MINIREVIEW

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

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Membrane-active proteins are able to bind lipid molecules, transfer lipids to proteins or to target membranes, and selectively perturb lipid bilayers (1). They differ in binding specificity and subcellular localization and also in their cytoplasmic, extracytoplasmic, and intercellular topology and functions. Lipid-binding proteins include, for example, cholesterol-binding proteins, like NPC-1 and -2 or ABCA1 (1). A subset of lipid-binding proteins has a major role in the lysosomal degradation of sphingolipids with short hydrophilic head groups. These extracytoplasmic proteins are the sphingolipid activator proteins (SAPs),[2] namely the saposins (Saps) and the GM2 activator protein. They act in the degradation of inner endosomal and lysosomal membranes. This process is favored by the low cholesterol and high bis(monoacylglycero)phosphate content of these inner membranes (2). The SAPs are membrane-perturbing and lipid-binding proteins with different specificities for the lipid to be bound and the enzyme-catalyzed reaction to be activated (2, 3). Inherited SAP deficiencies lead to sphingolipid and membrane storage diseases (4). SAPs not only facilitate glycolipid digestion but also act as transfer proteins by loading glycolipids on antigen-presenting molecules of the CD1 family (5). Other proteins of saposin-like structure are also able to enhance the permeability of membranes of target cells (see below). These proteins are used by different organisms for antimicrobial defense.

Lipid-binding Proteins in Membrane Digestion

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 intraendosomal vesicles and related lipid aggregates (8, 9). The limiting membrane, which separates the organelle from the cytosol, differs from the intraendosomal 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(monoacylglycero)phosphate (2, 12), also (erroneously) called lysobisphosphatidic 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 intraendosomal/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 vitro* 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 can cleave 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 intraendosomal/intralysosomal membrane pool.

The three-dimensional structures of β-hexosaminidase B (16, 17) and the nonglycosylated 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 with 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 Saposins—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 folds (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 Saps are found mainly in liver, lung, kidney, and spleen. The Sap precursor is targeted to the lysosomes through mannose 6-phosphate receptors, or sortilin (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 (30), storage of many 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-

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[2] The abbreviations used are: SAP, sphingolipid activator protein; Sap, saposin; SAPLIP, saposin-like protein; NK, natural killer; αGalCer, α-galactosylceramide; Cer, ceramide; GalNAc, *N*-acetyl-D-galactosamine; GM1, Galβ1,3GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glcβ1Cer; GM2, GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glcβ1Cer; GM3, NeuAcα2,3Galβ1,4Glcβ1Cer; GMM, glucose monomycolate; iGb3, isoglobotriaosylceramide (Galα1,3Galβ1,4Glcβ1Cer); LAM, lipoarabinomannan; NeuAc, *N*-acetylneuraminic acid; PIM, phosphatidyl-*myo*-inositol mannosides; MTTP, microsomal triglyceride transfer protein.

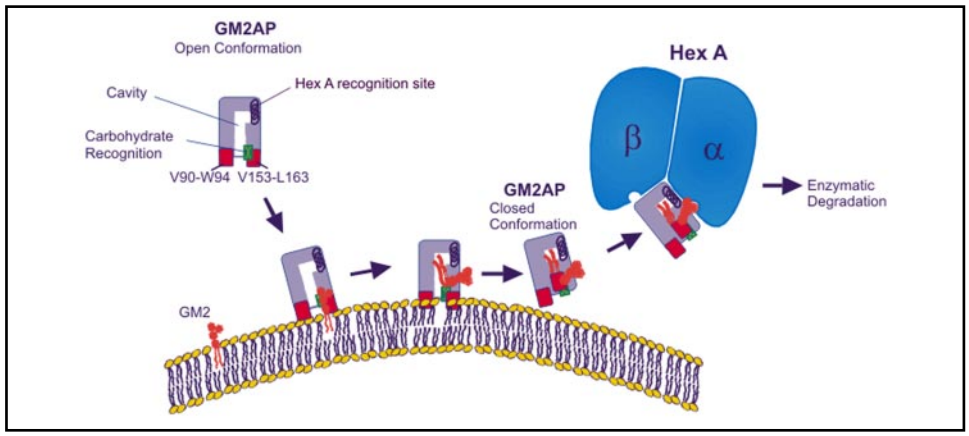

FIGURE 1. **Model of GM2 activator-stimulated hydrolysis of ganglioside GM2 by human β-hexosaminidase A (20).** The glycolipid binding site is lined by two hydrophobic (Val^{90}–Trp^{94} and Val^{153}–Leu^{163}) surface loops and a single short helix. The most flexible of the loops (Val^{153}–Leu^{163}) controls the entrance to the hydrophobic cavity allowing both an open and closed conformation. *GM2AP*, GM2 activator protein; *Hex A*, β-hexosaminidase A.

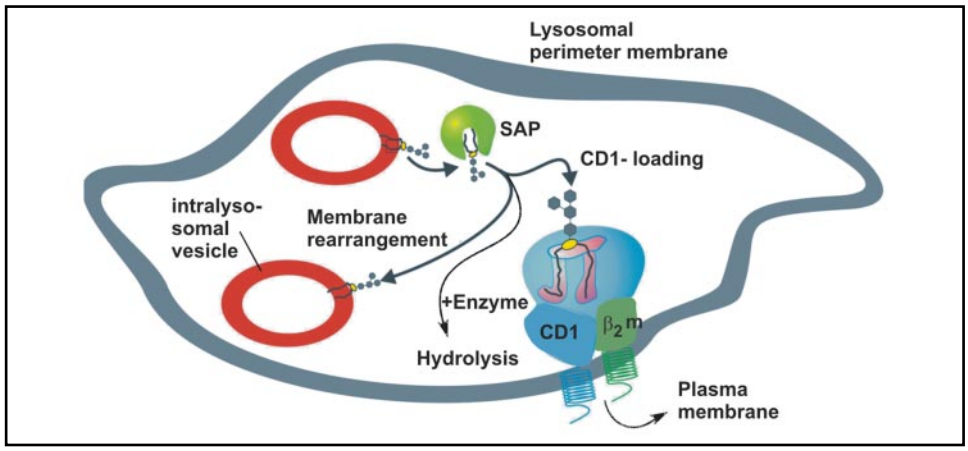

FIGURE 2. **Model of glycolipid loading onto CD1 molecules by lipid transfer proteins (modified from Ref. 2).** SAPs like Sap-C for CD1b (5) or Sap-A and the GM2 activator for CD1d (61) extract lipids from inner lysosomal membranes and transfer them to other membranes, to hydrolytic enzymes, or to CD1 molecules on the lysosomal perimeter membrane. Subsequently, lipid-loaded CD1 molecules are transported to the plasma membrane for subsequent T cell activation. The direct interaction between Sap-C and CD1b (5) is not shown. β_2m, β_2-microglobulin.

fatide by arylsulfatase A and of globotriaosylceramide 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 globotriaosylceramide (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 glucosylceramide-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 glucosylceramide-β-glucosidase in an allosteric 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 Vα14Jα18 in mice and Vα24JαQ in humans (49). NKT 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, glucose monomycolate (GMM), phosphatidyl-*myo*-inositol mannosides (PIM), and sulfoglycolipids are ligands for CD1b, bacterial mannophosphoisoprenoids for CD1c, and mycobacterial lipopeptides for CD1a (49–52). Microbial antigens for NKT cells include PIM, sphingolipids 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 antigen for $V\alpha14^+$ NKT cells (49). 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

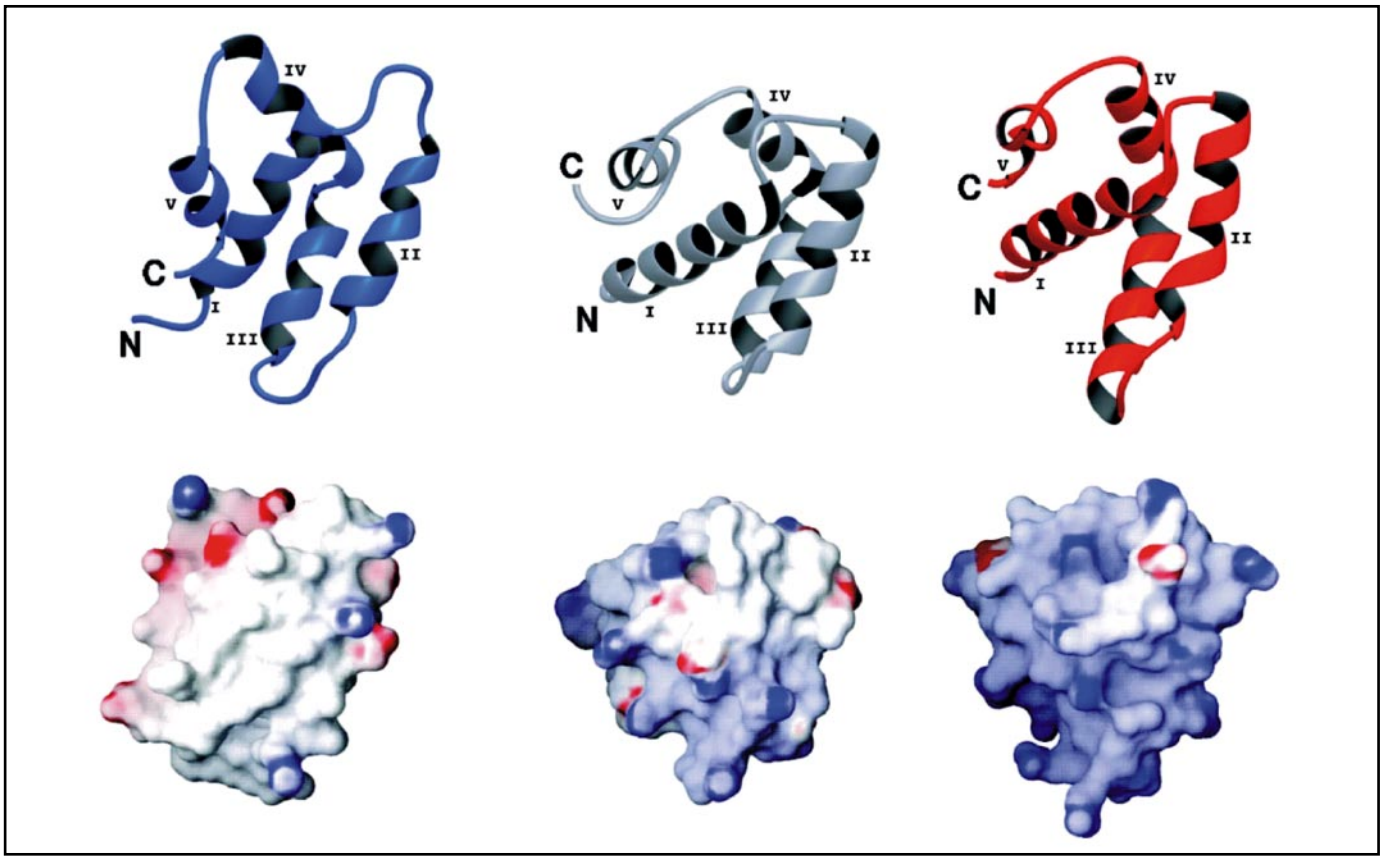

FIGURE 3. **Structures of amoebapore A (*left*), NK-lysin (*middle*), and granulysin (*right*).** This figure was reproduced with permission from Ref. 24. *Upper panel*, ribbon representations; the C and N termini are depicted in *black*. All molecules were superimposed onto C-α atoms of residues 30–60. Helices are numbered by *roman numerals*. *Lower panel*, the electrostatic potential of the molecular surface is shown in the same orientation as in the *upper panel*, indicating positive potential in *blue* and negative potential in *red*. The electrostatic potential maps were calculated and displayed with the GRASP program using standard parametrization (for details see Ref. 24).

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$^+$ NKT cells (61). This suggests that saposins are also important for loading of self-lipids on CD1d in late endosomes and lysosomes. Indeed, loading of iGb3 on CD1d in a cell-free assay is mediated by Sap-B (57). *In vitro* studies revealed that saposins, predominantly Sap-A and Sap-C, facilitate lipid exchange on CD1d (61). In hepatocytes, another lipid-binding protein, the microsomal triglyceride transfer protein (MTTP), has a complementary function in the endoplasmic reticulum to the lysosomal saposins for ligand binding to CD1d (63). Deletion of the MTTP gene or knocking down its expression abolishes NKT cell activation by hepatocytes and prevents NKT cell-mediated liver pathology. In conclusion, in addition to their basic function in lipid degradation and transport in lysosomes and endoplasmic reticulum, lipid-binding proteins are essential in antigen presentation and immunity.

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 naegleriapores from the free-living parasitic amoeboflagellate *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

bacterial membrane inside the digestive vacuoles of insatiable bacteria-phagocytosing cells (73). Such cells may also act as extremely potent but unprofessional killers of human host cells. Investigations in amoebapore-deficient amoebae 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 protozoan. The naegleriapores are organized as several isoforms in a prepromultipeptide structure, presumably destined to efficiently synthesize multiple antibacterial weapons (76). This is in contrast to amoebapores, 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 deglycosylation 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 to think that saposin-like proteins may have originated at the very early phylogenetic position of free-living amoebae. Subsequently, a plethora of variant isoforms may have evolved, which might have resulted in diverse functions such as broad spectrum antimicrobial activity and lipid binding cofactor activity for specific lysosomal enzymes.

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