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More Than a Pore: The Interplay of Pore-Forming Proteins and Lipid Membranes

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Abstract Pore-forming proteins (PFPs) punch holes in their target cell membrane to alter their permeability. Permeabilization of lipid membranes by PFPs has received special attention to study the basic molecular mechanisms of protein insertion into membranes and the development of biotechnological tools. PFPs act through a general multi-step mechanism that involves (i) membrane partitioning, (ii) insertion into the hydrophobic core of the bilayer, (iii) oligomerization, and (iv) pore formation. Interestingly, PFPs and membranes show a dynamic interplay. As PFPs are usually produced as soluble proteins, they require a large conformational change for membrane insertion. Moreover, membrane structure is modified upon PFPs insertion. In this context, the toroidal pore model has been proposed to describe a pore architecture in which not only protein molecules but also lipids are directly involved in the structure. Here, we discuss how PFPs and lipids cooperate and remodel each other to achieve pore formation, and explore new evidences of protein-lipid pore structures.

Keywords Pore-forming proteins · Protein folding in membrane · Lipid reorganization · Proteo-lipidic pore

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Abbreviations

PFPs	Pore-forming proteins
PFTs	Pore-forming toxins
CDCs	Cholesterol-dependent cytolysins
Chol	Cholesterol
AMPs	Antimicrobial peptides
SM	Sphingomyelin
CL	Cardiolipin
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
GPI	Glycosylphosphatidylinositol
MACPF/CDC	Complex-perforin/cholesterol-dependent cytolysin

Introduction: General Mechanism of Action of Pore-Forming Proteins (PFPs)

Many pathogenic organisms as well as fungi, sea anemones, or earthworms, produce toxic substances as a defense mechanism or as a means to attack the host at the onset of infection. Some of these virulence factors are commonly denominated pore-forming proteins (PFPs) due to their ability to pierce cellular membranes in the target cells (Bischofberger et al. 2012). A large subdivision of PFPs includes toxins, which are commonly referred to as pore-forming toxins (PFTs). Bacterial PFTs are the best-characterized family of PFPs, although eukaryotic PFTs such as actinoporins are also well studied (Álvarez et al. 2009; García-Ortega et al. 2011). Microorganisms produce PFTs in order to (i) obtain advantages in the competition with other microbes for the same resources (e.g., antimicrobial peptides or colicins) (Dalla Serra and Tejuca

Martínez 2011; Yeaman and Yount 2003), (ii) deliver proteins as an alternative to secretion systems (e.g., Gram-positive bacteria, such as *Streptococci*), or (iii) escape to the cytosol after entering the target cell by phagocytosis (e.g., listeriolysin O from *Listeria monocytogenes*) (Madden et al. 2001).

In animals, including humans, other PFPs simply form pores as part of their physiological action, which is usually also related to cell death. For example, Bax triggers apoptosis by inducing the permeabilization of the outer mitochondrial membrane (Cosentino and García-Sáez 2014; García-Sáez 2012) and perforin kills infected and malignant cells as part of the complement membrane attack complex (Pipkin and Lieberman 2007). Other examples are prion proteins involved in Alzheimer and Parkinson diseases, which have been shown to be able to alter membrane permeability (Kagan 2012). A large subset of PFPs perturbs the plasma membrane integrity in order to disrupt ion homeostasis of the host cell. Alternatively, their main target can be an intracellular component. In this case, the primary function of the pore-forming component is to facilitate the translocation across the membrane of other toxic constituents such as proteins with enzymatic activity (e.g., diphtheria and anthrax toxins) (Gonzalez et al. 2008; Iacovache et al. 2010).

Recent findings have proposed that PFPs form pores via similar mechanisms, which are independent of the structure or the source of the protein. This suggests that pore formation is an ancient form of attack that has been remarkably conserved (Iacovache et al. 2008). In general, PFPs are produced as soluble molecules and undergo a conformational change in order to insert into their target membrane, where they oligomerize and subsequently form water-filled pores. The pore structure is often formed by a ring of protein protomers that have rearranged their structure to generate an amphipathic surface large enough to drive spontaneous membrane insertion (Anderluh and Lakey 2008; Fradin et al. 2009; Parker and Feil 2005).

Beyond simply forming a pore, proteins and lipids act together at different levels during membrane damage. On the one hand, the lipid bilayer constitutes a suitable platform for the folding and oligomerization of PFPs. On the other hand, the effect of PFPs on the membrane involves a complex choreography of transformations before or during pore formation. These can include changes in lipid orientation, distribution, or fluidity, as well as variations in lipid phase organization (Álvarez et al. 2009; Barlic et al. 2004; Koller and Lohner 2014). Furthermore, and despite the fact that proteins are commonly seen as the main component of pores, lipids can be directly implicated in the formation and stabilization of the final pore structure (Fuertes et al. 2011; Gilbert et al. 2014; Qian et al. 2008).

PFPs have received special attention to investigate basic molecular mechanisms of protein insertion into lipid membranes and modulation of protein conformation by lipid binding. This superfamily of proteins has become an ideal model to study how soluble proteins interact with and oligomerize in membranes and induce lipid rearrangements. Due to the mechanistic similarities, new insight into the molecular aspects of PFPs assembly could help designing small molecules that promote or inhibit pore formation and, therefore, cell death in medically relevant situations. PFPs are also attractive systems for biotechnological applications in which their toxic action is targeted to specific membranes (Potrich et al. 2005; Tejuca et al. 2009).

This review focuses on the influence of the protein component on membrane structure and vice versa during pore formation by PFPs. We first describe the function of the lipid bilayer as the ideal environment for protein conformational changes, paying special attention to the definition of lipids as receptors of PFPs in the membrane. Then, we comment on the effect of PFPs on membrane organization, in addition to pore formation, which comprises lipid mixing, clustering, or domain remodeling, as well as lipid flip-flop. Finally, we discuss how lipids and proteins can act together to form the pore architecture.

Structural Classification of PFPs

Despite their highly divergent primary sequence, specific folds have been conserved throughout the evolution of PFPs. For this reason, it is useful to classify PFPs according to their main structural element in the final pore (Gouaux 1997) (Fig. 1; Table 1). PFPs are generally classified as α-PFPs (Fig. 1a, b, d) or β-PFPs (Fig. 1c, e) taking into account the α-helical or β-sheet structure of the membrane-integrated domain, respectively. Examples of α-PFPs are colicins (Fig. 1a) and ClyA, both from *Escherichia coli* (Lakey and Slatin 2001), actinoporins from sea anemones (Fig. 1b) (Álvarez et al. 2009), and the apoptotic protein Bax (Fradin et al. 2009; García-Sáez 2012). β-PFPs include staphylococcal α-toxin (Song et al. 1996), the protective antigen of anthrax toxin (Young and Collier 2007), the aerolysin family (Gurcel et al. 2006), and the family of cholesterol (Chol)-dependent cytolysins (CDC) (Fig. 1c) (Hotze and Tweten 2012; Tweten 2005).

The best-studied pores are those formed by β-barrels, which present high stability due to inter-strand hydrogen bonds. This allows a much more precise characterization of the oligomer architecture and of its intermediates by several techniques (Tilley et al. 2005; Tilley and Saibil 2006; Walker and Bayley 1995). Pioneering examples include the determination of the crystal structure of the pore of

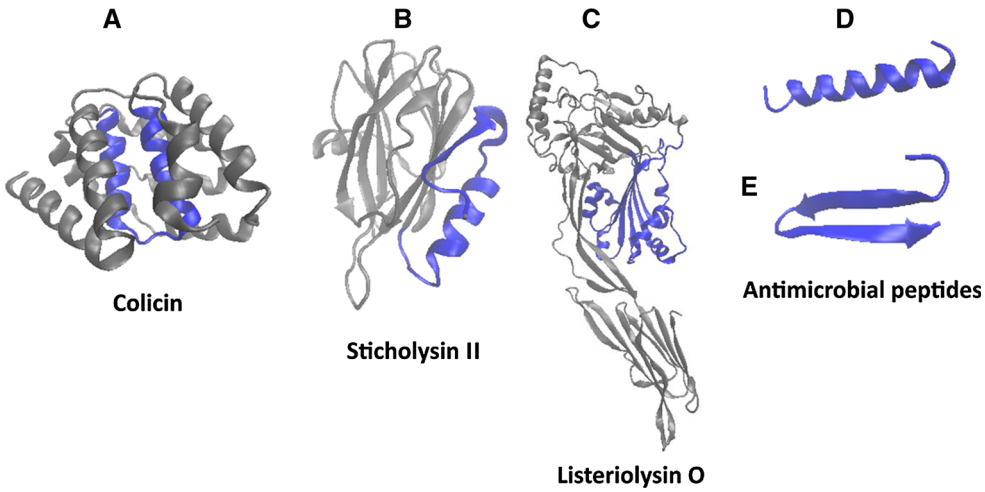

Fig. 1 3D structure of some representative PFPs and related antimicrobial peptides. **a** Water-soluble structure of colicin E1 (PDB: 2I88), **b** water-soluble structure of sticholysin II (PDB: 1O72), **c** water-soluble structure of listeriolysin O (PDB: 4CDB), **d** structure of magainin obtained in micelles as membrane mimetic system (PDB: 2MAG), **e** water-soluble structure of protegrin (PDB: 1PG1). The pore-forming domains are shown in *blue* (Color figure online)

Table 1 Pore-forming proteins (PFPs) classification and receptors

PFPs	Origen	Structural classification	Membrane targeting	Reference
Actinoporins	Sea anemones	α-PFP	Affinity for SM-containing membranes	(Álvarez et al. 2009; García-Ortega et al. 2011)
Bax	Humans	α-PFP	Affinity for negative charged membranes	(García-Sáez 2012)
Colicins	*E. coli*	α-PFP	Affinity for anionic lipids and binding to a protein receptor	(Cascales et al. 2007; Sobko et al. 2004)
Diphtheria toxin	*C. diphtheriae*	α-PFP	Affinity for anionic lipids and binding to a glycoprotein receptor	(Choe et al. 1992; Naglich et al. 1992)
Perfringolysin	*C. perfringens*	β-PFP	Affinity for Chol-containing membranes	(Hotze and Tweten 2012; Tweten 2005)
Protective antigen anthrax	*C. anthracis*	β-PFP	Binding to a protein receptor	(Young and Collier 2007)
Vibrio cholerae cytolysin	*V. cholerae*	β-PFP	Binding to carbohydrate receptor	(Saha and Banerjee 1997)

Staphylococcus aureus α-hemolysin (Mechaly et al. 2011; Song et al. 1996) or models of the pre-pore and pore structure of the CDC toxin perfringolysin O based on electron microscopy data (Dang et al. 2005; Rossjohn et al. 1997). On the contrary, most α-helical PFPs are believed to form flexible oligomeric structures, weakly associated and without a fixed stoichiometry, which makes their characterization difficult (Črnigoj Kristan et al. 2009; Subburaj et al. 2015). Consequently, studies aimed at obtaining 3D structures of α-PFPs pores often failed. In this sense, the description of the structure of the oligomeric pore of ClyA was considered exceptionally enlightening (Mueller et al. 2009). Recent efforts in this direction have allowed proposing a pore assembly pathway for α-PFPs based on the 3D structure of the actinoporin fragaceatoxin C at different stages during interaction with membranes (Tanaka et al. 2015) (further details in "Lipids and Proteins Acting Together: The Protein-Lipid Pore" section).

Antimicrobial peptides (AMPs) (e.g., magainin, melittin, or protegrin) are related compounds that share the ability with PFPs to permeabilize lipid membranes. In contrast with large PFPs, these are smaller molecules in which the lytic or pore-forming domain covers the entire length of the polypeptide (Fig. 1d, e). They generally exist in relatively unstructured or extended conformations in solution, but tend to adopt either an α-helical or β-sheet/-turn structure after interaction with the lipid bilayer (e.g., magainin, Fig. 1d). Other peptides adopt specific conformations in solution held by disulfide bonds (e.g., protegrin, Fig. 1e) (Yeaman and Yount 2003). Here, we revise some properties of small AMPs as simpler and classic examples of PFP-related compounds.

The Lipid Bilayer: Primary Target of PFPs

The contribution of lipids is relevant in every step of the mechanism of action of PFPs. In this section, we describe their role in PFPs binding to membranes and in the induction of changes in PFPs structure. We also discuss the main properties of the lipid bilayer that promote such transformations.

Once secreted, PFPs bind to their target membrane usually via a receptor exposed on the cell surface. Specific receptor interactions ensure high selectivity of the PFPs towards their target, which is essential for their function. Receptors for a variety of PFPs have been identified and they differ in their biochemical nature and specificity (Table 1). Transmembrane or lipid anchored proteins act as receptors in some cases (Nelson et al. 1997), but most often selectivity is achieved by the interaction with individual lipids or even clusters of lipids or domains (Álvarez et al. 2009; Cosentino and García-Sáez 2014; Valeva et al. 1997). Interaction with lipids allows targeting a broad range of host cells with similar membrane composition, while binding to a protein receptor permits restricting targeting to a narrow set of host membranes. Examples of PFPs recognized by protein receptors are colicins (Cascales et al. 2007), diphtheria toxin (Naglich et al. 1992), and the protective antigen of the anthrax toxin (Young and Collier 2007). Carbohydrates or glycoproteins are also able to function as receptors of PFPs as described for the Vcc toxin from *Vibrio cholera* (Saha and Banerjee 1997) or for aerolysin (Gordon et al. 1999). Some PFPs possess more than a single target in the membrane (Table 1). For instance, colicins (Cascales et al. 2007; Sobko et al. 2004) and diphtheria toxin (Choe et al. 1992) target the cell membrane through a mechanism involving binding to a specific protein receptor, but this process is accelerated by interaction with negatively charged lipids.

Here, we focus on the function of lipids as receptors of PFPs. Probably, the most studied case is the CDCs (Gonzalez et al. 2008; Hotze and Tweten 2012), whose affinity for membranes depends on the presence of Chol in the bilayer. Phospholipids like sphingomyelin (SM) or cardiolipin (CL), as well as unsaturated lipids, have also been described as lipid receptors of PFPs (Fradin et al. 2009; Gonzalez et al. 2008). For instance, SM has been proposed as the putative receptor for lysenin, a toxin from the earthworm *Eisenia fetida* (Yamaji-Hasegawa et al. 2003), and for actinoporins, which are toxins from sea anemones (Álvarez et al. 2009; García-Ortega et al. 2011). CL has been suggested to be essential for Bcl-2 proteins function during apoptosis, thereby participating in the regulation of programed cell death (Cosentino and García-Sáez 2014; Unsay et al. 2013), and Cyt δ-endotoxins seem to recognize unsaturated lipids (Thomas and Ellar 1983).

Lipids as "Membrane Receptors": Role of the Lipid Physical–Chemical Properties on Membrane Partitioning

Lipids are generally described as "membrane receptors" for a certain number of PFPs because they are their main targets on the membrane. According to the classical biochemical designation, receptors are characterized by highly specific binding to a ligand with a fixed stoichiometry and via specific sites. This description is appropriate when considering PFPs as ligands of membrane-embedded receptors of protein nature. However, conceptual differences arise when the definition of receptor is applied to the association of PFPs with membrane lipids. In this scenario, it is important to consider the collective properties of the lipids in the bilayer and the chemical origin of the interactions (i.e., hydrophobic or electrostatic) that drive PFP-bilayer association. One should consider PFPs interactions with a lipid assembly, the bilayer, rather than with the individual molecules comprising it (White et al. 1998). Therefore, the interaction of PFPs with membranes is often more complicated than the simple recognition of an individual lipid as a receptor. Each consecutive step in the process of pore formation could be influenced by a single physical–chemical property of the lipid molecule, by a combination of several of its features, or by the physical parameters arising from the collective nature of lipids in membranes (Álvarez et al. 2009).

The simplest mechanism by which PFPs preferentially interact with a target membrane is via electrostatic and hydrophobic interactions. Cluster of basic amino acids is often observed in PFPs like the members of the Bcl-2 family, the diphtheria toxin (Fradin et al. 2009), and actinoporins (García-Ortega et al. 2011). Additionally, clusters of aromatic amino acid residues, mainly Trp and Tyr, are also commonly found in their structures (e.g., actinoporins and CDCs) (Álvarez et al. 2009; Hotze and Tweten 2012). Consequently, preference for negatively charged membranes (e.g., CL-containing membranes) or raft components (e.g., SM or Chol-containing membranes) is observed for many PFPs (Table 1). From a mechanistic point of view, electrostatic interactions ensure the attraction to the negatively charged lipid membrane, while hydrophobic interactions seem to be relevant not only for membrane recognition but also for bilayer insertion.

Dual Role of Lipids as Receptors and Modulators of Membrane Properties Suitable for PFPś Interaction

Membrane properties vary with lipid composition. For instance, lipids like Chol and SM promote the creation of lipid domains with increased order and rigidity, while unsaturated lipids tend to partition to more disordered lipid phases, and lipids such as PE with relatively small head groups tend to adopt non-bilayer structures (Bayley 2009). In this section, we describe with classic examples how specific lipids can act as promoters of membrane properties convenient for membrane insertion more than as conventional receptors.

CDCs Family

CDCs are a family of β-PFPs secreted by several types of Gram-positive bacteria (e.g., perfringolysin O from *Clostridium perfringens*, listeriolysin O from *Listeria monocytogenes*, and pneumolysin O from *Streptococcus pneumonia*) that target animal cell membranes containing Chol or related sterols. Their common name arises from the fact that most CDCs bind to membranes by using Chol as their receptor. This is an elegant example of how PFPs selectivity can be ensured by binding to a lipid specific for mammalian cells (Alouf 2000; Hotze and Tweten 2012). The stoichiometry of CDC–Chol interaction is 1:1, which suggests the presence of a single Chol binding site in these toxins (Johnson et al. 1980). It is generally accepted that an undecapeptide and three loops located at the tip of domain 4 of CDCs contribute to membrane recognition. However, the pair Thr-Leu (490-491 in perfringolysin O) located in the loop 1 has recently been identified as the Chol binding motif for CDCs (Farrand et al. 2010). This simple binding motif is not unexpected, since the head group of Chol is also structurally simple when compared with other receptor molecules, like proteins. In fact, Chol recognition by CDCs seems to be restricted to the 3-β-hydroxyl group of the sterol, since toxin–lipid interaction is not affected when the Chol ring structure undergoes minor changes (Prigent and Alouf 1976).

The basis of this recognition is likely more complex than the simple encounter frequency between CDCs and individual Chol molecules as high concentrations of Chol (>40 %) are needed for pore formation (Hotze and Tweten 2012). Moreover, the membrane lipid environment also seems to play a role in the recognition. It is plausible that only a fraction of the total membrane Chol serves as a receptor for CDCs (Hotze and Tweten 2012). For example, studies with perfringolysin O have demonstrated that the accessibility of Chol to the toxin is dependent on the phospholipid structure. Lipids more tightly packed with Chol, such as those with large head groups, cause inefficient binding. This may be due to a sequestering effect that impairs the availability of the sterol to the toxins (Flanagan et al. 2009; Nelson et al. 2008). Furthermore, the availability of Chol in the membrane probably plays a major role in the binding process of CDCs. It is very likely that the binding of CDCs to membranes is triggered when the concentration of Chol exceeds the association capacity of the phospholipids, and such extra molecules of the sterol are then free to associate with the toxins (Flanagan et al. 2009). Lipid domains (or rafts) are also modulated by Chol levels and they have been proposed to act as a platform for CDCs binding. However, this is still under debate because SM is tightly packed with Chol in lipid rafts, and could confine the sterol avoiding toxin binding in natural membranes (Flanagan et al. 2009).

Actinoporins Family

Actinoporins (e.g., sticholysins, equinatoxin II, and fragaceatoxin C) are eukaryotic α-PFPs produced by sea anemones (Álvarez et al. 2009). SM has been proposed as their lipid receptor in the membrane. However, some studies have found that this phospholipid is not essential for actinoporins permeabilizing activity in liposomes (de los Rios et al. 1998). Moreover, the identification of a phosphocholine binding site in the 3D structure of different actinoporins questioned the nature of the structural elements that allow the discrimination of SM from phosphatidylcholine (PC) (Álvarez et al. 2009). It appears that actinoporins recognize SM both at the level of the head group and at the ceramide moiety (Álvarez et al. 2009; García-Ortega et al. 2011). Initially, it was believed that actinoporins possess a unique lipid binding site (Mancheño et al. 2003), but recent studies have found the presence of multiple lipid binding sites in their structure (Fig. 2). Therefore, lipid multivalency has emerged as a new concept for describing the interaction of PFPs with membranes. This property seems to be crucial to increase their membrane affinity. Importantly, SM not only acts as a lipid receptor of actinoporins on the membrane surface, but also as a structural element of the pore, where it plays the role of an assembly co-factor (Tanaka et al. 2015). In the context of the proteo-lipidic pore (see "Lipids and Proteins Acting Together: The Protein-Lipid Pore" section for details), the presence of SM is likely a unique feature of actinoporins, as it has not been observed in the crystal structures of the transmembrane pores of β-PFPs (Song et al. 1996) or of the other α-PFP ClyA (Mueller et al. 2009). The role of SM in the mechanism of action of actinoporins has also been investigated in the context of SM function as a modulator of membrane properties. Over the last years, it has been postulated that the affinity of

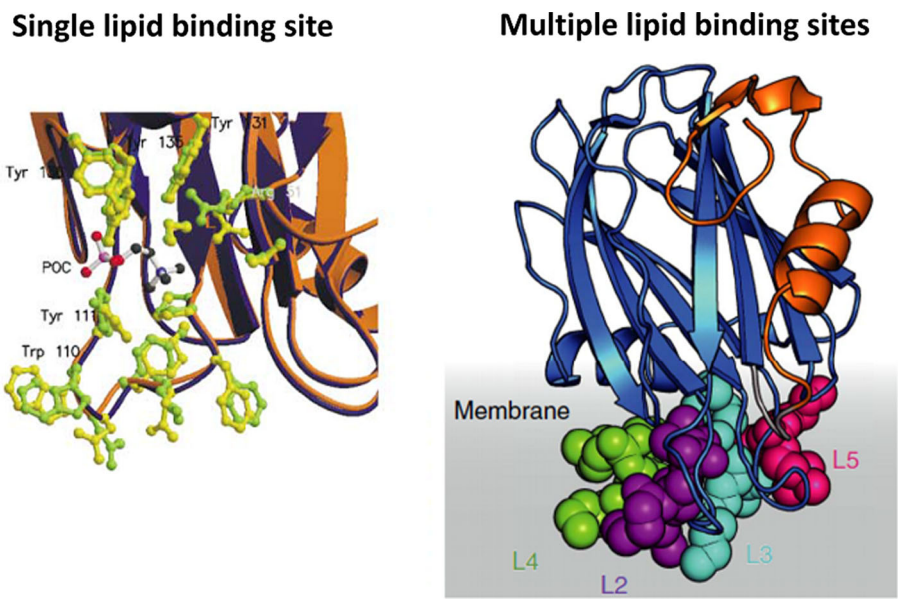

Fig. 2 Different lipid binding sites for actinoporins. The figure contrasts the concept of single lipid binding site, initially proposed for actinoporins by Mancheño et al. (2003) with the new concept of lipid multivalency (*right panel*) introduced by Tanaka et al. (2015) (Tanaka et al. 2015). *Left panel* POC binding site proposed for sticholysin II [adapted from (Mancheño et al. 2003)]. *Right panel* Multiple lipid binding sites proposed to fragaceatoxin C [adapted from (Tanaka et al. 2015)]. L2 and L3 (purple and cyan are proposed as high-affinity sites and are suited to recognize the solvent-exposed region of the lipids, the POC head group. L4 and L5 (*green* and *magenta*) represent low-affinity sites or probably high-affinity binding sites for lipids with head groups other than POC. An additional site (L1, not shown) able to bind non-annular lipids is supposed to have a key role as bridging lipids in the pore structure (Tanaka et al. 2015) (Color figure online)

actinoporins for membranes is greatly enhanced by the coexistence of lipid phases, and the role of SM was focused on its ability to form raft-like structures (Barlic et al. 2004; Schön et al. 2008). It is also likely that the environment is what determines its function as a receptor. For instance, lipid partners like Chol or Cer have an effect on the SM head group tilt, orientation, and dynamics, and consequently on actinoporins activity (Alm et al. 2015). We have generalized that once the membrane has a high availability of SM (>30 mol%), its phase state and rheological properties acquire a major role in the recognition of actinoporins (Pedrera et al. 2014).

The Pro-apoptotic Protein Bax

Bax plays a central role in the mitochondrial pathway of apoptosis and it is believed to participate directly in the permeabilization of the mitochondrial outer membrane that leads to cytochrome c release (García-Sáez 2012). Several groups have reported that the presence of CL, a specific mitochondrial lipid, and the active product of Bid (tBid) play a role in Bax activity (García-Sáez 2012; Kuwana et al. 2002; Lucken-Ardjomande et al. 2008). This is an elegant example about how simple lipids can function more as modulators of membrane properties than as classical receptors. This PFP is able to induce cytochrome c release from CL-deficient mitochondria (Gonzalvez et al. 2008), and it has been described that fragments corresponding to helices 5 and 6 of Bax exhibit similar pore activity to the full-length protein independently of CL (Garcia-Saez et al. 2006; Puech et al. 2003). This suggests that Bax itself contains the motifs required for pore formation and that CL and tBid are only regulators of Bax activity (García-Sáez 2012). Moreover, CL in conjunction with the other non-lamellar lipid phosphatidylethanolamine (PE) seems to be relevant for promoting the formation of contact sites between the inner and outer mitochondrial membranes (Cosentino and García-Sáez 2014; Gonzalvez et al. 2008; Lutter et al. 2000). Such contacts are likely optimal places for targeting Bcl-2 proteins to the mitochondrial membrane (Cosentino and García-Sáez 2014; Gonzalvez et al. 2008; Kuwana et al. 2002; Lutter et al. 2000). Indeed, CL seems to allow specific targeting of tBid to mitochondria and to promote tBid binding with interaction partners such as Bcl-xL (Lutter et al. 2000). Thus, activation of Bax by tBid takes place in cooperation with CL and results in pore formation leading to the release of apoptotic factors (Cosentino and García-Sáez 2014; Kuwana et al. 2002; Unsay et al. 2013). One should also keep in mind that the CL enrichment observed in the outer mitochondria membrane during apoptosis not only promotes local alterations in membrane curvature but also in charge, which may facilitate the recruitment of Bcl-2 proteins to mitochondria (Cosentino and García-Sáez 2014). Future work is needed

to clarify whether CL forms part of the pore organized by Bax and how this special lipid contributes to the different stages of Bax membrane permeabilization.

The Lipid Bilayer: A Platform for PFPs Structural Changes

Independently of its biochemical nature, binding to a receptor provides advantages for PFPs by guaranteeing specificity. PFPs binding to membranes often leads to a significant conformational change, which are characterized by partial loosening or even disappearance of the tertiary structure. This structural alteration helps subsequent membrane insertion and makes the protein competent for oligomerization (Bischofberger et al. 2012; Fradin et al. 2009). When PFPs associate with the membrane surface, they effectively increase their concentration by lowering their diffusion space from 3D in the extracellular medium to 2D on the cell surface (Gonzalez et al. 2008; Iacovache et al. 2010). Binding to specific regions of the membrane has the additional benefit of promoting even a higher increase in protein concentration. Many PFP receptors (either lipids or proteins) are indeed intrinsically concentrated, pre-clustered, or associated with membrane domains (Garcia-Saez et al. 2011; Lafont et al. 2004). Moreover, lipid-packing defects at the edges of lipid domains may favor PFPś insertion into the bilayer. This could be an efficient concentration strategy because it confines the proteins to a more limited space (Barlic et al. 2004; García-Sáez et al. 2007). Taking this into account, there is a general consensus that membrane features like lipid domains and domain edges strongly influence the ability of PFPs to oligomerize in the surface of the bilayer (Álvarez et al. 2009; Barlic et al. 2004; Bischofberger et al. 2012; Fradin et al. 2009).

Although PFPs form diverse types of structures in their soluble form, they share common and highly conserved organization (i.e., α- or β-hairpins) in the pore form (Fradin et al. 2009; García-Ortega et al. 2011). The lipid environment of the membrane is a biologically relevant component for these conformational changes, as it is the region of the cell that PFPs first contact (Ladokhin and White 1999; Wimley and White 2000). AMPs are a simple and classic example showing the role of the bilayer as an inductor of protein secondary structure. For most peptides, the lipid environment is a strong catalyzer of secondary structure formation since they are commonly very flexible and do not form regular secondary structures in aqueous solution (e.g., magainin, Fig. 1d). However, some peptides (e.g., protegrin, Fig. 1e) are folded in a specific conformation stabilized by intramolecular bonds (e.g., disulfide bonds). In any case, upon binding to their target membrane, AMPs undergo significant conformational changes that affect their antimicrobial activity (Epand and Vogel 1999; Yeaman and Yount 2003). Mechanistically relevant is the high energetic cost of dehydrating the peptide bond in addition to transferring it to a non-polar environment. These are claimed to promote large conformational changes in the peptide structure (Ladokhin and White 1999; Wimley and White 2000). Several studies have demonstrated that many linear antimicrobial peptides adopt α-helical or β-sheet structure in structure-promoting solvents like trifluorethanol, micelles, and lipid vesicles (Fig. 1d). The induced structure is commonly very amphipathic, and thus complementary to the nature of the lipid bilayer (Epand and Vogel 1999; Sitaram and Nagaraj 1999). Differences in the conformations adopted in the membrane seem to have an impact on AMPs selectivity. Furthermore, AMPs may have distinct antimicrobial conformers and undergo conformational phase transitions, self-association, or oligomerization within target cell membranes, as a means to achieve selective toxicity (Yeaman and Yount 2003).

α-PFPs

α-PFPs tend to have a high percentage of α-helical structure and typically organize into a so-called three-layer or sandwich structure, where a hydrophobic hairpin is part of the interior layer buried in the hydrophobic core of the protein (Fig. 1a). They can be considered as membrane proteins forced to adopt an inside–out configuration in solution in order to avoid contact between their pore-forming hydrophobic region and the water environment (Fradin et al. 2009). The membrane form of these proteins usually corresponds to a rearrangement of the helices present in the soluble form into a fold energetically compatible with the membrane (Bleicken et al. 2014; Fradin et al. 2009). The hydrophobic or amphipathic α-helices can generally exist within the lipid bilayer without the need to interact with other monomers. This is because all the peptide hydrogen bonds are satisfied within the α-helix via intra-chain interactions, which explains why α-PFPs sometimes seem to be able to form monomeric channels of variable size, and also involving lipids (for more details see "Lipids and Proteins Acting Together: The Protein-Lipid Pore" section) (Fradin et al. 2009).

Colicins/Bax Family

Colicins define the archetypal structure of α-PFPs (Fig. 1a). The pore-forming domain of these proteins consists of 10 α-helices arranged in three layers, with two hydrophobic helices (8 and 9) buried within the helical structure and surrounded by an outer shell of amphipathic helices that allows toxin solubilization (Parker et al. 1989,

1990). The packaging motif of the buried hydrophobic helices found in colicins is structurally shared with the apoptotic proteins of the Bcl-2 family (Muchmore et al. 1996), the diphtheria toxin (Choe et al. 1992), and δ-endotoxins from *Bacillus thuringiensis* (Li et al. 1996). Over the years, the consensus was that interaction of colicin-like proteins with membranes promoted a conformational change that released the hydrophobic or amphipathic central helical hairpin similar to the opening of an umbrella (Fig. 3a) (Anderluh and Lakey 2008; García-Sáez 2012). In the "umbrella" model, the hydrophobic α-helix and the amphipathic hairpin are proposed to be inserted perpendicular to the plane of the membrane, while the other helices are arranged on the surface and parallel to the membrane plane (Parker et al. 1990). Theoretically, in the "umbrella" mechanism, one monomer would be sufficient

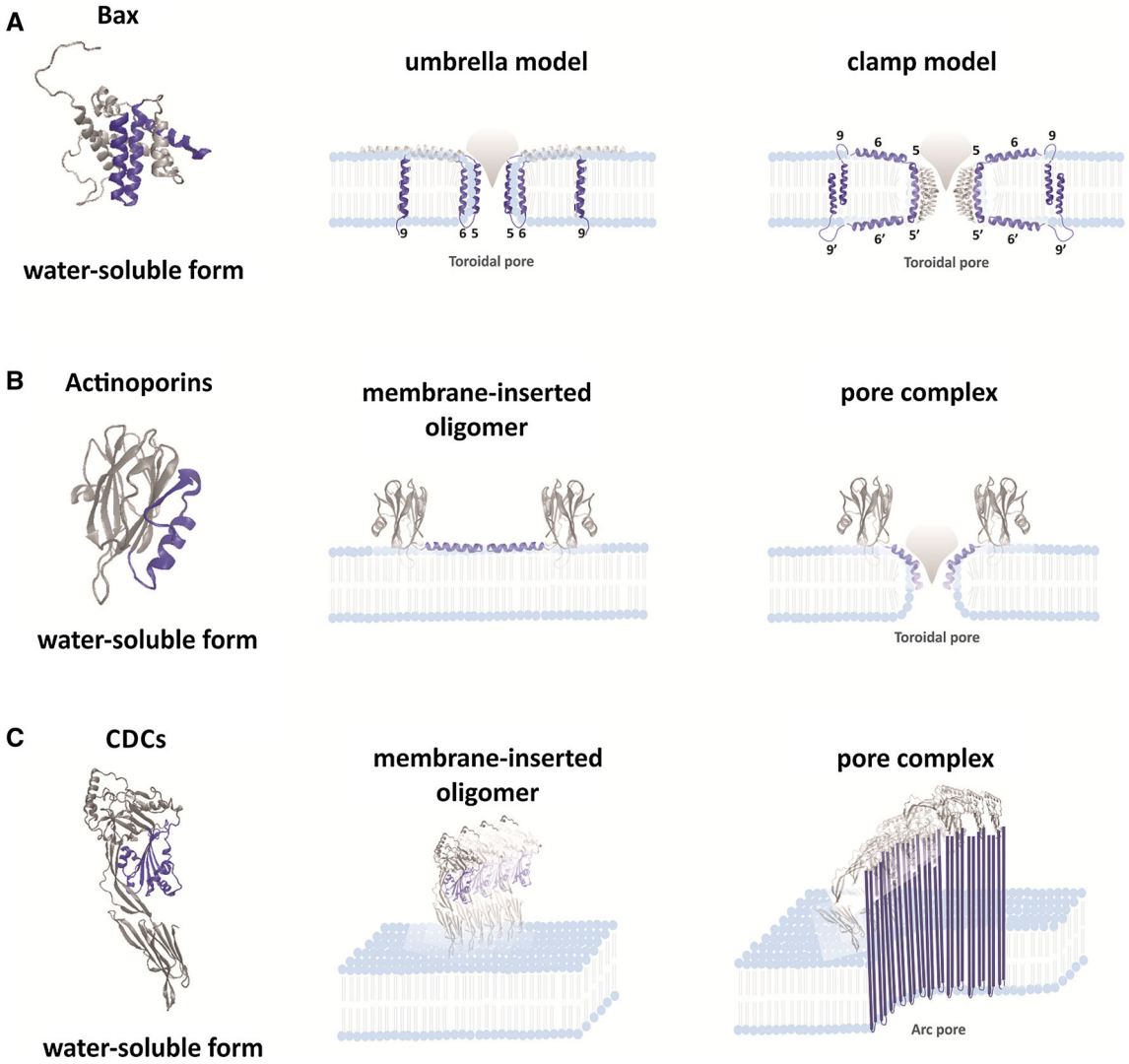

Fig. 3 Conformational changes trigged on PFPS upon binding to membranes. **a** Bax protein. The hydrophobic and the amphipathic helices (*blue*) are packaged within the helical bundle in solution. In the "umbrella" model (*left panel*), the hydrophobic helix (9) is too short to span the membrane but leads the initial anchoring of the proteins into the membrane. Amphipathic helices (5 and 6) insert a hairpin across the bilayer that forms the channel. Theoretically, a monomer or a dimer could be enough to form the pore (Cascales et al. 2007; García-Sáez 2012). In the clamp model (*right panel*), the hydrophobic helix (9) remains embedded into the bilayer but the hairpin formed by amphipathic helices (5 and 6) opens. Translocation across the membrane and dimerization is required (Bleicken et al. 2014). **b** Actinoporins are exceptional α-PFPs on account of their predominant β-structure. The N-terminal segment (*blue*) is the only capable of undergoing notable conformational changes upon binding to membranes (García-Ortega et al. 2011). **c** During β-PFPs interaction with membranes, the ordered arrangement of the pore is ensured by the lateral hydrogen bonding between the peptide bonds. Similar to the actinoporins, β-structures only need small hydrophobic portions to form a pore (*blue*) (Anderluh and Lakey 2008). For all models shown, the number of monomers is arbitrary and the regions undergoing the major conformational change upon binding to the membrane are shown in *blue*. In the toroidal pore representation (**a** and **b**), helices do not cross the lipid bilayer since their main role is to induce membrane curvature (Color figure online)

to form a pore. Indeed, the ability of such short helices to form a channel is mainly related to their effect on membrane thickness and the induction of curvature in the membrane associated with toroidal pore formation (further comments on this topic in "Lipids and Proteins Acting Together: The Protein-Lipid Pore" section) (Parker et al. 1990). Alternatively, the "penknife" model proposes that the hydrophobic hairpin is only shallowly inserted while laying more or less parallel to the membrane plane (Massotte et al. 1993). Another possibility is that the hydrophobic hairpin is in fact alternating between these two different conformations (Kienker et al. 1997).

In this regard, recent data from our group indicate that Bax core helices (2–5) maintain a similar conformation in the water-soluble and membrane-embedded states, while the rest of the protein undergoes a dramatic reorganization (Fig. 3a). The new "clamp" model assumes that Bax forms dimer units within an oligomer. Protein association takes place via a dimerization domain comprising helices 2–5, which lines the pore edge in a toroidal architecture in association with the lipids head groups. Such organization may require monomer translocation to the inner side of the membrane, which would challenge the paradigm of the multimerization mechanism of PFPs, as it does not include full proteins crossing the bilayer. According to this model, the most significant reorganization of Bax involves the partial opening of the hairpin of helices 5 and 6, which assembles into a clamp-like structure. In this topology, helix 6 is lying on the membrane interface, while helix 5 is located on the pore surface directly interacting with the lipids. The hydrophobic helix 9 adopts the typical transmembrane orientation as proposed in the "umbrella" model. The structural data on which the "clamp" model is based indicate that Bax forms dimer units, but the molecularity of the oligomeric species forming the pore is still unknown. These results disprove previous models ("umbrella" and "penknife"), which assumed the permanence of the hairpin upon Bax activation in membrane, as well as a transmembrane orientation of helices 5 and 6. However, all models agree that the central hairpin and the hydrophobic helix 9 are initially shielded from the bulk water in the soluble conformation, and undergo large structural changes upon Bax pore formation (Fig. 3a) (Bleicken et al. 2014).

Actinoporins

Actinoporins are the exception to this general pattern of α-PFPs, as their structure consists of a tightly folded β-sandwich composed of two β-sheets, each containing five strands (Fig. 1b). However, their pore-forming domain also consists of an amphipathic α-helix, preceded by a hydrophobic segment, both located at the N-terminus. This amphipathic N-terminal helix is connected to the β-sandwich by a short loop and it is attached to the main body of the protein in solution. Nonetheless, upon membrane interaction, a pseudo-rigid body movement takes place in the N-terminal segment that allows membrane penetration (Fig. 3b). This is the only portion of the protein that can be dislocated from the core of the protein without disrupting the general fold of the molecule, while the rest is kept invariable on the surface of the membrane in the final pore structure (Mancheño et al. 2003; Mechaly et al. 2011). The metamorphosis of a relatively small percentage of residues during pore formation described for actinoporins is an exceptional feature for α-PFPs. In contrast, this is a typical characteristic of β-PFPs (described in the next section). For instance, the pore structure of ClyA, the only other known of an α-PFP, shows a much greater conformational change during the assembly of the pore, which involves about 50 % of its entire sequence (Mueller et al. 2009).

β-PFPs

β-PFPs are inherently soluble proteins that are thought to form pores via transmembrane β-barrels. Because their hydrophobic content is very limited, they require oligomerization in order to form a hydrophobic surface large enough to insert in the membrane. β-PFPs need to be folded into a cylindrical structure containing multiple monomers of the protein to satisfy all of its peptide hydrogen bonds through inter-chain interactions and then insert into the membrane. In this case, each monomer usually contributes with only one or two β-hairpins to the pore (Fig. 3c). This arrangement is more efficient for spanning the membrane than the helical structures, which often need more residues participating in the pore. Additionally, β-strands only need to be hydrophobic on one side, which means that only about 50 % of the residues are water repellent. As a consequence, the transmembrane region can feature alternate runs of few hydrophobic amino acids (Anderluh and Lakey 2008; Fradin et al. 2009). As in the case of actinoporins (Fig. 3b), β-PFPs penetration into the membrane involves only a small portion of the protein sequence, while the membrane binding and other associated domains remain outside the membrane core in the pore assembly (Fig. 3c) (Anderluh and Lakey 2008).

Membrane Remodeling Induced by Proteins

As described, lipids are able to modulate the structure and activity of PFPs; conversely, these proteins are also able to modulate a number of membrane properties, like fluidity, lipid segregation, or curvature. We describe next the main

modifications induced by PFPs in the organization of the lipid bilayer during pore formation.

Lipid Domain Remodeling

Most current research on membrane remodeling involves lipid domains and is concentrated on the identification of dynamic processes triggered by proteins. The term "membrane domains" can be used in a very broad sense, covering a large variety of non-random assemblies of molecules in the membrane (Kusumi et al. 2004). Probably, one of the most controversial and studied membrane domains are the so-called raft domains (Mukherjee and Maxfield 2004). Rafts are defined as lipid/protein domains enriched in Chol and sphingolipids that act as signaling platforms in the plasma membrane of the cell (Dietrich et al. 2002). Remarkably, PFP receptors at the plasma membrane share the property of either being associated with lipid rafts (e.g., glycosylphosphatidylinositol (GPI)-anchored proteins for aerolysin) or being themselves components of rafts domains (e.g., Chol or SM for CDCs and actinoporins, respectively) (Bischofberger et al. 2012). In this review, we will concentrate on the effect of PFPs on this second case scenario.

The insertion of PFPs into the bilayer usually affects the natural organization of lipid membranes at some level. PFPs can promote the formation of domains by preferentially interacting with either lipid or protein components, by being sequestered into these regions or by being excluded from them. For instance, there is a structural motif in the human prion protein and the Alzheimer α-amyloid peptide (Mahfoud et al. 2002) with affinity for SM that is believed to target these proteins to raft domains. In this sense, domain formation has been shown to have a dramatic effect on the mechanism of action of CDCs (Hotze and Tweten 2012) and actinoporins (Barlic et al. 2004; Schön et al. 2008), due to preferential interaction of these toxins with raft lipidic components (as described above in "The Lipid Bilayer: Primary Target of PFPs" section).

Raft clustering has been related with the effect on membranes of the CDC toxins perfringolysin O (Waheed et al. 2001) and listeriolysin O (Gekara and Weiss 2004), as well as the actinoporin equinatoxin II (Garcia-Saez et al. 2011). Studies based on listeriolysin O found that GPI-anchored proteins, which normally exhibit a uniform distribution on cells, underwent clustering upon treatment with the toxin. In contrast, the non-raft marker transferrin receptor remained unaffected. Based on this, it was proposed that oligomerization of listeriolysin O monomers upon binding to Chol could induce raft clustering and affect plasma membrane signaling. Raft clustering might explain the induction of tyrosine phosphorylation observed in listeriolysin-treated cells. Signals triggered by this toxin strongly influenced the course of infection. Since rafts are ubiquitous targets for listeriolysin, this CDC might be able to act as a pleiotropic pseudocytokine/chemokine that triggers various host responses (Gekara and Weiss 2004). Additionally, the actinoporin equinatoxin II co-localized with immobile, microscopic domains that resembled stabilized rafts (Garcia-Saez et al. 2011). Upon binding to SM, the toxin oligomerized and formed pores that allowed the passage of small molecules (e.g., Ca^{2+}) into the cytosol. Subsequently, endocytosis and cytoskeleton dynamics were inhibited, which led to clustering of raft components into immobile domains of microscopic size. In parallel, disruption of membrane/cytoskeleton interactions and osmotic imbalance induced the growing of membrane blebs. Cells could not cope with stress and finally collapsed. To our best knowledge, this was the first time that such dramatic effect on membrane organization was directly visualized (Garcia-Saez et al. 2011). Despite these phenomenological observations, the purpose of PFPs targeting to lipid raft remains unclear so far. It has been proposed that lipid rafts promote pore activity and cell death by acting as concentration platforms or determining the target cell specificity (Garcia-Saez et al. 2011). It is tempting to speculate that raft clustering could be a general mechanism of PFPs that bind to raft components. This possibility would imply the evolution of PFPs to exploit the lateral organization of eukaryotic cells into lipid rafts for their toxic action.

In a completely different scenario, PFPs are also claimed to reduce line tension at the boundaries of lipid phases and to induce mixing of lipids from different regions of the membrane. Garcia-Saez et al. (2011) reported this effect for a peptide derived from the α-helix 5 of the apoptotic protein Bax. Liquid ordered domains normally show a circular morphology that indicates the existence of line tension between the two phases. This is due to the different thicknesses of the coexisting lipid phases, which leads to lipid tilt and curvature stress. When Bax-α5 peptide was added to the membrane, a dramatic change in the morphology of liquid ordered domains was observed. They became larger and more irregular, and the difference in thickness between the two phases decreased, all in line with a decrease in the line tension at the phase boundary. As domain interfaces contain a higher concentration of packing defects, they are expected to favor Bax-a5 binding. The peptide may stabilize the domain edge by releasing stress of curvature and thus reducing the line tension. Such an effect may be common for similar types of molecules, like AMPs, the pore-forming domain of bacterial toxins, and detergents, due to their preference for membrane interfaces (Puech et al. 2003). Moreover, lipid molecules at the interfaces seem to be intrinsically more

disordered, which offer less resistance to binding and pore formation (Barlic et al. 2004; García-Sáez et al. 2007). Ros et al. (2013) extended this hypothesis to actinoporins. It seems like the expansion of lipid disordered phases at the expense of ordered domains, with the resulting decrease in lipid packing at the borders of lipid rafts, turned these regions of the membrane into a more suitable environment for the membrane insertion.

Induction of Membrane Curvature and Lipid Flip-Flop

Pore formation in lipid membranes can also be modulated by curvature. It is well known that the predominant structure formed by the most abundant phospholipids in biological membranes is a bilayer formed by two opposite monolayers. However, lipids also have a certain propensity to form different phases, some of which are non-lamellar. For instance, lipids that tend to form bilayer structures include PC and SM, while PE is an example of lipids that prefer to form inverted hexagonal phases. This tendency depends on the intrinsic monolayer curvature of the lipid structure, which is determined by the size ratio between the head group and the acyl chains regions. Moreover, anionic phospholipids like phosphatidylglycerol, phosphatidic acid, and CL can also form non-lamellar lipid phases under extreme conditions like high ionic strength or in the presence of proteins like PFPs (Epand et al. 2015; Haney et al. 2010). The pore-forming segments of PFPs have been described as pore promoters based on their ability to induce or stabilize curvature at the pore edge (Fuertes et al. 2011).

Pore formation sometimes involves the formation of non-lamellar structures in the membrane (Lins et al. 2008). In this regard, the most classic mechanism described is the so-called toroidal lipid pore (see "Lipids and Proteins Acting Together: The Protein-Lipid Pore" section 6). This model postulates the bending of a usually planar lipid monolayer into a half torus. In this configuration, the membrane adopts both a positive curvature in the direction parallel to the pore axis, and a negative curvature, in the direction perpendicular to the pore axis. The generation of a pore is energetically expensive, which gives rise to a line tension at the edge. In agreement with this, the presence of lipids with intrinsic monolayer curvature (either positive or negative) stabilizes lipid pores (Haney et al. 2010). Negative curvature has a significant influence only in very small pores [e.g., pores formed by actinoporins (Tejuca et al. 2001; Valcarcel et al. 2001)]. On the contrary, positive curvature stress will increase with the pore radius and is usually the dominant effect in larger pores [e.g., Bax pores (Basanez 2002)].

Despite the difficulties in the determination of toroidal pore structures, curvature effects in the membrane have been considered indirect evidence of this mechanism. Concretely, the ability of a magainin 2 derivate to promote an increase in the lamellar to inverted hexagonal phase transition temperature of dipalmitoleoylphosphatidylethanolamine was a pioneer observation that was interpreted as induction of positive curvature in the mechanism of action of α-AMPs (Matsuzaki et al. 1998). This feature reflects the contribution of lipid molecules with predominant positive curvature at the pore edge, which requires energy and results in the observed increase of the phase transition temperature (Haney et al. 2010).

One additional implication of toroidal pore formation is that each pore behaves as a point of fusion between the inner and outer monolayers in a structure that favors the transbilayer or flip-flop movement of lipids, which otherwise is severely restricted. As a consequence, toroidal pore formation by PFPs facilitates the free diffusion of lipid molecules between the two leaflets of the bilayer. According to this, increased flip-flop of lipids was observed in vesicles in the presence of cytolytic peptides such as gramicidin (Classen et al. 1987), melittin (Rapson et al. 2011), and magainin (Nguyen et al. 2009). Transbilayer movement of lipids has been also shown for proteins like colicin E1 (Sobko et al. 2004), Bax (Epand et al. 2002, 2003), actinoporins (Anderluh et al. 2003; Valcarcel et al. 2001), and perforin (Metkar et al. 2015).

Lipids and Proteins Acting Together: The Protein-Lipid Pore

Proteins can exclusively participate in the architecture of the pores formed by PFPs (the so-called protein-lined pore) or, alternatively, they can be accompanied by lipids (the so-called toroidal or protein-lipid pore). In the first model, the pore-forming domains of PFPs are inserted in the membrane perpendicular to the plane of the lipid bilayer and form a ring delineating a water-filled channel. In the second model, the pore-forming domains stabilizing the pores are not necessarily in contact with each other but separated by lipids (Fradin et al. 2009). In this section, we describe in more detail the features of protein-lipid pores as examples of structures in which both proteins and lipids cooperate to form functional complexes in membranes.

A main feature of the toroidal pore model is that with assistance of peptides or pore-forming domains of PFPs, the membrane curves to form a torus-like channel. In this model, the walls of the channel are formed by both polypeptide chains and lipid head groups. To avoid the high energetic cost of exposing their hydrophobic acyl chains to the aqueous environment, lipids bend and form a highly curved, non-bilayer structure at the pore edge that connects the two monolayers of the membrane with a

continuous surface. Lipids can then easily exchange monolayers by simple diffusion at the pore. The role of the protein component is not so much to create an interfacial area between the hydrophobic core of the membrane and the water-filled channel, as to help to reduce the stress caused by membrane distortion and curvature formation. As a result, pore-forming domains are not required to span completely the bilayer (Gilbert et al. 2014; Qian et al. 2008; Yang et al. 2000). Not only AMPs but also larger PFPs have been proposed to form protein-lipid pores. The list includes proteins from the Bcl-2/colicin family (Basanez 2002), actinoporins (Valcarcel et al. 2001), and the membrane attack complex-perforin/cholesterol-dependent cytolysin (MACPF/CDC) superfamily (Gilbert et al. 2014; Metkar et al. 2015).

The toroidal pore concept was first proposed to explain experiments performed with α-helical AMPs like magainin (Fig. 1d) (Ludtke et al. 1996; Matsuzaki et al. 1996)) and melittin (both acquiring α-helical structures in membrane), as well as for protegrins (Fig. 1e) (which form a one-turn β-hairpin) (Yang et al. 2000). Visualization of toroidal pores has remained elusive for decades because unlike pores formed only by proteins, which form discrete and rigid structures, toroidal ones are highly variable (Črnigoj Kristan et al. 2009; Fradin et al. 2009). Since protein and lipids form the pore walls, it is not surprising that such pores are characterized by a typical broad conductance distribution (Črnigoj Kristan et al. 2009; Yang et al. 2001). Therefore, indirect observations have usually been taken as evidence for toroidal pore formation. Some examples include the induction of membrane curvature determined by differential scanning calorimetry or neutron magnetic resonance (^{31}P-NMR) (Anderluh et al. 2003; Matsuzaki et al. 1998), the transbilayer lipid flip-flop of fluorescent probes (Matsuzaki et al. 1996; Valcarcel et al. 2001), and the effect of lipids with intrinsic monolayer curvature on permeabilization activity (Basanez 2002; García-Sáez et al. 2005; Matsuzaki et al. 1996; Valcarcel et al. 2001). Data suggesting toroidal structure were also obtained by neutron scattering for magainin, melittin (Yang et al. 2001), and protegrin peptides (Yang et al. 2000). Probably, the most direct observation of a toroidal pore was obtained by Huang and coworkers, who used X-ray diffraction to visualize the electron density distribution of Br atoms in peptide-induced membrane pores (Fig. 4a) (Qian et al. 2008). In the presence of a peptide derived from Bax that forms toroidal pores (García-Sáez et al. 2005), the top lipid monolayer bent continuously into the lower layer at the pore edge. This study visualized the lining of the pore as an extension of the water–lipid chain interface and validated for the first time the toroidal pore model. Unfortunately, the distribution of the peptides with respect to the pore was not visible (Qian et al. 2008).

After decades of effort, the high-resolution structure of a protein-lipid pore was recently obtained for fragaceatoxin C, a member of the actinoporin family (Fig. 4b) (Tanaka et al. 2015). The mechanism of action of α-PFPs was revealed thanks to the determination of the crystal structure of different stages of the lytic process: the water-soluble state, the monomeric lipid-bound form, an assembly intermediate, and the fully assembled transmembrane pore in liposomes. These data clarified key aspects of the mechanism of action of actinoporins and revealed a critical role of lipids in the activation and in the architecture of PFPs. The pore structure consists of the N-terminal region of 8 FraC monomers and 24 molecules of lipids (identified as SM). Lipids are firmly bound to each protein chain and their head groups engage in numerous non-covalent interactions with conserved residues of the protein molecules. Protein–protein as well as protein–lipid interactions stabilize the pore structure, which explains the firm adhesion of the molecules in the pore. Moreover, the channel exhibits fenestrations or windows at the pore wall, a feature so far not documented for any other PFP. The amphipathic character of such windows may contribute to the passage of small molecules through the channel and also to the local disruption of the membrane lamellar structure by catalyzing the transbilayer movement of the lipids (Tanaka et al. 2015).

In general, it was widely accepted that α-PFPs insertion into membranes could be associated with lipid reorganization that resulted in the formation of a toroidal pore whose walls are lined by the hydrophilic face of amphipathic α-helices and the polar head groups of phospholipids (Qian et al. 2008). However, this concept has been challenged by new evidences demonstrating that not only α-helical peptides or proteins (e.g., antimicrobial peptides, pore-forming α5 helix of Bax, colicins, and Bax (García-Sáez et al. 2005)), but also those acquiring β-sheet folds in the membrane (e.g., the antimicrobial peptide protegrin, Alzheimer's Aβ1–41 peptides (Jang et al. 2013; Prieto et al. 2014), and proteins from the MACPF/CDCs family (Sonnen et al. 2014)) are able to form toroidal pores. Additional evidence obtained with the α-PFP, ClyA demonstrates that a protein-lined pore can be constructed exclusively with molecules arranged in an α-helical bundle (Mueller et al. 2009). Thus, the paradigm that toroidal pores are exclusive of α-PFPs has changed, although the nature of the lipid–protein and protein–protein interactions determining such pore architectures remains unclear. Gilbert and coworkers (Gilbert et al. 2014) recently defined two types of protein-lipid toroidal pores: the "matrix-type" and the "arc-type." The first one resembles more the "classical" toroidal pore concept and it is described as a defined structure in which proteins and lipid molecules are intercalated. The second model is understood as a less

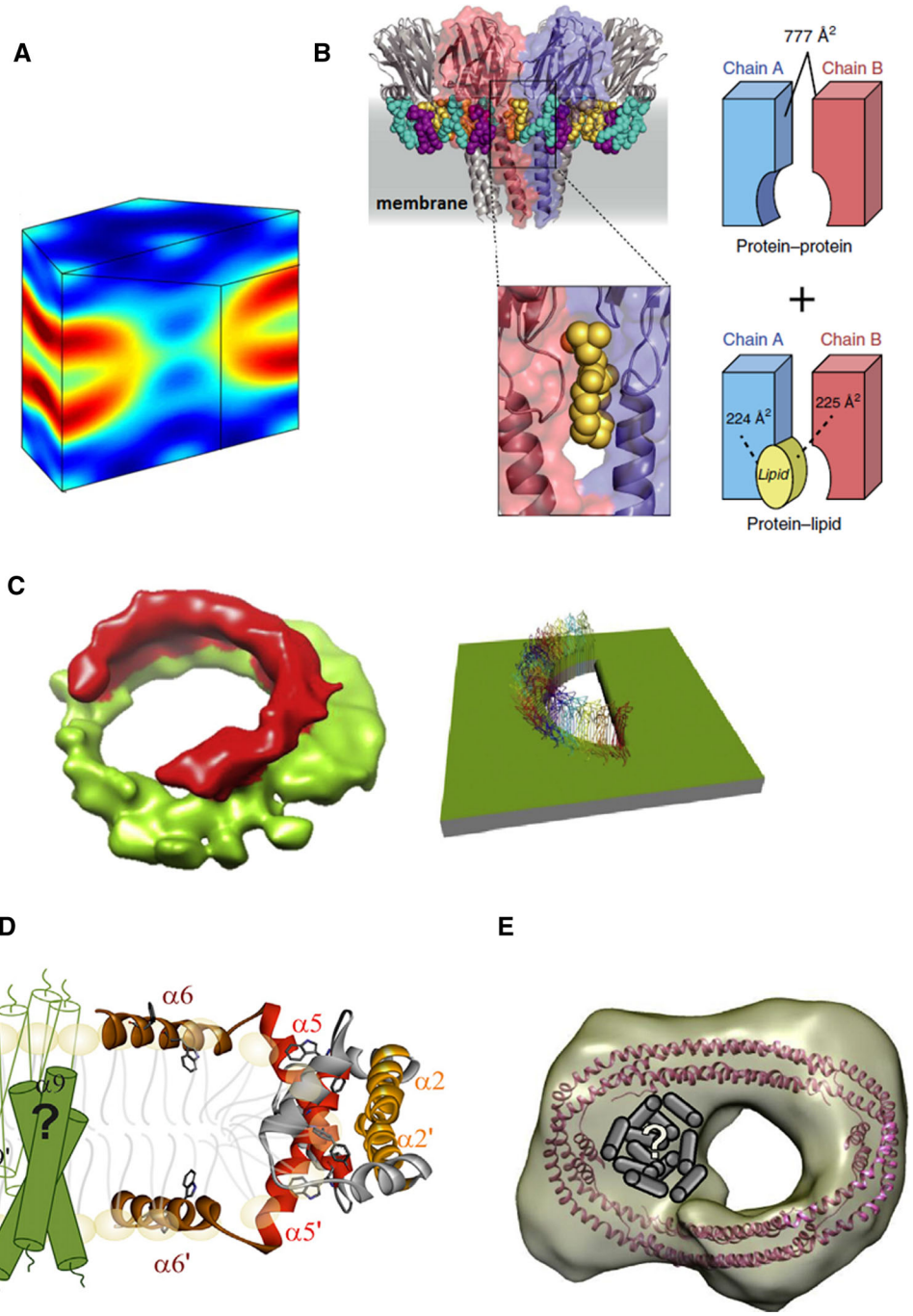

Fig. 4 Different protein-lipid pore structures. **a** Toroidal pore of Bax helix-5 peptide. X-ray diffraction structure of the pore formed by the peptide derived from the α5 of Bax [adapted from (Qian et al. 2008)]. The head groups (*yellow*) of non-lamellar lipid structures (*red*) form part of the structure of the channel in conjunction with the α-helices. **b** Pore structure of fragaceatoxin C. Lipids are part of the structure. *Top* View from within the membrane. The pore is constructed by N-terminal helices from 8 monomers of the protein and 24 molecules of lipids. Molecules of lipids are depicted in *yellow*, *purple*, and *cyan*. Zoom: Non-annular lipids are supposed to act as bridging structures between protein monomers. Both lipid–protein and protein–protein interactions stabilize the pore structure [adapted from (Tanaka et al. 2015)]. **c** Arc-shaped oligomer of pneumolysin. 3D sub-tomogram average map of the structure of the pore formed by pneumolysin [adapted from (Sonnen et al. 2014)]. The pore is formed by a protein oligomer (*red*) on one side and lipids in a toroidal arrangement (*green*) on the other side. (*Right panel*) Graphical representation of arc pores. The membrane must curve on the lipid side of the pore [adapted from (Gilbert et al. 2014)]. **d** Half of the toroidal pore formed by Bax dimer (clamp model). The dimerization domain is at the rim of a pore induced by Bax in the membrane, with helices 5 and 6 lying on the membrane surface. The hypothetical symmetric and transmembrane location of the dynamic helices 9 is sketched [adapted from (Bleicken et al. 2014)]. **e** Nanodisk with Bax [adapted from (Xu et al. 2013)]. 3D cryo-EM map of the Bax pore formed in a nanodisk. The question mark indicates the most likely location of the Bax protein, although there is uncertainty in the conformational state of Bax. The pore presumably evolves via a toroidal structure (Color figure online)

stable architecture in which amphipathic protein oligomers form an arc on one side of the pore and lipids located on the opposite side complete the toroidal structure. Pores formed by actinoporins are archetypal of "matrix-type" (Anderluh et al. 2003; Valcarcel et al. 2001), while MACPF/CDCs pores (like perforin and pneumolysin) represent the "arc-type" (Metkar et al. 2015; Sonnen et al. 2014). Direct evidences support the formation of pores by MACPF/CDCs oligomeric arcs (Fig. 4c). Moreover, a recent study of pores formed in liposomes by pneumolysin using cryo-electron tomography-identified arc structures (Sonnen et al. 2014). 3D sub-tomogram classification of pre-pores and pores sitting on intact vesicle membranes successfully demonstrated in situ the existence of pores formed at the interface between an oligomeric protein arc and a lipid membrane (Fig. 4c). Equivalent structures formed by perforin have been imaged by atomic force microscopy and deep-etch EM in intact homogeneous planar bilayers and in tumor cell membranes, respectively. The authors proposed that the observed protein arcs function as toroidal pores in whole cells, which explains the lipid flip-flop detected (Metkar et al. 2015).

Moreover, these two classifications are not mutually exclusive. In the case of Bax, the classical "matrix-type" model has been assumed over the years from results obtained in vitro with little structural information (Basanez 2002; Bleicken et al. 2014; García-Sáez et al. 2005; Garcia-Saez et al. 2006; Qian et al. 2008). Recently, we have proposed a 3D model (the "clamp" model described in "The Lipid Bilayer: A Platform for PFPs Structural Changes" section) for active Bax oligomers at the membrane based on double electron–electron resonance spectroscopy data obtained from liposomes and isolated mitochondria. We estimated that the two arms of the clamp are separated by 3 nm, a distance that fits well with the membrane thickness. The model assumes that the piercing domain pinches the membrane and stabilizes a toroidal pore structure (Figs. 3a, 4d). In addition, a study based on cryo-electron microscopy showed that even Bax monomers are able to induce pores in lipid nanodiscs, which supports the idea that this protein can also act according to the "arc-type" model. However, this study could not unambiguously locate Bax at the pore edge (Xu et al. 2013) (Fig. 4e). These data suggest the existence of more than one functional structure for pores formed by both lipids and proteins, which would explain the high dynamism observed for such channels.

Concluding Remarks

The lipid bilayer should be seen not only as the simple target of PFPs, but also as a suitable place for protein folding and oligomerization. PFPs are able to remodel the membrane structure even at the initial steps during the process of pore formation. Changes in the lipid bilayer morphology induced by PFPs include domain rearrangement and lipid lateral or transbilayer movements. Through these modifications, PFPs seem to adapt the membrane environment to achieve an efficient membrane penetration. Conversely, lipids promote the change in PFPs structure required for biological function. Moreover, proteins and lipids are able to act together in the formation of pore structures. Hence, pore formation can be understood as a dynamic process in which proteins and lipids cooperate to form complex structures in the bilayer with the final goal of permeabilization. To further understand pore formation by PFPs, the field is in need of methods that are able to determine the dynamics, structure, localization, and nature of lipids and protein molecules in the context of the pore. Ideally, these methods should be compatible with studies in living cells to verify their biological roles in physiological systems.

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References

Alm I, García-Linares S, Gavilanes JG, Martínez-del-Pozo Á, Slotte JP (2015) Cholesterol stimulates and ceramide inhibits Sticholysin II-induced pore formation in complex bilayer membranes. Biochim Biophys Acta (BBA) 1848:925–931. doi:10.1016/j.bbamem.2014.12.017

Alouf JE (2000) Cholesterol-binding cytolytic protein toxins. Int J Med Microbiol 290:351–356. doi:10.1016/S1438-4221(00)80039-9

Álvarez C, Mancheño JM, Martínez D, Tejuca M, Pazos F, Lanio ME (2009) Sticholysins, two pore-forming toxins produced by the Caribbean Sea anemone Stichodactyla helianthus: their interaction with membranes. Toxicon 54:1135–1147. doi:10.1016/j.toxicon.2009.02.022

Anderluh G, Lakey JH (2008) Disparate proteins use similar architectures to damage membranes. Trends Biochem Sci 33:482–490. doi:10.1016/j.tibs.2008.07.004

Anderluh G, Serra MD, Viero G, Guella G, Macek P, Menestrina G (2003) Pore formation by equinatoxin ii, a eukaryotic protein toxin, occurs by induction of nonlamellar lipid structures. J Biol Chem 278:45216–45223. doi:10.1074/jbc.M305916200

Barlic A, Gutierrez-Aguirre I, Caaveiro JMM, Cruz A, Ruiz-Arguello M-B, Perez-Gil J, Gonzalez-Manas JM (2004) Lipid phase coexistence favors membrane insertion of equinatoxin-ii, a pore-forming toxin from actinia equina. J Biol Chem 279:34209–34216. doi:10.1074/jbc.M313817200

Basanez G (2002) Bax-type apoptotic proteins porate pure lipid bilayers through a mechanism sensitive to intrinsic monolayer curvature. J Biol Chem 277:49360–49365. doi:10.1074/jbc.M206069200

Bayley H (2009) Membrane-protein structure: piercing insights. Nature 459:651–652. doi:10.1038/459651a

Bischofberger M, Iacovache I, van der Goot GF (2012) Pathogenic pore-forming proteins: function and host response. Cell Host Microbe 12:266–275. doi:10.1016/j.chom.2012.08.005

Bleicken S, Jeschke G, Stegmueller C, Salvador-Gallego R, García-Sáez AJ, Bordignon E (2014) Structural model of active Bax at the membrane. Mol Cell 56:496–505. doi:10.1016/j.molcel.2014.09.022

Cascales E et al (2007) Colicin biology. Microbiol Mol Biology Rev 71:158–229. doi:10.1128/MMBR.00036-06

Choe S, Bennett MJ, Fujii G, Curmi PMG, Kantardjieff KA, Collier RJ, Eisenberg D (1992) The crystal structure of diphtheria toxin. Nature 357:216–222. doi:10.1038/357216a0

Classen J, Haest CWM, Tournois H, Deuticke B (1987) Gramicidin-induced enhancement of transbilayer reorientation of lipids in the erythrocyte membrane. Biochemistry 26:6604–6612. doi:10.1021/bi00395a007

Cosentino K, García-Sáez AJ (2014) Mitochondrial alterations in apoptosis. Chem Phys Lipids 181:62–75. doi:10.1016/j.chemphyslip.2014.04.001

Črnigoj Kristan K, Viero G, Dalla Serra M, Maček P, Anderluh G (2009) Molecular mechanism of pore formation by actinoporins. Toxicon 54:1125–1134. doi:10.1016/j.toxicon.2009.02.026

Dalla Serra M, Tejuca Martínez M (2011) Pore-forming toxins. In: eLS. John Wiley & Sons Ltd, Chichester

Dang TX, Hotze EM, Rouiller I, Tweten RK, Wilson-Kubalek EM (2005) Prepore to pore transition of a cholesterol-dependent cytolysin visualized by electron microscopy. J Struct Biol 150:100–108. doi:10.1016/j.jsb.2005.02.003

de los Rios V, Mancheño JM, Lanio ME, Oñaderra M, Gavilanes JG (1998) Mechanism of the leakage induced on lipid model membranes by the hemolytic protein sticholysin II from the sea anemone Stichodactyla helianthus. Eur J Biochem 252:284–289. doi:10.1046/j.1432-1327.1998.2520284.x

Dietrich C, Yang B, Fujiwara T, Kusumi A, Jacobson K (2002) Relationship of lipid rafts to transient confinement zones detected by single particle tracking. Biophys J 82:274–284. doi:10.1016/S0006-3495(02)75393-9

Epand RM, Vogel HJ (1999) Diversity of antimicrobial peptides and their mechanisms of action. Biochim Biophys Acta (BBA) 1462:11–28. doi:10.1016/S0005-2736(99)00198-4

Epand RF, Martinou J-C, Montessuit S, Epand RM, Yip CM (2002) Direct evidence for membrane pore formation by the apoptotic protein Bax. Biochem Biophys Res Commun 298:744–749. doi:10.1016/S0006-291X(02)02544-5

Epand RF, Martinou J-C, Montessuit S, Epand RM (2003) Transbilayer lipid diffusion promoted by Bax: implications for apoptosis. Biochemistry 42:14576–14582. doi:10.1021/bi035348w

Epand RM, D'Souza K, Berno B, Schlame M (2015) Membrane curvature modulation of protein activity determined by NMR. Biochim Biophys Acta (BBA) 1848:220–228. doi:10.1016/j.bbamem.2014.05.004

Farrand AJ, LaChapelle S, Hotze EM, Johnson AE, Tweten RK (2010) Only two amino acids are essential for cytolytic toxin recognition of cholesterol at the membrane surface. Proc Natl Acad Sci 107:4341–4346. doi:10.1073/pnas.0911581107

Flanagan JJ, Tweten RK, Johnson AE, Heuck AP (2009) Cholesterol exposure at the membrane surface is necessary and sufficient to trigger perfringolysin O binding. Biochemistry 48:3977–3987. doi:10.1021/bi9002309

Fradin C, Satsoura D, Andrews DW (2009) Punching holes in membranes: how oligomeric pore-forming proteins and lipids cooperate to form aqueous channels in membranes. In: Faller R, Longo ML, Risbud SH, Jue T (eds) Biomembrane frontiers. Humana Press, Totowa, pp 223–262

Fuertes G, Giménez D, Esteban-Martín S, Sánchez-Muñoz OL, Salgado J (2011) A lipocentric view of peptide-induced pores. Eur Biophys J 40:399–415. doi:10.1007/s00249-011-0693-4

García-Ortega L, Alegre-Cebollada J, García-Linares S, Bruix M, Martínez-del-Pozo Á, Gavilanes JG (2011) The behavior of sea anemone actinoporins at the water–membrane interface. Biochim Biophys Acta 1808:2275–2288. doi:10.1016/j.bbamem.2011.05.012

García-Sáez AJ (2012) The secrets of the Bcl-2 family. Cell Death Differ 19:1733–1740. doi:10.1038/cdd.2012.105

Garcia-Saez AJ, Coraiola M, Serra MD, Mingarro I, Muller P, Salgado J (2006) Peptides corresponding to helices 5 and 6 of Bax can independently form large lipid pores. FEBS J 273:971–981. doi:10.1111/j.1742-4658.2006.05123.x

Garcia-Saez AJ, Buschhorn SB, Keller H, Anderluh G, Simons K, Schwille P (2011) Oligomerization and pore formation by equinatoxin II inhibit endocytosis and lead to plasma membrane reorganization. J Biol Chem 286:37768–37777. doi:10.1074/jbc.M111.281592

García-Sáez AJ, Coraiola M, Dalla Serra M, Mingarro I, Menestrina G, Salgado J (2005) Peptides derived from apoptotic Bax and Bid reproduce the poration activity of the parent full-length proteins. Biophys J 88:3976–3990. doi:10.1529/biophysj.104.058008

García-Sáez AJ, Chiantia S, Salgado J, Schwille P (2007) Pore formation by a Bax-derived peptide: effect on the line tension of the membrane probed by AFM. Biophys J 93:103–112. doi:10.1529/biophysj.106.100370

Gekara N, Weiss S (2004) Lipid rafts clustering and signalling by listeriolysin O. Biochem Soc Trans 32:712–714. doi:10.1042/BST0320712

Gilbert RJC, Serra MD, Froelich CJ, Wallace MI, Anderluh G (2014) Membrane pore formation at protein–lipid interfaces. Trends Biochem Sci 39:510–516. doi:10.1016/j.tibs.2014.09.002

Gonzalez MR, Bischofberger M, Pernot L, van der Goot FG, Frêche B (2008) Bacterial pore-forming toxins: the (w)hole story? Cell Mol Life Sci 65:493–507. doi:10.1007/s00018-007-7434-y

Gonzalvez F et al (2008) Cardiolipin provides an essential activating platform for caspase-8 on mitochondria. J Cell Biol 183:681–696. doi:10.1083/jcb.200803129

Gordon VM, Nelson KL, Buckley JT, Stevens VL, Tweten RK, Elwood PC, Leppla SH (1999) Clostridium septicum alpha toxin uses glycosylphosphatidylinositol-anchored protein receptors. J Biol Chem 274:27274–27280. doi:10.1074/jbc.274.38.27274

Gouaux E (1997) Channel-forming toxins: tales of transformation. Curr Opin Struct Biol 7:566–573. doi:10.1016/S0959-440X(97)80123-6

Gurcel L, Iacovache I, Van Der Goot F (2006) Aerolysin and related Aeromonas toxins. Academic Press, London

Haney EF, Nathoo S, Vogel HJ, Prenner EJ (2010) Induction of non-lamellar lipid phases by antimicrobial peptides: a potential link to mode of action. Chem Phys Lipids 163:82–93. doi:10.1016/j.chemphyslip.2009.09.002

Hotze EM, Tweten RK (2012) Membrane assembly of the cholesterol-dependent cytolysin pore complex. Biochim Biophys Acta 1818:1028–1038. doi:10.1016/j.bbamem.2011.07.036

Iacovache I, van der Goot FG, Pernot L (2008) Pore formation: an ancient yet complex form of attack. Biochim Biophys Acta (BBA) 1778:1611–1623. doi:10.1016/j.bbamem.2008.01.026

Iacovache I, Bischofberger M, van der Goot FG (2010) Structure and assembly of pore-forming proteins. Curr Opin Struct Biol 20:241–246. doi:10.1016/j.sbi.2010.01.013

Jang H, Connelly L, Teran Arce F, Ramachandran S, Kagan BL, Lal R, Nussinov R (2013) Mechanisms for the insertion of toxic, fibril-like β-amyloid oligomers into the membrane. J Chem Theory Comput 9:822–833. doi:10.1021/ct300916f

Johnson MK, Geoffroy C, Alouf JE (1980) Binding of cholesterol by sulfhydryl-activated cytolysins. Infect Immun 27:97–101

Kagan BL (2012) Chapter 9 Membrane pores in the pathogenesis of neurodegenerative disease. In: Teplow DB (ed) Progress in molecular biology and translational science, vol 107. Academic Press, London, pp 295–325

Kienker PK, Qiu X-Q, Slatin SL, Finkelstein A, Jakes KS (1997) Transmembrane insertion of the Colicin Ia hydrophobic hairpin. J Membr Biol 157:27–37. doi:10.1007/s002329900213

Koller D, Lohner K (2014) The role of spontaneous lipid curvature in the interaction of interfacially active peptides with membranes. Biochim Biophys Acta 1838:2250–2259. doi:10.1016/j.bbamem.2014.05.013

Kusumi A, Koyama-Honda I, Suzuki K (2004) Molecular dynamics and interactions for creation of stimulation-induced stabilized rafts from small unstable steady-state rafts. Traffic 5:213–230. doi:10.1111/j.1600-0854.2004.0178.x

Kuwana T et al (2002) Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell 111:331–342

Ladokhin AS, White SH (1999) Folding of amphipathic α-helices on membranes: energetics of helix formation by melittin. J Mol Biol 285:1363–1369. doi:10.1006/jmbi.1998.2346

Lafont F, Abrami L, van der Goot FG (2004) Bacterial subversion of lipid rafts. Curr Opin Microbiol 7:4–10. doi:10.1016/j.mib.2003.12.007

Lakey J, Slatin S (2001) Pore-forming colicins and their relatives. In: Van Der Goot FG (ed) Pore-forming toxins. Springer, Heidelberg, pp 131–161

Li J, Koni PA, Ellar DJ (1996) Structure of the mosquitocidal δ-endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and implications for membrane pore formation. J Mol Biol 257:129–152. doi:10.1006/jmbi.1996.0152

Lins L, Decaffmeyer M, Thomas A, Brasseur R (2008) Relationships between the orientation and the structural properties of peptides and their membrane interactions. Biochim Biophys Acta (BBA) 1778:1537–1544. doi:10.1016/j.bbamem.2008.04.006

Lucken-Ardjomande S, Montessuit S, Martinou J-C (2008) Contributions to Bax insertion and oligomerization of lipids of the mitochondrial outer membrane. Cell Death Differ 15:929–937. doi:10.1038/cdd.2008.9

Ludtke SJ, He K, Heller WT, Harroun TA, Yang L, Huang HW (1996) Membrane pores induced by magainin. Biochemistry 35:13723–13728. doi:10.1021/bi9620621

Lutter M, Fang M, Luo X, Nishijima M, Xie X-S, Wang X (2000) Cardiolipin provides specificity for targeting of tBid to mitochondria. Nat Cell Biol 2:754–761. doi:10.1038/35036395

Madden JC, Ruiz N, Caparon M (2001) Cytolysin-mediated translocation (CMT): a functional equivalent of type III secretion in gram-positive bacteria. Cell 104:143–152. doi:10.1016/S0092-8674(01)00198-2

Mahfoud R, Mylvaganam M, Lingwood CA, Fantini J (2002) A novel soluble analog of the HIV-1 fusion cofactor, globotriaosylceramide (Gb3), eliminates the cholesterol requirement for high affinity gp120/Gb3 interaction. J Lipid Res 43:1670–1679. doi:10.1194/jlr.M200165-JLR200

Mancheño JM, Martín-Benito J, Martínez-Ripoll MN, Gavilanes JG, Hermoso JA (2003) Crystal and electron microscopy structures of sticholysin II actinoporin reveal insights into the mechanism of membrane pore formation. Structure 11:1319–1328. doi:10.1016/j.str.2003.09.019

Massotte D et al (1993) Structure of the membrane-bound form of the pore-forming domain of colicin A: a partial proteolysis and mass spectrometry study. Biochemistry 32:13787–13794

Matsuzaki K, Murase O, Fujii N, Miyajima K (1996) An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. Biochemistry 35:11361–11368

Matsuzaki K, K-i Sugishita, Ishibe N, Ueha M, Nakata S, Miyajima K, Epand RM (1998) Relationship of membrane curvature to the formation of pores by magainin 2. Biochemistry 37:11856–11863. doi:10.1021/bi980539y

Mechaly AE, Bellomio A, Gil-Cartón D, Morante K, Valle M, González-Mañas JM, Guérin DMA (2011) Structural Insights into the oligomerization and architecture of eukaryotic membrane pore-forming toxins. Structure 19:181–191. doi:10.1016/j.str.2010.11.013

Metkar SS et al (2015) Perforin oligomers form arcs in cellular membranes: a locus for intracellular delivery of granzymes. Cell Death Differ 22:74–85. doi:10.1038/cdd.2014.110

Muchmore SW et al (1996) X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature 381:335–341. doi:10.1038/381335a0

Mueller M, Grauschopf U, Maier T, Glockshuber R, Ban N (2009) The structure of a cytolytic α-helical toxin pore reveals its assembly mechanism. Nature 459:726–730. doi:10.1038/nature08026

Mukherjee S, Maxfield FR (2004) Membrane domains. Annu Rev Cell Dev Biol 20:839–866. doi:10.1146/annurev.cellbio.20.010403.095451

Naglich JG, Metherall JE, Russell DW, Eidels L (1992) Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. Cell 69:1051–1061. doi:10.1016/0092-8674(92)90623-K

Nelson KL, Raja SM, Buckley JT (1997) The glycosylphosphatidylinositol-anchored surface glycoprotein Thy-1 is a receptor for the channel-forming toxin aerolysin. J Biol Chem 272:12170–12174. doi:10.1074/jbc.272.18.12170

Nelson LD, Johnson AE, London E (2008) How interaction of perfringolysin O with membranes is controlled by sterol structure, Lipid Structure, and Physiological Low pH: insights into the origin of perfringolysin O-lipid raft interaction. J Biol Chem 283:4632–4642. doi:10.1074/jbc.M709483200

Nguyen KT, Le Clair SV, Ye S, Chen Z (2009) Molecular interactions between magainin 2 and model membranes in situ. J Phys Chem B 113:12358–12363. doi:10.1021/jp904154w

Parker MW, Feil SC (2005) Pore-forming protein toxins: from structure to function. Prog Biophys Mol Biol 88:91–142. doi:10.1016/j.pbiomolbio.2004.01.009

Parker MW, Pattus F, Tucker AD, Tsernoglou D (1989) Structure of the membrane-pore-forming fragment of colicin A. Nature 337:93–96. doi:10.1038/337093a0

Parker MW, Tucker AD, Tsernoglou D, Pattus F (1990) Insights into membrane insertion based on studies of colicins. Trends Biochem Sci 15:126–129. doi:10.1016/0968-0004(90)90205-P

Pedrera L, Fanani ML, Ros U, Lanio ME, Maggio B, Álvarez C (2014) Sticholysin I–membrane interaction: an interplay between the presence of sphingomyelin and membrane fluidity. Biochim Biophys Acta 1838:1752–1759. doi:10.1016/j.bbamem.2014.03.011

Pipkin ME, Lieberman J (2007) Delivering the kiss of death: progress on understanding how perforin works. Curr Opin Immunol 19:301–308. doi:10.1016/j.coi.2007.04.011

Potrich C et al (2005) Cytotoxic activity of a tumor protease-activated pore-forming toxin. Bioconjug Chem 16:369–376. doi:10.1021/bc049873z

Prieto L, He Y, Lazaridis T (2014) Protein arcs may form stable pores in lipid membranes. Biophys J 106:154–161. doi:10.1016/j.bpj.2013.11.4490

Prigent D, Alouf JE (1976) Interaction of streptolysin O with sterols. Biochim Biophys Acta 443:288–300

Puech P-H, Borghi N, Karatekin E, Brochard-Wyart F (2003) Line thermodynamics: adsorption at a membrane edge. Phys Rev Lett 90:128304. doi:10.1103/PhysRevLett.90.128304

Qian S, Wang W, Yang L, Huang HW (2008) Structure of transmembrane pore induced by Bax-derived peptide: evidence for lipidic pores. Proc Natl Acad Sci 105:17379–17383. doi:10.1073/pnas.0807764105

Rapson AC, Hossain MA, Wade JD, Nice EC, Smith TA, Clayton HA, Gee Michelle MSL (2011) Structural dynamics of a lytic peptide interacting with a supported lipid bilayer. Biophys J 100:1353–1361. doi:10.1016/j.bpj.2011.01.026

Ros U et al (2013) The sticholysin family of pore-forming toxins induces the mixing of lipids in membrane domains. Biochim Biophys Acta (BBA) 1828:2757–2762. doi:10.1016/j.bbamem.2013.08.001

Rossjohn J, Feil SC, McKinstry WJ, Tweten RK, Parker MW (1997) Structure of a Cholesterol-Binding, thiol-activated cytolysin and a model of its membrane form. Cell 89:685–692. doi:10.1016/S0092-8674(00)80251-2

Saha N, Banerjee KK (1997) Carbohydrate-mediated regulation of interaction of Vibrio cholerae hemolysin with erythrocyte and phospholipid vesicle. J Biol Chem 272:162–167. doi:10.1074/jbc.272.1.162

Schön P, García-Sáez AJ, Malovrh P, Bacia K, Anderluh G, Schwille P (2008) Equinatoxin II permeabilizing activity depends on the presence of sphingomyelin and lipid phase coexistence. Biophys J 95:691–698. doi:10.1529/biophysj.108.129981

Sitaram N, Nagaraj R (1999) Interaction of antimicrobial peptides with biological and model membranes: structural and charge requirements for activity. Biochim Biophys Acta (BBA) 1462:29–54. doi:10.1016/S0005-2736(99)00199-6

Sobko AA, Kotova EA, Antonenko YN, Zakharov SD, Cramer WA (2004) Effect of lipids with different spontaneous curvature on the channel activity of colicin E1: evidence in favor of a toroidal pore. FEBS Lett 576:205–210. doi:10.1016/j.febslet.2004.09.016

Song L, Hobaugh MR, Shustak C, Cheley S, Bayley H, Gouaux JE (1996) Structure of staphylococcal α-hemolysin, a heptameric transmembrane pore. Science 274:1859–1865. doi:10.1126/science.274.5294.1859

Sonnen AF-P, Plitzko JM, Gilbert RJ (2014) Incomplete pneumolysin oligomers form membrane pores. Open Biol 4:140044. doi:10.1098/rsob.140044

Subburaj Y, Ros U, Hermann E, Tong R, Garcia-Saez AJ (2015) Toxicity of an α-pore-forming toxin depends on the assembly mechanism on the target membrane as revealed by single-molecule imaging. J Biol Chem 290:4856–4865. doi:10.1074/jbc.M114.600676

Tanaka K, Caaveiro JMM, Morante K, González-Mañas JM, Tsumoto K (2015) Structural basis for self-assembly of a cytolytic pore lined by protein and lipid. Nat Commun 6:6337. doi:10.1038/ncomms7337

Tejuca M, Dalla Serra M, Potrich C, Alvarez C, Menestrina G (2001) Sizing the radius of the pore formed in erythrocytes and lipid vesicles by the toxin sticholysin i from the sea anemone Stichodactyla helianthus. J Membr Biol 183:125–135. doi:10.1007/s00232-001-0060-y

Tejuca M, Anderluh G, Dalla Serra M (2009) Sea anemone cytolysins as toxic components of immunotoxins. Toxicon 54:1206–1214. doi:10.1016/j.toxicon.2009.02.025

Thomas WE, Ellar DJ (1983) *Bacillus thuringiensis* var israelensis crystal delta-endotoxin: effects on insect and mammalian cells in vitro and in vivo. J Cell Sci 60:181–197

Tilley SJ, Saibil HR (2006) The mechanism of pore formation by bacterial toxins. Curr Opin Struct Biol 16:230–236. doi:10.1016/j.sbi.2006.03.008

Tilley SJ, Orlova EV, Gilbert RJ, Andrew PW, Saibil HR (2005) Structural basis of pore formation by the bacterial toxin pneumolysin. Cell 121:247–256. doi:10.1016/j.cell.2005.02.033

Tweten RK (2005) Cholesterol-dependent cytolysins, a family of versatile pore-forming toxins. Infect Immun 73:6199–6209. doi:10.1128/IAI.73.10.6199-6209.2005

Unsay JD, Cosentino K, Subburaj Y, García-Sáez AJ (2013) Cardiolipin effects on membrane structure and dynamics. Langmuir 29:15878–15887. doi:10.1021/la402669z

Valcarcel CA et al (2001) Effects of lipid composition on membrane permeabilization by sticholysin I and II, two cytolysins of the sea anemone *Stichodactyla helianthus*. Biophys J 80:2761–2774. doi:10.1016/S0006-3495(01)76244-3

Valeva A, Palmer M, Bhakdi S (1997) Staphylococcal α-toxin: formation of the heptameric pore is partially cooperative and proceeds through multiple intermediate stages. Biochemistry 36:13298–13304. doi:10.1021/bi971075r

Waheed A et al (2001) Selective binding of perfringolysin O derivative to cholesterol-rich membrane microdomains (rafts). Proc Natl Acad Sci 98:4926–4931. doi:10.1073/pnas.091090798

Walker B, Bayley H (1995) Key residues for membrane binding, oligomerization, and pore forming activity of staphylococcal α-hemolysin identified by cysteine scanning mutagenesis and targeted chemical modification. J Biol Chem 270:23065–23071. doi:10.1074/jbc.270.39.23065

White SH, Wimley WC, Ladokhin AS, Hristova K (1998) Protein folding in membranes: determining energetics of peptide-bilayer interactions. Methods Enzymol 295:62–87

Wimley WC, White SH (2000) Designing transmembrane α-helices that insert spontaneously. Biochemistry 39:4432–4442. doi:10.1021/bi992746j

Xu X, Zhai D, Kim E, Swift M, Reed J, Volkmann N, Hanein D (2013) Three-dimensional structure of Bax-mediated pores in membrane bilayers. Cell Death Dis 4:e683. doi:10.1038/cddis.2013.210

Yamaji-Hasegawa A et al (2003) Oligomerization and pore formation of a sphingomyelin-specific toxin, lysenin. J Biol Chem 278:22762–22770. doi:10.1074/jbc.M213209200

Yang L, Weiss TM, Lehrer RI, Huang HW (2000) Crystallization of antimicrobial pores in membranes: magainin and protegrin. Biophys J 79:2002–2009

Yang L, Harroun TA, Weiss TM, Ding L, Huang HW (2001) Barrel-stave model or toroidal model? A case study on melittin pores. Biophys J 81:1475–1485. doi:10.1016/S0006-3495(01)75802-X

Yeaman MR, Yount NY (2003) Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev 55:27–55. doi:10.1124/pr.55.1.2

Young JAT, Collier RJ (2007) Anthrax toxin: receptor binding, internalization, pore formation, and translocation. Ann Rev Biochem 76:243–265. doi:10.1146/annurev.biochem.75.103004.142728