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The Immunological Functions of Saposins

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Abstract

Saposins or sphingolipid activator proteins (SAPs) are small, nonenzymatic glycoproteins that are ubiquitously present in lysosomes. SAPs comprise the five molecules saposins A–D and the GM2 activator protein. Saposins are essential for sphingolipid degradation and membrane digestion. On the one hand, they bind the respective hydrolases required to catabolize sphingolipid molecules; on the other hand, saposins can interact with intralysosomal membrane structures to render lipids accessible to their degrading enzymes. Thus, saposins bridge the physicochemical gap between lipid substrate and hydrophilic hydrolases. Accordingly, defects in saposin function can lead to lysosomal lipid accumulation. In addition to their specific functions in sphingolipid metabolism, saposins have membrane-perturbing properties. At the low pH of lysosomes, saposins get protonated and exhibit a high binding affinity for anionic phospholipids. Based on their universal principle to interact with membrane bilayers, we present the immunological functions of saposins with regard to lipid antigen presentation to CD1-restricted T cells, processing of apoptotic bodies for antigen delivery and cross-priming, as well as their potential antimicrobial impact.

1. INTRODUCTION

Cells perform endocytosis to capture extracellular material and to remove used plasma membrane components. From endosomes, macromolecules can be either recycled to the plasma membrane or delivered to lysosomes for subsequent destruction. Lysosomes are acidic membrane-enclosed organelles representing the terminal degradative compartment of the endocytic pathway. They contain more than 60 different soluble hydrolytic enzymes specialized in the degradation of macromolecules. In addition, lysosomes are equipped with sphingolipid activator proteins (SAPs) that belong to the large and divergent family of saposin-like proteins (SAPLIPs). SAPLIP domains have been identified in relatively small proteins of about 80 amino acids in length, including the lung surfactant-associated protein B (SP-B), the tumorolytic proteinsNK-lysin and granulysin, cytolytic proteins from amoeba, and several plant aspartic proteases. SAPs comprise the five molecules saposins A–D and the GM2 activator protein. Saposins A–D are produced in acidic endosomal compartments upon sequential proteolytic cleavage of their single polypeptide precursor termed prosaposin (pSAP). Saposins are nonenzymatic, acidic, heat-stable, and protease-resistant molecules of about 8–11 kDa with essential functions in sphingolipid degradation and membrane digestion. The modes of action of SAPs include binding and stimulation of glycosidases required for sphingolipid degradation, as well as interaction with intralysosomal membranes to render lipids accessible to their respective degrading enzymes. Thus, saposins bridge the physicochemical gap between membrane lipid substrates and water-soluble hydrolases.

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Moreover, the multimolecular association of saposins, lipid bilayers, and CD1 glycoproteins facilitates the loading of CD1 molecules with lipid antigens for subsequent activation of lipid-reactive T cells. In this chapter, we present the current knowledge pertaining to the biology of saposins in lysosomal sphingolipid degradation and membrane digestion and highlight its implications for immunological processes, such as lipid antigen presentation, processing of apoptotic bodies for cross-priming, and direct antibiotic function.

2. SAPOSINS IN LYSOSOMAL GLYCOSPHINGOLIPID DEGRADATION AND MEMBRANE DIGESTION

Glycosphingolipids (GSLs) are a class of lipids present in the plasma membrane of eukaryotic cells (Kolter and Sandhoff, 2005). Structurally, GSLs share a common hydrophobic ceramide moiety that acts as a membrane anchor, which is coupled to a hydrophilic oligosaccharide chain. GSLs play important roles in the structural organization of membranes and cellular interactions with microbes or toxins (Hakomori, 1981; Karlsson, 1989). GSLs are generated along the secretory pathway by the sequential, combinatorial addition of monosaccharide units, starting with the initial elongation of ceramide by glycosidic binding of either D-glucose or D-galactose in β-configuration. In particular, β-D-glucosylceramide undergoes elongation upon the stepwise action of specific glycosyltransferases to produce GSLs with complex oligosaccharide chains, such as gangliosides, globosides, and cerebrosides (Ichikawa and Hirabayashi, 1998). Alternatively, the addition of a phosphorylcholine moiety to ceramide produces sphingomyelin, a major constituent of the membrane of nerve cells and a dominant species among sphingolipids (Hooghe-Peters *et al.*, 1979). Eventually, sphingolipids reaching the plasma membrane become a part of structural microdomains enriched in cholesterol (Simons and Ikonen, 1997). Degradation of sphingolipids is initiated upon internalization of membrane patches through diverse mechanisms including endocytosis, phagocytosis, or autophagy, and commences in acidified compartments of the endosomal route for terminal degradation in lysosomes (Luzio *et al.*, 2007). In contrast to soluble molecules, degradation of membranes is a more delicate process since the limiting membrane of lysosomes must remain intact to avoid the leakage of potentially hazardous enzymes into the cytosol. Thus, prior to reaching lysosomes, sphingolipids are sorted to intraendosomal membranes and degraded on the surface of intralysosomal vesicles (ILVs) upon exposure to soluble glycosidases (Sandhoff and Kolter, 1996). Sphingolipid degradation proceeds in a sequential pathway that assures the stepwise removal of monosaccharide units from the nonreducing end of the oligosaccharide chain (Fig. 2.1). Ultimately, ceramide is disassembled to sphingosine and fatty acid for subsequent reuse in metabolic pathways. As sphingolipid degradation proceeds, the length of the sugar headgroup inevitably shrinks in size, thereby becoming less accessible to water-soluble glycosidases. To overcome this physicochemical obstacle and to bring sphingolipids and their respective enzymes in close proximity, mammalians possess five saposins encoded by two genes (Rorman *et al.*, 1992). The first gene encodes pSAP, the common precursor to the four saposins A–D. The second gene encodes the GM2AP. The physiological significance of saposins in stimulating sphingolipid degradation is underscored by multiple reports of human sphingolipidoses with mutations in the *pSAP* gene leading to deficiencies of saposin function (O'Brien and Kishimoto, 1991). The five SAPs

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share a high degree of structural homology. However, SAPs show diverse ligand-binding properties and exist in multiple structural states that account for their distinct modes of action in sphingolipid degradation and membrane interaction.

2.1. Prosaposin

pSAP is a 524-amino acid glycoprotein that contains a 16-residue signal peptide sequence and five glycosylation sites. Importantly, pSAP is the common precursor to the four saposins A–D (Furst *et al.*, 1988; O'Brien *et al.*, 1988). In humans, pSAP exists as an intracellular molecule of 68 kDa and a major extracellular form of 73 kDa. Accordingly, pSAP is intracellularly targeted to lysosomes either via mannose-6-phosphate receptors or by sortilin (Lefrancois *et al.*, 2003). Alternatively, pSAP can be secreted and reendocytosed by mannose-6-phosphate receptors, low density lipoprotein receptor-related protein (LRP), or mannose receptors (Hiesberger *et al.*, 1998). Expression of pSAP and individual saposins is virtually ubiquitous and conserved among mammalian species (Kishimoto *et al.*, 1992). This is not surprising considering their important functions in sphingolipid degradation. Protein expression analyses revealed high concentrations of pSAP in the adult liver and body fluids, especially in the brain, semen, milk, serum, pancreatic juice, and bile (Kolter and Sandhoff, 2005). Further, pSAP and saposins are expressed in cells of hematopoietic origin and nerve cells (Kondoh *et al.*, 1993; Sano *et al.*, 1989). pSAP is sequentially processed from its N-terminal end, starting with the cleavage of the SAP-A domain (Hiraiwa *et al.*, 1993). The mechanism through which pSAP is processed to the four saposins remains incompletely understood. Early studies suggested that pSAP proteolysis occurs at low pH and requires the action of proteases susceptible to inhibition by pepstatin A (Hiraiwa *et al.*, 1993). One of these candidate proteases was later shown to be cathepsin D (Hiraiwa *et al.*, 1997). *In vitro*, unprocessed pSAP can stimulate the degradation of sphingolipids to a similar extent like SAP-B, SAP-C, and SAP-D act on their respective enzymes (Kishimoto *et al.*, 1992). However, considering that pSAP proteolysis occurs in acidic compartments, its contribution to lysosomal sphingolipid degradation might be of limited physiological relevance. However, pSAP has been proposed to represent a neurotrophic factor and might also be involved in the transport of gangliosides (Hiraiwa *et al.*, 1992; O'Brien *et al.*, 1994). Point mutations in pSAP have been identified in patients lacking all four saposins, a disease referred to as combined SAP deficiency (Harzer *et al.*, 1989; Hulkova *et al.*, 2001). pSAP-deficient individuals and $pSAP^{-/-}$ mice show similar clinicopathological features and die during the neonatal period or at the age of 3–4 weeks, respectively, due to multiple organ failure (Fujita *et al.*, 1996). Analysis of pSAP-deficient cells reveals numerous electron-dense membrane inclusions, and $pSAP^{-/-}$ mice show massive accumulation of GSLs such as ceramide, β-glucosylceramide (β-GlcCer), β-galactosylceramide (β-GalCer), sulfatides, galabiaosylceramide (Ga_2Cer), lactosylceramide (LacCer), globotriaosylceramide (Gb3), and the ganglioside GM3 (Bradova *et al.*, 1993; Fujita *et al.*, 1996).

2.2. Saposin A

SAP-A activates the hydrolysis of β-GalCer by galactosylceramide b-galactosidase (Fig. 2.1) (Morimoto *et al.*, 1989). Accordingly, mice that carry a point mutation in the SAP-A domain of pSAP, and thus lack SAP-A expression, show tissue accumulation of β-GalCer (Matsuda *et al.*, 2001). SAP-A deficiency forms the basis of a chronic late-onset form of

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globoid cell leukodystrophy that resembles the disease of patients carrying genetic deficiency in galactosylceramide β-galactosidase (Krabbe disease) (Spiegel *et al.*, 2005). Under acidic conditions, SAP-A mobilizes lipids from liposomes in a reaction that is enhanced by bis(monoacylglycero) phosphate (BMP) or decreased by cholesterol (Locatelli-Hoops *et al.*, 2006). The crystal structure of human SAP-A has been resolved in its closed monomeric saposin-fold conformation and consists of four amphipathic α-helices folded in the shape of an oblate ellipsoid (Ahn *et al.*, 2006). Charged residues are located on the surface of the molecule, whereas conserved hydrophobic residues are directed toward a small cavity. Elucidation of the SAP-A structure in its open, lipid-binding conformation remains to be determined.

2.3. Saposin B

SAP-B has been the first activator protein identified (Mehl and Jatzkewitz, 1964). SAP-B is required to stimulate the breakdown of sulfatide by arylsulfatase A, Gb3 and Ga2Cer by α-galactosidase A, and LacCer by galactosylceramide β-galactosidase (Fig. 2.1). Accordingly, the latter GSLs are present in abnormally high concentrations in the urine of SAP-B-deficient patients and accumulate in the tissues of SAP-$B^{-/-}$ mice (Li *et al.*, 1985; Sun *et al.*, 2008). Further, SAP-B cooperates with GM2AP in the degradation of the ganglioside GM2 *in vitro* (Wilkening *et al.*, 2000). SAP-B can be considered as a nonspecific activator protein. Accordingly, SAP-B stimulates the hydrolysis of ceramide-free glycolipids by diverse glycosidases from animals, plants, and microorganisms (Li *et al.*, 1988). These special properties might explain as to why SAP-B stimulates the degradation of a broader spectrum of sphingolipids compared to other SAPs. Additionally, SAP-B binds and transfers the anionic phospholipid phosphatidylinositol (PI) between biological membranes (Ciaffoni *et al.*, 2006). Inherited defects of SAP-B lead to an atypical form of metachromatic leukodystrophy (MLD) with late infantile or juvenile onset (Kretz *et al.*, 1990; Schlote *et al.*, 1991; Wenger *et al.*, 1989). The crystal structure of human SAP-B displays a shell-like homodimer that consists of two V-shaped monomers (Ahn *et al.*, 2003). The concave inner surface of each monomer is lined with hydrophobic residues that create a large lipid-binding cavity when two monomers are associated. In its open conformation, the SAP-B dimer can directly interact with lipid membranes, promote the reorganization of lipid alkyl chains, and extract lipid substrates upon transition to the closed conformation.

2.4. Saposin C

SAP-C has been primarily described by O'Brien and colleagues as an activator of β-GlcCer degradation by glucosylceramide β-glucosidase (Fig. 2.1) (Ho and O'Brien, 1971). SAP-C might also stimulate the degradation of ceramide, β-GalCer, and galactosylsphingosine (Harzer *et al.*, 1997). Inherited deficiency of SAP-C causes a variant juvenile form of Gaucher disease (type III) with marked storage of β-GlcCer (Christomanou *et al.*, 1989; Matsuda *et al.*, 2004; Schnabel *et al.*, 1991). SAP-C exhibits a characteristic dual mode of action in sphingolipid degradation. Similar to SAP-A, SAP-C stimulates its respective enzyme partner in an allosteric manner (Berent and Radin, 1981). In parallel, SAP-C can directly bind anionic phospholipids to destabilize membranes and to promote the association of β-glucosylceramidase with its lipid substrate for subsequent degradation (Vaccaro *et al.*, 1993). At low pH, SAP-C additionally triggers the fusion of vesicles containing anionic

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phospholipids *in vitro* (Vaccaro *et al.*, 1994, 1995; Wang *et al.*, 2003). Using SAP-C mutants, the fusogenic activity could be mapped to the lysine-rich amino-terminal portion of the molecule, indicating that electrostatic interactions between negatively charged lipids and positively charged saposin residues might be required (Qi and Chu, 2004). Finally, evidence suggests that SAP-C functions as a neurotrophic factor by stimulating neurite outgrowth and increasing choline acetyltransferase activity in neurons (O'Brien *et al.*, 1995). The structure of SAP-C in its closed conformation reveals a homodimer of boomerang-shaped intertwining monomers in an open, extended conformation, with solvent-exposed hydrophobic pockets (Hawkins *et al.*, 2005; Rossmann *et al.*, 2008). Accordingly, a "clip-on" model of vesicle fusion has been proposed. At lysosomal pH, SAP-C dimers unfold their hydrophobic pockets to interact with anionic phospholipids of membrane bilayers. Ultimately, SAP-C molecules, inserted into opposing lipid vesicles, clip one another through domain swapping, thus bringing the vesicles close enough for fusion.

2.5. Saposin D

SAP-D is the most abundant saposin in normal tissues with concentrations threefold higher than other SAPs (O'Brien and Kishimoto, 1991). SAP-D promotes the hydrolysis of ceramide by acid ceramidase *in vivo* (Fig. 2.1), as demonstrated by the accumulation of α-hydroxyl fatty acid ceramides in the kidneys and the cerebellum of SAP-$D^{-/-}$ mice (Matsuda *et al.*, 2004). Consequently, SAP-D-deficient animals show renal tubular degeneration and hydronephrosis, as well as progressive loss of Purkinje cells in the cerebellum, leading to ataxia. To date, inherited SAP-D deficiency has not been identified in humans. Of note, the phenotype of $SAPD^{-/-}$ mice does not resemble human ceramidase deficiency (Farber disease) or its murine counterpart characterized by early embryonic lethality (Li *et al.*, 2002; Matsuda *et al.*, 2007). Similar to SAP-C, SAP-D poorly binds sphingolipids, but displays high affinity for anionic phospholipids at lysosomal pH (Tatti *et al.*, 1999). However, SAP-C and SAP-D are functionally different. SAP-D is a membrane disrupter, whereas SAP-C fuses lipid bilayers (Ciaffoni *et al.*, 2001). SAP-D spontaneously binds to membranes that contain anionic lipids, including BMP, PI, and phosphatidylserine (PS), in a reversible, pH-driven fashion (Ciaffoni *et al.*, 2003). By destabilizing membranes, SAP-D allows the formation of small vesicles derived from larger liposomes *in vitro* (Ciaffoni *et al.*, 2001). Multivesicular or multilamellar bodies found in the lumen of acidic cellular compartments are specifically enriched for BMP (Kobayashi *et al.*, 1998, 2002). Therefore, it is tempting to speculate that SAP-D might regulate the homeostasis of internal endolysosomal membranes. SAP-D exists as a substrate-free closed helix bundle or in a V-shaped, open, and ligand-bound configuration in the presence of lipids. Prior to interaction with lysosomal membranes, SAP-D remains in a monomer–dimer equilibrium in the closed conformation. The acidic pH of lysosomes dramatically increases the surface hydrophobicity of SAP-D, thereby allowing the positively charged amino acids at the bottom of SAP-D to associate with the surface of intralysosomal membranes enriched in negatively charged lipids. The top of SAP-D subsequently rotates by 180° along its axis, thus positioning its hydrophobic residues into the membrane bilayer, and exposing positively charged residues to the solvent. Thereafter, SAP-D changes its configuration to the closed conformation, which forms the mechanistic basis for lipid extirpation from the bilayer. Ultimately, SAP-D leaves the membrane with bound lipid (Rossmann *et al.*, 2008).

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2.6. The GM2 activator

The GM2AP is the fifth member of the SAP family. GM2AP is larger than saposins A–D, with a molecular weight of 20 kDa in its mature lysosomal form (Furst and Sandhoff, 1992). To reach lysosomes, newly synthesized GM2AP uses the major intracellular mannose-6-phosphate-mediated trafficking route (Rigat *et al.*, 1997). Mannose-6-phosphate receptors also allow the endosomal recapture of GM2AP from extracellular fluids upon endocytosis (Rigat *et al.*, 1997). The saposin function of GM2AP is required to stimulate the degradation of GM2 by β-hexosaminidase A (Hex-A) *in vivo* (Fig. 2.1) (Conzelmann and Sandhoff, 1979). Deficiency in GM2AP leads to the AB variant of GM2 gangliosidosis, an atypical form of Tay–Sachs disease with characteristic tissue accumulation of GM2 and related GSLs in neuronal lysosomes (Conzelmann and Sandhoff, 1978). GM2AP acts as a lipid transfer protein *in vitro* as indicated by its capacity to extract and carry GSLs from donor to acceptor liposomes (Conzelmann *et al.*, 1982). The structure of monomeric GM2AP consists of an eight strand, cup-shaped, antiparallel β sheet (Wright *et al.*, 2000). The monomer 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 (Wright *et al.*, 2003). The most flexible of the loops contains the substrate-binding site and controls the entrance to the cavity to facilitate an open or a closed conformation. Accordingly, open, empty GM2AP binds to target membranes by using its hydrophobic loops and penetrates into the hydrophobic region of the bilayer. Subsequently, the lipid recognition site of the activator can interact with the substrate and insert its ceramide portion into the hydrophobic cavity. At this point, the lipid-loaded activator may change to the closed conformation, allowing the complex to leave the membrane in a soluble state. Finally, GM2AP exposes GM2 to the water-soluble enzyme Hex-A for subsequent degradation in the lysosomal lumen (Kolter and Sandhoff, 2005).

2.7. Topology

The inner leaflet of the limiting lysosomal membrane is covered with a thick glycocalix composed of glycoproteins. This layer allows the lysosome to resist the low lumenal pH, and protects the inner limiting membrane from digestion by acid hydrolases or destabilization through lipid-binding molecules. Therefore, membrane constituents targeted for lysosomal degradation have to be sorted to small lumenal vesicles formed from endosomes by the inward budding of the limiting membrane into the lumen. Vesicle-rich endosomes are referred to as multivesicular endosomes or multivesicular bodies (MVBs) (Piper and Luzio, 2001). This topological transition exposes macromolecules originating from the outer leaflet of the plasma membrane to the endosomal lumen. Hence, the subsequent fusion of an MVB with a lysosome renders macromolecules accessible to hydrolases. Following this principle, intraendosomal vesicles serve as important devices in the delivery of used plasma membrane proteins and lipids to lysosomes (Futter *et al.*, 1996; Sandhoff and Kolter, 1996). Recently, the biogenesis and function of MVBs and ILVs has become a major focus for cell biologists, especially since the discovery of endosomal sorting complexes required for transport (ESCRT) (Luzio *et al.*, 2007). Yet, the sequence of events leading to their formation remains poorly understood. Three lipids play critical roles in the biogenesis and function of MVBs and ILVs. Firstly, phosphatidylinositol-3-phosphate is

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enriched on the cytosolic face of endosomes, and evidence suggests that it might be required for the formation of MVBs (Futter *et al.*, 2001; Odorizzi *et al.*, 1998). Secondly, cholesterol is highly enriched on intraendosomal vesicles, where it stabilizes the membrane (Hornick *et al.*, 1985). By contrast, cholesterol is almost absent from ILVs as it is removed from lysosomes by the Niemann–Pick disease protein C1 (NPC1) and NPC2 (Friedland *et al.*, 2003; Mobius *et al.*, 2003). Considering the negative impact of cholesterol on SAP-A- and SAP-B-mediated lipid mobilization from liposomes, physiological depletion of cholesterol from ILVs might favor lipid mobilization by SAPs (Locatelli-Hoops *et al.*, 2006; Remmel *et al.*, 2007). Thirdly, the anionic lipid BMP plays a crucial role in the topology of ILVs and is derived from mitochondrial cardiolipin during the process of autophagy, or BMP is produced from phosphatidylglycerol (PG) in the endoplasmic reticulum (ER) (Hullin-Matsuda *et al.*, 2009). In contrast to cholesterol, BMP is specifically enriched on ILVs, where it can account for up to 70% of the total membrane phospholipid content (Kobayashi *et al.*, 1998, 2002). BMP functions in the formation of ILVs since BMP-containing liposomes can spontaneously form inward-budding profiles in a pH-inducible manner (Matsuo *et al.*, 2004). Remarkably, the presence of BMP in liposomes strongly enhances the stimulatory capacity of all five SAPs (Chu *et al.*, 2005; Ciaffoni *et al.*, 2003; Locatelli-Hoops *et al.*, 2006; Remmel *et al.*, 2007; Wilkening *et al.*, 2000). Taken together, these findings support a model in which the gradual depletion of cholesterol and subsequent integration of BMP into ILVs renders them more accessible for SAPs. Moreover, BMP enhances SAP-stimulated degradation of GSLs by specific glycosidases. Notably, the membrane-perturbing properties of SAP-C potentially mediate the fusion of multiple ILVs in order to promote their subsequent disruption by SAP-D.

3. SAPOSINS FACILITATE LIPID PRESENTATION TO CD1-RESTRICTED T LYMPHOCYTES

The majority of studies on antigen presentation have concentrated on molecules encoded by the major histocompatibility complex (MHC). However, over the past two decades, it has become evident that other molecules can also trigger T cell responses, including the family of lipid-presenting CD1 molecules. In addition to proteins, lipid antigens extend the spectrum of determinants that are potentially recognized by the immune system, and thus amplify the diversity of immune responses to fight intruders, for example, in the context of infection of the host with lipid-rich pathogens. In contrast to protein processing to peptides, cellular lipid acquisition challenges the host due to the physicochemical properties of fats. Accordingly, hydrophobic lipid antigens have to be extracted from aggregates or membranes and solubilized for subsequent transport. Subsequently, lipids are loaded onto specific antigen-presenting molecules such as CD1 proteins. For both steps, helper molecules like saposins are required (Fig. 2.2). On the other hand, some lipids have to be further subjected to structural editing, or processing, to reveal antigenic epitopes otherwise unavailable for recognition by T cells. In the following, we highlight the predominant roles played by saposins and lysosomal glycosidases in both processes.

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3.1. Characteristics of antigen-presenting CD1 molecules

The organization of the CD1 complex is similar to MHC class I. Each *CD1* gene contains three exons encoding separate extracellular domains (α1, α2, α3) that noncovalently associate with β2-microglobulin (β2m) to form a stable transmembrane heterodimer of approximately 50 kDa (Brigl and Brenner, 2004). Five separate genes (*CD1A*, *CD1B*, *CD1C*, *CD1D*, and *CD1E*) are mapping to chromosome 3 in humans. In mice, CD1 maps to chromosome 1 and consists of two genes (*CD1D1* and *CD1D2*). On the basis of sequence homology, CD1 proteins fall into one of two groups: group 1 CD1 molecules (CD1a, CD1b, and CD1c) and group 2 CD1 molecules (CD1d), with CD1e representing an intermediate. While CD1a-d proteins are mainly expressed on the surface of antigen-presenting cells (APCs), CD1e is exclusively found as a soluble molecule in lysosomes, and therefore cannot present antigens to CD1-restricted T cells (Angenieux *et al.*, 2005). The subdivision of CD1 molecules correlates with functional differences. Accordingly, group 1 molecules present lipid antigens to T lymphocytes, while group 2 molecules display antigens to Natural Killer T (NKT) cells. Structurally, CD1 molecules contain a main hydrophobic groove that can be prolonged by further pockets (Moody *et al.*, 2005). Group 1 CD1 molecules are mainly expressed by cortical thymocytes and myeloid cells, such as dendritic cells (DCs) and Langerhans cells (Dougan *et al.*, 2007a). In addition, CD1c proteins are also expressed by marginal zone B cells and a fraction of peripheral blood B lymphocytes (Delia *et al.*, 1988). In humans, group 2 CD1d molecules are found on cells of the myeloid lineage as well as on few nonlymphoid cells. However, cortical thymocytes and mantle zone B cells in lymph nodes exhibit the highest levels of CD1d expression (Exley *et al.*, 2000). In mice, CD1d expression depends on the *CD1D1* gene. CD1d molecules are mainly expressed by APCs, including hepatic stellate cells (Winau *et al.*, 2007). In addition, cortical thymocytes as well as some activated T cells express CD1d proteins (Dougan *et al.*, 2007a). Expression of CD1d on the surface of APCs is enhanced by proinflammatory cytokines, such as interferon-β (IFN-β), IFN-γ, and TNF-α, as well as by toll-like receptor (TLR)-2 and TLR-4 ligands (Skold *et al.*, 2005). The peroxisome proliferator-activated receptor-γ (PPAR-γ) controls CD1d expression by triggering retinoic acid synthesis in human DCs (Szatmari *et al.*, 2006). Furthermore, a number of pathogens modulate the extent of CD1d expression. Viruses such as Kaposi's sarcoma-associated herpes virus (KSHV) and HIV downregulate the expression of CD1d on the plasma membrane (Andre *et al.*, 2005; Sanchez *et al.*, 2005). Finally, herpes simplex virus-1 (HSV-1) and vesicular stomatitis virus (VSV) can cause downmodulation of CD1d by suppressing CD1d recycling (Raftery *et al.*, 2006; Yuan *et al.*, 2006).

3.2. Intracellular trafficking of CD1 molecules

In analogy to MHC-I molecules, CD1 heavy chains are folded and associated with β2m within the lumen of the ER, in a process that involves calnexin and calreticulin, but excludes transporters associated with antigen processing (TAP) (Kang and Cresswell, 2002; Sugita *et al.*, 2007). At this stage, the pocket formed by the α1 and α2 helices of the heavy chain can be loaded with hydrophobic spacers such as neutral phospholipids to stabilize the nascent CD1 molecule (Gadola *et al.*, 2002). Accordingly, ER-resident lipid exchange proteins such as the microsomal triglyceride transfer protein (MTP) could fulfill this loading function (Dougan *et al.*, 2005, 2007b). From the ER, CD1 molecules transit along the secretory

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pathway until they reach the plasma membrane, where they follow a major pathway of internalization subdivided into distinct intracellular trafficking routes. The type of endocytic compartment through which CD1 molecules transit largely depends on the presence of the amino acid sequence Y-X-X-Z (tyrosine-X-X-bulky hydrophobic residue) in the cytoplasmic moiety of the heavy chain (Chiu *et al.*, 2002). At the plasma membrane, this motif mediates recruitment of the adaptor molecule AP-2 by CD1b, CD1c, and CD1d molecules, and promotes their subsequent internalization through clathrin-coated pits (Sever, 2003). Following their traffic to endosomes, CD1b and CD1d molecules are further sorted to late acidic endocytic compartments by the recruitment of AP-3 through the same cytoplasmic motif (Sugita *et al.*, 2002). CD1b is almost exclusively located in lysosomes, whereas CD1c that does not bind AP-3 shows a broad localization throughout the endosomal pathway (Sugita *et al.*, 2007). Notably, there is no evidence for targeting motifs in the cytoplasmic tail of CD1a proteins. Nevertheless, they are found in recycling endosomes and traffic back to the plasma membrane in an ARF6-dependent manner (Sugita *et al.*, 1999). Finally, CD1e displays unique features in comparison to other CD1 molecules. Upon assembly, CD1e molecules are targeted to lysosomes without reaching the plasma membrane, where they exist in a cleaved soluble form and participate in the processing of microbial glycolipid antigens (de la Salle *et al.*, 2005). Ubiquitination of the cytoplasmic tail is a prerequisite for CD1e proteins to target lysosomes (Maitre *et al.*, 2008). In the endocytic system, CD1 molecules exchange lipids acquired in the secretory pathway with self or foreign lipids, and, except for CD1e, traffic to the plasma membrane where they display their lipid cargo to CD1-restricted T cells. Hence, the divergent trafficking routes of CD1 molecules may reflect an evolutionary adaptation to face the diversity of intracellular pathogen lifestyles through detection by the immune system.

3.3. CD1-restricted T cells

Michael Brenner and colleagues provided the first evidence that human CD1 molecules present lipid antigens to induce T cell responses (Beckman *et al.*, 1994). The cognate antigen presented in the context of CD1b proved to be mycolic acid, a lipid contained in the cell wall of *Mycobacterium tuberculosis*. Intriguingly, T lymphocytes restricted by group 1 CD1 molecules demonstrate favored reactivity with lipid antigens originating from the cell wall of mycobacteria. *M. tuberculosis* is the etiologic agent of tuberculosis, one of the most ancient and life-threatening infectious diseases worldwide (Kaufmann, 2006). Since humans represent the principal reservoir of *M. tuberculosis*, it is tempting to speculate that group 1 CD1-restricted T cells might have developed as a result of coevolution of host and pathogen to provide specific protection against *M. tuberculosis* infection. Both hydrophobic peptides and lipids or glycolipids from mycobacteria can be presented by CD1 molecules. Accordingly, didehydroxymycobactin (DDM), a lipopeptide structurally related to siderophores, is presented to T cells in the context of CD1a (Moody *et al.*, 2004). Mannosylated phosphatidylinositides (PIMs), including lipoarabinomannan (LAM), and diacylated sulfoglycolipids (Ac2SGLs) activate T cells when presented through CD1b (Gilleron *et al.*, 2004; Sieling *et al.*, 1995). Further, glucose monomycolate (GMM), produced upon interaction of biosynthetic pathways of host and pathogen, activates T cells restricted by CD1b (Moody *et al.*, 2000a). Finally, hexosyl-1-phosphoisoprenoids stimulate CD1c-dependent T cells (Beckman *et al.*, 1996; Moody *et al.*, 2000b). Lymphocytes

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restricted by group 1 CD1 molecules are found in all the major phenotypic subsets of T cells, including single-positive $CD4^+$ and $CD8^+$ T cells, as well as double-negative (DN) $CD4^-$ $CD8^-$ T lymphocytes (Porcelli *et al.*, 1992; Rosat *et al.*, 1999; Sieling *et al.*, 2000). Upon activation, CD1-restricted T cell clones develop T_H1 effector functions dominated by the production of IFN-γ and TNF-α (Rosat *et al.*, 1999; Sieling *et al.*, 1999). In addition, DN and $CD8^+$ CD1-restricted T cells exert potent cytotoxic functions toward *M. tuberculosis*-infected macrophages through Fas–Fas ligand interactions or the release of granulysin, respectively (Stenger *et al.*, 1997). Using these mechanisms, CD1b-restricted T cells effectively kill *M. tuberculosis*-infected macrophages in a CD1b-dependent manner (Stenger *et al.*, 1997). A feature shared by all CD1-restricted T cells is the basal recognition of CD1 molecules in the absence of foreign lipids (Porcelli *et al.*, 1989). Autoreactive responses by T cells can stimulate the maturation of DCs toward a proinflammatory phenotype, which may play a critical role with regard to the ensuing generation of adaptive immune responses (Spada *et al.*, 2000; Vincent *et al.*, 2002). Recognition of sulfatide or GM1 by CD1-restricted T cells could form the basis of this autoreactivity (Shamshiev *et al.*, 2000, 2002). In contrast to CD1b molecules, CD1a and CD1c are largely excluded from acidic subcellular compartments, and their antigen-presenting functions are not affected upon inhibition of endosomal acidification (Briken *et al.*, 2000; Porcelli *et al.*, 1992; Sieling *et al.*, 1995). Optimal binding of PIMs, GMM, and LAM require an acidic environment in which the α-helices of CD1b can partially unfold (Ernst *et al.*, 1998). Although these findings highlight an interesting mechanism that allows access of lipid antigens to the CD1b groove only upon their trafficking to the proper compartment, they cannot account for a model in which CD1b itself extracts lipids from membranes and thus, chaperoning helper molecules have to fill this gap. In this context, SAP-C has been identified as the critical saposin required for recognition of microbial lipid antigens by CD1b-restricted T cells (Winau *et al.*, 2004b). Accordingly, human pSAP-deficient fibroblasts expressing CD1b failed to present mycolic acid, GMM, and LAM for activation of antigen-specific CD1b-restricted T cell clones. Moreover, T cell responses could be restored upon fibroblast reconstitution with SAP-C but not other SAPs (Winau *et al.*, 2004b). The underlying mechanism involved SAP-C-mediated extraction of LAM from membranes and subsequent transfer to CD1b (Fig. 2.2), as indicated by coprecipitation experiments identifying a direct interaction between SAP-C and CD1b (Winau *et al.*, 2004b). These findings demonstrated saposins as a missing link in antigen presentation of lipids to group 1 CD1-restricted T cells, and suggest that SAP-C dysfunctions potentially have adverse consequences concerning T cell immunity in infectious diseases like tuberculosis.

3.4. CD1d-restricted natural killer T cells

In contrast to humans, mice lack genes encoding group 1 CD1 molecules. Therefore, their repertoire of lipid-specific T cells is solely represented by lymphocytes restricted to CD1d molecules, namely NKT cells. Unique features of NKT cells include usage of an invariantly rearranged T cell receptor (TCR) a chain (Vα14-Jα18 in mice, Vα24-Jα18 in humans) paired with a limited set of TCR βchains, expression of diverse surface receptors characteristic for NK cells, and functional autoreactivity toward CD1d-expressing APCs *in vitro* (Bendelac *et al.*, 1995; Budd *et al.*, 1987; Dellabona *et al.*, 1994; Fowlkes *et al.*, 1987; Porcelli *et al.*, 1993). Further, NKT cells express intermediate levels of TCR at the cell

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surface and a phenotype of activated/memory T cells in naive and germ-free mice, as well as in human cord blood, which may reflect the consequence of continuous basal TCR stimulation with self antigens (D'Andrea *et al.*, 2000; Park *et al.*, 2000; van Der Vliet *et al.*, 2000). NKT cells are a heterogeneous population and dominated by a subset that reacts with the marine sponge-derived GSL antigen α-galactosylceramide (α-GalCer) presented by CD1d proteins (Chen *et al.*, 1997; Kawano *et al.*, 1997). By definition, NKT cells that respond to α-GalCer are referred to as invariant NKT (iNKT) cells. iNKT cells show exclusive usage of the TCR rearrangement Vα14-Jα18 coupled to Vβ8, Vβ7, or Vβ2 in mice, or the rearrangement Vα24-Jα18 associated with Vβ11 in humans, and promptly produce IFN-γ and interleukin-4 (IL-4) upon activation (Bendelac *et al.*, 2007). By contrast, NKT cells that fail to respond to α-GalCer stimulation are referred to as noninvariant NKT cells, or type II NKT cells. Notably, type II NKT cells use diverse TCRs, and owing to the lack of specific markers to track them, their biology remains poorly understood.

3.4.1. Invariant NKT cells—The relevance of iNKT cells to diseases such as cancer, infection, or autoimmunity, has been extensively reviewed elsewhere (Bendelac *et al.*, 2007; Godfrey *et al.*, 2004). One of the most exciting features in the biology of iNKT cells pertains to the modes of their activation. In the face of infection, it has become evident that the host can use several pathways to activate iNKT cells. Firstly, activation may result from direct, TCR-mediated recognition of microbial lipid antigens presented in the context of CD1d-expressing APCs. Known antigens include α-glucuronosylceramides and α-galacturonosylceramides from *Sphingomonas* spp., diacylglycerols from *Borrelia burgdorferi*, the causative agent of Lyme disease, and phosphatidylinositol tetramannoside from *M. tuberculosis* (Fischer *et al.*, 2004; Kinjo *et al.*, 2005, 2006; Mattner *et al.*, 2005; Sriram *et al.*, 2005). Following a second pathway predominantly triggered by infection with Gram-negative lipopolysaccharide (LPS)-positive bacteria, such as *Salmonella typhimurium*, iNKT cells become activated upon recognition of self antigens presented by LPS-exposed DCs in an IL-12-dependent manner (Brigl *et al.*, 2003). In addition to TLR-4-mediated activation of iNKT cells triggered by LPS, several other axes of DC sensitization through TLR ligation have been identified (De Libero *et al.*, 2005). Accordingly, stimulation of DCs through the nucleic acid sensor TLR-9 results in the subsequent activation of iNKT cells (Paget *et al.*, 2007). In the latter case, neosynthesized β-linked self GSLs and type I interferons provided by DCs were strictly required. Accordingly, stimulation of DCs through TLR-4, TLR-7, or TLR-9, could influence the expression of various glycosyltransferases involved in the biogenesis of GSLs (Paget *et al.*, 2007; Salio *et al.*, 2007). Importantly, blocking the *de novo* generation of GSLs abrogated the responses by iNKT cells. Finally, increased expression of CD1d/GSL complexes, representing ligands for the iNKT cell invariant TCR, could be visualized at the surface of APCs stimulated with LPS or a TLR-8 agonist (Salio *et al.*, 2007). Thus, microbe-exposed APCs remodel the repertoire of self GSLs to produce dominant species that can be recognized by iNKT cells. How signals relayed through pattern recognition receptors can lead to selective induction of GSL antigens without compromising cellular lipid homeostasis remains to be clarified. Lastly, several recent studies indicate that iNKT cells could become activated in a pure cytokine-driven fashion without requirement for TCR tickling by CD1d–self-lipid complexes (Montoya *et al.*, 2006; Nagarajan and Kronenberg, 2007). Accordingly, MCMV-infected

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DCs could activate iNKT cells in an IL-12-dependent, but CD1d-independent manner (Tyznik *et al.*, 2008). In analogy to human CD1b, murine CD1d molecules primarily localize to lysosome-associated membrane protein 1 (LAMP-1)-positive organelles, indicating a trafficking route that includes late acidic compartments for acquisition of self lipids (Chiu *et al.*, 2002). Of note, deletion of the AP-3-binding motif in the cytoplasmic tail of CD1d (CD1-TD) depletes the molecule from late endosomal and lysosomal compartments, while surface expression of the mutant molecule is slightly increased (Chiu *et al.*, 1999). This tail modification leads to severe functional consequences specifically affecting iNKT cells. Accordingly, CD1-TD-expressing APCs fail to present self lipids and exogenous α-GalCer to iNKT cell hybridoma, and CD1-TD knock-in mice show impaired production of iNKT cells in the thymus, which results in profound defects of iNKT cells in peripheral organs (Chiu *et al.*, 2002). Taken together, these findings identified late endocytic compartments as primary sites where CD1d molecules acquire self lipids or exchange self lipids with foreign antigens. Further, the endosomal route proved to be essential in the generation of CD1d–self-lipid complexes that are recognized by thymocytes, which ultimately facilitates iNKT cell development. Finally, these findings also suggested that lipid exchanges between CD1d and membranes might require helper molecules located in late endosomal and lysosomal compartments. First evidence supporting this hypothesis derived from studies performed with $pSAP^{-/-}$ mice, which are devoid of all four SAPs and selectively lack iNKT cells (Zhou *et al.*, 2004a). *In vitro*, DCs and thymocytes from $pSAP^{-/-}$ mice failed to stimulate iNKT cell hybridoma, but showed intact functions in the presentation of self lipids to noninvariant NKT cell hybridoma, as well as a normal capacity to process exogenous proteins for activation of various antigen-specific MHC-IIrestricted T cells. In cell-free assays, recombinant SAP-A and SAP-C showed the highest efficiency in the exchange of trisialoganglioside GT1 loaded onto CD1d molecules with PS or sulfatide contained in liposomes, using an experimental pH that corresponded to lysosomes. However, SAPs alone could not extract GT1 bound to CD1d. By contrast, GM2AP extracted the ganglioside from CD1d but did not replace it (Zhou *et al.*, 2004a). Using murine pSAP-deficient cell lines transduced with human CD1d as APCs, another study concluded that recognition of α-GalCer by a human iNKT cell line could be enhanced by reintroduction of human pSAP. However, pSAP did not increase autoreactive responses by the iNKT cell line (Kang and Cresswell, 2004). Finally, reintroduction of mutant pSAP constructs, each lacking one of the four saposins, revealed that SAP-B-expressing APCs most efficiently enhanced α-GalCer presentation to NKT cells (Yuan *et al.*, 2007). However, no individual SAP proved to be absolutely essential in that process. In humans and mice, SAP-B seems to play a dominant role in the exchange of CD1d-bound self lipids acquired in the secretory pathway with self or foreign lipids present in lysosomes.

3.4.2. Noninvariant NKT cells—Similar to iNKT cells, type II NKT cells show reactivity to CD1d molecules expressed by APCs. In contrast to iNKT cells, type II NKT cells recognize CD1d-bound lipids that are loaded along the secretory pathway (Chiu *et al.*, 1999). The myelin-derived self GSL sulfatide, which previously has been identified as a self antigen comparably presented by CD1a, CD1b, and CD1c molecules, is specifically recognized by a subset of type II NKT cells (Jahng *et al.*, 2004; Shamshiev *et al.*, 2002). Identification of this subpopulation using sulfatide-loaded CD1d tetramers revealed its

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specific enrichment in the central nervous system during experimental autoimmune encephalomyelitis (EAE). Interestingly, sulfatide treatment prevented antigen-induced EAE in wild type, but not in CD1d$^{-/-}$ mice. The underlying mechanism involved sulfatide-reactive type II NKT cells that prevented the production of IFN-γ and IL-4 by pathogenic myelin oligodendrocyte glycoprotein (MOG)-reactive T cells (Jahng *et al.*, 2004). More recent studies explored the requirements for type II NKT cell hybridoma recognition of sulfogalactosylsphingosine (lysosulfatide), a sulfatide derivative lacking the fatty acid constituent (Roy *et al.*, 2008). In this context and according to findings that type II NKT cells specialize in the recognition of antigens acquired along the secretory pathway, pSAP deficiency had no impact on the recognition of lysosulfatide by type II NKT cell hybridoma. However, at acidic pH, SAP-C enhanced the recognition of plate-bound CD1d molecules loaded with lysosulfatide by noninvariant NKT cells (Roy *et al.*, 2008).

4. SAP$_S$ STIMULATE THE PROCESSING OF LIPID ANTIGENS BY LYSOSOMAL GLYCOSIDASES

The lysosomal system is of considerable biomedical importance since its alterations are associated with numerous human diseases. To date, more than 50 monogenic human diseases that are primarily associated with lysosomal dysfunction have been identified, and the majority of these conditions are classified as lysosomal storage disorders (LSDs). LSDs are caused by deficiencies in membrane proteins that transport degradation products out of the lysosome, or they are due to defects in molecules involved in the processing or trafficking of lysosomal proteins and GSLs (Fig. 2.1). Pathways of GSL production and degradation have recently attracted the attention of lipid immunologists since analyses of mouse models of human LSDs have uncovered unexpected antigen-processing defects with major impact on CD1-restricted T cell responses.

4.1. Hexosaminidase B

The mouse model of Sandhoff disease that lacks the β-subunit of hexosaminidase A and hexosaminidase B (Hexb$^{-/-}$ mice) has been instrumental in the elucidation of an endogenous antigen recognized by iNKT cells, namely, the GSL isoglobotriaosylceramide (iGb3) (Zhou *et al.*, 2004b). *In vivo* analyses revealed a specific lack of iNKT cells in Hexb$^{-/-}$ mice, which suggested that functions of hexosaminidases in the catabolism of GSLs are required for the production of iNKT cell agonists in the thymus. In line with this postulate, APCs from Hexb$^{-/-}$ mice expressing CD1d molecules failed to stimulate autoreactive responses by iNKT cell hybridoma, whereas responses to α-GalCer were preserved. By testing the potential antigenicity of several GSL species belonging to the globo-, isoglobo-, and neolacto-series, which are produced in lysosomes upon the action of hexosaminidases (Fig. 2.1), only iGb3 could stimulate murine and human iNKT cells (Zhou *et al.*, 2004b). Hence, by removing the terminal *N*-acetyl-β-$_D$-galactosamine residue from iGb4, Hex-B produces iGb3 that is recognized by iNKT cells. Possibly due to missing self on CD1d molecules, either by lack of generation (Hexb$^{-/-}$) or by deficient CD1d loading (pSAP$^{-/-}$) of endogenous antigens, both knock-out strains fail to develop iNKT cells. Therefore, potential iGb3–SAP–CD1d interactions have been proposed. Accordingly, SAP-B could exchange CD1dbound GT1 with free iGb3 or iGb4 (Zhou *et al.*, 2004b). However, while

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immunological and novel biochemical evidence points toward iGb3 as the natural self antigen, its physiological role remains vividly challenged by several studies in humans and mice (Christiansen *et al.*, 2008; Gadola *et al.*, 2006; Li *et al.*, 2009; Porubsky *et al.*, 2007; Speak *et al.*, 2007). Recently, SAP-B$^{-/-}$ mice were described to accumulate Gb3 in various tissues, which is in agreement with previous findings that SAP-B is required to activate the degradation of Gb3 by 903B1;-galactosidase A (Sun *et al.*, 2008). Since globosides and isoglobosides use the same degradation pathway, SAP-B$^{-/-}$ mice could provide an interesting model to test the possible accumulation of iGb3 or other potential endogenous antigens for iNKT cells.

4.2. α-Galactosidase A

First evidence uncovering the antigen-processing component of α-galactosidase A (α-Gal-A) derived from a study in which APCs from a-Gal-A$^{-/-}$ mice (Fabry disease) failed to present the synthetic disaccharide antigen Gala(1→2) α-GalCer to iNKT cell hybridoma (Prigozy *et al.*, 2001). Accordingly, removal of the terminal galactose residue by lysosomal α-Gal-Awas required to expose the α-GalCer epitope to the invariant TCR. In the catabolism of globosides, α-Gal-A functions downstream of Hex-B to produce LacCer from Gb3 (Fig. 2.1). In addition, α-Gal-A degrades the galactolipid Ga_2Cer to β-GalCer (Ohshima *et al.*, 1997). Interestingly, α-Gal-A shows a broader substrate specificity than previously expected, since it can also remove terminal galactose residues bound in the a(1→3) configuration. Therefore, iGb3 that contains a terminal a(1→3)-branched galactose could represent a physiological substrate for α-Gal-A. Consequently, enzyme deficiency in α-Gal-A activity could lead to iGb3 accumulation. Unexpectedly, α-Gal-A$^{-/-}$ mice demonstrated a lack of iNKT cells (Prigozy *et al.*, 2001; Zhou *et al.*, 2004b). In contrast to Hexb$^{-/-}$ mice, this deficit was only partial and specific for iNKT cells located in peripheral organs. Since Fabry and Sandhoff diseases have different etiologies and display diverse patterns of GSL storage, a generalized defect of iNKT cell selection has been proposed in LSD mice. Analyses of knock-out mouse models of Tay–Sachs disease in which hexosaminidase A is lacking (HexA$^{-/-}$ mice), Sandhoff disease (Hexb$^{-/-}$ mice), GM1 gangliosidosis (β-Gal$^{-/-}$ mice), and mice deficient for the endosomal transmembrane protein NPC1 involved in cholesterol homeostasis, revealed reduced frequencies as well as functional defects of iNKT cells (Gadola *et al.*, 2006; Schumann *et al.*, 2007). The hypothesis has been proposed that the degree of iNKT cell deficiency in each mouse model could be related to the extent of lipid stored, irrespective of specific lipid entities. Therefore, lipid storage itself could exert a nonspecific negative impact on the selection of thymic iNKT cell precursors. Upon immunological analysis of α-Gal-A$^{-/-}$ mice, we found specific loss of peripheral iNKT cells in accordance with previous reports (Zhou *et al.*, 2004b). These defects were the direct consequence of iNKT cells chronically exposed to self GSLs (unpublished observations). Moreover, DCs from a-Gal-A$^{-/-}$ mice inducedCD1d-dependent production of IFN-γ and IL-4 by iNKT cells in the absence of exogenous antigen. Additionally, wild-type DCs treated with an inhibitor of α-Gal-A elicited NKT cell activation. Further, reconstitution of α-Gal-A-deficient DCs with recombinant enzyme, or iGb3 blocking in Fabry DCs, abrogated iNKT cell responses. In a more recent study analyzing iNKT cells in diverse animal models of LSDs, in which GSLs, glycosaminoglycans, or both accumulate, defective iNKT cell development could only be

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observed in mice affected by combined deficiency in sulfatase activity. However, these defects were generalized to other T cell subsets. By contrast, mice with single lysosomal enzyme deficiencies showed normal iNKT cell development (Plati *et al.*, 2009). In conclusion, constitutive or induced deficiency in α-Gal-A activity leads to accumulation of endogenous self antigens such as iGb3 for subsequent activation of iNKT cells.

4.3. α-Mannosidase

A decisive function of α-mannosidase in the processing of carbohydrate antigens has been clarified in the human system. Accordingly, DCs from a patient with congenital deficiency in α-mannosidase failed to present mycobacterial hexamannosylated phosphatidyl-myo-inositides (PIM_6) to a CD1b-restricted T cell line (de la Salle *et al.*, 2005). In detail, the enzyme is required for the stepwise degradation of PIM_6 to PIM species that contain fewer mannose residues, including the stimulating antigen PIM_2. Importantly, the generation of stimulating PIM_2 species required assistance by soluble CD1e molecules (de la Salle *et al.*, 2005). In contrast to SAPs, which are ubiquitously expressed and primarily required in the catabolism of GSLs, CD1e is mainly expressed in immune cells, and could therefore specifically act as an immunological lipid transfer protein (Angenieux *et al.*, 2005). In analogy to saposins, whether CD1e uses a “solubilizer” or “liftase” mode of action remains to be clarified.

5. SAPOSINS DISRUPT VESICLES RELEASED BY APOPTOTIC CELLS

5.1. Implications for apoptosis

Upon hypoxia, trauma, or the effect of noxious substances, cells die by necrosis, which involves depletion of the intracellular ATP stores associated with cell swelling and rupture of cellular organelles (Winau *et al.*, 2005). Ultimately, necrotic cells burst and release their organellar and cytosolic content into the surrounding tissue, which subsequently causes inflammation. In sharp contrast to necrosis, apoptosis represents a regulated form of cell death that prevents inflammatory responses under physiological conditions (Winau *et al.*, 2005). Accordingly, apoptotic cells shrink, condense their DNA and organelles prior to fragmentation, release membrane blebs, and finally disintegrate into apoptotic bodies. Thus, apoptosis avoids cell leakage and secondary harmful inflammation, and represents a “silent” way of death that cells undergo during development and tissue homeostasis (Ravichandran and Lorenz, 2007). However, the silencing feature of apoptosis can be overridden in the context of infection or cancer, when apoptotic bodies become vehicles for antigens and tumor- or pathogen-associated molecular patterns that trigger immune responses (Winau *et al.*, 2004a). A hallmark of apoptosis is the exposure of PS, which is normally confined to the inner leaflet of the cytoplasmic membrane in living cells, on the surface of apoptotic cells (Savill *et al.*, 2002). Further, apoptotic cells release high amounts of chemoattractant nucleotides and lysophosphatidylcholine (LPC), which subsequently recruit phagocytes (Elliott *et al.*, 2009). Capture and subsequent internalization of apoptotic bodies involves specific recognition of externalized PS by diverse phagocytic receptors, including the scavenger receptor CD36, brain angiogenesis inhibitor 1 (BAI1), T cell immunoglobulin and mucin domain-containing molecule 4 (TIM-4), Mer tyrosine kinase, and stabilin-2 (Ravichandran and Lorenz, 2007). Finally, apoptotic bodies are incorporated into

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phagosomes, which subsequently mature, and eventually fuse with lysosomes for terminal degradation. Elucidation of these clearance pathways is of great interest since removal of apoptotic cells by DCs bears important implications for the establishment of immune tolerance (Albert *et al.*, 1998a, 2001; Kawane *et al.*, 2006). While rapid progress has been made toward the understanding of molecular processes inherent to the delivery, recognition, and engulfment of apoptotic bodies, the mechanism of the critical final processing step, describing their lysosomal disintegration, remains largely unexplored. Ultrastructural examination of apoptotic vesicles by electron microscopy reveals similar features to ILVs. Therefore, we anticipated that the special mode of action of saposins on intralysosomal membranes could be used to disrupt apoptotic vesicles located in lysosomes, following phagocytosis by macrophages or DCs (Fig. 2.2). Of note, high content of anionic phospholipids in ILVs favors their solubilization upon functional interaction with specific SAPs in lysosomes (Ciaffoni *et al.*, 2001). We propose that PS on apoptotic bodies deploys its actual specific function inside the phagocytes, namely, as molecular target for saposins in lysosomes to facilitate disintegration of apoptotic vesicles (Fig. 2.2, unpublished observations).

5.2. Saposins facilitate antigen cross-presentation

Presentation of peptide antigens by MHC molecules to T lymphocytes classically comprises two major pathways. Following the MHC-I pathway, endogenous proteins that are synthesized inside the cells, such as antigens produced by viruses, are primarily located in the cytosol. Subsequently, the multienzyme complex of the proteasome degrades the proteins into peptide fragments, which are translocated into the ER through TAP (Goldberg and Rock, 1992; Shepherd *et al.*, 1993). After loading of MHC-I molecules in the ER assisted by the peptide-loading complex, consisting of tapasin, calreticulin, and Erp57, MHC-I-peptide complexes are transported to the cell surface of the APC for specific recognition by $CD8^+$ T cells (Degen *et al.*, 1992; York and Rock, 1996). By contrast, exogenous antigens derived from pathogenic bacteria, for example, are endocytosed by APCs for subsequent degradation by cathepsins in late endosomal / lysosomal compartments, prior to loading onto MHC-II molecules. Subsequently, $CD4^+$ T cells recognize complexes of MHC-II and peptide exposed on the APC surface. However, exogenous antigens can also be presented by MHC-I molecules in a process termed cross-presentation (Vyas *et al.*, 2008). Accordingly, the respective antigen crosses from the endosomal route to the MHC-I pathway. The activation of $CD8^+$ T cells by cross-presented antigens is referred to as cross-priming, and DCs are APCs uniquely equipped for cross-presentation (Bevan, 1976; Guermonprez *et al.*, 2002). To date, multiple mechanisms for the cellular pathway of cross-presentation have been proposed, which are likely not mutually exclusive (Vyas *et al.*, 2008). Moreover, several antigen vehicles have been described to have cross-priming abilities, including proteins, peptides, and heat-shock proteins (HSP) chaperoning peptides (Srivastava, 2002). In addition, apoptotic cells represent a potent device to deliver antigens to the cross-presentation pathway. Notably, apoptotic bodies derived from tumors or host cells infected with viruses induce vigorous $CD8^+$ T cell responses (Albert *et al.*, 1998b). Further, macrophages infected with mycobacteria release apoptotic vesicles that are engulfed by uninfected bystander DCs for cross-priming of $CD8^+$ T cells (Schaible *et al.*, 2003; Winau *et al.*, 2006). Ultimately, immunization with apoptotic

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vesicles released by mycobacteria-infected macrophages elicits $CD8^+$ T cell responses and protects against tuberculosis (Winau *et al.*, 2006). Therefore, apoptotic bodies as mediators of antigen cross-presentation are part of a unique detour pathway that promotes T cell responses in tumor and infection immunity (Winau *et al.*, 2004a). However, it remains unclear as to how antigens enclosed in apoptotic bodies become accessible for cross-presentation to $CD8^+$ T cells. Our previous findings suggested that successful cross-priming requires pSAP-dependent processing of apoptotic vesicles in recipient DCs (Winau *et al.*, 2006). Thus, we propose that saposins unseal apoptotic bodies for antigen delivery in DCs and subsequent $CD8^+$ T cell responses (Fig. 2.2).

6. SAP-LIKE PROTEINS IN ANTIMICROBIAL DEFENSE

The family of SAPLIPs comprehends heterogeneous and functionally divergent proteins that share a conserved motif of six cysteine residues associated by three disulfide bonds (Munford *et al.*, 1995). This motif forms the characteristic “saposin fold” that allows SAPLIPs to interact with lipids (Bruhn, 2005). The SAPLIP domain can be regarded as an ancestral molecule since it is present both in humans and in one of the most primitive eukaryotes, namely amoebozoans. *Entamoeba histolytica* is a prototypical pathogenic amoebozoan that produces amoebapores. These molecules belong to the family of SAPLIPs and exert cytolytic activities against bacteria and human cells, by forming pores in the target cell membrane as a killing principle (Leippe *et al.*, 1994a, b). *E. histolytica*is the etiologic agent of human amoebiasis, and recent evidence suggests that amoebapores could be responsible for the tissue destruction upon infection (Bracha *et al.*, 2003). To date, 19 genes encoding SAPLIPs have been identified in *E. histolytica*. Considering that the primary function of amoebapores is the destruction of phagocytosed bacteria for nutritional purposes, a diversity of SAPLIPs might be required for subsequent digestive steps. Interestingly, SAPLIPs with similar lytic functions have been described in humans and other mammals. These include human granulysin and porcine NK-lysin that share the highest degree of homology among members of the SAPLIP family. Similar to amoebapores, granulysin and NK-lysin show a broad spectrum of antimicrobial activity, killing parasites, bacteria, and fungi (Ernst *et al.*, 2000; Leippe, 1995; Pena *et al.*, 1997; Stenger *et al.*, 1998). In combination with perforin, granulysin released by T cells kills *M. tuberculosis*in macrophages by affecting the integrity of its cell wall, leading to subsequent osmotic bacterial lysis (Stenger *et al.*, 1998). NK-lysin is predominantly stored by T cells and NK cells in cytosolic granules, and is released upon activation (Andersson *et al.*, 1995). In addition, NK-lysin was found to be lytic against YAC-1 tumor targets, and especially potent at killing tumor cell lines that had increased surface levels of PS (Andersson *et al.*, 1995; Schroder-Borm*et al.*, 2005). By contrast, granulysin has been more extensively studied with regard to its tumorolytic functions against human T cell lymphoma of the Jurkat type (Kaspar *et al.*, 2001). Moreover, several studies could unequivocally demonstrate the importance of granulysin and antimicrobial proteins in various infectious diseases (Heusel *et al.*, 1994; Kagi *et al.*, 1994; Lowin *et al.*, 1994; Stenger *et al.*, 1998). To date, the structures of six SAPLIPs have been resolved, starting with the first crystal of NK-lysin (Liepinsh *et al.*, 1997). They all show the same α-helical fold of five helices connected by three disulfide bonds. Surprisingly, the predicted modes of action by lytic SAPLIPs suggest extremely

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diverse mechanisms used to achieve membrane permeabilization. In its active state, the pore-forming amoebapore A is a dimer stabilized by electrostatic interactions that involve a unique, centrally positioned histidine residue (Andra and Leippe, 1994). One side of the dimer is exclusively hydrophobic, which allows its insertion into membranes. Once docked, the protein oligomerizes to create ring-like pores that resemble channels (Gutsmann *et al.*, 2003). The histidine residue functions as a pH-dependent switch. Activation of this switch occurs at low pH and allows the dimerization that is crucial for the cytolytic function (Andra and Leippe, 1994). Considering its function, it is therefore not surprising that this residue is highly conserved among all amoebapore isoforms. By contrast, NK-lysin and granulysin permeabilize membranes in a monomeric state through an electrostatic process termed electroporation (Miteva *et al.*, 1999). Concerning both SAPLIPs, membrane recruitment occurs through interactions of positively charged residues in the SAPLIP and negatively charged phospholipids in the target membrane. Subsequent conformational changes allow the two halves of the molecules to slit the membrane in a scissor-like fashion, ultimately causing osmotic lysis (Anderson *et al.*, 2003). In *Naegleria fowleri*, the SAPLIP naegleriapores are encoded in larger multipeptide precursor structures, each potentially giving rise to multiple glycosylated naegleriapore-like molecules (Herbst *et al.*, 2004). Finally, functional characterization of a novel SAPLIP in *E. histolytica*, namely SAPLIP 3, revealed fusogenic activities similar to SAP-C in mammalians (Winkelmann *et al.*, 2006). Accordingly, we highlight the possibility that saposins might possess conserved evolutionary traits of ancient weapons which potentially mediate direct antimicrobial functions (Fig. 2.2).

7. CONCLUSIONS

Decades ago, saposins have been considered as negligible test tube activators of lysosomal glycosidases. Today, saposins emerge as critical components of membrane homeostasis and unexpected actors on the scene of immunology. In addition to their well-established capacities in sphingolipid degradation and membrane digestion, saposins also fulfill important immunological functions. Based on their universal principle to interact with membrane bilayers in lysosomes, SAPs exert versatile helper functions further defined by their respective interaction partners. In this context, saposins can mobilize lipids from membranes and associate with lipid-degrading enzymes, in order to degrade or generate antigenic epitopes. Further, saposins interact with antigen-presenting molecules to facilitate the loading of lipid antigens onto CD1 proteins for subsequent activation of lipid-reactive T lymphocytes. Additionally, the membrane-perturbing properties of SAPs can mediate the disintegration of apoptotic bodies for antigen delivery in APCs and subsequent cross-presentation. Finally, saposins could have antimicrobial functions through direct membrane attacks on pathogens.

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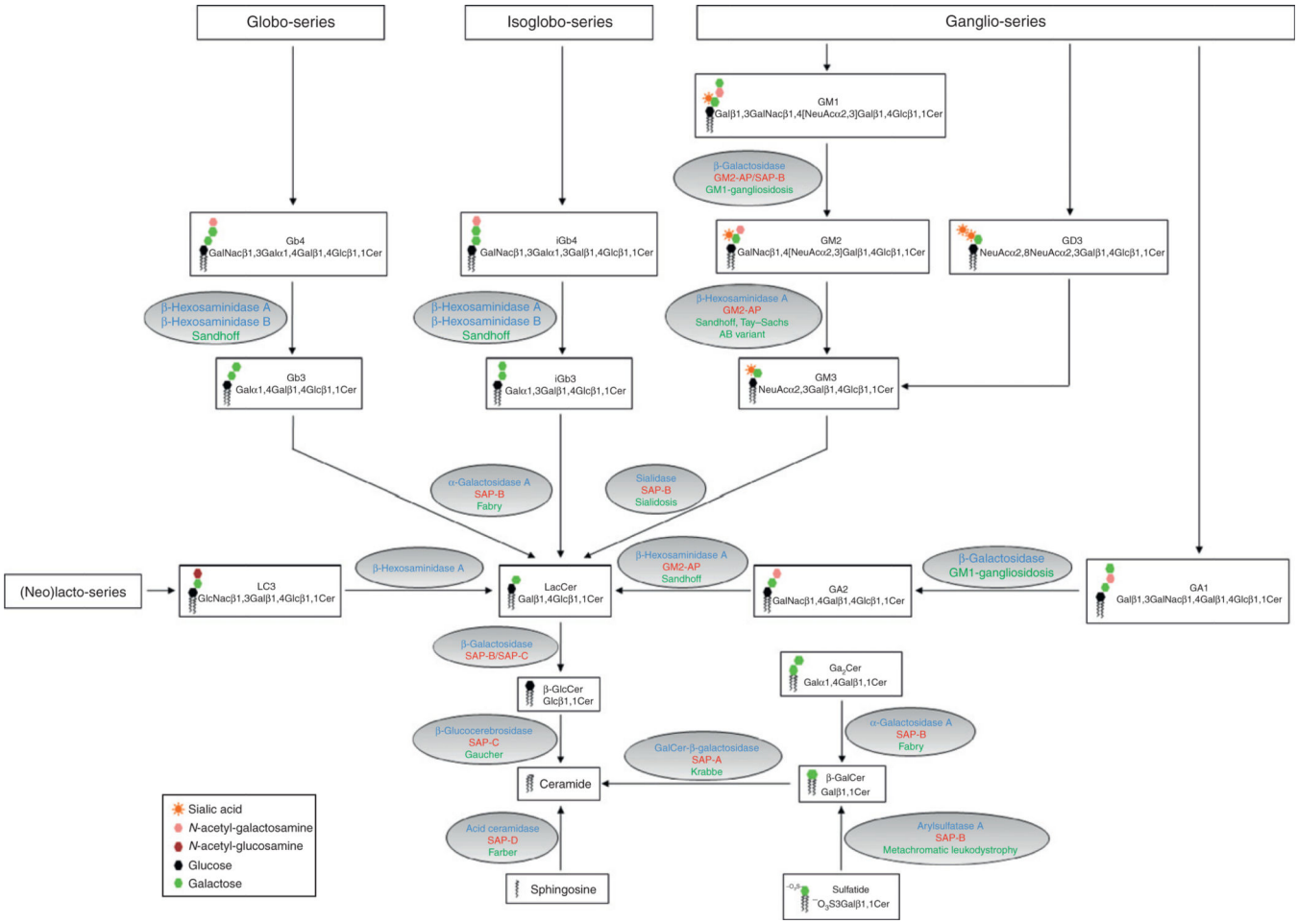

Figure 2.1.

Pathways of GSL degradation. The graph depicts the degradative pathways of various GSL species, including the globo-, isoglobo-, ganglio-, and (neo)lacto-series. The names, formula, and structural icons of the respective GSLs are shown in the boxes. The ovals contain the enzymes (depicted in blue) and the saposins (shown in red) that are involved in the corresponding lipid degradation step. The associated lysosomal storage disease due to enzyme or saposin deficiency or both is indicated in green.

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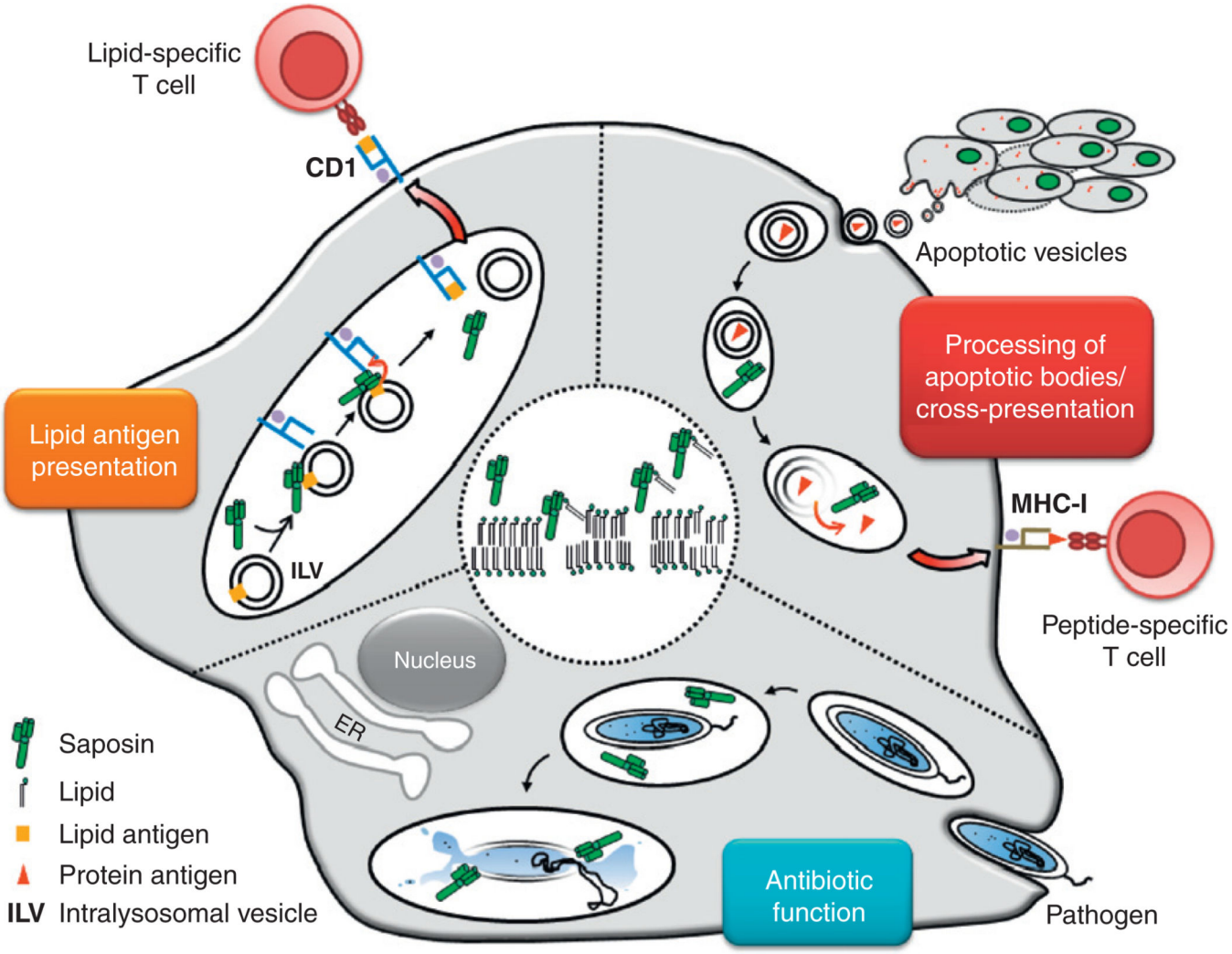

Figure 2.2.

Immunological functions of saposins. The central circle demonstrates the universal mechanism of saposin action on lipid bilayers. Accordingly, saposin molecules insert into lysosomal membranes to induce bilayer disintegration and lipid extraction. Further, the graph represents the three immunological consequences of this mechanism. (1) Lipid antigen presentation: In lysosomes of APCs, saposins interact with intralysosomal vesicles (ILVs) that contain lipid antigens. Subsequently, SAPs facilitate the loading of CD1 molecules with lipid antigens to activate CD1-restricted lipid-reactive T cells. (2) Processing of apoptotic bodies / cross-presentation: Apoptotic bodies derived from tumor or infected cells are engulfed by phagocytes such as DCs or macrophages. Protein antigens contained in apoptotic vesicles are liberated in lysosomes upon membrane disintegration induced by saposins. This leads to antigen delivery from apoptotic bodies for subsequent cross-presentation of antigens to MHC-I-restricted $CD8^+$ T cells. (3) Antibiotic function: Pathogens like intracellular bacteria are phagocytosed by macrophages or DCs. At the acidic

pH of lysosomes, saposins unfold their antimicrobial effects through direct attack on the membranes of pathogens.

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