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Review

Lysosomal degradation of membrane lipids

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ABSTRACT

The constitutive degradation of membrane components takes place in the acidic compartments of a cell, the endosomes and lysosomes. Sites of lipid degradation are intralysosomal membranes that are formed in endosomes, where the lipid composition is adjusted for degradation. Cholesterol is sorted out of the inner membranes, their content in bis(monoacylglycero)phosphate increases, and, most likely, sphingomyelin is degraded to ceramide. Together with endosomal and lysosomal lipid-binding proteins, the Niemann–Pick disease, type C2-protein, the GM2-activator, and the saposins sap-A, -B, -C, and -D, a suitable membrane lipid composition is required for degradation of complex lipids by hydrolytic enzymes.
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1. The stomach of the cell

Within the acidic compartments, the endosomes and lysosomes, macromolecules, complex lipids, and oligosaccharides are degraded into their building blocks by hydrolytic enzymes [1]. The resulting catabolites are exported from the lysosome and re-used in cellular metabolism. This export is mediated by transport proteins present in the limiting lysosomal membrane [2,3]. In general, these transporters make use of the energy conserved in the proton gradient along the perimeter membrane and co-transport small molecules or ions together with protons. Many transporters, among them those for amino acids, cystine, dipeptides, inorganic ions, sialic acids, and inositol, have been characterized. Only recently, transport proteins for hexoses (GLUT-8) [4,5] and for cobalamin [6] were identified. Cobalamin, essential for degradation of branched chain amino acids, odd chain fatty acids, and C1-metabolism, is delivered to the cell via the lysosomes, which illustrates the role of lysosomes for cellular nutrition.

Soon after their recognition, lysosomes have been regarded as a kind of intracellular stomach that provides the cell with nutrients. Not only cobalamin-bound cobalt-ions, also other metal ions like iron ions [7], which are essential for many cellular processes from oxygen-transport to respiration are delivered or recycled through the endo/lysosomal compartment. While several of the transport proteins for the degradation products of water-soluble macromolecules have been identified, together with their role in human diseases such as cystinosis and Salla-disease (sialic acid transporter deficiency), putative transport proteins for the final degradation products of membrane lipids, fatty acids and sphingosine, are not known. These substances serve not only as building blocks in membrane recycling, but can also be further degraded in the cytoplasm, or can be receptor ligands or precursors thereof and participate in the regulation of cell function. In dependence of their degree of saturation, fatty acids are ligands of nuclear receptors [8] and sphingosine-1-phosphate [9] derived from sphingosine acts as a first and second messenger.

About 25 proteins that reside within the limiting lysosomal membrane have been characterized in structural and functional terms, including pathobiochemical aspects [10,11], but their function is not known in every case. In addition, about 60 proteins, which are present in the lumen of the lysosome, are known, many of them for a long time, also because of their role in monogenic

Abbreviations: BMP, bis(monoacylglycero)phosphate; ESCRT, endosomal sorting complexes required for transport; FRET, fluorescence or Förster resonance energy transfer; GM1, GM2, GM3, ganglioside-nomenclature according to Svennerholm, compare Fig. 1 for structures; GSL, glycosphingolipid; LLBPs, lysosomal lipid-binding proteins; MVBs, multivesicular bodies; NBD, 4-nitrobenzo-2-oxa-1,3-diazol; NPC1, Niemann–Pick disease, type C1-protein; NPC2, Niemann–Pick disease, type C2-protein; ORP, oxysterol binding protein-related protein; PAF, platelet activating factor; PtdIns, phosphatidylinositol; sap, saposin; SAP, sphingolipid activator protein (GM2-activator, sap-A–sap-D); SCP-2, sterol carrier protein-2; *sn*1, *sn*2, stereospecific numbering of glycerolipids according to IUPAC

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human diseases that result from the deficiency of one of them. Recently, new lysosomal proteins have been discovered in proteomic studies [12]. Among them are the *N*-acylsphingosine amidohydrolase, an acid ceramidase-like protein that acts as a *N*-acylethanolamine-hydrolyzing acid amidase, ribonucleases 1, 6 and T2, and proteins of currently unknown function such as acid sphingomyelinase-like phosphodiesterase 3a, arylsulfatases G and K, or mannose-6-phosphate protein P76.

2. pH-value

A characteristic feature of the acidic compartments is the low pH of endosomes (pH 5–6) and lysosomes (pH 4.6–5) [13]. This pH is generated by the action of V-type ATPases and serves various functions [14]. For example, after receptor-mediated endocytosis, internalized cargo such as LDL, but not transferrin, is released from its receptor, which, in turn, is recycled to the plasma membrane or – together with other proteins of the plasma membrane – sorted to intraluminal vesicles by a complex molecular machinery and then degraded in the lysosomes [15]. Soluble proteins that are targeted in the TGN to lysosomes dissociate at the acidic pH from their receptor, usually a mannose-6-phosphate receptor, but also, in the case of glucosylceramide-beta-glucosidase, the membrane protein LIMP-2 [16]. Also, the maturation from endosomes to lysosomes, and the formation of intraluminal membranes at the stage of late endosomes, the multivesicular body, requires acidic pH (and calcium ions) [17]. The lysosomal pH is also required not only for optimal activity of hydrolytic enzymes in the lumen, but can contribute to their stability in the presence of proteolytic enzymes: due to their isoelectric points, some soluble lysosomal enzymes such as acid sphingomyelinase are positively charged in the acidic environment of the lysosome. As polycations, they adhere to the surface of intralysosomal membranes that bear a negative charge due to the presence of the acidic lysosomal lipid bis(monoacylglycero)phosphate (BMP; Fig. 1). In this form, they are protected from attack by acid hydrolases. Their release from these membranes by cationic amphiphilic drugs like desipramine (Fig. 1) leads to their premature degradation [18,19] in the case of acid sphingomyelinase with the consequence of a drug-induced lipidosis. Compared to lysosomes, the higher pH of early, but not of late endosomes can be attributed to the action of Na,K-ATPase, which, like V-ATPase, is electrogenic, contributes to the positive potential of the lumen and thereby limits proton influx [20]. Other factors such as regulation of V-ATPase by its membrane environment, chloride channels, and luminal buffer systems might additionally account for the lower acidification of endosomes [21] and the vacuole of yeast [22]. In cell culture, endosomal pH can vary between different cell types, and also values below pH 4 are theoretically possible [23].

3. Ions from the outside: iron

Together with the peptide portion of many proteins, metal ions such as iron [24], copper [25], and zinc are recycled during autophagy in the lysosomes or taken up from the outside. Zinc and copper ions can be bound to lysosomal metallothionein from which they are released during acidification. Non-dividing cells can meet their requirement for iron ions largely by degradation of iron-containing proteins after autophagy. For this reason, and also for the removal of aberrant proteins, autophagy is a vital process and required for the survival especially for neuronal cells, as indicated by the observation that loss of Atg7, a gene required for autophagy, leads to rapid neurodegeneration in mice [26].

Exogenous supply of iron ions and their delivery through the endolysosomal system is essential for dividing cells. After uptake of transferrin, the decrease of the endosomal pH leads to the release of iron ions from receptor-bound transferrin. These ions leave the endolysosomal compartment via the divalent metal-transporter-1 to the cytoplasm or via membrane contacts with the outer mitochondrial membrane. Regulation of the amount of ferritin and

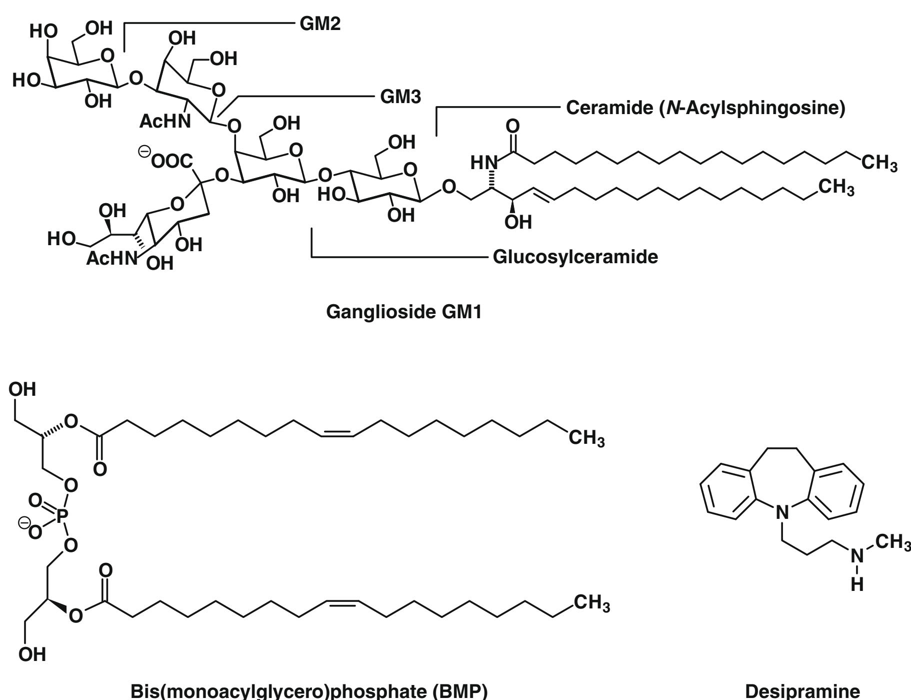

Fig. 1. Structures of selected glycosphingolipids (gangliosides GM1, GM2, GM3, glucosylceramide, ceramide), of bis(monoacylglycero)phosphate (BMP), and desipramine. Please note that the acyl residues are on the *sn*2-positions of both glycerol backbones according to the proposed biosynthesis of BMP [67], but that acyl migration is known to occur from the *sn*2- to the *sn*3-position.

transferrin-receptor by cytoplasmic iron concentrations ensures minimal levels of free iron ions to prevent the formation of hydroxyl radicals via the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot} \quad (1)$$

While the primary reduction product of dioxygen, the hyperoxide (superoxide) radical $O_2^{-\cdot}$, is not particularly reactive, the OH-radical is one of the most reactive free radical species known. Hydrogen peroxide required for the Fenton reaction is produced by respiratory chain enzymes or oxidoreductases and is present at low concentrations. The presence of reducing agents in the lysosol favors Fe(II) formation at acidic pH. Hemosiderin, apparently an intralysosomal aggregation- and degradation product of ferritin, and eventually also of other iron-rich non-degradable proteins within lipofuscin accumulate, so that certain lysosomal populations can develop sensitivity towards oxidative stress. This can lead to oxidation and misfolding of proteins, to peroxidation of lipids present in the limiting membrane with subsequent leakage, to impaired autophagy, and might constitute a factor for ageing [27]. Oxidative stress mediated by iron ions might account for the observation that tissues from Niemann–Pick disease, type C1-protein (NPC1) knockout mice show increased levels of potentially atherogenic cholesterol autoxidation products [28] with hydroxy groups in positions 5, 6, or 7, while the levels of enzymatically formed 27-hydroxycholesterol are decreased [29].

4. Lysosomal diseases

Defects in enzymes and proteins required for lysosomal catabolism lead to inherited diseases, the lysosomal storage diseases. They can be classified according to the stored substances, as sphingolipidoses, mucopolysaccharidoses, mucolipidoses, glycoprotein- and glycogen storage diseases [30,31]. Impairment of membrane degradation is especially visible in defects of sphingolipid and glycosphingolipid catabolism. In contrast to other lipid classes, a whole group of diseases is known, which is caused by defects in this pathway. A reason for this is that glycosphingolipids are degraded in a sequential manner. Understanding of pathogenesis [32], and improvements in diagnosis and therapy of sphingolipidoses [33] is an ongoing challenge. Investigation of human patients [34], genetically engineered mammals [35], and other organisms [36] allowed insight into various aspects of lysosomal catabolism, including membrane metabolism and transport. A characteristic feature of several sphingolipidoses is that the primary defect leads to incomplete degradation of complex lipids. Due to their insolubility, they co-precipitate other hydrophobic substances, lipids and proteins, as secondary storage products [37]. This kind of traffic jam [38,39] occurs, e.g., in Niemann–Pick disease, type C, in the sense that NPC1 dysfunction leads to the accumulation not only of cholesterol, but also of sphingomyelin, glycosphingolipids, sphingosine, and others in multilamellar storage bodies [40]. Sphingosine causes also calcium depletion and a subsequent defect of endocytosis, as demonstrated for NPC1-disease, in which sphingosine accumulation appears to be one of the first measurable effects after induction of the disease [41].

Traffic jam can impair lysosomal function, such as nutrient delivery through the endolysosomal system. For example, iron homeostasis is impaired in animals of two glycolipid storage diseases, the GM1- and the 0-variant of the GM2 gangliosidoses, Sandhoff-disease. Supplementation of the animals with iron ions improved their condition and increased their life expectancy by nearly 40% [42]. This illustrates the role of the endolysosomal system as a stomach and adds another factor that contributes to the pathogenesis of these diseases. Another observation of relevance for both the understanding of lysosomal storage diseases and the function of lysosomes is that autophagy can be blocked as a general feature of lysosomal storage or, alternatively, can be increased, e.g., investigation of mouse models of Multiple Sulfatase Deficiency and Mucopolysaccharidosis, type IIIA, showed accumulation of polyubiquitinated proteins and of dysfunctional mitochondria. Neurodegeneration in storage diseases and in unrelated late-onset neurodegenerative disorders might share altered autophagy as common mechanism [43,44].

Most lysosomal storage diseases are caused by the primary defect of a lysosomal protein. However, defects in proteins of non-lysosomal localization can also lead to a lysosomal disease, which is the case in multiple sulfatase deficiency, mucolipidosis II/III, and Niemann–Pick disease, type C1, a defect in endosomal cholesterol efflux [45]. In addition, several developmental disorders with neurodegenerative phenotypes can be caused by defects in functionally unrelated enzymes, which affect lysosomal function, such as Alzheimer, Parkinson, Huntington, and Down syndrome. Accordingly, the endolysosomal system has been called a "genetic hotspot" for neurodegenerative diseases [46]. Accumulation and abnormal processing of the Amyloid precursor protein, a feature characteristic for Alzheimer disease, occurs also in NPC-disease, type 1, which indicates that disturbance of endosomal membrane turnover leads to an Alzheimer-like pathology [47].

5. Lysosomal degradation of membranes

Membranes are essentially composed of amphiphilic lipids and proteins. Complex lipids such as glycerolipids and sphingolipids as well as proteins have to be degraded within the process of endosomal/lysosomal membrane digestion, so that the final degradation products can leave the compartment. While soluble macromolecules such as proteins, glycoproteins, or oligosaccharides can be degraded directly by soluble enzymes, the degradation of membranes requires a much more complex disintegration and degradation system [48]. Lysosomal digestion of membranes is essential for cellular membrane homeostasis. Breakdown-products of glycosphingolipids are continuously recycled and re-utilised in salvage processes [49].

In late endosomes, membrane proteins and lipids targeted for degradation undergo a sorting process to intraendosomal membranes, which are degraded in the lysosomes, while the integrity of the lysosomal limiting membrane has to be preserved. However, pathophysiological factors can lead to the permeabilization of the lysosomal perimeter membrane: Higher concentrations of the membrane-active substances fatty acids and sphingosine can lead to damage of the perimeter membrane and subsequent necrosis or apoptosis [50]. Therefore, the release of these hydrophobic products of bulk lipid degradation has to proceed in a controlled fashion. This is achieved by a buffering mechanism according to which these hydrophobic substances are liberated within a twodimensional lipid environment in a controlled fashion. Soluble enzymes lead to their release in a reversible way in the sense that they catalyze also the corresponding back-reaction: Acid ceramidase cleaves the degradation product of most sphingolipids and glycosphingolipids, ceramide, into sphingosine and fatty acid in a reversible way. If the concentrations of these cleavage products are high, the reverse reaction can be catalyzed, since both components are present in the bilayer and diffusion into the lysosol is not fast enough [51]. Especially the accumulation of the cationic amphiphile sphingosine would impair other lysosomal degradation steps, presumably in a similar manner as by the cationic amphiphile desipramine (see above). A similar effect is exerted by acid phospholipase A2, the enzyme required for the lysosomal degradation of glycerolipids: this enzyme also catalyzes the reverse reaction, the formation of glycerophospholipid from its

lysolipid and fatty acid. It is also able to acylate other hydrophobic alcohols present in intralysosomal membranes, for example ceramide [52], but also exogenously added alcohols [53].

Destabilization of the lysosomal membrane can be associated with oncogenic transformation. Its permeabilization in response to TNFalpha can occur in a caspase-dependent and also -independent way with the consequence of apoptotic cell death [54].

A view on the molecular mechanisms of membrane digestion reveals a complex machinery composed of lipids of inner lysosomal membranes and lysosomal proteins. This system ensures selective degradation of inner membranes (review: [48,55]).

6. Entry pathways

Components of cellular membranes reach the lysosomes from the plasma membrane via endocytosis or from the trans Golgi network. Also lysosomal proteins are sorted via these routes [56]. Foreign membranes and membrane components enter the cell via endocytosis, e.g., after phagocytosis or via receptor-mediated endocytosis of lipoproteins. During endocytosis, eukaryotic cells internalize areas of the plasma membrane, in part together with foreign material, via different mechanisms and deliver them to early endosomes [57–60]. As an alternative to endocytosis, membranes, macromolecules, but also whole organelles can enter lysosomes by the different pathways of autophagy [61–63]. After fusion of the autophagosome with endosomes or lysosomes, macroautophagy requires degradation of the single membrane of the autophagic body, which separates the engulfed cytoplasmic space from the lysosol. At least in the yeast vacuole, this has to occur in a pH-dependent manner [64], before the autophagosomal content can be degraded by the lysosomal hydrolases. Autophagy is of special relevance for membrane degradation since it appears to be a source of BMP (erroneously also called lysobisphosphatidic acid). This negatively charged lipid is highly enriched in the inner membranes of lysosomes and is required for degradation of their lipid components (see below). It is derived from phosphatidylglycerol generated in the ER and from cardiolipin of mitochondria [65–69], which reaches the lysosome as a component of mitochondria by macroautophagy.

7. Lysosomal sphingolipid degradation

Major insight into the process of membrane digestion came from the investigation of glycosphingolipid catabolism. Glycosphingolipids (Fig. 1) are ubiquitously expressed on eukaryotic cell surfaces. They are composed of a hydrophobic ceramide moiety and an extracytoplasmic oligosaccharide chain [70]. 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 [71]. A great variety of glycosphingolipid structures is known, which depend on species and cell type. Neuronal cells are especially rich in acid glycosphingolipids of the ganglio-series. This is the reason why defects in ganglioside degradation affect especially the nervous system.

After endocytosis, glycosphingolipids are degraded on the surface of intralysosomal membranes in a sequential pathway. Soluble glycosidases cleave off monosaccharide units from the non-reducing end of the glycosphingolipid oligosaccharide chain in a stepwise manner. For the degradation of substrates with short oligosaccharide chains of less than four sugars, these enzymes need the assistance of small glycoprotein cofactors, the sphingolipid activator proteins, the saposins A–D and the GM2 activator protein [72]. Results from in vitro-experiments indicate that in addition to enzymes and activator proteins, an appropriate membrane lipid composition is required for degradation. Most hydrolases are water-soluble polycations at a lysosomal pH of less than 5.0. They bind to negatively charged intraendosomal and intralysosomal membranes, but in the absence of activator proteins, they are not able to degrade their membrane-bound substrates. An exception is acid sphingomyelinase, which hydrolyses membrane-bound sphingomyelin slowly even in the absence of an activator protein, which is obviously due to its *N*-terminal saposin-homology domain [73].

For glycerophospholipids [74] and for non-glycosylated sphingolipids like ceramide and sphingomyelin [75], also non-lysosomal degradation steps are known, which apparently do not need the assistance of an additional activator protein. A cytoplasmic glucosylceramide-cleaving enzyme, which is not deficient in Gaucher's disease, contributes to the degradation of the cytoplasmic glucosylceramide pool [76,77].

During lysosomal membrane degradation, the integrity of the limiting membrane has to be preserved. Historically, this led to the assumption of two distinct pools of membranes, which have to be present in the late endosomal/lysosomal compartment [78]. Ultrastructural examination of cells from patients with defects of glycosphingolipid degradation showed that non-degradable lipids accumulate as multi-vesicular storage bodies in diseases like the GM1-gangliosidosis [79] or the combined sphingolipid activator protein deficiency [80]. This gave the first hint for the assumption of a topological differentiation of the two membrane pools [78].

The model that membrane components – lipids and proteins – reach the lysosomal compartment either as intraendosomal membranes, or as part of the limiting membrane (Fig. 2) has been supported by a series of observations [81]. Both membranes differ in their lipid- and protein composition. The lysosomal leaflet of the limiting membrane is covered with a thick glycocalix that protects the membrane from the attack by the membrane-degrading enzymes present in the lysosol. This glycocalix is formed by lysosomal integral membrane proteins, which are highly *N*-glycosylated with polylactosamine units and therefore highly resistant towards lysosomal digestion [82]. Apparently the degrading enzymes present within the lumen of the lysosome, cannot access their substrates through this glycocalix. Already 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 [83], while it is easily degraded during the constitutive process of membrane turnover [84].

Years after the model of inner membranes as degradation platforms for membranes has been proposed, proteins coordinating the generation of inner vesicles in endosomes have been identified (reviews: [85,86]) The formation of multivesicular bodies (MVBs) starts with inward budding of the limiting endosomal membrane and requires the sequential action of three endosomal sorting complexes required for transport, ESCRT-I,-II,-III [87]. One sorting signal sufficient for protein targeting to inner membranes is the ubiquitinylation of proteins [88]. This signal appears to be conserved between yeast and higher eukaryotes, but also ubiquitin-independent factors have been reported (review: [89]).

8. Functional aspects of membrane lipid sorting in the endocytic pathway

8.1. Anionic lipids as activators of sphingolipid degradation

The lipid composition of cellular membranes is precisely regulated and highly specific for different cell types, organelles, and the two leaflets of the lipid bilayer [90]. During endocytosis and maturation of endosomes, the luminal pH decreases, and the com-

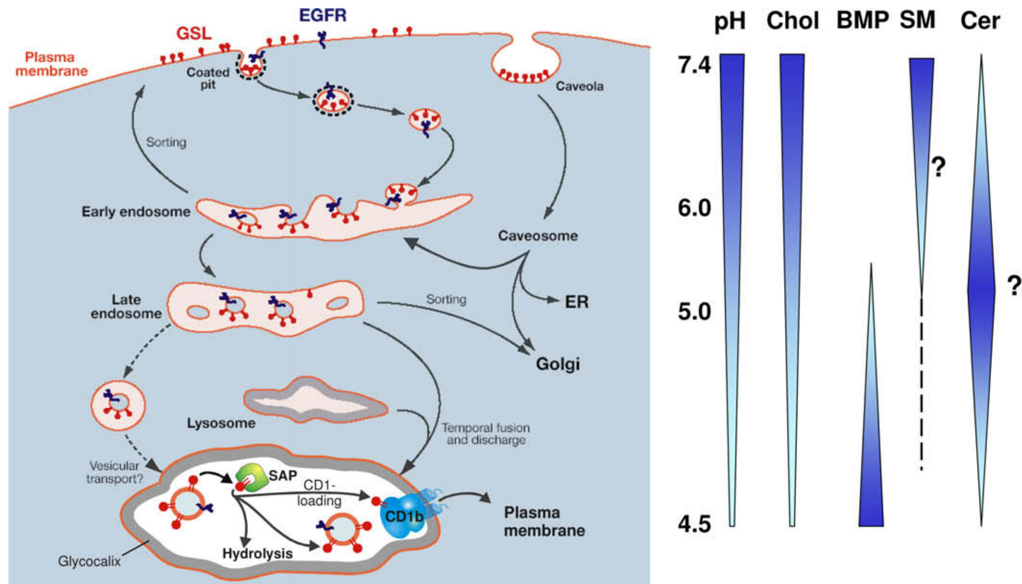

Fig. 2. Model of endocytosis and lysosomal digestion of membranes. Glycosphingolipids (GSL) are highlighted on the plasma membrane (PM) and on internal membranes, and gradients of pH, cholesterol (Chol), BMP, sphingomyelin (SM), and ceramide (Cer; hypothetical) are shown (modified from: [55,48]).

position of the internal membranes changes. While early endosomes resemble the plasma membrane in their composition, membrane-stabilizing cholesterol, other sterols, and phosphatidylserine are sorted out, and BMP levels increase drastically. In BHK cells, two populations of intralysosomal membranes were found, and in one of them, BMP accounted for up to 70% of total phospholipids [91]. BMP is not present in the limiting lysosomal membrane [115], but is required for sphingolipid degradation on inner membranes of the acidic compartments.

In addition to that of bulk lipids, also levels of phosphoinositides change from phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) on the plasma membrane to PtdIns3P on early endosomes, and PtdIns(3,5)P2 on late endosomes [92]. Sphingolipid-containing membranes of autophagocytic bodies should contain lower amounts of cholesterol, but metabolic precursors of BMP, and might be degraded in a similar manner.

BMP (Fig. 1) is a characteristic anionic phospholipid on the surface of intralysosomal membranes. Its unusual sn1,sn1′-configuration leads to its higher resistance to the action of phospholipases than normal phospholipids [93]. Together with smaller amounts of other anionic lipids like phosphatidylinositol [94] and dolichol phosphate [95], it accounts for the negative charge of intralysosomal membranes and is also required for the formation of intraendosomal membranes. The fatty acid composition of BMP is tissue-dependent, with oleic acid as the major acyl residue of BMP from BHK cells, while highly unsaturated BMP is found in testis and rat uterine stromal cells (W.W. Christie, www.lipidlibrary.co.uk/Lipids/lysobpa/index.htm). A protein that binds to BMP-containing membranes [96], Alix/AlP1, plays a role in sorting to inner membranes [97,98] intramolecular vesicle fission, and fusion during lysosome maturation [99,100]. Alix knockdown leads to a decrease in levels of BMP and cellular cholesterol; also the amount of intra-endolysosomal membranes is reduced [101].

In vitro results indicate that packing and conformation of BMP favors budding, stabilization [102], and fusion [91] of vesicles at acidic pH. Moreover, different endosomal and lysosomal lipid transfer proteins require an optimal lipid composition of the membrane they have to interact with, as in the case of the NPC2-protein that extracts sterols from membranes (see below). Saposin A [103] and saposin B [104] extract membrane lipids best from cholesterol-poor and BMP-rich liposomes, while neutral lipid bilayers are resistant towards solubilization even at acidic pH values. 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 [105]. In addition, negatively charged lysosomal lipids also stimulate the interfacial hydrolysis of membrane-bound ganglioside GM1 by GM1-β-galactosidase [106], of ganglioside GM2 by β-hexosaminidase A [105], of the sulfated gangliotriaosylceramide SM2 by β-hexosaminidases A and S [107] in the presence of the GM2-activator protein, of sphingomyelin by acid sphingomyelinase [73], and of ceramide by acid ceramidase [108]. Glucosylceramide degradation by glucosylceramide-β-glucosidase is enhanced in the presence of saposin C in membranes containing negatively charged lipids such as phosphatidylserine, phosphatidylglycerol, and phosphatidic acid [109–111].

Enzymes like acid sphingomyelinase [18] and acid ceramidase [112] are released from intralysosomal membranes after treatment with the cationic amphiphile desipramine, and are rapidly degraded. Also the activity of acid phospholipase A2 towards membranes is increased when they contain negatively charged lipids and is reduced in the presence of a cationic amphiphilic drug, amiodarone [113].

8.2. Cholesterol, sphingomyelin, and ceramide

Since saposins and the GM2-activator required for membrane degradation extract lipids best from cholesterol-poor and BMP-rich membranes, intraendosomal membranes have to adjust their lipid composition, so that they can be degraded after their delivery to lysosomes. In endosomes, cholesterol is removed from inner membranes by the proteins NPC1 and NPC2 [114] (Fig. 3). Cholesterol, the major lipid molecular species of the plasma membrane and in membranes of early endosomes is nearly completely absent in inner lysosomal membranes [115]. It enters the endosomal compartment via membrane recycling, non-vesicular pathways, or lipoprotein uptake, and can leave the endosomes to the Golgi apparatus, the ER, or can return to the plasma membrane [116]. While exogenous cholesterol can traffic to intracellular sites within a few

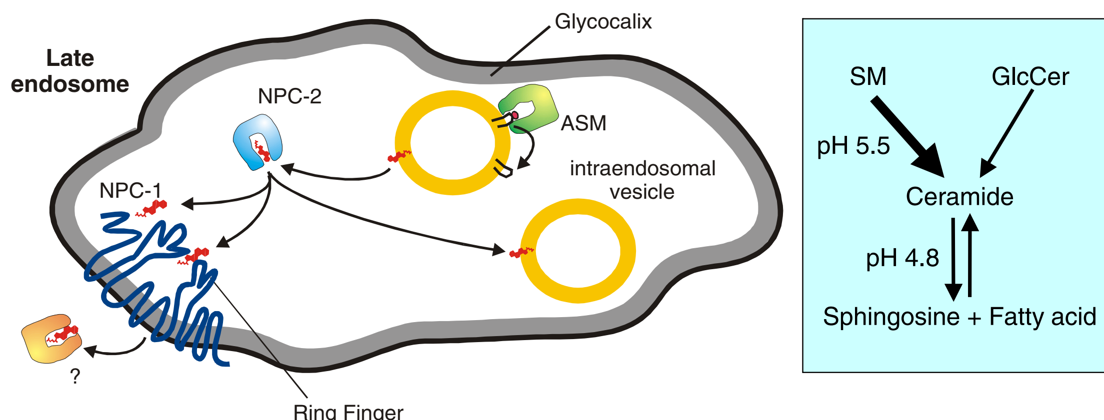

Fig. 3. Model for lipid sorting on the stage of late endosomes: at pH 5, acid sphingomyelinase degrades sphingomyelin and produces ceramide on the surface of endosomal vesicles. Decrease of sphingomyelin and increase of ceramide enhances the availability of cholesterol on inner membranes and facilitates its binding to NPC2 (Abdul-Hammed, Breiden, Adebayo, Babalola, Schwarzmann, and Sandhoff, unpublished), which transfers cholesterol to the N-terminal domain of NPC1 according to Kwon et al. [206]. NPC1 enables cholesterol transfer through the glycocalix. Bulk production of glucosylceramide by degradation of most glycosphingolipid series occurs at lower pH, presumably on later stages of lysosomal maturation. Ceramide formation is reversible to ensure controlled formation of potentially harmful sphingosine and fatty acids (see text).

minutes, the spontaneous sterol exit from isolated lysosomes or purified lysosomal membranes is more than 100-fold slower than that from intact cells [117]. This exit from lysosomes can be drastically stimulated by a cytoplasmic binding protein, sterol carrier protein-2 (SCP-2) [118].

Experiments using exogenous addition of ganglioside GM1 derivatives bearing a photoaffinity label to cultured fibroblasts and other cell types showed a high degree of crosslinking to cholesterol, which decreases during endocytosis from 89% to less than 5% of all lipid derivatives, crosslinking with BMP was elevated from 0% to 45%, whereas crosslinked phosphatidylcholine and sphingomyelin remained nearly unchanged (von Coburg and Sandhoff, unpublished). These results are in agreement with those obtained by electron microscopy [115,119,120]. For the molecular aspects of cholesterol exit from endosomes, see the chapter on NPC2 below.

During the maturation of intraendosomal/intralysosomal membranes, the levels of ceramide should increase at the expense of sphingomyelin, which is rapidly degraded to ceramide by acid sphingomyelinase. According to a simplified view, sphingomyelin, a cholesterol-binding lipid, is replaced by ceramide, a cholesterol-competitor [121], during the processing of inner membranes in the late endosomes. Actually, the effect of the concentration of these lipids on membrane properties and domain formation is much more complicated: At low cholesterol concentrations, ceramide segregates into gel domains, which disappear, when cholesterol levels increase. This is independent of the presence of sphingomyelin. Also, ceramide is more soluble in cholesterol-rich fluid membranes than in cholesterol-poor ones, and the solubility of cholesterol in ceramide domains is low [122]. Ceramide, the common product of glycosphingolipid- and sphingomyelin catabolism can stabilize lipid phases, i.e., microdomains, more efficiently than cholesterol [121,123,124].

In endosomes, ceramide is produced largely by sphingomyelinase-catalyzed cleavage of sphingomyelin (Fig. 3). Glucosylceramide is formed during the degradation of most glycosphingolipid series found in mammals [70], but in the lysosomes. Therefore, glucosylceramide cleavage cannot be expected to contribute significantly to the ceramide pool on intraendosomal membranes. There, ceramide, a "membrane-intercalating agent" that can replace cholesterol via enhancing its thermodynamic activity (escape potential) in the bilayer, should facilitate cholesterol loading on NPC2, analogous to an increased cholesterol transfer on cyclodextrin, as determined in vitro [125]. On the other hand, sphingomyelin inhibits cholesterol transfer by NPC2 (Abdul-Hammed, Breiden, Adebayo, Babalola, Schwarzmann, and Sandhoff, unpublished). This points to a major role of acid sphingomyelinase for cholesterol exit. Cholesterol is transferred through the glycocalix of the perimeter membrane after its transfer to NPC1 (see below) and is further transported and processed.

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 that converts glucosylceramide into ceramide and acid sphingomyelinase that converts sphingomyelin into ceramide have pH-optima of about 5.5 [126,73,75], while the pH-optimum of acid ceramidase is in the range of 3.8–4.2 in the presence of saposin D [108,127]. This should lead to a continuous increase of ceramide levels within the intralysosomal membranes during the process of endosomal maturation. The replacement of sphingomyelin by ceramide should facilitate cholesterol exit out of this membrane population by the cholesterol-carrier NPC2.

8.3. Lysosomal lipid-binding proteins are required for membrane degradation

In vivo, the lysosomal degradation of inner membranes is dependent on the presence of certain proteins and lipids. In addition to water-soluble hydrolases, lysosomal lipid-binding proteins (LLBPs) and acidic lipids, especially BMP, are required for membrane degradation. Lipid-binding- and transfer proteins of different, also non-endo/lysosomal subcellular localization are crucial for the metabolism of different lipid classes, such as fatty acids, sphingolipids [128,129], and intracellular trafficking and metabolism of cholesterol. They include other proteins of saposin-like structure [130], CERT, a protein that transfers ceramide from the ER to Golgi membranes [131], fatty acid-binding proteins (review: [132]), or the glycolipid binding protein, a cytosolic transfer protein for glycolipids of unknown function [133]. Although lipid-metabolizing enzymes and lipid-antigen-presenting CD1-proteins can be regarded as lipid-binding proteins [134,135], LLBPs in the narrow sense encompass the saposins A–D and the GM2 activator protein. An endosomal lipid-binding protein is NPC2. LLBPs play a role in membrane fusion, disintegration, and metabolism, and also in the transfer of lipids to proteins

of the CD1-family, which present them to receptors on natural killer cells (review: [136]).

It has been known for more than 30 years that glycosphingolipid degradation requires the presence of certain of LLBPs, the sphingolipid activator proteins [72]. While other membrane components like phospholipids can apparently be degraded without additional cofactors that make their lipid ligands accessible for the degrading enzymes, sequential hydrolytic cleavage of glycosphingolipids with short carbohydrate chains of four or less sugars is critically dependent on the presence of lysosomal lipid-binding proteins, and of negatively charged lysosomal lipids (see above). In the absence of detergents that are able to solubilize lipids, membrane-bound glycosphingolipids with short carbohydrate chains are not sufficiently accessible to the water-soluble enzymes present in the lysosol in the absence of activator proteins [137,106]. In vitro, synthetic water-soluble glycosphingolipids (GSLs) with either short chain fatty acids or with no fatty acids (lysoGSLs) can already be hydrolyzed by the water-soluble enzymes in the absence of SAPs. Sphingolipid activator proteins known to date are encoded by two genes: one gene encodes the GM2-activator protein, the other encodes prosaposin, also called SAP-precursor protein (review: [72]). This protein is posttranslationally processed to four homologous mature proteins, the saposins A–D or saps A–D. These activator proteins act on the intraendosomal/intralysosomal membrane pool and lead to the selective degradation of membrane lipids without impairment of the perimeter membrane and therefore of lysosomal integrity.

8.4. Saposins

Saposins are water-soluble lysosomal lipid-binding and -transfer glycoproteins of about 8–11 kDa molecular weight (for review see: [72]). They belong to a family of saposin-like proteins with conserved three-dimensional folds [130] and occur as homo- and heterodimers and -oligomers. The solution structures of NK-lysine [138], of the pore-forming peptide of *Entamoeba histolytica* [139], of saposinC [140], and the x-ray structures of unglycosylated human recombinant saposin A and saposin C [141], saposin B [142], saposin C, and saposin D [143] are known and show high structural similarity.

Saposins share lipid-binding- and membrane perturbing properties. For example, the protozoan parasite *Entamoeba histolytica* expresses pore-forming proteins of saposin-like structure, the amoebapores. Like eukaryotic NK-lysin and granulysin, these proteins are able to permeabilize the membranes of target cells, which accounts for their antimicrobial activity [144]. Although the four mammalian saposins share a high degree of homology and similar properties, they act differently and show different specificities.

8.5. Prosaposin

Prosaposin, a 70 kDa glycoprotein, is proteolytically processed to the mature activator proteins in the late endosomes and lysosomes [145–147]. Initially, the non-signal aminoterminal peptide preceding the saposin A-domain is cleaved off, then saposin A is released with initial formation of a sap B–D trimer that is subsequently cleaved via sapB/C and sapC/D dimers to the mature saposins [148]. Prosaposin is intracellularly targeted to the lysosomes via mannose-6-phosphate receptors, by sortilin [149], or secreted and re-endocytosed after binding to mannose-6-phosphate receptors, LRP, or mannose receptors [150].

Rare mutations in the start codon of the prosaposin gene lead to a complete deficiency of the protein and of all four mature saposins. Since prosaposin is rapidly cleaved in the acidic compartments, it can be assumed that the protein itself plays no role for membrane digestion. Prosaposin deficiency in human patients and mice [151] causes simultaneous storage of many sphingolipids and glycosphingolipids accompanied by a dramatic accumulation of intralysosomal membranes. In cultured cells, this storage can be completely reversed by treatment with human prosaposin, as demonstrated in prosaposin deficient fibroblasts [152].

8.6. Saposin A

Saposin A is required for the degradation of galactosylceramide by galactosylceramide-β-galactosidase. Genetically engineered mice that carry a mutation in the saposin A-domain of the Sap-precursor accumulate galactosylceramide and suffer from a late-onset variant form of Krabbe disease [153].

8.7. Saposin B

Saposin B was the first activator protein that had been identified as so-called sulfatide-activator in 1964 [154]. It mediates the degradation of sulfatide by arylsulfatase A, of globotriaosylceramide and digalactosylceramide by α-galactosidase A, of other glycolipids [155], for example together with the GM2-activator protein for the degradation of ganglioside GM1 [106].

The X-ray structure shows a shell-like homodimer that encloses a large hydrophobic cavity [142]; the monomers are composed of four amphipathic α-helices arranged in a long hairpin that is bent into a simple V-shape. Like in the case of the GM2-activator, two different conformations of the saposin B-dimers were visible, and a mode of action similar to that of the GM2-activator has been proposed: The open conformation is supposed to interact directly with the membrane, to promote a reorganization of the lipid alkyl chains, and to extract the lipid ligand, which is accompanied by a change to the closed conformation. The substrate is then exposed to the enzyme in a water-soluble activator–lipid complex [156], consistent with the previous observation that saposin B can act as a lipid-transport protein [157].

The inherited defect of saposin B leads to an atypical form of metachromatic leukodystrophy [158] with accumulation of sulfatides, digalactosylceramide, and globotriaosylceramide (review: [72]). Saposin B-knockout mice display enhanced levels of hydroxy and non-hydroxy fatty acid-containing sulfatides especially in brain and kidney [159]. The interaction of saposins A and -B with immobilized lipids was characterized by surface plasmon resonance [103,104]. Glycosylated saposins bind to lipid bilayers at acidic pH and are able to extract lipids. The disintegration of liposomes is increased by high BMP levels, whereas cholesterol stabilized the membranes. The carbohydrate part of saposins turned out to be crucial for their function, since unglycosylated proteins showed increased binding, but reduced extraction capacity. Variant proteins without glycosylation site from patient lost their lipid-extraction capacity almost completely. X-ray and NMR structure determination showed variations in the structure motif common for saposins on different conditions such as changes of pH value, addition of detergents, and of lipid ligands [160–162]. Data from atomic force microscopy favor a multistep mechanism in lipid mobilization by saposins: after formation of a transient layer on the surface of intraluminal membranes, a conformational change occurs that allows interaction of hydrophobic amino acid residues with the acyl chains embedded in the bilayer [163]. Other potential functions of saposin B are only beginning to emerge, e.g., as a binding protein for coenzyme Q10 in urine and cultured cells [164] and in vitro also for gamma-tocopherol [165].

8.8. Saposin C

Saposin C has been initially isolated from spleen of patients of Gaucher disease. It occurs as a homodimer and is required for

the lysosomal degradation of glucosylceramide by glucosylceramide-β-glucosidase [166]. Saposin C also renders protease-resistance to glucosylceramide-β-glucosidase inside the cell [167]. The solution structure of saposin C [140] consists of five tightly packed α-helices that form the half of a sphere. All charged amino acids are solvent-exposed, while the hydrophobic residues are contained within the protein core. In contrast to the mode of action of the GM2-activator and of saposin B, saposin C can directly activate glucosylceramide-β-glucosidase as an allosteric effector [109,166,168]). Saposin C binds to membranes in a pH-controlled, reversible manner [169], supports the interaction of the enzyme with its membrane-bound substrate in the presence of anionic phospholipids, and is able to destabilize such vesicles [137]. Saposin C deficiency leads to an abnormal juvenile form of Gaucher disease with an accumulation of glucosylceramide [170,171]. Atomic force microscopy suggests the view that saposin C locally alters membrane regions preferentially at edges of membrane defects for subsequent attack by the saposin C-β-glucosidase complex [172].

8.9. Saposin D

Saposin D stimulates lysosomal ceramide degradation by acid ceramidase in cultured cells [173] and in vitro [108]. It is able to bind to vesicles containing negatively charged lipids and to solubilize them at an appropriate pH [174]. Saposin D-deficient mice accumulate ceramides with hydroxylated fatty acids mainly in the brain and in the kidney [175]. Mice with a combined deficiency of saposin C and saposin D store glucosylceramide and alpha-hydroxy fatty acid-containing ceramides; in addition, also prosaposin trafficking is impaired, and levels of saposins A and B are reduced [176].

8.10. The GM2-activator

The GM2-activator is a glycoprotein with a molecular weight of 17.6 kDa in its deglycosylated form [72] and is required for the in vivo degradation of ganglioside GM2 by β-hexosaminidase A [177]. β-Hexosaminidase A can cleave glycolipid substrates on membrane surfaces only if they extend far enough into the aqueous phase. In the absence of detergents, the degradation of ganglioside GM2 occurs only in the presence of the GM2 activator protein.

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 [178]. The X-ray structure of the non-glycosylated protein shows a hydrophobic cavity for the ceramide moiety of ganglioside GM2 [179,180]. A model for its mode of action that considers also data from photoaffinity labeling is shown in Fig. 4 [181]. It forms stoichiometric, water-soluble glycolipid–protein complexes that are the physiological Michaelis–Menten-substrates of β-hexosaminidase A [177]. As concluded from sedimentation experiments, the GM2-activator is able to extract ganglioside GM2, but also other lipids, in a pH-dependent fashion from membranes. BMP increases the extraction efficiency, and less than 15% of the activator protein remains membrane-associated. Remarkably, the binding characteristics of the GM2-activator are altered by the presence of a His-tag [182].

In vitro, the GM2-activator acts as a lipid transfer protein that can carry lipids from donor to acceptor liposomes [178]. In Langmuir experiments, the GM2-activator protein is able to penetrate into a phospholipid monolayer, but only when the lateral pressure is below a critical value of 15–25 mN/m, dependent on the lipid composition [183]. The transfer capacity of GM2AP was quantified in a liposomal fluorescence or Förster resonance energy transfer (FRET) assay [184] based on novel NBD-labeled glycolipid substrates [185].

In addition to its function as a ganglioside transfer protein, the GM2-activator binds also other lipids like phosphatidylcholine and platelet activating factor (PAF) and inhibits its action [186]. The observation that also lysoPAF was detected in the X-ray structure of the protein–lipid complex raises the possibility that the GM2-activator might also display hydrolase activity towards this lipid [187]. This has also been observed for phosphatidylcholine, which can be cleaved by the activator in a phospholipase A2-like manner with a pH-optimum of 6 [188]. GM2-activator orthologs might serve different functions in other organisms. In *Drosophila*, a homologous proteins acts as a pheromone binding protein [189], and in a nematode, *Trichinella spiralis*, a secreted protein apparently without the domain required for binding to hexosaminidase does not stimulate GM2-degradation by β-hexosaminidase A, but inhibits PAF-induced chemotaxis [190].

8.11. NPC2

Niemann–Pick disease protein C2 (NPC2) is a late endosomal lipid-binding protein [191], and together with proteins like Insig, SCAP, LXR, HMG-CoA-reductase, or NPC1, it belongs to the group of cholesterol-binding proteins, whose members have different subcellular localization [192]. An inherited disorder, in which intracellular traffic of cholesterol is impaired, is Niemann–Pick disease, type C (NPC) [193,194]. NPC is a neurodegenerative disorder characterized by accumulation of cholesterol in endolysosomes, and secondarily also of neutral and acidic glycolipids, especially gangliosides GM3 and GM2 [195], sphingomyelin (less than in Niemann–Pick disease, types A and B), BMP, and phospholipids [194].

NPC is due to mutations in the genes encoding the NPC1- [196] or the NPC2-protein [197]. NPC1-deficiency accounts for 95% of NPC-cases [198]; the other cases are due to mutations in the gene encoding the NPC2-protein [199]. This protein has been identified before as a lysosomal glycoprotein that can bind cholesterol with high affinity in a 1:1 stoichiometry [200] as well as other sterols [201]. In vitro, it acts as a transport protein for cholesterol to and from liposomes [202,203]. Lysosomal conditions (high BMP content of the membranes, low pH value) stimulate the transport between donor and acceptor liposomes [204]. NPC2 most likely mediates cholesterol transport between intraendosomal membranes and from the internal membranes to the NPC1-protein, an integral protein of the perimeter membrane. Crystal structures of NPC2 with cholesterol sulfate [205] and of the *N*-terminal domain of NPC1 in complex with 25-hydroxy-cholesterol suggest the following model [206]: NPC2 binds cholesterol so that its isooctyl side chain is buried in a hydrophobic cleft and its hydroxyl group is exposed to the aqueous surroundings. The *N*-terminal domain of NPC1, however, binds cholesterol in the opposite orientation: the hydroxyl-group is buried in the domain, and the hydrophobic isooctyl side chain is exposed. Therefore, cholesterol can be transferred from NPC2 to NPC1 without re-orientation. This is subsequently inserted into the lysosomal perimeter membrane, from where it enters sites in the cytoplasm by an unknown mechanism. The observation that NPC1 might be required for copper transport out of endosomes might also account for the pathogenesis of NPC1-disease [207].

The fact that insolubility of storage material in lipidoses determines the phenotype is illustrated by the fact that oral treatment of the NPC1 disease model mice with 2-hydroxypropyl-beta-cyclodextrin, which is able to extract and solubilize cholesterol and other lipids, is able to reverse the lysosomal transport defect [208]. Since NPC2 acts only as a sterol transfer protein, it is not clear how NPC2 might be involved in loading of a glycolipid on CD1d, as reported for the only known of the selecting ligands of NKT cells in the thymus, isoglobotrihexosylceramide [209,210].

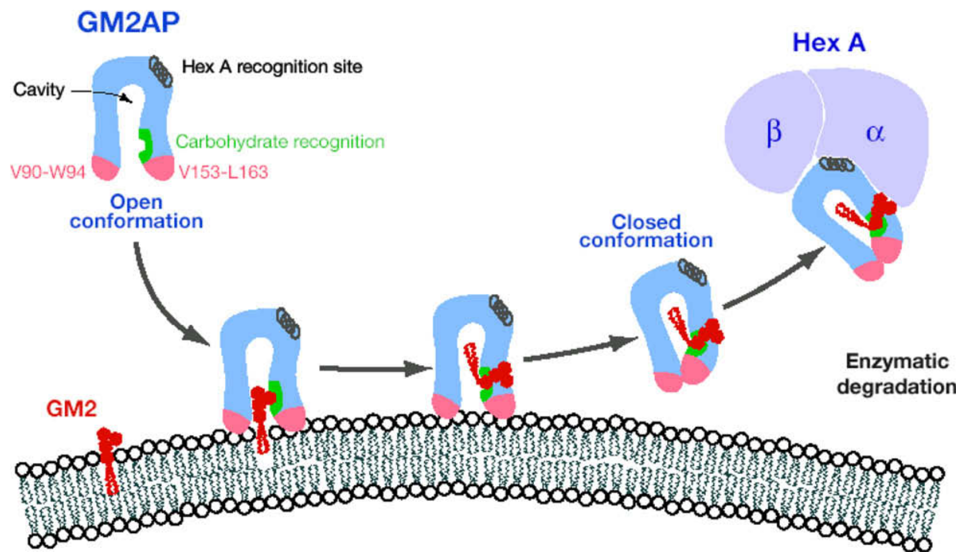

Fig. 4. Model for GM2-activator stimulated hydrolysis of ganglioside GM2 by human β-hexosaminidase A [181]. The GM2-activator contains a hydrophobic cavity for binding of the ceramide portion of GM2 or other lipids. One of the surface loops contains the substrate-binding site (V153–L163) and controls the entrance to the cavity. In the open conformation, the empty activator binds to the membrane with the aid of hydrophobic loops and penetrates into the hydrophobic region of the bilayer. The lipid recognition site of the activator can interact with the substrate, and its ceramide moiety moves inside the hydrophobic cavity. The lipid loaded activator can change to the closed conformation, becomes more water soluble and might leave the membrane. In solution or at the membrane surface, it present gangliosides GM2 or GM1 to water-soluble hydrolases.

8.12. Lipid-antigen presentation

The lysosome serves not only diverse housekeeping functions, but also plays a role in immune responses, e.g., for the presentation of exogenous and endogenous antigens [211]. Intracellular material present in the cytoplasm is delivered to lysosomes by autophagy, their degradation products loaded on MHC class II proteins, and presented by them to T-lymphocytes [212]. In addition to their role in membrane degradation, lysosomal lipid-binding proteins are required for the presentation of lipid and glycolipid antigens. In humans, they are needed for loading of the four MHC-I-like glycoproteins CD1a–d that present lipid antigens to T-cells. A fifth protein, CD1e, is synthesized as an integral membrane protein, from which a soluble lipid binding domain is released by proteolysis within the lysosomes of mature dendritic cells [213]. In addition to the saposins and the GM2-activator (review: [136]), a major role in the transfer of lipid antigens to membrane-resident CD1-proteins has to be attributed to this protein [214,215].

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