Lipid-binding Proteins in Membrane Digestion, Antigen Presentation, and Antimicrobial Defense

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Lipid-binding proteins are required for the lysosomal digestion of a subset of membrane components, the glycosphingolipids. Glycosphingolipids are ubiquitously present on eukaryotic cell surfaces, where they form cell type-specific and development-dependent patterns (6). They are composed of a hydrophobic ceramide moiety and an extracytoplasmic oligosaccharide chain (4). The digestion of membranes requires their delivery into the lumen of the endosomal compartment, which starts with the formation of clathrin-coated pits, non-clathrin-coated pits, caveolae, and others (7). During endocytosis, the membrane fraction that is destined for degradation buds into the lumen of the acidic compartment as intraluminal vesicles and related lipid aggregates (8, 9). The limiting membrane, which separates the organelle from the cytosol, differs from the intraluminal membranes by its lipid composition and is protected from degradation by glycoproteins (e.g. LAMP-1) that are highly N-glycosylated with polylactosamine units (10). Therefore, two membrane pools in the endosomal compartment have to be distinguished in structural and functional terms, the perimeter membrane with a slow turnover, and the internal membrane structures that are the major site of digestion. These membranes are visible in the microscope as multivesicular bodies (11), accumulate in patients with sphingolipid storage diseases (3), and are specifically enriched in a lysosomal lipid, bis(O-monoacylglycerol)phosphate (2, 12), also erroneously called lysobisphosphatic acid. In addition to other lipids, this negatively charged lipid is required for the digestion of intralysosomal membranes and is essential for the action of SAPs (2).

It has been known for more than 30 years that in vivo degradation of some glycosphingolipids requires the presence of SAPs. These proteins act on the intraluminal/intralysosomal membrane pool and lead to the selective degradation of membrane lipids without impairment of lysosomal integrity. The GM2 activator The GM2 activator is a glycoprotein of about 22 kDa and acts as a lipid transfer protein in vivo that can carry lipids from donor to acceptor liposomes (13). The GM2 activator is required for in vivo degradation of ganglioside GM2 by β-hexosaminidase A (14). β-Hexosaminidase A cleaves glycolipid substrates on membrane surfaces only when 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. Film balance experiments revealed that this protein is only able to insert into lipid monolayers when the lateral pressure is below a critical value of about 25 millinewtons/m (15). The lateral surface pressure of most biological membranes is significantly higher (30–35 millinewtons/m). Apparently this factor also ensures that the GM2 activator can only interact with the intraluminal/intralysosomal membrane pool.

The three-dimensional structures of β-hexosaminidase B (16, 17) and the non-glycosylated GM2 activator (18, 19) have been solved by x-ray diffraction. The GM2 activator contains a hydrophobic cavity that can be closed by a flexible loop. Based on this structural information (18, 19) and on photoaffinity labeling experiments (20), a mechanism of action has been proposed, also taking earlier considerations into account (8) (Fig. 1). The lipid-free activator in its open conformation binds two hydrophobic loops to the membrane and penetrates the bilayer. Subsequently, the lipid recognition site of the activator interacts with the ganglioside substrate, the ceramide moiety of which inserts into the hydrophobic cavity. By moving the flexible hydrophobic loop, the lipid-loaded activator changes from the open conformation to the more hydrophilic closed one. Finally, the activator-ganglioside complex is recognized by the water-soluble enzyme as the real Michaelis-Menten substrate (14).

The inherited deficiency of the GM2 activator results in the AB variant of GM2 gangliosidosis, in which ganglioside GM2 accumulation in neuronal cells leads to the early death of the patients (21). The Saps The Saps or saposins A–D are four acidic, enzymatically inactive, heat-stable, and protease-resistant glycoproteins of about 8–11 kDa (3). They belong to a family of saposin-like proteins (SAPLIPs) with lipid-binding and membrane-perturbing properties and with conserved three-dimensional structures (22). Three-dimensional structures of the saposin family members have been established, including NK-lysin (23), the pore-forming peptide of Entamoeba histolytica (24), Sap-C (25), and the x-ray structure of nonglycosylated human recombinant Sap-B (26).

Prosaposin—All four saposins are derived from the Sap precursor, or prosaposin, by proteolytic processing in late endosomes and lysosomes (3). Prosaposin is a 70-kDa glycoprotein detected mainly in body fluids, brain, heart, and muscle. Mature Sap are found mainly in liver, lung, kidney, and spleen. The Sap precursor is targeted to the lysosomes through mannose 6-phosphate receptors, or sortin (27, 28), or it is secreted and re-endocytosed by mannose 6-phosphate receptors, low density lipoprotein receptor-related protein, or mannose receptors (29).

To date, two different mutations in four human patients have been reported that lead to a complete deficiency of the whole Sap precursor protein. In human patients with Sap precursor deficiency as well as in Sap precursor knock-out mice, storage of some sphingolipids has been observed, including ceramide, glucosylceramide, lactosylceramide, ganglioside GM3, galactosylceramide, sulfatides, digalactosylceramide, and globotriaosylceramide, accompanied by a dramatic accumulation of inner membranes in late endosomes and lysosomes. Storage can be completely reversed by treatment with human Sap precursor, as demonstrated in prosaposin-deficient fibroblasts (31).

Sap-A—Sap-A is required for the degradation of galactosylceramide by galactosylceramide–β-galactosidase in vivo. Mice that carry a mutation in the Sap-A domain of the Sap precursor and therefore lack mature Sap-A accumulate galactosylceramide. The phenotype of these mice resembles a late onset form of galactosylceramide–β-galactosidase deficiency (Krabbe disease) (32). Recently, a human disease also has been described that resembles Krabbe disease but is caused by a singular defect of Sap-A (33).

Sap-B—Sap-B has been identified as the first sphingolipid activator protein, the sulfatide activator, in 1964 (34). It is required for the degradation of sul-
fatide by arylsulfatase A and of globotriosylceramide and digalactosylceramide by α-galactosidase A in vivo. Patients with Sap-B deficiency accumulate these substrates in the urine (35). In association with GM2 activator protein, Sap-B is also required for the degradation of membrane-bound ganglioside GM1 by water-soluble β-galactosidase (36, 37).

Like the GM2 activator, Sap-B acts as physiological detergent but shows a broader specificity than the GM2 activator. Its crystal structure shows a shell-like homodimer that encloses a large hydrophobic cavity (26). Like the GM2 activator, Sap-B dimers occur in two different conformations. Thus, a similar mechanism for its action has been proposed. The open conformation is believed to interact directly with the membrane, to promote reorganization of the lipid alkyl chains, to extract the lipid substrate, and to change into the closed conformation. Thereby, the substrate could be exposed to the enzyme in a water-soluble activator-lipid complex (38), consistent with the observation that Sap-B can act as a lipid transport protein (39).

The inherited defect of Sap-B leads to an atypical form of metachromatic leukodystrophy, with late infantile or juvenile onset (40). The disease is characterized by accumulation of sulfatides, digalactosylceramide, and globotriosylceramide (3).

Sap-C—Sap-C has initially been isolated from spleens of patients with Gaucher disease (41). It also occurs as a homodimer and is required for the lysosomal degradation of glucosylceramide by glucosylceramidase-β-glucosidase (41). The solution structure of Sap-C (25) consists of 5 tightly packed α-helices that form a hemisphere. In contrast to GM2 activator and Sap-B, Sap-C can directly activate glucosylceramidase-β-glucosidase in an astatic manner (41) and bind to the antigen-presenting molecule CD1b (see below). Sap-C also supports the interaction of the enzyme with the substrate embedded in vesicles containing anionic phospholipids, and Sap-C is able to destabilize these vesicles (42). Binding of Sap-C to phospholipid vesicles is a pH-controlled, reversible process (43). Sap-C deficiency leads to an abnormal juvenile form of Gaucher disease and an accumulation of glucosylceramide (44).

Sap-D—Sap-D stimulates lysosomal ceramide degradation by acid ceramidase in cultured cells (45) and in a liposomal assay (46). Sap-D binds vesicles containing negatively charged lipids and solubilizes them at an appropriate pH (47). Sap-D-deficient mice accumulate ceramides with hydroxylated fatty acids mainly in the brain and in the kidney (48).

Lipid-binding Proteins in Immunity

Conventional T cells recognizing peptides in the context of major histocompatibility complex class I and II molecules are major effectors in antimicrobial immunity. However, unconventional T cell populations are also activated upon infection. One of these populations responds to lipid antigens presented by CD1 molecules, which are encoded by genes located outside of the major histocompatibility complex locus (49). In humans, the group I CD1 molecules, CD1a, CD1b, and CD1c, restrict αβ T cell receptor-bearing T cells. These T cells primarily secrete interferon-γ and have cytolytic function (49), characterizing them as prime effector T cells in infections with intracellular bacteria such as Mycobacterium tuberculosis. The group II CD1 molecule CD1d restricts natural killer (NK) T cells. These T cells bear an evolutionary conserved T cell receptor using the invariant α-chains Vα14-Jα18 in mice and Vα28-JαQ in humans (49). NK T cells probably fulfill regulatory as well as effector functions according to their dual secretion of interferon-γ and interleukin-4 and their cytolytic activity.

The lipids presented by CD1 molecules are mostly of mycobacterial origin suggesting their specialized function to pick up and present bacterial lipids. Mycobacterial lipoarabinomannan (LAM), mycolic acids, glucos monomycolate (GMM), phosphatidylinositol mannosides (PIM), and sulfoglycolipids are ligands for CD1b, bacterial mannophosohosphoinositol lipids for CD1c, and mycobacterial lipopeptides for CD1a (49–52). Microbial antigens for NK T cells include PIM, sphenolipids from Ehrlichia and Sphingomonas species, and lipophosphoglycan from parasites of the genus Leishmania (53–56). α-Galactosylceramide (αGalCer), derived from a marine sponge and therefore of questionable physiological relevance to mammals, binds CD1d and is a strong agonist for Vα14 T cell receptor-bearing T cells. Moreover, self-lipids represent ligands for CD1 molecules such as ganglioside GM1 for CD1b and isoglobotriaosylceramide (iGb3) for CD1d (49, 57).

Recent studies shed light on the molecular machinery underlying glycolipid presentation. Crystal structures of CD1 molecules suggest that fatty acid chains of amphipathic lipids bind the hydrophobic antigen-binding groove between the α1 and α2 domain and that the T cell receptor recognizes the
hydrophilic head group (58–60). Whereas CD1a and CD1d have two hydrophobic pockets to bind two acyl chains, CD1b has a series of four hydrophobic channels accommodating long fatty acids such as mycolates (58–60). Lipid presentation by CD1b, CD1c, and CD1d requires trafficking of these molecules through late endosomes/lysosomes (49). There, lysosomal lipid-binding proteins are involved in bridging the lipid-water interface to load lipids into the CD1 antigen-binding groove (Fig. 2). Saposin-negative cells expressing CD1b were unable to activate T cells specific for LAM, GMM, and mycolic acid. More importantly, antigen presentation was fully restored by exogenous Sap-C but no other saposins. Thus, Sap-C is indispensable for the presentation of mycobacterial lipids by CD1b (5). This protein extracts mycobacterial lipids from membranes and, based on co-immunoprecipitation studies, directly interacts with CD1b (5). Saposins are also involved in loading of lipids onto CD1d (57, 61, 62). Moreover, loading of exogenous αGalCer on CD1d is facilitated by GM2 activator protein (61). One study showed that only recognition of exo- but not endogenous lipids by NKT cells is blocked in the absence of saposins (62). However, another study demonstrated that prosaposin-deficient mice lack Vα14 T cells specific for LAM, GMM, and mycolic acid. More importantly, antigen presentation was fully restored by exogenous Sap-C but no other saposins. Thus, Sap-C is indispensable for the presentation of mycobacterial lipids by CD1b (5). This protein extracts mycobacterial lipids from membranes and, based on co-immunoprecipitation studies, directly interacts with CD1b (5). Saposins are also involved in loading of lipids onto CD1d (57, 61, 62). Moreover, loading of exogenous αGalCer on CD1d is facilitated by GM2 activator protein (61). One study showed that only recognition of exo- but not endogenous lipids by NKT cells is blocked in the absence of saposins (62). However, another study demonstrated that prosaposin-deficient mice lack Vα14 T cells specific for LAM, GMM, and mycolic acid.

**Lipid-binding Proteins in Antimicrobial Defense**

In many species from protozoa to man, saposin-like proteins have been identified as putative gene products by data base searches. Although their biological function is often unknown, some saposin-like proteins from amoeboid protozoans and from mammalian cytotoxic lymphocytes can kill foreign cells by membrane permeabilization (64, 65). For example, the pore-forming proteins amoebapores from the enteric parasite *Entamoeba histolytica* and *Naegleria fowleri* (66) are both well known human pathogens that can cause life-threatening diseases. Saposin-like proteins from NK cells and cytotoxic T-lymphocytes of pigs and humans are NK-lysin and granulysin (67). The genes of their precursors have been cloned, and for the majority the three-dimensional structures have been solved (23, 24, 68). Although the amoebic and mammalian proteins originate from evolutionarily very distant organisms, they share a variety of properties: (i) the mature polypeptides are about 80 residues in length and comprise the SAPLIP domain only, (ii) they are stored as active molecules in cytoplasmic granules of the producing cell, (iii) they display antimicrobial activity but are also cytolytic toward mammalian cells, and (iv) they are transferred intracellularly to the phagolysosome, for example, in the case of phagocytosed bacteria, or they are released extracellularly onto the target cell membrane by granule exocytosis in a contact-dependent cytolytic reaction.

However, clear differences between the amoebapores and the mammalian effector peptides became apparent when their tertiary structures were solved (Fig. 3). Whereas the cationic mammalian effector peptides act more superficially on the membranes of bacteria to permeabilize them, the amoebapores are more hydrophobic and appear to insert into the phospholipid bilayer. Here, one histidine residue can trigger the pH-dependent formation of an active dimer (24) and ultimately the creation of an oligomeric, most likely hexameric, pore inside the target cell membrane (for a review, see Ref. 69).

Comprehensive functional and structural studies have been conducted with the natural proteins and with shortened analogs thereof. For example, the monitoring of the biological activity of the protein toward different natural targets such as Gram-positive and Gram-negative bacteria and eukaryotic cells and the use of artificial minimalistic systems such as liposomes and planar lipid bilayers were studied (70–72). Saposin-like proteins combat growth of engulfed bacteria by permeabilizing the
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bacterial membrane inside the digestive vacuoles of inatissible bacteria-phagocy-
tosing cells (73). Such cells may also act as extremely potent but unprofessional
killers of human host cells. Investigations in amoebicidal-deficient amoeba tested
in animal model systems indicate that saposin-like proteins play a key role in
pathogenesis (74). In lymphocytes as professional killer cells, it appears that the
lytic effector proteins, particularly granulysin, are part of the machinery that kills
intracellular pathogens such as mycobacteria (75) and that the proteins have a role
in apoptotic cell death of tumor cells (for a review, see Ref. 67).

A distinct situation was found in the amoeboid N. fowleri, a free-living proto-
zoan. The naegleriapores are organized as several isoforms in a prepropeptide-
tide structure, presumably destined to efficiently synthesize multiple antibacterial
weapons (76). This is in contrast to amoeboids, NK-lysin and granulysin, but
comparable to the saposins. Moreover, some of the naegleriapores were found to be
N-glycosylated, which is again comparable to the saposins. Although deglyco-
sylation did not affect the biological activity of naegleriapores, it may well be that
similarly as in Sap-B and Sap-D, glycosylation improves the correct folding of the
peptides and therefore the stability against proteolytic degradation. It is tempting
therefore the stability against proteolytic degradation. It is tempting to think that saposin-like proteins may have originated at the very early phylogene-

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