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Review

Mode of action of antimicrobial proteins, pore-forming toxins and biologically active peptides (Hypothesis)

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Abstract

Antimicrobial peptides and pore-forming toxins are important effectors in innate immune defence reactions. But their mode of action, comprising the insertion into cholesterol-containing membranes is not known. Here we explore the mechanical implications of pore-formation by extracellular protein assemblies that drive cellular uptake reactions by leverage-mediated (LM) processes, where oligomeric adhesion molecules bent membrane-receptors around 'hinge'-like lipophorin particles. The interactions of antimicrobial peptides, pore-forming toxins and biologically active proteins with LM-assemblies provide a new paradigm for the configurational specificity and sterical selectivity of biologically active peptides.

Key words: antimicrobial peptides; pore-forming toxins; peptide hormones; endocytosis; lipophorin; cholesterol; lipid rafts; channel formation; lectins

Introduction

Antimicrobial proteins are effectors of innate immunity with unique structural properties mediating mainly pore-forming activities in membranes (Boman, 2000). Since its discovery more than thirty years ago (Boman *et al.*, 1974; Faye *et al.*, 1975), peptides with antibacterial activities have been divided into a number of different categories (Boman, 2003), including alpha-helical peptides, peptides with cysteine bonds and peptides enriched in one or more amino acids. A unique feature of anti-microbial peptides is their ability to permeate and disrupt target membranes (Shai, 2002).

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Antimicrobial peptides are believed to kill microorganisms via non-receptor-mediated mechanisms, although some peptides, such a nisin Z bind to bacterial cell wall components (Breuking *et al.*, 1999). According to (Shai, 2002) monomeric peptides that have random structures gain amphipathic structures and form oligomers in solution such that the hydrophobic regions are buried in the lumen of the oligomer and the hydrophilic regions are exposed to the solution. Upon reaching the membrane the organization is reversed. The hydrophobic regions are exposed to the lipid constituents of the membrane, and the hydrophilic regions are either segregated in the lumen of the oligomer (if the peptide oligomerizes and inserts into the membrane via the 'barrel' mechanism (Ehrenstein and Lecar, 1977), or exposed to the solution (if the peptides lay on the surface of the membrane and insert via the 'carpet' mechanism (Pouny and Shai, 1992). Two different mechanisms of peptide insertion have been implicated in related antimicrobial peptides (Chen *et al.*, 2003), where cecropin B molecules, (with one amphipathic and one hydrophilic á-helix) may be inserted into the

membrane in a concentration-dependent 'barrel-stave' mechanism, whereas synthetic cecropin B3 molecules (with two hydrophobic á-helices) may disrupt the membrane by a 'carpet-like' lysis mechanism.

Although many peptide properties have been described within the framework of the two models, a fundamental question remains: why are these peptides specific to bacterial membranes, but not damaging to most eukaryotic membranes? One argument is that the positive net charge of antibacterial peptides enables binding and permeation of negatively charged phospholipid membranes of bacteria but not to zwitterionic membranes, which are the major constituents of the outer leaflet of erythrocytes and other eukaryotic membranes (Shai, 2002). But there are important exceptions, which suggest that it is not the charge alone, but that the secondary and tertiary structure of the peptide is also crucial for insertion into the membrane. This is apparent in some antimicrobial peptides that are active in bacteria and in mammalian cells, such as melittin, its hybrid cecropin A permutations and its diastereomeric analogs (Merrifield *et al.*, 1995b). It appears that neither the direction of the peptide bond, nor the turn of the helix in d-enantiomers (Merrifield et al., 1995a) interferes with the membrane-lytic mechanism of the peptides (Boman, 2003). This and other examples (Staubitz *et al.*, 2001) suggest that chiral and sequence-specific determinants are not required for membrane-disrupting activity, while target specificity is strongly influenced by the overall physico-chemical nature of the analogues (Merrifield *et al.*, 1995b; Staubitz *et al.*, 2001).

Apart from the observation that changes in a peptide's sequence are more likely to destroy its activity to eukaryotic than to prokaryotic cells, there are no identifiable amino acid sequences that are responsible for the specificity (Hancock and Rozek, 2002). Another argument put forward to explain peptide-specificities against bacterial membranes is that cholesterol, which is present in eukaryotic membranes, protects against the action of antibacterial peptides (Boman, 2003). Although this has been confirmed in artificial membrane systems, it raises the question of why a large portion of antibacterial peptides, such as defensins (Hoffmann and Reichart, 2002), dermaseptins (Shai, 2002), cathelicidines (Zanetti *et al.*, 1997), pardaxin analogs (Shai, 2002), and tachystatin (Osaki *et al.*, 1999) are also active against fungal membranes, which contain ergosterol. Also difficult to understand in the context of current models are the non-lytic modes of action, such as the transfer of some peptides through the membrane into the underlying cytoplasm, where active peptides interfere with a diverse range of metabolic processes (Hancock and Rozek, 2002). In fact, some argue that the lytic action may not be the primary cause of death for bacteria (Boman, 2003), since antibacterial peptides may have already irreversibly impaired the viability of bacteria before the apparent disruption of the membrane.

Membrane trafficking an Achilles heel?

Although most experiments are performed with artificial membrane systems comprising regular-shaped vesicles, it is very likely that *in vivo* membrane disruptions by peptides are instigated during lipid-bilayer disturbances, such as cellular processes involving extreme membrane curvature and membrane fusion. This is relevant for membrane trafficking in higher organisms, but also applies to prokaryotes during cell division. The *in vitro* studies mentioned above suggest that membrane lipid composition is crucial for susceptibility to peptide insertion. Artificial membranes lacking cholesterol are more susceptible to peptide insertions than cholesterol-containing membranes (Boman, 2003). Furthermore, insertion of amphiphilic molecules forming nanotubes into cholesterol-free lipid bilayers has been modelled around hydrophobic-hydrophilic matching using molecular dynamics simulations (Lopez *et al.*, 2004). Thus the presence of cholesterol and sphingolipids appear to be a major barrier for the insertion of peptides into the membranes. This may explain why bacteria are susceptible to most peptides, while animals and plants are not. In this context it is interesting to note that higher organisms with intensive membrane trafficking perform non-clathrin mediated uptake reactions in special membrane domains, called 'lipid rafts', which represent accumulations of cholesterol and sphingolipids (Brown and London, 1998; Ikonen, 2001). The fact that uptake reactions involve reorganisation of the membrane bilayer makes membrane trafficking vulnerable to peptide attacks. But if these membrane domains are perceived to protect against pore-forming toxins, why do many toxins interact with receptors in lipid rafts (Kurzchalia, 2003; Zhuang *et al.*, 2002)? Is it possible that pore-forming toxins and channel-forming peptides exploit the protein machinery of cellular uptake reactions to achieve insertion into the membrane?

Pore-forming toxins

An alternative mechanism of channel formation involves the membrane insertion of pore-forming toxins with â-barrel peptides (Lesieur *et al.*, 1997) by putative leverage-mediated (LM) uptake reactions (Schmidt and Theopold, 2004). In line with the model, receptors are assembled around ring-shaped lipoproteins, such as lipophorin or hexamerin. Oligomeric adhesion molecules, such as lectins, cross-link receptors using membrane-distal mucin-domains thereby bending receptors around hinge-like lipoproteins. In this model the configuration of the complex provides the leverage to curve the membrane and internalise receptors in a cluster of LM-complexes affecting cell shape changes (Schmidt and Theopold, 2004) and adhesive properties of the cell (Schmidt and Schreiber, in press).

Since many pore-forming toxins, such as crystal toxins from *Bacillus thuringiensis* (*Bt*-toxins), are lectins, the insertion into the membrane may be mediated by an LM-uptake reaction (Schmidt and Theopold, 2004). In the LM-scenario, the ring-shaped pore complex is formed before or during the assembly of receptors (Fig. 1), which are bend around the hinge-like lipoprotein by the oligomeric pore-forming complex (Fig. 2). This is in contrast to the current assumption that Bt-toxin molecules are inserted into the membrane as monomers by a receptor-mediated

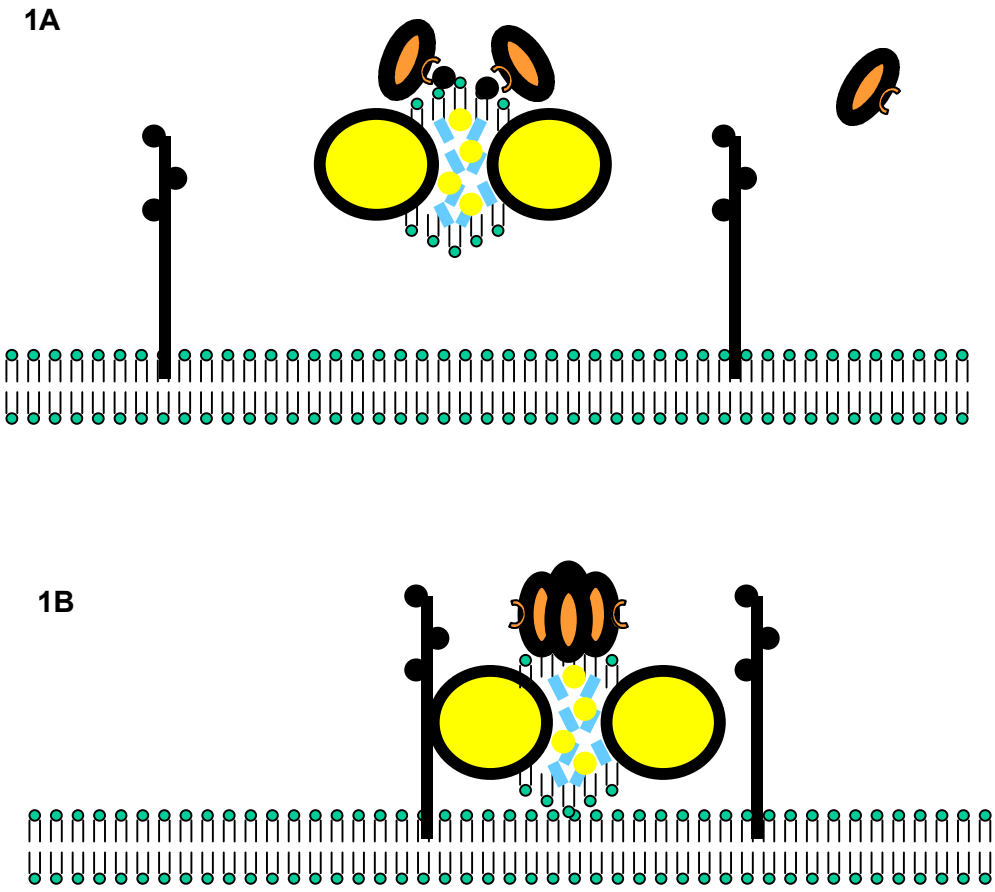

Fig. 1 Schematic illustration of receptor-assemblies with the potential to provide configurational energy by leverage-mediated mechanisms. A) Lipoproteins, such as lipophorin, contain ring-shaped proteins filled with lipids (Canavoso *et al*., 2001), including phospholipids (green), cholesterol (blue) and diacylglycerols (yellow). Lipophorin particles can bind to membrane-bound receptors to form lipophorin complexes (Theopold and Schmidt, 1997). Alternatively, lectins may bind to glycolipids or glycoproteins on lipid particles to form lectin-lipophorin complexes, before interacting with receptors. (glycodeterminants are indicated by black dots) B) Formation of a receptor complex with the potential to create configurational energy by a leverage-mediated (LM) process.

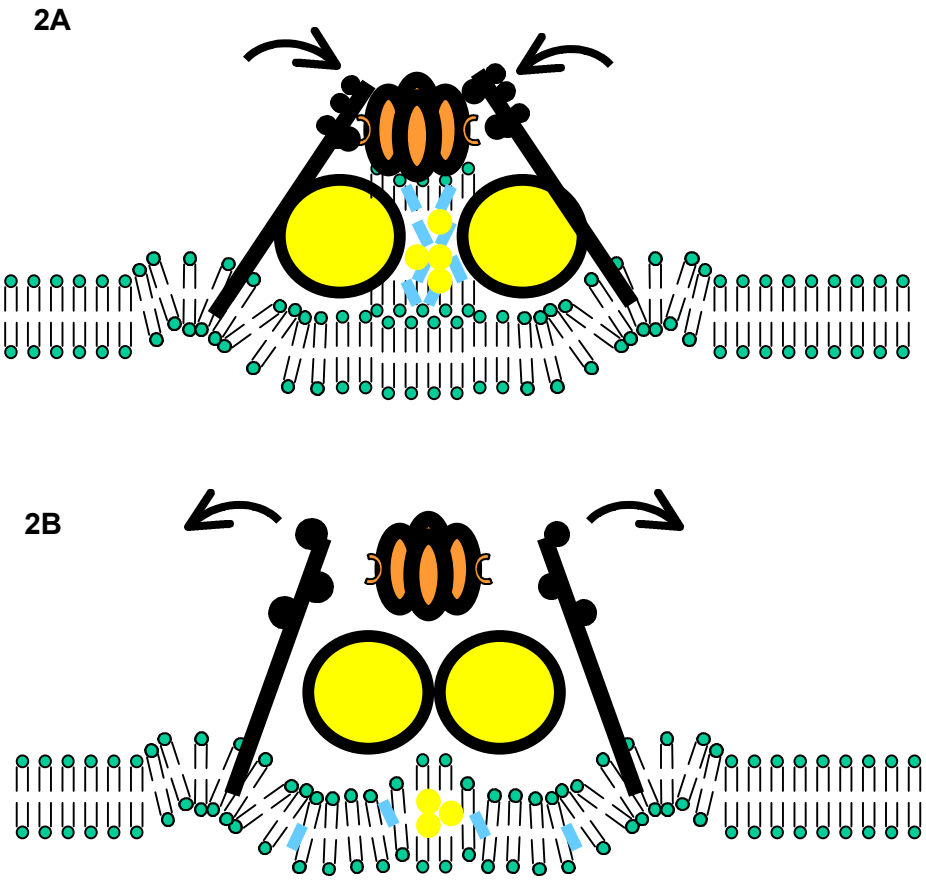

Fig. 2 Schematic illustration of leverage-mediated uptake reactions. LM-uptake reactions are mediated by oligomeric adhesion molecules, such as lectins. A) Assemblies, consisting of lipoproteins and multimeric lectins, interact with membrane-anchored molecules, such as membrane receptors, lipid anchored glycoproteins or glycolipids molecules. As a result of the LM-process the lipid particle is pushed into the underlying membrane thereby 'unloading' the lipophorin without the need to internalize the complex. Note that multiple LM-complexes predict membrane domains enriched in particle-derived lipids, such as cholesterol that may alter the local composition and protect the membrane against antibacterial peptide attack. B) Putative 'shuttle' mechanism: if the lipophorin particle changes shape in the process of lipid unloading, the resulting complex may not support a leverage-mediated uptake reaction and the complex unravels before internalization occurs. The unloaded lipophorin particles may then be released to become loaded on the gut membrane by an unknown process.

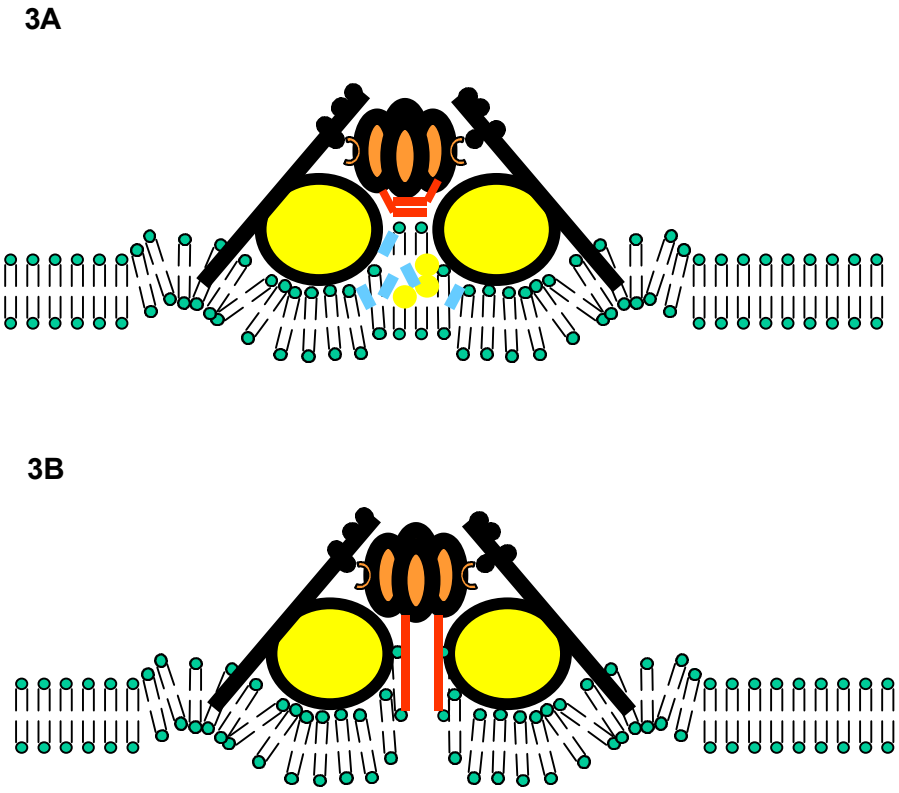

Fig. 3 Insertion of pore-forming toxins into the membrane by a putative LM-mechanism. A) LM-complex comprising oligomeric lectins containing amphipathic alpha-helices (red), which can engage in uptake reactions like other lectins. B) Leverage-mediated uptake reactions may push the amphipathic peptide into the lipid layer opening a membrane gap to the cytoplasm. This allows ions and water to pass from the endosome into the cytoplasm, causing osmofragility in some lectins (Pande ***et al***., 1998) and pores in endotoxins. Note that the pore-forming peptides can be covalently attached to the oligomeric adhesion molecule (e.g. pore-forming toxin, such as Bt-toxin). Alternatively, the active peptide may be assembled with the LM-complex without being covalently attached to any components by its space-filling properties. In this case the active peptide may be toxic and form a pore, such as mellittin, or interfere with LM-functions by altering cellular processes and signalling functions as observed with biologically active peptides.

reaction and only assembled into pore-forming oligomeric complexes once inside the membrane bilayer (de Maagd *et al.*, 2001). However, it has been shown that some mature *Bt*-toxins form tetrameric complexes when processed *in vitro* (Ma *et al.*, 2005), which would enable multiple interactions with receptors and lipoproteins before membrane insertion. Another intriguing observation is that some plant lectins increase osmofragility (Pande *et al.*, 1998), which in itself is not damaging to membranes, but may provide clues for our understanding of how pore-forming toxins are inserted into the membrane (Schmidt and Theopold, 2004). Since many pore-forming toxins are lectins, which recognise mucin-like glycoprotein-receptors (Armstrong *et al.*, 1996; Kuwahara *et al.*, 2000) and glycolipids (Griffitts *et al.*, 2005), such ring-shaped adhesion complexes could potentially be internalised by an LM-mechanism (Schmidt and Theopold, 2004), where the structural features of the complex predict a disruption of the lipid bilayer (Fig. 2).

In this context the structure of pore-forming toxins can be viewed as oligomeric lectins that have amphipathic peptides with antibacterial properties covalently attached. Indeed, secondary structure predictions, helical wheel/net diagrams and molecular mechanics calculations of membrane-inserting peptides from the *Bt*-toxin, form a strongly amphiphilic alpha-helix and show haemolytic activity *in vitro* comparable to that of bee venom peptide melittin (Szabo *et al.*, 1993). Similar results were obtained with the isolated á4-loop-á5 hairpin from the Bt-toxin, which showed that this peptide is extremely active compared to the isolated helices or their mixtures, indicating the complementary role of the two helices and the need for the loop for efficient insertion into membranes (Gerber and Shai, 2000). The concept that pore-forming toxins are oligomeric adhesion molecules with covalently attached antibacterial peptides is compatible with the idea that antibacterial peptides exploit LM-uptake reactions and are inserted into the membrane together with oligomeric adhesion molecules. A prediction of this model is that some antibacterial peptides can potentially overcome the cholesterol barrier of the membrane by assembling into the LM-machinery and become inserted into the membrane during LM-reactions. The fact that antibacterial peptides targeting eukaryotic cells are attached to oligomeric adhesion molecules, whereas peptides targeting prokaryotes are not, could indicate that peptides are effective in prokaryotes without the help of LM-mechanisms. Alternatively, prokaryotic uptake mechanisms may be different (e.g. in the absence of cholesterol) and less selective against active peptides.

Lipid exchange

In higher organisms cholesterol is transported between cells by a ring-shaped protein complex of apolipophorins that stabilize a mix of other hydrocarbons, such a phospholipids and diacylglycerols (DAGs). These lipid particles are in general internalised by cells via endocytosis reactions, except in insects, where lipophorin particles can act as a reusable shuttle by taking up lipid and delivering it to target tissues without internalization and degradation of the particle. For example, a single lipophorin particle, synthesized in the fat body, can take up dietary lipid at the midgut in the form of DAG, diffuse through the hemolymph to the fat body, and deliver the DAG for storage in the fat body without being internalised and degraded. This same particle can then return to the midgut surface and repeat the process (Canavoso *et al.*, 2001). How the lipophorin complex is able to release cholesterol and other lipids into the underlying cell membrane is not known. One of the possible implications of the LM-model is that an LM-uptake mechanism may provide the extracellular energy that is required to merge zwitterionic lipid layers.

In this scenario, the lipid-loaded lipophorin complex is pushed into the membrane as receptors form linkages with an oligomeric adhesion molecule (Fig. 2). While the nature and identity of receptors and adhesion molecules involved in lipophorin unloading is not known at this stage, recent observation suggest that lipophorin particles carry glycolipids and lipid-anchored glycoproteins that can interact with lectins and membrane receptors. For example, when lectins were mixed with cell-free hemolymph from lepidopteran insects and lipophorin separated on density gradients, lectins were enriched in the lipophorin fractions (Sarjan, 2002). Conversely when lipophorin fractions where analysed on Western blots with lectins a number of glycoproteins were found to be co-purified with lipophorin particles. One of these proteins, a ca 50 kDa protein was identified (Fabbri, 2003) and shown to belong to a group of chitinase-like molecules known as imaginal disc growth factors (IDGFs) (Asgari and Schmidt, 2004) with possible lectin-like properties (Homma *et al.*, 1996; Kawamura *et al.*, 1999; Li and Aksoy, 2000) that are conserved in other invertebrates (Akalal and Nagle, 2001) and vertebrates (Riazi *et al.*, 2000). More recently, lipid-anchored morphogens, such as Wingless and Hedgehog, have been found in association with *Drosophila* lipophorin particles and shown to be morphogen carriers in the extracellular space of imaginal discs (Panakova *et al.*, 2005).

It is therefore conceivable that lipophorin particles are pushed onto the membrane by functional LM-complexes, and in the process may allow the lipid moieties to mix, releasing the lipids into the underlying membrane (Fig. 2A). In this context a reversible shuttle function of lipophorin particles is possible if apolipophorin changes its conformation as a result of lipid depletion of the particle. In this case the hinge-like properties of lipophorin may be lost, disrupting the internalisation process and releasing the unloaded lipophorin (Fig. 2B).

While this is hypothetical, the importance of a lipophorin shuttle based on LM-mechanisms is that it makes specific predictions that can be experimentally tested. For example, mature crystal toxin from *B. thuringiensis* was co-purified with lipophorin particles on density gradients (Fig. 4). Since apolipophorin is not recognised by the toxin on Western blots this implies that the toxin binds to other glycodeterminants, such as lipid-anchored glycoproteins (Luo *et al.*, 1999; Panakova *et al.,* 2005) and glycolipids (Griffitts *et al.*, 2005; Hatakeyama *et al.*, 1999; Inoue *et al.*, 2001; Nedelkoska and Benjamins, 1998; Sandvig *et al.*,

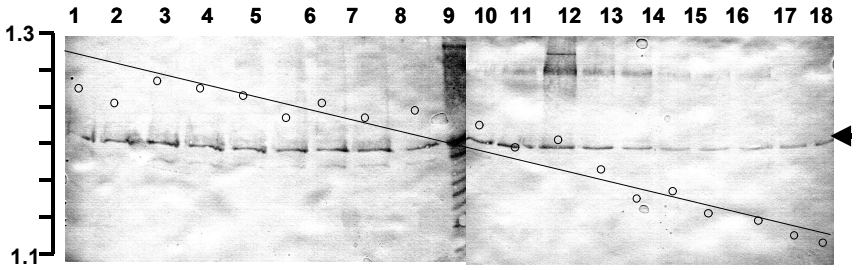

Fig. 4 Low density gradient of cell-free hemolymph (plasma) from *Galleria mellonella* larvae mixed with mature Bt-toxin (Cry1Ac). Aliquots of fractions were analysed on Western blots using anti-Cry1Ac antibodies. A 69 kDa monomer was found predominantly in high density fractions (arrowhead) together with a minor 60 kDa protein resulting from over-digestion of the pro-toxin. In addition, monomers were also found in low-density regions of the gradient between fractions 11 and 14, where apolipophorin I and II subunits peak in addition to a minor peak between fractions 16 and 20. Apart from monomers, oligomeric forms of the toxin, such as trimers and tetramers, are enriched in the lipophorin fractions (Sarjan, 2002). It is not clear whether pre-existing oligomers preferentially bind to lipophorin particles or whether monomers are bound to particles and form oligomers on the particle as observed in other systems (Park *et al.*, 2005).

1989). Although the identity of lectin-binding proteins from lipophorin particles in the gut lumen remains to be determined, this observation could suggest toxicity mechanisms at the gut lining involving lipophorin-mediated lipid exchange or uptake. Conversely, the fact that lipophorin (Li *et al.*, 2002) and other lipid carrying storage proteins (Ma *et al.*, 2005) are known to be involved in coagulation reactions could potentially be responsible for the observed aggregation reactions in the gut lumen (Ma *et al.*, 2005). The sequestration of toxin molecules in the gut lumen by immune-related coagulation reactions can also explain the observed tolerance to the toxin by immune induction (Rahman *et al.*, 2004; Gunning *et al.*, 2005; Ma *et al.*, 2005).

Configurational specificity

If LM-uptake reactions are the driving force for the insertion of pore-forming complexes into membranes, the functioning of such multi-protein complexes will depend on the correct configurational compilation of the protein assembly. For example, only oligomeric adhesion molecules that are able to generate leverage by interacting with membrane receptors across a hinge-like protein will be able to curve the membrane (Fig. 2A), a prerequisite for the insertion of amphipathic peptides into the membrane (Fig. 3). In this context the role and specificity of antibacterial peptides that are inserted into cholesterol-containing membranes may depend on structural requirements, which allow the peptide to intercalate into gaps provided by the LM-assemblies comprising oligomeric adhesion molecules, membrane-receptors and hinge-like proteins (Fig. 3A), without damaging the functionality of the complex. This implies that the observed peptide-specificity may be based on space-filling rather than protein-binding properties and that biologically active peptides are able to specifically interact with LM-assemblies without the need to bind to any individual proteins or receptors. The outcome of this interaction may be the formation of a damaging pore, but also non-toxic ion flux or alteration of the LM-uptake process, which can modify cell behavior and signaling. In fact the evolution of eukaryotic protein assemblies with LM-uptake properties may have been under selection pressure to protect against peptides with lytic properties, which makes cholesterol-containing lipoproteins attractive components in the process. If the lipid exchange during uptake reduce the toxicity of some antibacterial peptides and instead reduce the effect to modulate LM-uptake and signaling reactions, this may explain, why some antibacterial peptides function as biologically active peptide in vertebrates, such as vaso-active intestinal peptide hormones (VIP), PR-39 and other non-lytic peptides (Boman, 2003).

In summary, we propose that toxin-producing pathogens exploit a cellular uptake mechanism involving receptor rearrangements by a leverage-mediated process. The mechanical and configurational specifications of these LM-assemblies predict new types of protein specificity based on space-filling properties rather than protein-binding interactions.

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