saposins, sphingolipids

Abstract

Sphingolipids and glycosphingolipids are membrane components of eukaryotic cell surfaces. Their constitutive degradation takes place on the surface of intra-endosomal and intra-lysosomal membrane structures. During endocytosis, these intra-lysosomal membranes are formed and prepared for digestion by a lipid-sorting process during which their cholesterol content decreases and the concentration of the negatively charged bis(monoacylglycero)phosphate (BMP)—erroneously also called lysobisphosphatidic acid (LBPA)—increases. Glycosphingolipid degradation requires the presence of water-soluble acid exohydrolases, sphingolipid activator proteins, and anionic phospholipids like BMP. The lysosomal degradation of sphingolipids with short hydrophilic head groups requires the presence of sphingolipid activator proteins (SAPs). These are the saposins (Saps) and the GM2 activator protein. Sphingolipid activator proteins are membrane-perturbing and lipid-binding proteins with different specificities for the bound lipid and the activated enzyme-catalyzed reaction. Their inherited deficiency leads to sphingolipid- and membrane-storage diseases. Sphingolipid activator proteins not only facilitate glycolipid digestion but also act as glycolipid transfer proteins facilitating the association of lipid antigens with immunoreceptors of the CD1 family.

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PRINCIPLES OF LYSOSOMAL MEMBRANE DIGESTION

Lysosomal Degradation of Complex Biomolecules

The constitutive degradation of macromolecules and of smaller substances that are composed of cleavable building blocks occurs in the acidic subcellular compartments, the endosomes and the lysosomes. Cellular and foreign components reach these organelles via different routes, by endocytosis, phagocytosis, autophagy, or direct transport. Inside the lysosomes, hydrolytic enzymes with acidic pH-optima cleave macromolecules such as proteins, polysaccharides, and nucleic acids, but also substances with complex structures such as glycoconjugates and phospholipids. The building blocks formed during degradation are able to leave the lysosomes either via diffusion or with the aid of specialized transport systems. Outside the lysosomal compartment, for instance in the cytosol, the building blocks can be utilized for the resynthesis of complex molecules or can be further degraded to provide metabolic energy. Inheritable disorders affecting proteins acting in these degradation pathways lead to lysosomal storage diseases and are characterized by the accumulation of nondegradable enzyme substrates. They can be classified according to the stored substances, e.g., as sphingolipidoses, mucopolysaccharidoses, mucolipidoses, glycoprotein and glycogen storage diseases (Suzuki 1994).

Lysosomal Degradation of Membranes

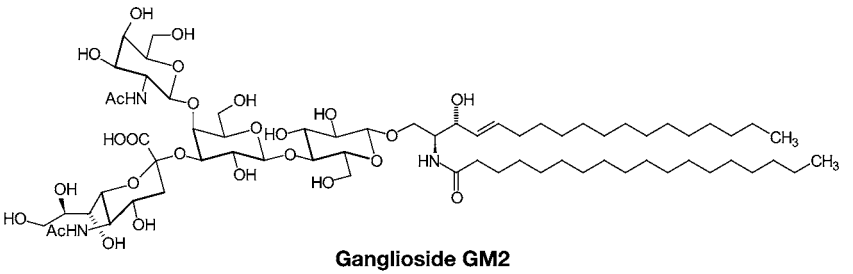
Membranes are essentially composed of amphiphilic lipids and proteins. Both components have to be degraded in the course of membrane digestion inside lysosomes. Proteins and glycoproteins, glycerolipids, sphingolipids, and glycosphingolipids are cleaved into their building blocks: amino acids, monosaccharides, sialic acids, glycerol, fatty acids, and a sphingoid base, e.g., sphingosine. Also, cholesterol is liberated during this process. In contrast to the lysosomal degradation of soluble macromolecules such as proteins and oligosaccharides, in the case of membrane digestion the question arises: Why and in which way are some membranes within the lysosomes degraded, whereas other membranes, the limiting membranes of endosomes and lysosomes, remain unaffected and

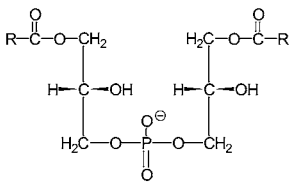
survive. An examination of the molecular mechanisms of membrane digestion reveals a complex machinery composed of lipids of inner lysosomal membranes and lysosomal proteins that ensures selective degradation of inner membranes containing foreign material and components of the former plasma membrane. Sorting of membrane lipids during endocytosis and the maturation of endosomes, together with the selectivity of degrading enzymes and activator proteins, turns out to be crucial for this process. Basic insight into this highly complex process came from the investigation of inherited diseases that result from defects of glycosphingolipid catabolism; essentials of this metabolic pathway are summarized below.

Sphingolipids and Glycosphingolipids

Major insight into the process of membrane digestion came from the investigation of glycosphingolipid catabolism. Glycosphingolipids (**Figure 1**) are ubiquitously expressed on eukaryotic cell surfaces. They are composed of a hydrophobic ceramide moiety and an extracytoplasmic oligosaccharide chain (Kolter & Sandhoff 1999). Combination of different carbohydrate residues, anomeric linkages, and additional modifications of the carbohydrate and lipid moiety lead to a variety of naturally occurring glycosphingolipids that are biosynthetically formed in a combinatorial manner (Kolter et al. 2002). Glycosphingolipid structures depend on species and cell type. They can be classified into series that are characteristic for a group of evolutionarily related organisms. Neuronal cells, especially of the central nervous system, are rich in glycosphingolipids of the ganglio-series, the sialic acid-containing gangliosides. Their lysosomal degradation is particularly well understood on the molecular level and is discussed in more detail below.

It is believed that glycosphingolipids of the plasma membrane, together with cholesterol, the phosphosphingolipid sphingomyelin, and glycosylphosphatidyl-anchored proteins, segregate into functional microdomains, often called rafts (Simons & Ikonen 1997, Brown & London 2000, Munro 2003). The raft





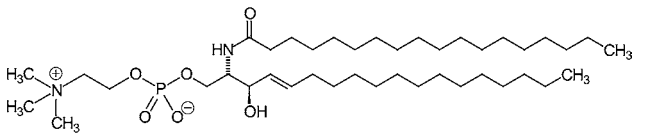


Figure 1

Structures of ganglioside GM2, bis(monoacylglycero)phosphate (BMP), and sphingomyelin.

concept originated from the differential solubilities of membrane components in detergent-containing solutions, but there is still no convincing proof for their existence under physiological conditions (Heerklotz 2002, Heerklotz et al. 2003).

Glycosphingolipid biosynthesis starts with the formation of ceramide at the membranes of the endoplasmic reticulum (ER) (Merrill 2002) and continues at the membranes of the Golgi apparatus with the stepwise addition of single carbohydrate residues (Kolter et al. 2002). Addition of a glucose residue in β-glycosidic linkage to ceramide occurs at the cytoplasmic face of the Golgi apparatus; then, glucosylceramide undergoes a transversal membrane translocation, and the carbohydrate chain is elongated by membrane-resident glycosyltransferases with their active sites in the lumen of the Golgi apparatus. As a consequence, the oligosaccharide moieties of most complex glycosphingolipids face the extracytoplasmic space on the plasma membrane and the lumen of cellular organelles.

Glycosphingolipid biosynthesis is coupled to the intracellular movement of its biosynthetic intermediates and final products to the plasma membrane (van Meer & Lisman 2002). The combinatorial variety of naturally occurring glycolipids can be largely attributed to the combination of glycosyltransferase activities found in different species and cell types.

Similar to phospholipids (Opekarová & Tanner 2003), glycosphingolipids can influence the activity of integral membrane proteins (Yamashita et al. 2003). The activities of peripheral membrane proteins also can be influenced by the lipid composition of the membrane. Because such membrane-binding proteins are crucial for lysosomal sphingolipid degradation, the influence of the lipid composition of the internal membranes of the lysosomal compartment on their functions has to be discussed.

The investigation of lysosomal sphingolipid degradation led to the discovery of principles governing membrane digestion, and key topics of this pathway are discussed in more detail below.

LYSOSOMAL SPHINGOLIPID DEGRADATION

The lysosomal degradation of glycosphingolipids is a sequential pathway that starts with the stepwise release of monosaccharide units from the nonreducing end of the oligosaccharide chain (**Figure 2**). These reactions are catalyzed by exohydrolases with acidic pH-optima. Several of these enzymes need the assistance of small glycoprotein cofactors, the SAPs (Sandhoff et al. 2001). In addition to enzymes and activator proteins, the membrane must have the right lipid composition in order to be degraded. This means a low cholesterol content and the presence of the negatively charged lysosomal lipid BMP.

The stepwise cleavage of the hydrophilic head groups from glycosphingolipids ultimately generates sphingosine, fatty acids, monosaccharides, sialic acids, and sulfate. These final degradation products are able to leave the lysosome. Members of other glycosphingolipid series enter the degradation pathway of gangliosides at the lactosylceramide stage. Glycosphingolipids of the gala-series, but also of sphingomyelin, are degraded to ceramide.

Most hydrolases are water-soluble polycations at a lysosomal pH of less than 5.0. They bind to negatively charged membranes, but hardly work on their membrane-bound substrates. A notable exception is acid sphingomyelinase, which slowly hydrolyzes membrane-bound sphingomyelin even in the absence of an activator protein, presumably due to its N-terminal saposin-homology domain (Linke et al. 2001a).

Non-glycosylated sphingolipids, such as ceramide and sphingomyelin (Goni & Alonso 2002), have non-lysosomal degradation steps that apparently do not need the assistance of an activator protein. A cytoplasmic glucosylceramide-cleaving enzyme, which is not deficient in Gaucher's disease,

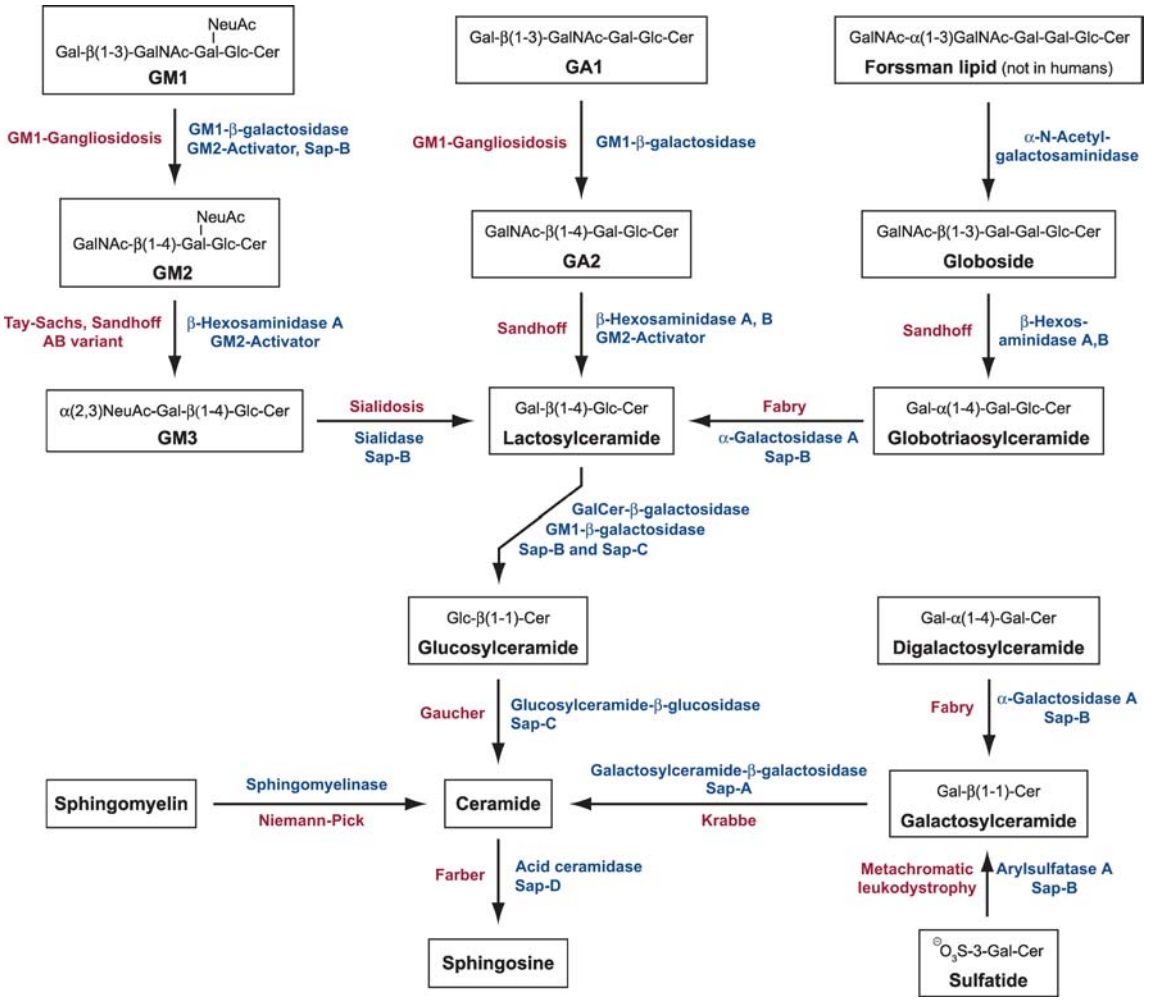


Figure 2

Degradation of selected sphingolipids in the lysosomes of the cells (modified from Kolter & Sandhoff 1998). The eponyms of individual inherited diseases (shown in red) are given. Activator proteins required for the respective degradation step in vivo are indicated. Variant AB: AB variant of GM2 gangliosidosis (deficiency of GM2-activator protein).

contributes to the degradation of the cytoplasmic glucosylceramide pool (van Weely et al. 1993).

Topology

For lysosomal membrane digestion, plasma membrane components are transported to the lysosomes within the process of endocytotic membrane flow. Vesicles of the endosomal/lysosomal compartment are formed via different routes, starting with the formation of clathrin-coated pits, non-clathrin-coated pits, caveolae, and others (Maxfield & McGraw 2004). The observation that the integrity of lysosomal- and endosomal-limiting membranes is preserved during the process of lysosomal degradation led necessarily

to the assumption that two distinct pools of membranes must be present in the endosomal/lysosomal compartment (Fürst & Sandhoff 1992). Early reports about the ultrastructural examination of cells derived from patients with defects of glycosphingolipid catabolism indicated storage of intralysosomal lipid aggregates: nondegradable lipids accumulate as multivesicular storage bodies (MVB) in diseases such as the GM1-gangliosidosis (Suzuki & Chen 1968) or combined Sap deficiency (Harzer et al. 1989). These reports gave the first hint of a topological differentiation of the two membrane pools (Fürst & Sandhoff 1992).

The following model for the topology of endocytosis and membrane digestion was initially proposed in 1992 (Fürst & Sandhoff 1992) and has been further supported by a series of observations (Sandhoff & Kolter 1996). According to this view, plasma membrane components, lipids and proteins, reach the lysosomal compartment either as intra-endosomal membranes or as part of the limiting membrane (**Figure 3**). Both membranes differ in their lipid and protein

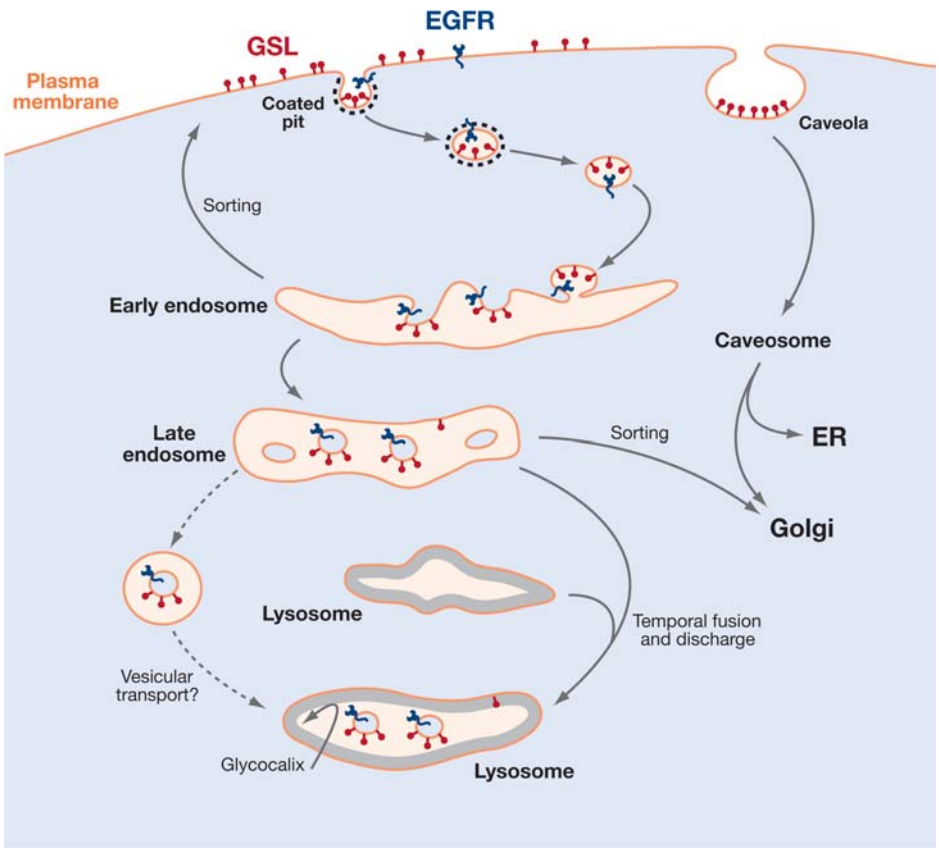


Figure 3

Model of endocytosis and lysosomal digestion of membranes (modified from Sandhoff & Kolter 1996). Glycosphingolipids (GSL) are highlighted on the plasma membrane and on internal membranes. Parts of the plasma membrane, including GSLs, are incorporated into the membranes of intra-endosomal vesicles and membrane structures during endocytosis. The vesicles reach the lysosomal compartment when late endosomes are transiently fused with primary lysosomes and are degraded there. The lysosomal perimeter membrane is protected from degradation by a thick glycocalix. EGFR: epidermal growth factor receptor.

composition. The lysosomal leaflet of the limiting membrane is covered with a thick glycocalix that protects the membrane from attack by the membrane-degrading enzymes present in the lysosol. This glycocalix is formed by lysosomal integral and peripheral membrane proteins, which are highly N-glycosylated with polylactosamine units and therefore highly resistant toward lysosomal digestion (Eskelinen et al. 2003). Apparently, the degrading enzymes present within the lumen of the lysosomes cannot easily access their substrates through this glycocalix. Indeed, more than 30 years ago it was demonstrated that after its incorporation into the limiting lysosomal membrane, plasma membrane-derived ganglioside GM3 is protected from degradation (Henning & Stoffel 1973), whereas it is easily degraded during the constitutive process of membrane turnover (Tettamanti 2004). In addition to this membrane pool resistant to degradation, components originating from the plasma membrane transiently occur as components of intra-endosomal/intra-lysosomal membranes. The major part of membrane digestion has to proceed on the surface of these internal membrane structures. This second pool originates from the plasma membrane and reaches the lumen of the endosomes as intra-endosomal vesicles or other lipid aggregates and not as part of the perimeter membrane. Vesicles of this type accumulate in patients with sphingolipid-storage diseases (see above) and have been described by other groups in normal cells (e.g., van der Goot & Gruenberg 2002).

The occurrence of intra-endosomal membranes is not necessarily restricted to cells with impaired membrane digestion; they are regularly visible in the microscope as MVBs. MVB formation starts with inward budding of the limiting endosomal membrane (Hopkins et al. 1990). Lipids, as well as proteins, are sorted to either the internal or the limiting membrane. One sorting signal sufficient for protein targeting to internal vesicles of MVBs is the ubiquitinylation of cargo proteins (Katzmann et al. 2001). This signal appears to be conserved between yeast and higher eukaryotes, but ubiquitin-independent factors have also been reported (Umebayashi 2003). This membrane segregation is accompanied by lipid sorting that prepares the internal membranes for the attack by the lysosomal degradation system (see below).

SPHINGOLIPID ACTIVATOR PROTEINS STIMULATE SPHINGOLIPID DEGRADATION

In vivo, the lysosomal degradation of membranes is dependent on certain proteins and lipids. It has been known for more than 30 years that glycosphingolipid degradation requires the presence of SAPs (for review, see Sandhoff et al. 2001). Whereas other membrane components, for example phospholipids, can apparently be degraded without cofactors that make them accessible to the degrading enzymes, degradation of glycosphingolipids with short carbohydrate chains of four or fewer sugars is critically dependent not only on lysosomal glycosidases but also on activator proteins and negatively charged lysosomal lipids. According to the topology of endocytosis discussed above, lysosomal enzymes cleave sphingolipid substrates that are part of intra-endosomal and intra-lysosomal membrane structures. In the absence of detergents that are able to solubilize the lipids, glycosphingolipids with short carbohydrate chains are not sufficiently accessible to the water-soluble enzymes present in the lysosol in the absence of membrane-perturbing activator proteins (Wilkening et al. 1998, 2000). In vitro, synthethic water-soluble GSLs with either short-chain fatty acids or no fatty acids (lysoGSLs) can already be hydrolyzed by the water-soluble enzymes in the absence of SAPs. Two genes are known to encode the SAPs: one encodes the GM2-activator protein, the other encodes the Sap-precursor protein, also called prosaposin (Sandhoff et al. 2001). This protein is post-translationally processed to

four homologous mature proteins, Saps A-D, or saposins A-D. These activator proteins act on the intra-endosomal/intra-lysosomal membrane pool and lead to the selective degradation of membrane lipids without impairment of lysosomal integrity. Inherited deficiency of either lysosomal enzymes or SAPs leads to the accumulation of nondegradable membranes within the lysosomal compartment and to the development of sphingolipid-storage diseases (Kolter & Sandhoff 1998, Suzuki & Vanier 1999, Platt & Walkley 2004, Winchester 2004).

The GM2-Activator

The GM2-activator is a glycoprotein with a molecular mass of 17.6 kDa in its deglycosylated form (Sandhoff et al. 2001). It acts as a cofactor essential for the in vivo degradation of ganglioside GM2 (**Figures 1, 2, 4**) by β-hexosaminidase A (Conzelmann & Sandhoff 1979). β-hexosaminidases are dimeric isoenzymes formed by combination of two subunits, α and β, which differ in their substrate specificity (Hepbildikler et al. 2002). β-hexosaminidase A can cleave glycolipid substrates on membrane surfaces only if they extend far enough into the aqueous phase. Therefore, in the absence of detergents, the degradation of ganglioside GM2 occurs only in the presence of the GM2 activator protein. The crystal structure of β-hexosaminidase B (Maier et al. 2003, Mark et al. 2003) has been solved, so now the question of how the members of the three-component system might interact can be addressed on the molecular level.

The inherited deficiency of the GM2-activator protein leads to the AB variant of GM2-gangliosidoses, in which lipid accumulation in neuronal cells leads to the early death of the patients (Conzelmann & Sandhoff 1978). An X-ray crystallographic structure of the non-glycosylated protein expressed in *Escherichia coli* is available (Wright & Rastinejad 2000, Wright et al. 2003). According to this, the GM2-activator contains a hydrophobic cavity that harbors the ceramide moiety of ganglioside GM2. A detailed model of this mechanism, based on earlier considerations (Fürst & Sandhoff 1992), structural information (Wright et al. 2003), and photoaffinity labeling (Wendeler et al. 2004), is shown in **Figure 4**. To present ganglioside GM2 or related glycosphingolipids (e.g., GM1; Wilkening et al. 2000) to the active site of the corresponding degrading enzyme, the GM2-activator has to insert into the bilayer of intra-lysosomal lipid vesicles and lift the glycolipid out of the membrane. Therefore, it can be regarded as a weak detergent with high selectivity, which as a "liftase" (Fürst & Sandhoff 1992) forms stoichiometric, water-soluble glycolipid-protein complexes that are the physiological Michaelis-Menten substrates of β-hexosaminidase A (Conzelmann & Sandhoff 1979). Similar to other SAPs, the GM2-activator protein acts as a lipid transfer protein in vitro (**Figure 5*a***, **Table 1**) that can carry lipids from donor to acceptor liposomes (Conzelmann et al. 1982). The transfer properties of activator proteins are crucial for the loading of lipid antigens to the immunoreceptors of the CD1 family (see below, **Table 1**). As is discussed below, the lipid composition of the GM2-containing membrane, as well as their lateral pressure, is important for degradation and for ensuring that only ganglioside GM2 in the internal membranes is digested.

In addition to the SAPs, other lipid-binding proteins are known (Malinina et al. 2004), including additional proteins of saposin-like structure (Munford et al. 1995); CERT, a protein that transfers ceramide from the ER to Golgi membranes (Hanada et al. 2003); fatty acid–binding proteins (Coe & Bernlohr 1998); and immunoreceptors of the CD1 family (see below). The three-dimensional structure of the complex between a cytoplasmic glycosphingolipid transfer protein, which can transport lactosylceramide, and its ligand has been reported

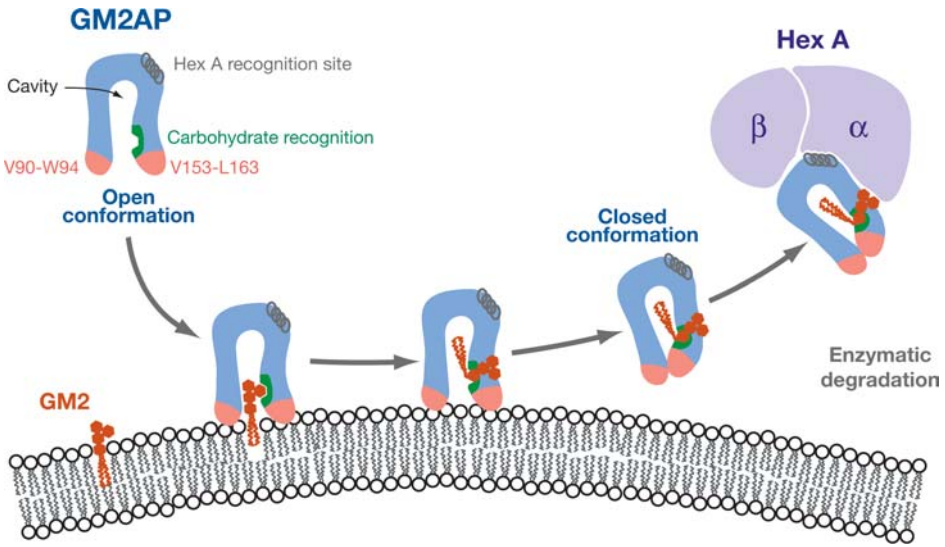


Figure 4

Model for GM2-activator-stimulated hydrolysis of ganglioside GM2 by human β-hexosaminidase A (Wendeler et al. 2004). The GM2AP contains a hydrophobic cavity, with dimensions that can accommodate the ceramide portion of GM2 and other lipids, lined by surface loops and a single short helix. The most flexible of the loops contains the substrate-binding site (V153–L163) and controls the entrance to the cavity, so that two conformations are possible: one open and one closed. The open empty activator binds to the membrane by using the hydrophobic loops and penetrates into the hydrophobic region of the bilayer. Then the lipid recognition site of the activator can interact with the substrate, and its ceramide portion can move inside the hydrophobic cavity. At this point, the conformation of the lipid-loaded activator may change to the closed one, thus the complex becomes more water soluble and leaves the membrane, exposing GM2 to the water-soluble enzyme to be degraded.

(Malinina et al. 2004). Immunoreceptors of the CD1 family present lipid antigens to T-lymphocytes (see below).

Saposins

The Saps or saposins A–D are four acidic, not enzymatically active, heat-stable and protease-resistant glycoproteins of about 8–11 kDa (for review, see Sandhoff et al. 2001). They belong to a family of saposin-like proteins with conserved three-dimensional folds (Munford et al. 1995), of which the solution structures of NK-lysin (Liepinsh et al. 1997) and the pore-forming peptide of *Entamoeba histolytica* (Hecht et al. 2004), Sap-C (de Alba et al. 2003), and the X-ray crystallographic structure of unglycosylated human recombinant Sap-B (Ahn et al. 2003) are known.

The proteins of this group carry out diverse functions, but share lipid binding- and membrane-perturbing properties. For example, the protozoan parasite *E. histolytica* expresses pore-forming proteins with a saposin-like structure, the amoebapores. Similar to eukaryotic NK-lysin and granulysin, these proteins are able to permeabilize the membranes of target cells, which accounts for their antimicrobial activity (Gutsmann et al. 2003). Although the four Saps share a high degree of homology and similar properties, they act differently and show different specificities.

Sap-A. Sap-A is required for the degradation of galactosylceramide by

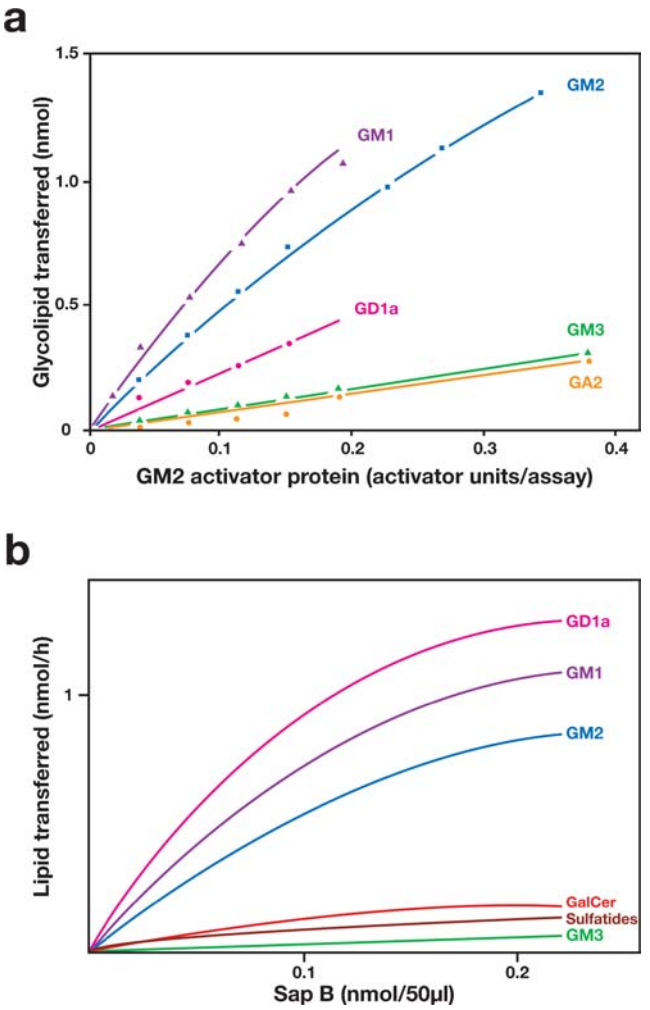


Figure 5

(*a*) Transfer of various glycolipids from donor to acceptor liposomes by the GM2-activator protein. Donor liposomes (250 nmol of lipids) containing 2 mol% of the respective glycolipid (5 nmol) were incubated with an equal amount of acceptor liposomes, 2 μmol of citrate buffer, pH 4.2, 4 μg of bovine serum albumin, and the amount of activator protein indicated in a total volume of 40 μl for 30 min at 37°C. Negatively charged acceptor liposomes were separated from uncharged donor liposomes on DEAE-cellulose columns. Controls were run without activator protein and substracted (Conzelmann et al. 1982). (*b*) Transfer of glycosphingolipids from acceptor to donor liposomes by Sap-B. Donor liposomes (250 nmol lipid) containing 2 mol% of the respective glycosphingolipids were incubated with an equal amount of acceptor liposomes, cytochrome *c* (10 μg) and increasing amounts of activator protein in a total volume of 50 μl, 50 mM citrate, pH 4.0, at 37°C for 1 h. Negatively charged acceptor liposomes were separated from uncharged donor liposomes on small DEAE-cellulose columns. Controls were run without activator proteins and the values subtracted. Modified after (Vogel et al. 1991).

galactosylceramide-β-galactosidase in vivo (**Figure 2**), convincingly demonstrated by the phenotype of mice carrying a mutation in the Sap-A domain of the Sap-precursor. These animals accumulate galactosylceramide and suffer from a late-onset form of Krabbe disease (Matsuda et al. 2001). To date, only one human patient has been reported with an isolated defect of Sap-A (Spiegel et al. 2005).

Sap-B. Sap-B was the first activator protein to be identified (Mehl & Jatzkewitz 1964). It mediates the degradation of sulfatide by arylsulfatase A and of globotriaosylceramide and digalactosylceramide by α-galactosidase A in vivo (**Figure 2**). This has been demonstrated in patients with Sap-B deficiency, where these substrates are found in the urine (Li et al. 1985). Obviously, a small fraction of these amphiphilic and barely water-soluble substances can escape the lysosomes and are detected in the urine after massive accumulation. Sap-B is also required for the degradation of other glycolipids (Li et al. 1988); for example, it cooperates with the GM2-activator protein in the degradation of ganglioside GM1 (Wilkening et al. 2000).

Similar to the GM2-activator, Sap-B acts as a physiological detergent but shows a broader specificity. The crystal structure shows a shell-like homodimer that encloses a large hydrophobic cavity (Ahn et al. 2003); the monomers are composed of four amphipathic α-helices arranged in a long hairpin that is bent into a simple V-shape. As in the GM2-activator, there are two different conformations of the Sap-B dimers, and a similar mechanism for its action has been proposed: The open conformation should interact directly with the membrane, promote a reorganization of the lipid alkyl chains, and extract the lipid substrate accompanied by a change to the closed conformation. Thus the substrate could be exposed to the enzyme in a water-soluble activator-lipid complex (Fischer & Jatzkewitz 1977),

TABLE 1 Lysosomal lipid transfer proteins (modified from Sandhoff et al. 2001)

	GM2-activator	Sap-A	Sap-B	Sap-C	Sap-D	Prosaposin	NPC-2
Subcellular site	le, lys	le, lys	le, lys	le, lys	le, lys	Extracellular, ER, Golgi	le, lys
Acts as lipid transfer protein	Yes	?	Yes	(Yes)	?	?	(Yes)
Stoichiometric complexes	Yes (1:1)	?	Yes	Yes	?	?	?
Solubilizes lipids with BMP and other anionic PL	Yes	?	Yes	Yes	(Yes)	?	?
Defect leads to storage of	GM2, GA_2	GalCer (k.o. mouse)	Sulfatides, Gbose3Cer	GlcCer	Hydroxylated Ceramides (k.o. mouse)	Cer, most GSL and inner lysosomal membranes	Chol.
Interaction with exohydrolases	HexA	?	?	Glucosylceramide-β-glucosidase	?	?	?
Lipid antigen transfer to	CD1d?	?	CD1d	CD1b	?	?	?

le, late endosomes; lys, lysosomes.

consistent with the previous observation that Sap-B can act as a lipid-transport protein (**Figure 5*b***; Vogel et al. 1991). The inherited defect of Sap-B leads to an atypical form of metachromatic leukodystrophy, with late infantile or juvenile onset (Kretz et al. 1990). The disease is characterized by accumulation of sulfatides, digalactosylceramide, and globotriaosylceramide (Sandhoff et al. 2001).

Sap-C. Sap-C is a homodimer and was initially isolated from the spleens of patients with Gaucher disease (Ho & O'Brien 1971). It is required for the lysosomal degradation of glucosylceramide by glucosylceramide-β-glucosidase (Ho & O'Brien 1971) (**Figure 2**). In addition, Sap-C renders glucosylceramide-β-glucosidase more protease-resistant inside the cell (Sun et al. 2003). The solution structure of Sap-C (de Alba et al. 2003) consists of five tightly packed α-helices that form half of a sphere. All charged amino acids are solvent-exposed, whereas the hydrophobic residues are contained within the protein core. In contrast to the mode of action of the GM2-activator and of Sap-B, Sap-C can directly activate glucosylceramide-β-glucosidase in an allosteric manner (Ho & O'Brien 1971, Berent & Radin 1981, Fabbro & Grabowski 1991). Sap-C also supports the interaction of the enzyme with the substrate embedded in vesicles containing anionic phospholipids, and Sap-C is able to destabilize these vesicles (Wilkening et al. 1998). Binding of Sap-C to phospholipid vesicles is a pH-controlled, reversible process (Vaccaro et al. 1995). Sap-C deficiency leads

to an abnormal juvenile form of Gaucher disease and an accumulation of glucosylceramide (Christomanou et al. 1986, Schnabel et al. 1991).

Sap-D. Sap-D stimulates lysosomal ceramide degradation by acid ceramidase in cultured cells (Klein et al. 1994) and in vitro (Linke et al. 2001b). Moreover, it stimulates acid sphingomyelinase-catalyzed sphingomyelin hydrolysis, but this seems not to be necessary for the in vivo degradation of sphingomyelin (Morimoto et al. 1988, Linke et al. 2001a). The detailed physiological function and mode of action of Sap-D is unclear. It is able to bind to vesicles containing negatively charged lipids and to solubilize them at an appropriate pH (Ciaffoni et al. 2001). Sap-D-deficient mice accumulate ceramides with hydroxylated fatty acids mainly in the brain and in the kidney (Matsuda et al. 2004).

Prosaposin. All four saposins are derived from a single protein, the Sap-precursor, or prosaposin, which is proteolytically processed to the mature activator proteins in the late endosomes and lysosomes (Fürst et al. 1988, O'Brien et al. 1988, Nakano et al. 1989). Prosaposin is a 70 kDa glycoprotein detected mainly uncleaved in brain, heart, and muscle, whereas mature Saps are found in all organs tested for so far, but mainly in liver, lung, kidney, and spleen. The Sap-precursor also occurs in body fluids such as milk, semen, cerebrospinal fluid, bile, and pancreatic juice. The Sap-precursor is either intracellularly targeted to the lysosomes via mannose-6-phosphate receptors or by sortilin (Lefrancois et al. 2003), or it can be secreted and re-endocytosed by mannose-6-phosphate receptors, low-density, lipoprotein receptor–related protein (LRP), or mannose receptors (Hiesberger et al. 1998).

To date, two different mutations in four human patients have been reported that lead to a complete deficiency of the whole Sap-precursor protein, and consequently of all four Saps. Because the Sap-precursor is proteolytically processed efficiently within the acidic compartments, it can be assumed that the unprocessed protein plays no role in membrane digestion. Inherited deficiency of the protein, however, was an indispensable tool in the elucidation of the specificity of individual saposins (Sandhoff et al. 2001). In human patients with Sap-precursor deficiency, but also in the Sap-precursor knockout mice (Fujita et al. 1996), there is simultaneous storage of many sphingolipids, including ceramide, glucosylceramide, lactosylceramide, ganglioside GM3, galactosylceramide, sulfatides, digalactosylceramide, and globotriaosylceramide, accompanied by a dramatic accumulation of intra-lysosomal membranes. This storage can be completely reversed by exogenous treatment with human Sap-precursor, as demonstrated in prosaposin-deficient fibroblasts (Burkhardt et al. 1997).

Activator Proteins in Lipid Antigen Presentation

In addition to their function as enzyme cofactors, SAPs play an important role in the presentation of lipid and glycolipid antigens. It is now established that CD1 immunoreceptors present lipid antigens to T cells. However, these lipids first must be removed from the membranes in which they are embedded to allow loading of CD1 molecules (**Figure 6**). The human genome encodes four MHC-I-like glycoproteins (CD1a-d) that present lipid antigens to T cells. A fifth gene encodes CD1e, which is synthesized as an integral membrane protein and from which a soluble lipid-binding domain is released by proteolysis within the lysosomes of mature dendritic cells (Angénieux et al. 2005). A possible function of this protein might also be lipid transfer, but this has not been proven to date. The three-dimensional structures of protein-lipid-complexes between human CD1b and two lipids, phosphatidylinositol and ganglioside GM2 (Gadola et al. 2002), and between CD1a

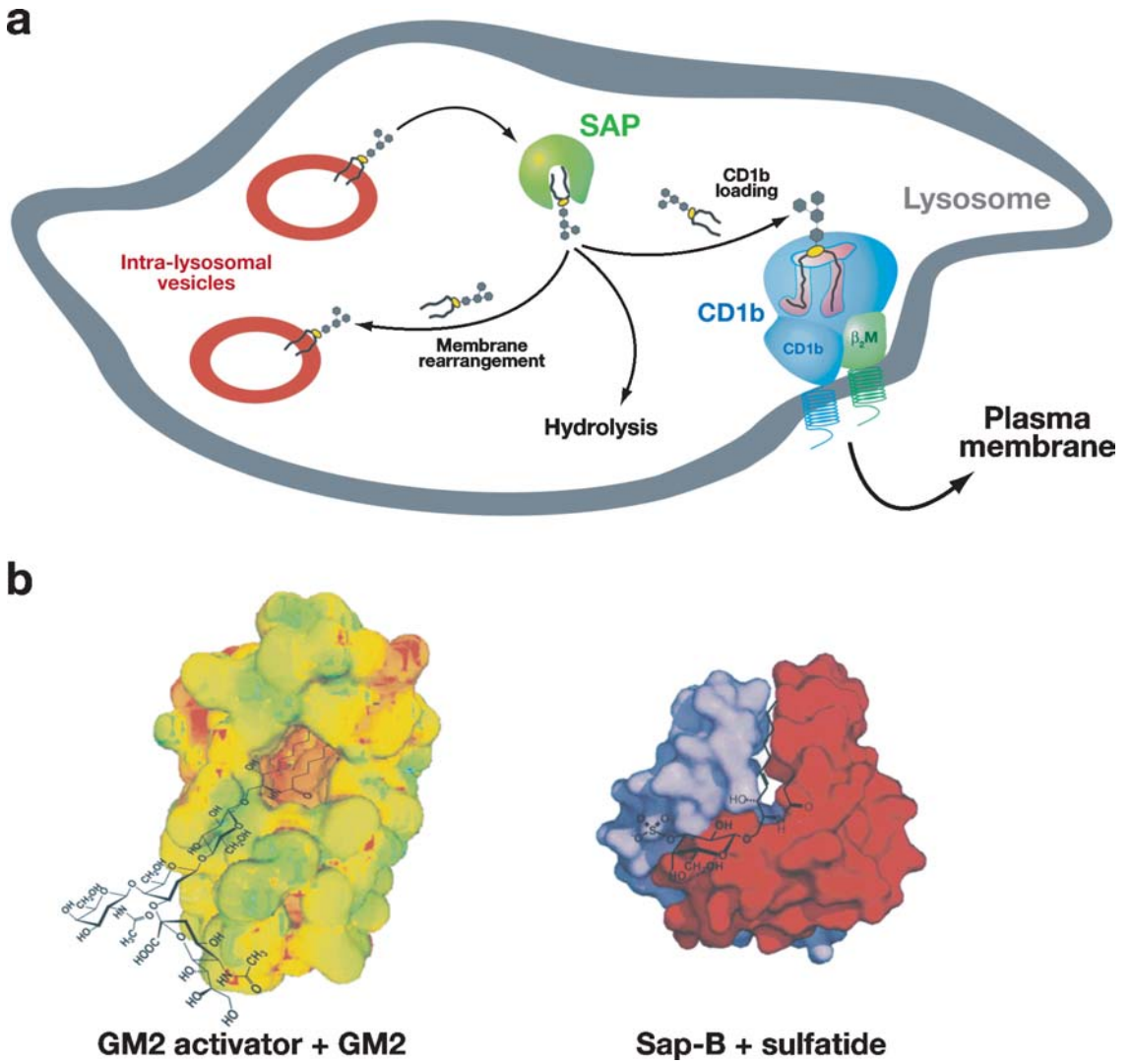


Figure 6

Model of the presentation of glycolipid antigens to immunoreceptors of the CD1 family by lipid transfer proteins. SAPs, such as Sap-B, or the GM2-activator, extract lipids or glycolipids from inner lysosomal membranes and transfer them to other membranes, to hydrolytic enzymes, or to immunoreceptors of the CD1 family on the perimeter membrane. Lipid-loaded CD1 proteins then travel back to the plasma membrane and activate lipid-antigen-specific T-lymphocytes.

and sulfatide (Zajonc et al. 2003) have been reported. There is evidence that SAPs acting as lipid transfer proteins (**Table 1**) participate in this loading process within the acidic compartments of the cell. Antigen presentation by human CD1b (Winau et al. 2004), as well as human (Kang et al. 2004) and mouse CD1d (Zhou et al. 2004), have been studied: Human CD1b especially requires Sap-C to present different types of glycolipid antigens. In vitro,

all saposins can exchange phosphatidylserine bound to murine CD1d against glycosphingolipids, but with different activity. The state of our knowledge in this regard is incomplete. Further research is required to reach a better understanding of the mechanisms involved in this process.

ANIONIC LIPIDS AS ACTIVATORS OF SPHINGOLIPID DEGRADATION

Intra-Endosomal and Intra-Lysosomal Membranes as Degradation Platforms

During maturation of endosomes, the luminal pH value decreases and the composition of the internal membranes changes. Not only the protein composition (Umebayashi 2003) but also the lipid composition is adjusted during this process so that the internal membranes in the acidic compartments of the cell are prepared for degradation. Their lipid composition differs considerably from that of plasma membrane or the limiting membranes of cellular organelles. Membrane-stabilizing cholesterol is continuously removed during this process, and the content of the negatively charged lysosomal lipid, BMP (erroneously also named as lysobisphosphatidic acid), increases (**Figures 1, 7*b***). BMP is not present in the limiting lysosomal membrane, but it stimulates sphingolipid degradation on inner membranes of the acidic compartments (see below). Sphingolipid-containing membranes of autophagocytic bodies should contain less cholesterol but should contain metabolic precursors of BMP and might be degraded in a similar manner.

Cholesterol

Cholesterol is enriched in the plasma membrane and in membranes of early endocytic organelles but not in the lysosomes (Umebayashi 2003, Friedland et al. 2003). As indicated by immuno-electronmicroscopical examination of human B-lymphocytes (Möbius et al. 2003), about 80% of the cholesterol detected in the endocytic pathway is present in the recycling compartments and in internal membranes of early and late endosomes. On the other hand, it is nearly completely absent in inner lysosomal membranes (**Figure 7*a***).

Data on the differential lipid composition of the internal endo/lysosomal membranes have been obtained with the aid of exogenous addition of ganglioside GM1 derivatives bearing a photoaffinity label and fluorescence- or biotin-labels to cultured cells and subsequent monitoring of endocytosis by fluorescence microscopy (Möbius et al. 1999a,b; von Coburg 2003). Membrane segregation into the intra-endosomal membrane pool has been demonstrated in cultured human fibroblasts, where biotin-labeled ganglioside GM1 derived from the plasma membrane is mainly targeted to intralysosomal structures and much less to the lysosomal perimeter membrane (Möbius et al. 1999a).

Analysis of the molecular lipid environment of a short-chain, photoactivatable ganglioside GM1 derivative in the plasma membrane showed an unexpectedly high amount of cholesterol (80%) and comparatively low amounts of phosphatidylcholine (17%) and sphingomyelin (7%). During endocytosis, coupling to cholesterol continuously decreased down to 46%, whereas the amounts of cross-linked phosphatidylcholine increased to 31% and those with sphingomyelin reached up to 23%. Using a long-chain derivative of photoactivatable ganglioside GM1 also enabled examination of its lipid environment in the inner membranes of the lysosomes in cultured human fibroblasts (von Coburg 2003). The amount of cross-linking products with cholesterol dropped down to 1% of all lipid derivatives. Cross-linking with BMP was elevated to 45%, whereas cross-linked phosphatidylcholine and sphingomyelin remained nearly unchanged at values of 31% and 22%,

respectively. Similar results were obtained in other cell types, suggesting an efficient sorting of membrane lipids during endocytosis. These results are in agreement with those obtained by electron microscopy (Möbius et al. 1999a, 2003). Other cross-linking experiments using cells derived from patients with NPC1 (see below) yielded 25% of coupled cholesterol in the inner membranes of lysosomes (von Coburg 2003).

Niemann-Pick disease, type C. An inherited disorder, in which intracellular traffic of cholesterol is impaired, is Niemann-Pick disease, type C (NPC) (Patterson et al. 2001, Patterson 2003). Although the molecular details underlying this disorder are far from clear, this disease might shed some light on unexplained aspects of endocytotic lipid sorting. The disease is a neurodegenerative disorder characterized by accumulation of cholesterol and, secondarily also of other membrane components in the lysosomal compartment. Endosomal-lysosomal storage of unesterified cholesterol, neutral glycolipids such as glucosylceramide and lactosylceramide, acidic glycolipids, especially gangliosides GM3 and GM2 (Zervas et al. 2001), sphingomyelin (less than in Niemann-Pick disease, types A and B), BMP, and phospholipids occurs in liver, spleen, brain, and other organs (Patterson et al. 2001).

NPC is an inherited disorder in which mutations in the genes encoding the NPC1 (Carstea et al. 1997) or NPC2 protein (Naureckiene et al. 2000) have been detected. NPC1-deficiency accounts for 95% of NPC cases (Millat et al. 1999), whereas the other cases are from mutations in the HE1 gene encoding the NPC2 protein (Millat et al. 2001). This protein was previously identified as a lysosomal glycoprotein that can bind cholesterol with high affinity in a 1:1 stoichiometry (Okamura et al. 1999). In agreement with results from in vitro experiments (C. Arenz & K. Sandhoff, unpublished results), it probably acts as a cholesterol carrier (**Table 1**) and regulates cholesterol transport from the internal membranes to the NPC1 protein, an integral protein of the perimeter membrane. The NPC1 gene product has 13–16 predicted transmembrane domains and a sterol-sensitive domain with homologies to HMG-CoA-reductase. A strain of mutant BALB/c mice has been used as an animal model for NPC. The mice show storage of unesterified cholesterol and impaired processing of exogenous cholesterol (Pentchev et al. 1984, Liu et al. 2000). Cultured cells derived from these animals show

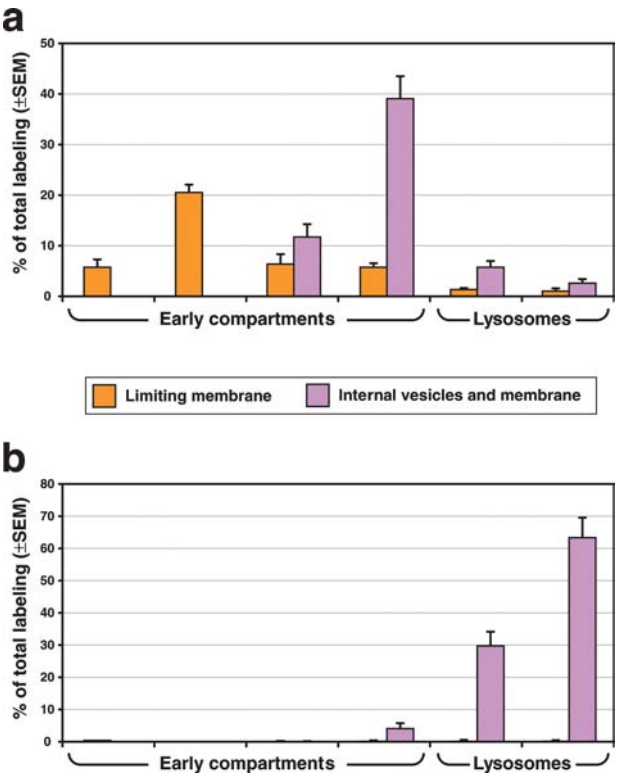


Figure 7

(*a*) Relative distribution of cholesterol in human B-lymphocytes in membranes of different types of endocytic organelles. Cryosections of human B-lymphocytes, which had internalized BSA-gold for different time points, were labeled with biotinylated Θ-toxin and antibiotin antibodies followed by protein A-gold (Möbius et al. 2003). (*b*) Distribution of BMP in human B-lymphocytes in membranes of different types of endocytic organelles. Cryosections of human B-lymphocytes, which had internalized BSA-gold for different time points, were labeled with a monoclonal antibody against BMP followed by rabbit antimouse antibodies and protein A-gold (Möbius et al. 2003).

elevated levels of unesterified cholesterol if they are fed with low-density lipoprotein (Pentchev et al. 1986). In skin fibroblasts from NPC patients, the accumulating cholesterol is colocalized with BMP-rich late intra-endosomal membrane structures (Kobayashi et al. 1999). The NPC1 protein is suggested to transport lipophilic molecules through membranes and presumably also out of the endosomal-lysosomal system (Davies et al. 2000).

Ceramide

We assume that during the maturation of intra-endosomal/intra-lysosomal vesicles the levels of ceramide, a cholesterol-competitor (London & London 2004), increase at the expense of sphingomyelin, a cholesterol-binding lipid (see below). Ceramide, the common product of glycosphingolipid and sphingomyelin catabolism, can stabilize lipid phases, i.e., microdomains, more efficiently than cholesterol (Massey 2001, Xu et al. 2001, London & London 2004). Although cholesterol is removed from the internal membranes, the ceramide content of the internal membranes most likely increases. During endosomal maturation, the luminal pH decreases. The enzymes that lead to generation of ceramide have higher pH-optima than acid ceramidase, which converts ceramide into sphingosine that is positively charged at the pH of the lysosol. Glucosylceramide-β-glucosidase, which converts glucosylceramide into ceramide, and acid sphingomyelinase, which converts sphingomyelin into ceramide, have pH optima of about 5.5 (Osiecki-Newman et al. 1988, Goni & Alonso 2002), whereas the pH optimum of acid ceramidase is in the range of 3.8–4.2 in the presence of Sap-D (Bernardo et al. 1995, Linke et al. 2001b). This should lead to a continuous increase of ceramide levels within the intra-lysosomal membranes during the process of endosomal maturation. The replacement of cholesterol by ceramide might facilitate cholesterol exit out of this membrane population by NPC2.

Bis(monoacylglycero)phosphate

BMP is a characteristic anionic phospholipid of the acidic compartments of the cell. It is biosynthetically formed during the degradation of phosphatidylglycerol and cardiolipin (Amidon et al. 1996, Brotherus et al. 1974), presumably on the surface of intra-lysosomal vesicles. It has an unusual sn1,sn1′ configuration, which accounts for its higher resistance to the action of phospholipases than normal phospholipids (Matsuzawa & Hostetler 1979). Other anionic lipids, such as phosphatidylinositol (Kobayashi et al. 1998) and dolichol phosphate (Chojnacki & Dallner 1988), albeit in smaller amounts than BMP, are also found within the lysosomal compartment.

In vivo, enzymatic hydrolysis of most membrane-bound sphingolipids is stimulated not only by sphingolipid activator proteins, but also by BMP. The percentage of BMP increases starting from late endosomes to lysosomes, where the maximal amount is found (**Figure 6*b***). Moreover, BMP was shown to be mainly present in internal membranes; therefore, it distinguishes these membranes from the perimeter membrane (Möbius et al. 2003).

Biophysical Properties

The size, the lateral pressure, and the composition of intra-lysosomal vesicles are contributing factors in the degradation of glycolipids. The diameter of intra-lysosomal vesicles has been determined in tissues from sphingolipid activator protein-deficient patients to be in the range of 50 to 100 nm (Bradova et al. 1993). The lateral surface pressure of most biological membranes is in the range of 30 to 35 mNm^{-1} (Marsh 1996, Maggio et al. 2002). This high lateral pressure seems to contribute to the protection of limiting membranes from inappropriate

degradation because in vitro experiments showed that the GM2-activator protein is only able to penetrate into a phospholipid monolayer when the lateral pressure is below a critical value of 15 to 25 mNm^{-1} depending on the lipid composition (Giehl et al. 1999). Even if no data are available on the lateral pressure of intra-lysosomal vesicles, the combination of size and composition can be expected to lower this pressure below this critical value.

Functional Aspects of Membrane Lipid Sorting in the Endocytic Pathway

High amounts of BMP and low amounts of membrane-stabilizing cholesterol in internal lysosomal membranes appear to be required for the degradation of glycosphingolipids. The presence of BMP in these vesicles increases the ability of the GM2-activator to solubilize lipids (Werth et al. 2001). In addition, negatively charged lysosomal lipids drastically stimulate the interfacial hydrolysis of membrane-bound ganglioside GM1 by GM1-β-galactosidase (Wilkening et al. 2000); ganglioside GM2 by β-hexosaminidase A (Werth et al. 2001); the sulfated gangliotriaosylceramide SM2 by β-hexosaminidases A and S (Hepbildikler et al. 2002) in the presence of the GM2-activator protein; sphingomyelin by acid sphingomyelinase (Linke et al. 2001a); and ceramide by acid ceramidase (Linke et al. 2001b). Furthermore, in the presence of Sap-C, a drastic enhancement of glucosylceramide degradation by glucosylceramide-β-glucosidase is produced by negatively charged model lipids such as phosphatidylserine, phosphatidylglycerol, and phosphatidic acid (Berent & Radin 1981, Sarmientos et al. 1986, Salvioli et al. 2000).

Taken together, degradation of an individual (glyco)sphingolipid (**Figure 2**) embedded in intra-lysosomal membrane structures requires the appropriate combination of hydrolytic enzyme, activator protein, and lysosomal lipid. This ensures that substrates are cleaved only when they are part of membranes destined for degradation.

Inner lysosomal membranes represent the main site of membrane degradation in eukaryotic cells. Their specific lipid profile, low cholesterol, and high BMP content ensure their degradation by water-soluble hydrolases and membrane-perturbating SAPs without affecting the limiting membrane.

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