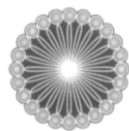# Topology of glycosphingolipid degradation

Konrad Sandhoff and Thomas Kolter

Glycosphingolipids (GSLs) form cell-type-specific patterns on the surface of eukaryotic cells. Degradation of plasma-membrane-derived GSLs in the lysosomes after internalization through the endocytic pathway is achieved through the concerted actions of hydrolysing enzymes and sphingolipid activator proteins. The latter are proteins necessary for the degradation of GSLs possessing short oligosaccharide chains. Some activator proteins bind to GSLs and form water-soluble complexes, which lift out of the membrane and give the water-soluble hydrolysing enzymes access to the regions of the GSL that would otherwise be obscured by the membrane. The inherited deficiency of both lysosomal hydrolases and sphingolipid activator proteins gives rise to sphingolipid storage diseases. An analysis of these diseases suggests a new model for the topology of endocytosis and lysosomal digestion, which is discussed in this article.

Glycosphingolipids (GSLs) are components of the plasma membrane of eukaryotic cells[1]. Each GSL contains a hydrophobic ceramide moiety, which acts as a membrane anchor, and a hydrophilic, extracellular oligosaccharide chain. Variations in the type, number and linkage of sugar residues in the oligosaccharide chain give rise to the wide range of naturally occurring GSLs. These form cell-type-specific patterns at the cell surface that change upon cell growth, differentiation, viral transformation and oncogenesis[2]. GSLs interact at the cell surface with toxins, viruses and bacteria, as well as with membrane-bound receptors and enzymes[3]. They are also involved in cell-type-specific adhesion processes, and, in addition, lipophilic products of GSL metabolism, such as sphingosine and ceramide, play a role in signal-transduction events[4]. Finally, GSLs form a protective layer on biological membranes, protecting them from inappropriate degradation.

The biosynthesis[5] of sphingolipids takes place in the endoplasmic reticulum (ER) and the Golgi apparatus. The initial steps, converting L-serine into dihydroceramide, occur at the cytosolic face of the ER. At present, the exact subcellular localization of the desaturation step leading from dihydroceramide to ceramide is not known. Ceramide is then modified by the addition of a phosphorylcholine moiety to form sphingomyelin or by the stepwise addition of sugar residues to form glycosphingolipids. The formation of sphingomyelin occurs at the lumenal face of the Golgi apparatus. In glycosphingolipid synthesis, the first glucosyl residue is added at the cytosolic face of the Golgi apparatus[6]. The stepwise introduction of other sugar residues seems to be restricted to the lumenal face of Golgi membranes. The products of these reactions are then transported to the plasma membrane by exocytosis.
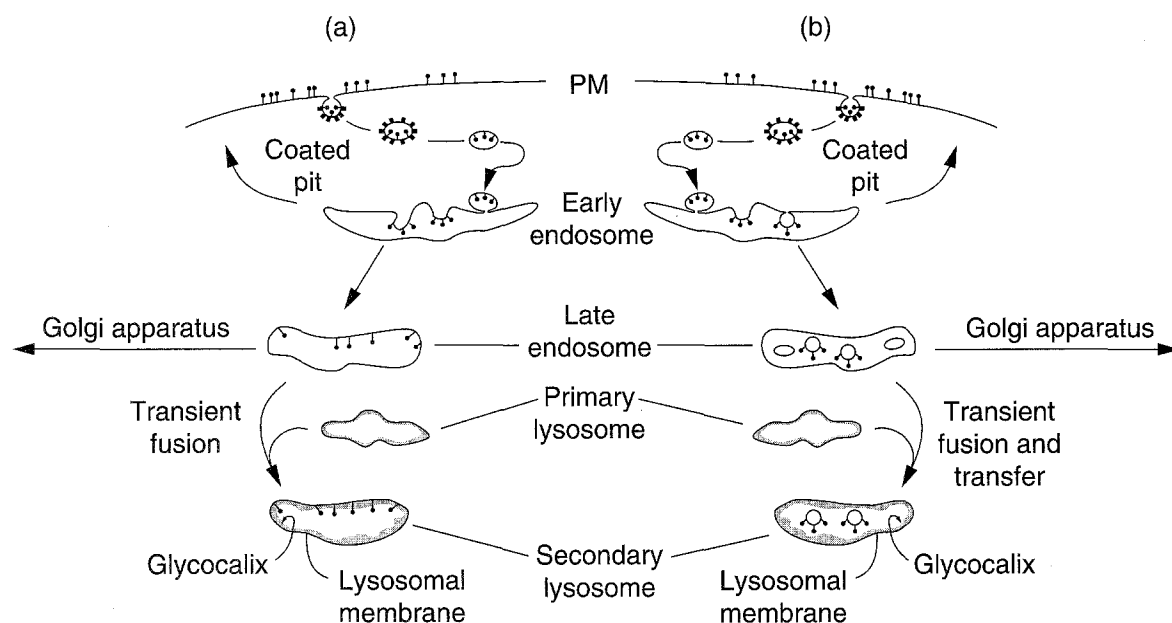
Degradation of GSLs occurs in lysosomal compartments within the cell[7]. Fragments of the plasma-membrane-containing GSLs destined for degradation are endocytosed and traffic through the endosomal compartments to the lysosome. Here, hydrolysing enzymes cleave sugar residues sequentially to produce ceramide, which is deacylated to sphingosine. This can leave the lysosome and re-enter the biosynthetic pathway or can be degraded further. To date, all characterized, inherited diseases of GSL metabolism affect GSL degradation, and none has been described that affects biosynthesis of GSL. Presumably, severe disorders affecting GSL biosynthesis would result in incorrect GSL patterns on the cell surface, and these in turn would affect morphogenesis and embryogenesis and result in embryonic lethality. A study of the inherited disorders in GSL degradation, the lysosomal storage diseases, has provided insights into the processes involved in GSL degradation.

Topology of endocytosis and lysosomal digestion

Catabolism of complex GSLs derived from the plasma membrane occurs in the lysosomes after endocytosis. In the conventional model, components of the plasma membrane reach the lysosomal compartment by endocytic membrane flow via the early and late endosomes (Ref. 8; Fig. 1). During this vesicular flow, molecules in the membrane are subjected to a sorting process that directs some of the molecules to the lysosomal compartment, others to the Golgi apparatus and yet others back to the plasma membrane[9-12]. After a series of vesicle-budding and -fusion events through the endosomal compartment, the membrane fragments reach the lysosomal compartment. As a consequence of endocytic membrane flow, constituents of the plasma membrane are incorporated into the lysosomal membrane. Lysosomal degradation of former components of the plasma membrane must then proceed selectively within the lysosomal membrane, without destroying it. It is difficult to see how this could occur, especially as the inner leaflet of the lysosomal membrane is covered with a thick glycocalix composed of glycoproteins, the so-called limps (lysosomal integral membrane proteins) and lamps (lysosomal associated membrane proteins)[13].

In an alternative model for the topology of endocytosis (Ref. 14; Fig. 1), components of the plasma membrane pass through the endosomal compartment as intraendosomal vesicles that become intralysosomal vesicles on reaching the lysosomes. The vesicles are formed initially in the early endosome

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(a)   (b)

PM

Coated   Coated
pit   pit

Early
endosome

Golgi apparatus   Late   Golgi apparatus
                       endosome

Transient   Primary   Transient
fusion   lysosome   fusion and
                                        transfer

Glycocalix   Secondary   Glycocalix
        Lysosomal   lysosome   Lysosomal
        membrane   membrane

FIGURE 1

Two models for the topology of endocytosis and lysosomal digestion of glycosphingolipids (GSLs) derived from the plasma membrane. (a) Conventional model: degradation of GSLs derived from the plasma membrane occurs selectively within the lysosomal membrane. (b) Alternative model: during endocytosis, glycolipids of the plasma membrane become incorporated into the membranes of intraendosomal vesicles (multivesicular bodies). The vesicles are transferred into the lysosomal compartment when the late endosome fuses with primary lysosomes. PM, plasma membrane; ↑, glycosphingolipid.

by selective invagination (budding-in) of endosomal membranes enriched for components of the plasma membrane. The surrounding endosome then passes along the endocytic pathway by way of the normal, successive events of membrane fission and fusion. The intraendosomal vesicles, however, are carried along as passengers and, normally, do not undergo fusion and fission. When the vesicles reach the lysosome, glycoconjugates originating from the outer leaflet of the plasma membrane face the lysosol on the outer leaflet of intralysosomal vesicles and, thus, are in the correct orientation for degradation. According to this model, there is no glycocalix blocking the action of hydrolases. This hypothesis is supported by a series of observations:
• Multivesicular bodies are observed at the level of the early and late endosomal reticulum[12,15–17].
• The epidermal growth factor receptor derived from the plasma membrane and internalized into lysosomes of hepatocytes is not integrated into the lysosomal membrane[18].
• Spherical multivesicular bodies are the predominant endocytic compartments in HEp-2 cells. Moreover, these multivesicular bodies mature within 60 to 90 min into lysosomes that still contain internal vesicles[19].
• Multivesicular storage bodies accumulate in cells from patients with sphingolipid storage diseases. They were observed almost 30 years ago in cerebral cells from patients with GM1 gangliosidosis[20]. Multivesicular storage bodies also occur in cells (e.g. Kupffer cells and fibroblasts) of a patient with another sphingolipid storage disease – giving a combined activator protein deficiency[21,22]. Analysis of cultivated fibroblasts taken from this patient shows

that the affected storage vesicles are in the late endosomal or lysosomal compartments. These compartments are still functionally active, except that they are unable to degrade sphingolipids that have short oligosaccharide head groups. After complementation of the medium of these cells with the missing precursor of sphingolipid activator protein, not only is the degradation block abolished, but also the number and size of the storage organelles are reduced (J. K. Burkhardt, S. Hüttler, A. Klein, W. Möbius, A. Habermann, G. Griffiths and K. Sandhoff, unpublished).

To date, budding-in of vesicles at the early endosome stage has not been observed by microscopy. This membrane fission event is thought to occur since it would explain both the occurrence of intralysosomal vesicles and the selective degradation of GSLs derived from the plasma membrane, in contrast to those of the lysosomal membrane.

Within the lysosome, degradation of GSLs occurs by the stepwise action of specific acid exohydrolases (Ref. 7; Fig. 2). More than ten different exohydrolases are involved in this process. If any of these enzymes is deficient, the corresponding lipid substrate accumulates and is stored in the lysosomal compartment. In the inherited sphingolipid storage diseases, accumulation of lipids occurs mainly in those cell types and organs in which the lipids are predominantly synthesized. In Tay–Sachs disease, for example, β-hexosaminidase A is deficient. This causes accumulation of the ganglioside GM2 in neuronal cells, the main sites for synthesis of gangliosides (sialic acid-containing GSLs). In this autosomal recessive, lethal disease, the neuronal cells swell and eventually die[23,24].
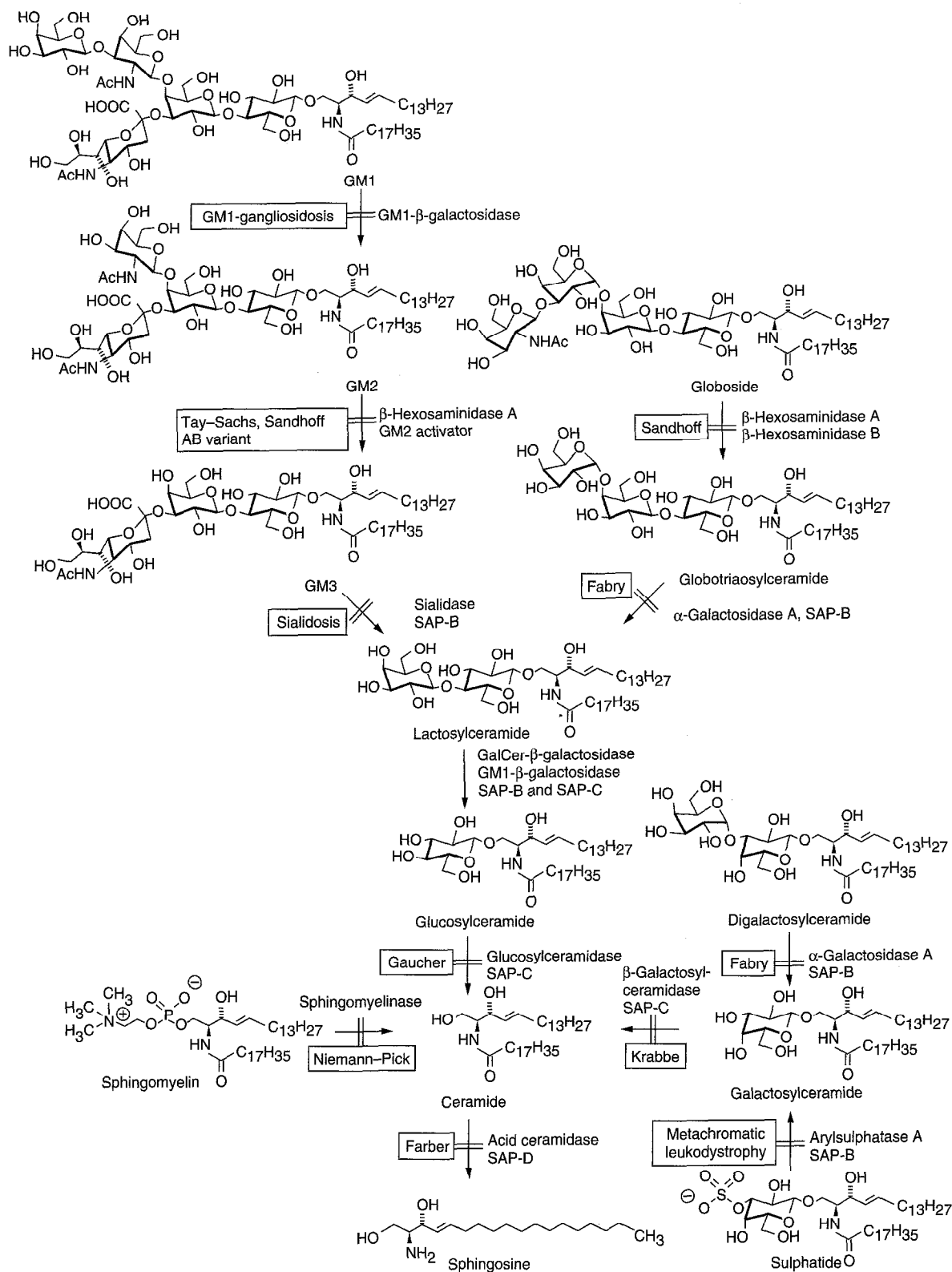
GM1

GM1-gangliosidosis — GM1-β-galactosidase

GM2

Globoside

Tay–Sachs, Sandhoff AB variant — β-Hexosaminidase A / GM2 activator

Sandhoff — β-Hexosaminidase A / β-Hexosaminidase B

GM3

Globotriaosylceramide

Sialidosis — Sialidase / SAP-B

Fabry — α-Galactosidase A, SAP-B

Lactosylceramide

GalCer-β-galactosidase
GM1-β-galactosidase
SAP-B and SAP-C

Glucosylceramide

Digalactosylceramide

Gaucher — Glucosylceramidase / SAP-C

Fabry — α-Galactosidase A / SAP-B

β-Galactosyl-ceramidase SAP-C

Krabbe

Galactosylceramide

Sphingomyelinase

Sphingomyelin

Niemann–Pick

Ceramide

Farber — Acid ceramidase / SAP-D

Metachromatic leukodystrophy — Arylsulphatase A / SAP-B

Sphingosine

Sulphatide

FIGURE 2

Lysosomal sphingolipid degradation (modified, with permission, from Ref. 7). Sphingolipid activator proteins, exohydrolases and the eponyms of known storage diseases are shown. AB variant, AB variant of GM2 gangliosidosis (deficiency of GM2 activator protein); SAP, sphingolipid activator protein. Heterogeneity within the ceramide moiety due to varying degrees of saturation, hydroxylation and chain length is not shown.

For glycosphingolipids with carbohydrate chains of more than four sugar residues, the presence of an enzymatically active exohydrolase is sufficient for degradation *in vivo*. However, detailed biochemical analysis of sphingolipidoses, such as metachromatic leukodystrophy and Tay–Sachs disease, demonstrate that the *in vivo* degradation of some glycolipids involves not only exohydrolases but also a second

type of effector molecule[25]. In these cases, lipid storage is observed in patients without any enzyme deficiency, but deficient in a protein cofactor. The degradation of membrane-bound GSLs possessing short oligosaccharide chains requires the cooperation of an exohydrolase and a SAP (for sphingolipid activator protein). By using two components in this way, the plasma membrane may be protected from inappropriate degradation. Any missorted hydrolases that end up in the extracellular space, albeit at a low concentration, are prevented from damaging the cell by the low extracellular concentration of lysosomal activator proteins. Furthermore, the neutral pH on the cell surface is not optimal for lysosomal hydrolases as they mainly require a low pH for full activity. Several SAPs are now known, including the GM2 activator and the saposins. A detailed study of these proteins has illuminated some of their main features and has indicated the mechanisms by which they facilitate digestion of GSLs in the lysosome.

The GM2 activator and its role in lysosomal digestion

The enzymatic degradation of ganglioside GM2, the main storage material in Tay–Sachs disease, requires β-hexosaminidase A and a lysosomal ganglioside-binding protein, the GM2 activator[26]. The GM2 activator binds ganglioside GM2, as well as related gangliosides, and forms water-soluble complexes (mostly in a 1:1 molar ratio). It functions *in vitro* as a ganglioside-transfer protein, transferring gangliosides from donor membranes to acceptor membranes. The GM2 activator behaves as a 'liftase', recognizing GM2 within the membrane and, by binding to it, lifting the lipid out of the bilayer and presenting it to the water-soluble β-hexosaminidase A for degradation (Fig. 3, mode 1). However, it is also possible that the activator–lipid complex leaves the membrane and the enzymatic reaction takes place in free solution (Fig. 3, mode 2). It is unknown whether the GM2 activator acts *in vivo* according to mode 1 or mode 2, as depicted in Figure 3. Also, formation of the ternary complex presumably involves a protein–protein interaction between the GM2 activator and β-hexosaminidase A (Ref. 27).

Point mutations within the structural gene encoding the GM2 activator have been identified in two patients with the AB variant of GM2 gangliosidosis (reviewed in Refs 24 and 25). The mutated proteins are proteolytically labile, resulting in some of them being degraded in the ER and others in the Golgi compartment. The resulting loss of the GM2 activator causes a block to GM2 degradation in the lysosomes, as demonstrated in metabolic studies of cultivated fibroblasts taken from the patients. The block can be bypassed by feeding native or recombinant GM2 activator to the culture medium of the mutant fibroblasts[28].
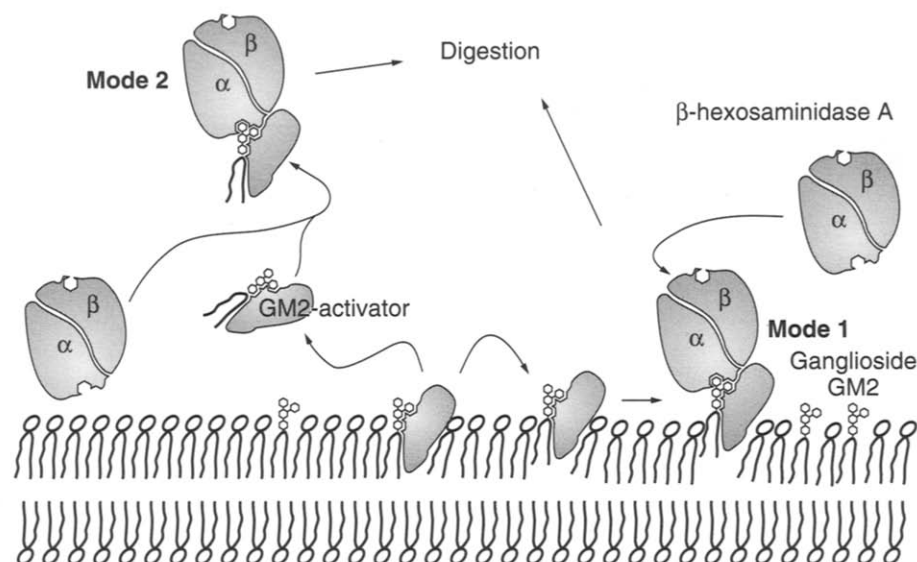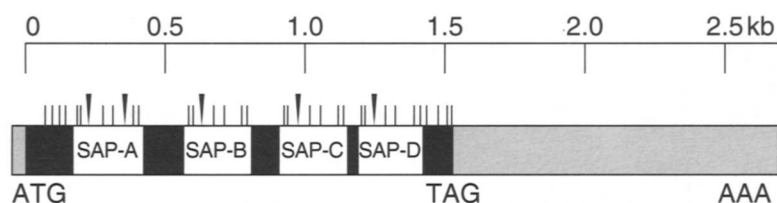

FIGURE 3

Model for the GM2-activator-stimulated degradation of ganglioside GM2 by human β-hexosaminidase A (modified, with permission, from Ref. 14). Water-soluble β-hexosaminidase A does not degrade membrane-bound ganglioside GM2, which has a short carbohydrate chain, in the absence of GM2 activator or appropriate detergents. The GM2 activator binds one molecule of ganglioside GM2 and lifts it a few angstroms out of the membrane. In this position, this activator–lipid complex can be reached and recognized by water-soluble β-hexosaminidase A, which cleaves the substrate (mode 1). Alternatively, the water-soluble activator–lipid complex may leave the membrane completely and the enzymatic reaction may take place in free solution (mode 2).

Other activator proteins

The first activator protein was identified in 1964 as a protein that is necessary for the hydrolytic degradation of glycosphingolipids carrying a sulphuric ester group (sulphatides) by lysosomal arylsulphatase A (Ref. 29). This sulphatide activator, SAP-B (saposin B), is a small, lysosomal glycoprotein consisting of 80 amino acids, with an N-linked carbohydrate chain and three disulphide bridges[30]. Like the GM2 activator, it binds GSLs but has a broader specificity. In some respects, it behaves similarly *in vitro* to the GM2 activator; that is, it recognizes and binds several different GSLs on the surface of micelles by forming a stoichiometric complex and then transfers them to the membranes of acceptor liposomes[14,25]. SAP-B can also present GSLs as substrates to water-soluble enzymes[31]. The inherited deficiency of the sulphatide activator leads to a lysosomal storage disease that resembles metachromatic leukodystrophy. However, unlike typical metachromatic leukodystrophy, not only sulphatide, but also additional glycolipids (e.g. globotriaosyl ceramide) accumulate owing to a blockage of degradation at several points in the catabolic pathway (Ref. 25; Fig. 2).

The sulphatide activator has sequence homology with three further activator proteins, or saposins: the Gaucher factor (SAP-C), SAP-A and SAP-D. Molecular-biological analysis has revealed that these four activator proteins are formed by proteolytic processing of a common precursor protein, the SAP precursor, or prosaposin (Refs 32–35; Fig. 4). The domain structure of the precursor protein suggests that the activators may have evolved by gene duplication. The

SAP-A   SAP-B   SAP-C   SAP-D

ATG   TAG   AAA

FIGURE 4

Structure of the cDNA for SAP precursor (modified, with permission, from Ref. 14). The cDNA encodes a sequence of 524 amino acids (or 527 amino acids, see Ref. 43), including a signal peptide of 16 amino acids. The four domains on the precursor, SAP-A, -B, -C and -D (Ref. 34), correspond to the mature proteins found in human tissues. SAP-A is also known as saposin A; SAP-B is also known as saposin B, SAP-1 or sulphatide activator; SAP-C is also known as SAP-2, saposin C or glucosylceramidase activator protein; SAP-D is also known as saposin D or component C. The positions of the cysteine residues are marked by vertical bars and the positions of the N-glycosylation sites by arrowheads.

four activator proteins, SAP-A, -B, -C and -D show homology to each other and have some similar properties, but differ in their function and their mechanism of action[14,25]. Their physiological functions are only partially clarified to date; most of our knowledge of them has emerged from studies of patients with atypical lipid storage diseases. A deficiency of SAP-C causes a juvenile variant of Gaucher disease, in which glucosyl ceramide accumulates in spite of normal levels of glucosylceramidase[36,37]. In one patient with a complete deficiency of the whole SAP-precursor protein caused by a homoallelic mutation within the start codon (changing AUG to UUG; Ref. 22), there is simultaneous storage of many sphingolipids, including ceramide, glucosylceramide, lactosylceramide, ganglioside GM3, galactosylceramide, sulphatides, digalactosylceramide and globotriaosylceramide (Ref. 38; see Fig. 2 for structures).

Functional analysis of sphingolipid activator proteins

The storage of GSLs can be demonstrated in cultivated mutant cells by pulse–chase experiments[39]. Storage substances in cells from different patients can be visualized by biosynthetic labelling of glycolipids with [14C]serine, followed by long chase periods. As previously mentioned, the glycolipids that accumulate reflect the types of GSLs synthesized in each cell type. In the case of fibroblasts, glucosylceramide accumulates in cells from patients deficient in SAP-C, ganglioside GM2 accumulates when there is deficiency of the GM2 activator, and ceramide, glucosylceramide, lactosylceramide and ganglioside GM3 all accumulate when the SAP precursor is deficient. Cell-culture systems, such as these, are not only suitable for diagnostic purposes, they also allow the elucidation of the function of the different activator proteins. Although the four SAPs are highly homologous, their differences are revealed in these experiments. If purified SAP-B is fed to the cells deficient in SAP precursor, the storage of lactosylceramide is abolished, but ceramide and glucosylceramide still accumulate. By contrast, when SAP-D is fed to the cells, ceramide accumulation is prevented. These experiments give the first hint to the

in vivo function of this activator protein[39]. As expected, if the mutant cells are fed with the SAP precursor in nanomolar concentrations, storage of all these glycolipids is abolished and the aberrant accumulation of intralysosomal membrane structures is prevented.

These studies on cultured cells from patients with different storage diseases support our hypothesis concerning the function of SAPs. In their absence, membrane-bound GSLs with short oligosaccharide chains are not readily accessible for digestion by water-soluble exohydrolases. By analogy to a razor blade, the hydrolases can only attack oligosaccharide chains of GSLs that protrude far enough from the membrane into the aqueous phase. This is no problem for long-chain oligosaccharides, but, for the degradation of GSLs with short sugar chains, the assistance of activator proteins is required. Some of them act as binding proteins or liftases, forming a water-soluble complex with the lipid and hence raising the lipid out of the membrane. However, the function of activator proteins is not necessarily restricted to binding lipids in this way. For example, SAP-C can activate directly glucosylceramide β-glucosidase (Ref. 40) and the GM2 activator interacts specifically with β-hexosaminidase A (Ref. 27).

The simultaneous deficiency of the four SAPs, as seen in the patient described earlier, causes an intraendosomal and intralysosomal accumulation of vesicles and other membrane fragments (multivesicular bodies) and supports our view of the topology of endocytosis and lysosomal digestion. Furthermore, the occurrence of storage bodies in the combined activator protein deficiency suggests a possible function of glycolipids. The increased concentrations of glycolipids within distinct membranes appears to protect the vesicles from degradation by phospholipases, proteases and other hydrolases that occur in high concentration in the lysosomes. In general, glycoconjugates form a protective layer on the anticytosolic face of biological membranes, protecting them from enzymatic degradation. Specific interaction may also play a role in protecting biological membranes; for example, at a molar concentration of >25%, ganglioside GM1 protects phospholipid vesicles completely from the degradation by phospholipase A2 (Ref. 41). The protective effect of GM1 on neuronal membranes may explain how treatment of patients who have Alzheimer's disease type II with ganglioside GM1 can partially attenuate the disease progression[42].

Future directions

Future research will concentrate on elucidating both the fine details of the endocytic pathway for plasma membrane fragments containing GSLs and the mechanism of action of the SAPs. How does the cell decide whether membrane components join the conventional endocytic pathway or become incorporated into an intralysosomal vesicle? How do the activator proteins interact with the glycolipids, does the activator enter the membrane or does the interaction occur at the surface? Is activator action dependent on physical disturbance of biological membranes?

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These and other questions will be tackled by using cultured cells from human patients and also by studying the knockout mouse mutants that are currently being developed. Finally, a better understanding of the mode of action and other aspects of glycosphingolipid degradation will hopefully lead to therapeutic advances in the treatment of lipid storage diseases.

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