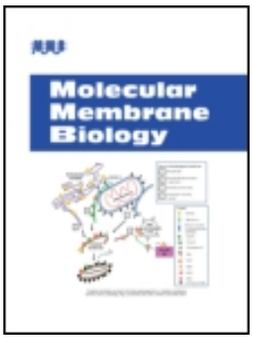

Molecular Membrane Biology

ISSN: 0968-7688 (Print) 1464-5203 (Online) Journal homepage: informahealthcare.com/journals/imbc20

Non-permanent proteins in membranes: when proteins come as visitors (Review)

Félix M. Goñi

To cite this article: Félix M. Goñi (2002) Non-permanent proteins in membranes: when proteins come as visitors (Review), Molecular Membrane Biology, 19:4, 237-245, DOI: 10.1080/0968768021000035078

To link to this article: https://doi.org/10.1080/0968768021000035078

Published online: 09 Jul 2009.

Molecular Membrane Biology, 2002, **19,** 237–245

Taylor&Francis
healthsciences

Non-permanent proteins in membranes: when proteins come as visitors (Review)

Félix M. Goñi*

Unidad de Biofísica (CSIC-UPV/EHU), and Departamento de Bioquímica, Universidad del País Vasco, Aptdo. 644, 48080 Bilbao, Spain

Summary

The present review introduces the concept of 'non-permanent membrane proteins', to encompass the wide variety of proteins that are not found in a stable membrane-bound form under physiological conditions, yet they interact with the membrane at some stage of their specific course of action. Non-permanent membrane proteins can be codified by the cell's own genome, or else they may arise from a foreign genome. Non-permanent membrane proteins can be classified, according to the reversibility of the membrane interaction, into those with reversible and irreversible (very long-lived) membrane contacts. According to the nature (strength) of the interaction, non-permanent membrane proteins may be divided into those that interact weakly with the membrane, in an extrinsic-like form, and those that interact strongly with the membrane. The latter can in turn be classified into those that cause and those that do not cause covalent modification of the lipids, the latter behaving, after interacting with the membrane, in the way of the conventional intrinsic proteins. Multiple examples are provided for the different groups of non-permanent membrane proteins, and a more detailed description is given of three of them, representative of different groups, namely TrwD from plasmid R388, *E. coli* α-haemolysin and *B. cereus* sphingomyelinase.

Keywords: cell membranes, membrane proteins, lipid-protein interactions, non-permanent membrane proteins.

Introduction

Along with its many virtues, the Singer-Nicolson model of biomembrane structure has one serious (and almost inevitable) limitation, in that it offers an *equilibrium* view of the membrane. The Singer-Nicolson membrane is an isolated system in the thermodynamic sense, no exchange of matter or energy with the environment being allowed. Of course, the situation in the cell is very different, with hormones, growth factors, metabolic substrates and end-products and a host of other molecules endlessly reaching and leaving the membrane at the rhythm marked by the physiological events. In the isolated membrane, proteins are there forever, as they have always been, either integral (intrinsic), solvated by the lipid hydrocarbon moieties, or peripheral (extrinsic), bound to the lipids and/or integral proteins through electrostatic and polar forces. In the living cell, a number of membrane proteins are indeed permanently bound to the lipid bilayer, either as integral or as peripheral proteins, while others will contact the membrane only under certain conditions, thereby remaining membrane-bound or returning promptly to the aqueous medium to which they belong. The present review deals with this kind of proteins that interact only briefly with the cell membrane, and with those that, becoming only occasionally in contact with the membrane, are irreversibly bound to it when they do. One or the other will interact in a more or less specific way with the bilayer. One or the other will cause some degree of bilayer perturbation. In both cases, the nature of the interaction/perturbation will be directly related to the physiological or pathological role of the proteins. This heterogeneous group of molecules will be designated as *non-permanent* membrane proteins.

The subject of proteins that can exist either free or membrane-bound has been studied in the past by several workers. Wilson (1978) called them 'ambiquitous proteins', and was perhaps the first to present in a systematic way the idea that variation in intracellular distribution may represent a regulatory mechanism to suit changing metabolic needs. Burn (1988) introduced the concept of 'amphitropic proteins' to encompass the wide group of proteins that associate reversibly with membranes under certain physiological conditions. Later, Bazzi and Nelsestuen (1993) exemplified in protein kinases C and the annexins the paradigm of proteins that are found either in soluble or membrane-bound forms, their change in location having important physiological consequences. Also among the precedents of this study, the work by Wimley and White (1996) should be mentioned. The latter authors achieved a quantitative description of the partitioning of peptides into membrane interfaces, by constructing an 'interfacial hydrophobicity' scale that has found important applications afterwards. They also noted that membrane partitioning promotes formation of secondary structure in the peptide and computed the coupling of structure formation to partitioning.

This review will include a proposed classification of the non-permanent membrane proteins, with a few examples given of each group. A short methodological section will follow, outlining laboratory methods more or less specifically designed for this kind of study. Finally, the membrane interactions of three 'visitor' proteins that are currently being studied in the laboratory will be described in some detail. It will be seen that, in the society of biomembranes, every visitor has a specific purpose in mind, only not every purpose is one that suits the interests and well-being of the host.

An attempt at taxonomy

The variety of biological objects and their ability to adapt to new environments by changing both structure and function make their classification a hopeless task. The following classification of non-permanent membrane proteins should, thus, be taken only as a first approximation. In fact, two classifications are proposed, perhaps with the hope that their respective failures may mutually compensate.

*e-mail: gbpgourf@lg.ehu.es

Molecular Membrane Biology
ISSN 0968-7688 print/ISSN 1464-5203 online
http://www.tandf.co.uk/journals
DOI: 10.1080/0968768021000035078

Non-permanent membrane proteins may be classified according to the *reversibility of the membrane contact* (table 1) into:

Proteins that bind the bilayers reversibly

These proteins have in common that, within a time scale compatible with the turnover time of membrane components (up to tens of minutes), the protein binds the membrane and then comes back to the aqueous medium. This group encompasses a large variety of proteins, with widely diverging kinetics of membrane binding. They are often, but not always, proteins with specific lipid binding sites. At the limit of the fast exchange, one should mention the ceramide-activated protein phosphatases 1 and 2A (Chalfant *et al*. 2001). In fact, these proteins have never been isolated in a membrane-bound form, but they contain a ceramide-binding site in their catalytic sub-unit. Thus, considering the highly hydrophobic character of ceramides, either they bind ceramide through transient, fast docking to the membrane, or else a cytosolic ceramide transfer protein, yet undetected, exists.

Other ceramide-binding proteins are known to exist transiently in the membrane-bound form, such as ceramide-activated protein kinase (Zhang *et al*. 1997), some isoenzymes of protein kinase C (Diaz-Meco *et al*. 1996, Huwiler *et al*. 1998), or c-Raf-1 (Huwiler *et al*. 1996). It has been proposed that ceramide binds proteins in this group through their cysteine-rich domains (van Blitterswijk 1998).

A large number of proteins are known that transiently bind the cell membranes and have a specific binding site for diacylglycerol (see Goñi and Alonso (1999) for a review). Chief among these are the protein kinase C isozymes belonging to the so-called 'conventional' and 'novel' families. In these enzymes, binding of diacylglycerol, in addition to causing protein activation, induces membrane binding of the protein that exists otherwise in the cytosol. The movement from cytosol to membrane is called translocation and constitutes an important event in signal transduction (Jaken and Parker 2000).

Another important group of proteins that transiently bind the cell membranes is constituted by the so-called 'lipid transfer proteins' (Wirtz 1997). These are intracellular proteins with the capacity to bind a lipid from one membrane and release it to a different one. Some recent results have revealed their role as mediators between lipid metabolism and cell functions. For example, yeast SEC14p protein, identical to mammalian phosphatidylinositol transfer protein, plays an important role in vesicle flow both in the budding reaction from the trans-Golgi network and in the fusion reaction with the plasma membrane. SEC14p links vesicle budding to the regulation of phosphatidylcholine biosynthesis in the Golgi, with the apparent purpose of maintaining an optimum phosphatidylinositol/ phosphatidylcholine ratio in the Golgi complex.

Several enzymes involved in phospholipid metabolism are, by conventional standards, cytosolic, although they exert their catalytic properties in the membrane-bound state. The various phospholipases are often good examples of cytosolic or extracellular proteins that become transiently docked to membranes in order to perform their catalytic roles (see below). All of the above enzymes possess lipid binding sites, either regulatory or catalytic, thus their transient binding to membranes must be mediated by those specific sites. This is not the case, however, of the transiently membrane-bound CTP:phosphocholine cytidyltransferase, an important enzyme in the synthesis of phosphatidylcholine whose substrates and products are water-soluble and has no specific lipid binding site (Davies *et al*. 2001). Studies in which membrane binding and activity of the purified enzyme were measured in lipid vesicles (Arnold *et al*. 1997) showed that membrane binding of the cytidyltransferase required anionic lipids. Diacylglycerol interacts synergistically with the anionic phospholipids to stimulate both binding and activation, in a way that resembles the situation with protein kinase C. Other proteins are known that, without possessing specific binding sites, are still able to bind transiently the cell membranes in the course of their physiological mechanism of action. This would be the case for proteins as diverse as the insect apolipoprotein apolipophorin III (Garda *et al*. 2002) or the small G-proteins Rho A and ADP-ribosylation factor. The latter two become membrane-associated in the presence of GTP (Brown *et al*. 1993, Kahn *et al*. 1993). In some cases, the physiological correlation is not evident, e.g. glyceraldehyde-3-phosphate dehydrogenase is a soluble glycolytic enzyme that contains hydrophobic patches on the molecular surface and has been reported to destabilize and fuse phospholipid vesicles under certain conditions (Morero *et al*. 1985).

Table 1. A classification of non-permanent membrane proteins.

Type	Examples	References
(a) According to the reversibility of the membrane contact.		
(1) Proteins that interact reversibly with the membrane.	Phospholipid transfer protein.	Wirtz (1997)
	Ceramide-activated protein kinase.	Zhang *et al*. (1997)
(2) Proteins with very long-lived (irreversible) contacts.	Bacterial toxins (e.g. aerolysin).	Buckley (1999)
	Blood coagulation factors.	Wu *et al*. (2002)
(b) According to the nature (strength) of the interaction.		
(1) Proteins that interact weakly with the membrane (extrinsic-like)	TrwD.	Rivas *et al*. (1997)
	Protein kinases C.	Jaken and Parker (2000)
(2) Proteins that interact strongly with the membrane:		
(2.1) Without covalent modification of the lipid (intrinsic-like).	RTX toxins.	Welch (2002)
	Complement proteins.	Wang *et al*. (2000)
(2.2) With covalent modification of the lipid.	Phospholipases.	Montes *et al*. (2002)
	Enzymes of lipid metabolism.	Davies *et al*. (2001)

Bacteriophage M13 offers an interesting and rather unique example of a protein that becomes inserted into the cell membrane in the way of the integral proteins, yet insertion is reversible. During viral replication, the major coat protein of M13 (protein VIII) accumulates in the host cell membrane, in the form of an integral protein (Stopar *et al*. 2002). Inside the cell, the newly synthesized phage DNA is protected transiently by protein V. Viral extrusion occurs without lysis of the host cell and, simultaneously, protein V is released to the cytoplasm and protein VIII is taken up from the membrane. Protein VIII appears to exist in two conformations, α (or viral form) and β (or membrane form) (Stopar *et al*. 2002).

Proteins that bind the bilayers irreversibly

In some cases, proteins that are not permanent constituents of the membrane become associated with it in an irreversible way. This occurs most often with proteins that are not encoded by the own cell genome, i.e. proteins from parasitic or toxic organisms. To mention but a few examples, this is the case of equinatoxin II from the sea anemone *Actinia equina* (Macek and Lebez 1988), α-haemolysin from *Escherichia coli* (Coote 1996) or aerolysin from *Aeromonas hydrophila* (Buckley 1999).

In all these cases, after insertion, the proteins behave exactly like any other integral protein in the cell membrane. Note that the toxins are released as soluble proteins to the aqueous medium and the mechanism by which they undergo the transition from soluble (water-soluble) to integral (lipid-soluble) proteins is a fascinating mystery and a difficult one to unravel.

The above concepts can be illustrated with the example of aerolysin, a 47 kDa channel-forming protein that contributes to the pathogenicity of *Aeromonas hydrophila*, a bacterium associated with intestinal and deep wound infections (Buckley 1999). The toxin is secreted in the form of an inactive precursor called proaerolysin that can be proteolytically processed to aerolysin by a number of proteases including trypsin and furin. The active form of the toxin is a water-soluble molecule that can spontaneously oligomerize, producing heptamers that may then insert into lipid bilayers, giving rise to discrete hydrophilic channels. On the basis of the crystal structure of proaerolysin, which reveals extensive β-structure, it seems likely that the oligomeric form of the toxin contains an amphipathic β-barrel analogous to that observed in the heptamer of *Staphylococcus aureus* α-toxin (Song *et al*. 1996). Aerolysin has no apparent affinity for lipids until it has oligomerized, but the oligomer contains an exposed hydrophobic surface, presumably the outside of the amphiphathic barrel, and can insert directly from solution. How such a large structure as the barrel penetrates a lipid bilayer is a largely unexplored puzzle. Alonso *et al*. (2000) have proposed that the aerolysin oligomer may overcome the barrier of the polar interfacial region by destabilizing the bilayer locally, causing the formation of non-lamellar structures. Using liposomes as the host membranes, those authors found that the inclusion of lipids that facilitate the lamellar-to-inverted hexagonal phase transition enhanced aerolysin insertion, whereas the opposite occurred when lipids that stabilize the lamellar phase were present.

An additional aspect that complicates the taxonomy of non-permanent membrane proteins is that there are examples of proteins that can bind lipid bilayers either reversibly or irreversibly, depending on the composition and physical properties of the bilayer. This is the case of *E. coli* α-haemolysin that binds reversibly bilayers in the gel state and irreversibly those in the liquid-crystalline state (Bakás *et al*. 1996). Equinatoxin II binds reversibly pure phosphatidylcholine bilayers, but irreversible insertion occurs when sphingomyelin is incorporated in the bilayer composition (Caaveiro *et al*. 2001). However, binding to cell membranes is almost always irreversible, and this is why these toxins are best classified within the group of non-permanent membrane proteins that become irreversibly bound.

The previous paragraphs have shown examples of bacterial or viral proteins that become part of the host cell membrane. The opposite may occur as well, when the host tries to attack an unwanted parasite, as in the case of complement-mediated bacterial killing. Complement proteins, of which more than 20 are known, exist in the blood as part of the innate immune system. Proteins 5b and 9 of the complement system form the so-called 'membrane attack complex' that binds the outer membrane of Gram-negative bacteria. The bactericidal activity of complement is dependent upon C9, but currently it is not understood how this protein translocates across the periplasm and dissipates the potential across the inner membrane (Wang *et al*. 2000).

In some cases, proteins encoded by the same organism may irreversibly bind its cell membranes. This is the case of several proteins involved in blood coagulation, e.g. factor X (Wu *et al*. 2002), factor VIII (Brinkman *et al*. 2002) or factor V (Kalafatis and Mann 2001). To mention but one example, factor V circulates in plasma as a single chain procofactor (330 kDa), from which derive the two chains, respectively 94 kDa and 74 kDa, of the active form, factor Va. Factor Va facilitates activation of prothrombin to α-thrombin by factor Xa. For this purpose, the serine protease factor Xa and factor Va assemble on a membrane surface in the presence of calcium ions. Insertion of factor Va in lipid bilayers containing neutral and acidic phospholipids has been studied by Koppaka and Lentz (1996). Membrane association of factor Va appears to be a complex process involving both chains of the protein, changes in lipid packing and in protein conformation. Interestingly, membrane binding also facilitates proteolytic degradation of factor Va (Kalafatis and Mann 2001).

Non permanent membrane proteins can also be classified according to the *nature (strength)* of their interaction with the host membrane (table 1).

Proteins that interact weakly with the membrane

These are proteins that remain membrane-bound through non-covalent forces other than the hydrophobic bond. Electrostatic and polar forces are the most relevant in this case. This group of proteins overlaps almost exactly with those that reversibly bind the cell membranes: many ceramide- and diacylglycerol-activated proteins involved in

cell signalling belong to it. However, phospholipases and other enzymes of lipid metabolism that induce covalent modification of membrane lipids should not be included here, since for the most part they bind the membrane through strong, transient, hydrophobic forces.

Proteins that interact strongly with the membrane

The proteins in this group are bound to the membrane mainly, but not exclusively, through hydrophobic forces. This is not to say that hydrophobic forces are particularly strong. When hydrophobic interactions are individually considered they are actually rather weak (of the order of 5 kJ/mol), their strength coming from the fact that, within the non-polar membrane matrix, a multitude of hydrophobic interactions join their forces providing an overall strong bond for the incoming protein. Among the non-permanent proteins that interact strongly with the membrane, an important distinction must be made between:

- proteins whose interaction does not lead to covalent modification of the membrane lipids, and
- proteins whose interaction with membranes does lead to covalent modification of the lipids.

In other words, strongly bound non-permanent membrane proteins may or may not have an enzyme activity on the lipids. Among the proteins devoid of enzyme activity on lipids are the toxins mentioned above: equinatoxin II, aerolysin, RTX toxins and many others, but note that there are bacterial toxins with phospholipase activity. The latter, together with the physiological phospholipases (including sphingomyelinase) and other enzymes of lipid metabolism constitute the second sub-group.

Methodological aspects

Detection and quantification of non-permanent protein binding to membranes requires a specific methodology, of which only an outline can be given here.

The classical method for measuring protein binding involves the use of ^{125}I-derivatized proteins (Bolton 1986). This is usually a reliable method and its main drawback is the requirement of a radioactive isotope. Inherent to this method is the use of an excess unlabelled protein that displaces the radioactive one from non-specific binding sites, so that radioactivity measurements provide data on high-affinity binding. Unfortunately, adding an excess toxin or an excess lipase to a cell system may be too damaging to the cell, so that the method becomes impractical in those cases.

A variety of methods are based on fluorescence. Koppaka and Lentz (1996) have comparatively studied a series of them as applied to the binding of proteins (specifically blood coagulation factor Va) to phospholipid bilayers. Studies from this laboratory have followed binding of *E. coli* α-haemolysin and its precursor prohaemolysin to liposomal membranes through changes in the intrinsic tryptophan fluorescence of the proteins (Ostolaza and Goñi 1995, Soloaga *et al*. 1996).

Other methods require the direct measurement of free and bound protein after it has been equilibrated with the membrane. Free and bound protein must be physically separated, then quantitatively assayed. Separation can be achieved, among other methods, by filtration or centrifugation. Caaveiro *et al*. (2001) measured the reversible and irreversible binding of equinatoxin II to liposomes with a filtration method. Pereira *et al*. (1997) developed a flotation method, in which the protein and membrane mixture is deposited at the bottom of a sucrose gradient. In a centrifugal field, membrane-bound proteins float, while free protein remains at the bottom of the gradient. The method has been applied to assess the proportion of TrwD, a protein involved in bacterial conjugation, that binds liposomal membranes (Machón *et al*. 2002). In general, methods that require the physical separation of free and bound protein have the intrinsic drawback that the shear stress induced by the separation method can perturb the free-bound equilibrium. However, the fact that the proportions of free and bound protein are directly measured provides a degree of reliability to these techniques.

Cortajarena *et al*. (2001) have developed a method for measuring the specific binding of *E. coli* α-haemolysin to erythrocyte membranes at sub-lytic toxin concentrations. The cells were incubated with the toxin and washed with hypotonic buffer. The pellet was resuspended in SDS-PAGE buffer and heated for 10 min at 100°C. Cell-associated toxins were quantified in immunoblots by comparing densitometric scans of bound toxin signals with calibrated signals of varying amounts of purified toxin electrophoresed and blotted under identical conditions. The binding data were analysed using an equation described by Gutfreund (1972) that relates the fractional saturation Y (equivalent to bound haemolysin/maximal bound haemolysin) with the total concentration of toxin as follows:

$$\frac{1}{1-Y} = \frac{1}{K_d}\left(\frac{\text{total toxin conc.}}{Y}\right) - \frac{\eta}{K_d}$$

The equation holds for the case of a toxin binding to a single class of sites. In this case, the slope provides an estimate of the dissociation constant K_d of the toxin-receptor complex, while the intersection with the α-axis gives η, the concentration of the binding sites.

Elusive protein-membrane contacts may be detected using photoactivatable lipid derivatives. For example, a technique has been developed that could help in detecting cytosolic ceramide target proteins (Wickel *et al*. 1999). The procedure uses a combination of ceramide affinity chromatography and a photoactivatable ^{125}I-labelled ceramide analogue, and should be able to identify cytosolic proteins with specific ceramide binding sites. In fact, this method has allowed the identification of the first endosomal ceramide target, cathepsin D, that colocalizes with, and may mediate downstream signalling effects of, acid sphingomyelinase (Heinrich *et al*. 1999).

Specific examples

TrwD from plasmid R388

An example of a protein that interacts weakly and reversibly with lipid bilayers, yet with clearly detectable effects, is TrwD,

a protein involved in bacterial conjugation. TrwD is encoded by conjugative plasmid R388. This plasmid contains one of the simplest gene organizations known for a conjugative transfer system (Bolland *et al*. 1990). The *trwD* gene is located in PIL_w, the R388 region involved in pilus formation. Many TrwD homologues are known, and many have functions unrelated to conjugation. TrwD was cloned and purified as a glutathione S-transferase (GST)-TrwD fusion protein (Rivas *et al*. 1997). The fusion protein was functionally active, as shown by genetic complementation assays. It displayed ATP hydrolase activity that was essential for R388 conjugation.

The precise effect of TrwD on bilayer architecture is defined by the experiments in figure 1 (Machón *et al*. 2002). The protein induces vesicle aggregation, leakage and intervesicular lipid mixing. Aggregation usually occurs as a mechanism by which to avoid contacts between water and hydrophobic molecules. This is, e.g. the mechanism of liposome aggregation due to the *in situ* production of diacylglycerol by phospholipase C (Basañez *et al*. 1996). In the present case, TrwD binding to (and perhaps partial insertion into) the lipid bilayer may lead to exposure of the membrane hydrophobic matrix. Alternatively, the protein may expose additional hydrophobic areas on its surface upon binding of the bilayer. Any of these phenomena would explain the observed aggregation.

Leakage of vesicular contents occurs concomitantly with aggregation. Leakage is probably a mere consequence of the lipid rearrangements secondary to protein insertion, as occurs with magainins (Matsuzaki 1998) or with *E. coli* α-haemolysin (Soloaga *et al*. 1999). A third effect of TrwD on liposomes is to induce intervesicular lipid mixing. This phenomenon requires an intimate degree of contact between vesicles, beyond the mere aggregation, that excludes water molecules from the vesicle contact area. In this case, lipid mixing never goes beyond 50% of the total lipid. In addition, it is limited to the outer monolayer (figure 1(*c*)) and is not accompanied by mixing of aqueous contents (figure 1(*b*)). Hence, TrwD appears to induce a phenomenon that has been called hemifusion (Chernomordik *et al*. 1995) or close apposition (Viguera *et al*. 1993) of vesicles, i.e. mixing of outer lipid monolayers but not of the inner compartments.

Yeo *et al*. (2000) have elucidated the crystal structure of hexameric HP0525 from *Helicobacter pylori*, which is another member of the VirB11 family and is related to TrwD. The predicted secondary structure of TrwD for the N-terminal domain is the same as the calculated structure for HP0525 (Machón *et al*. 2002). It is, thus, reasonable to assume that the two proteins have the same overall folding pattern in this region, including membrane binding through the N-terminal domain. Yeo *et al*. (2000) suggested that hexameric HP0525 spans the membrane and forms an ATP-dependent pore. In their view, the small hole formed by the pore in its closed form would be ~10 Å in diameter. However, a pore of this size would permit complete release of the vesicular aqueous contents in milliseconds (Nir and Nieva 2000). This is in contrast with the rather slow rates of leakage, compared to the rates of protein-induced vesicle aggregation seen in figure 1(*d*). The fact that TrwD-induced leakage is ATP-independent also speaks against the notion that the

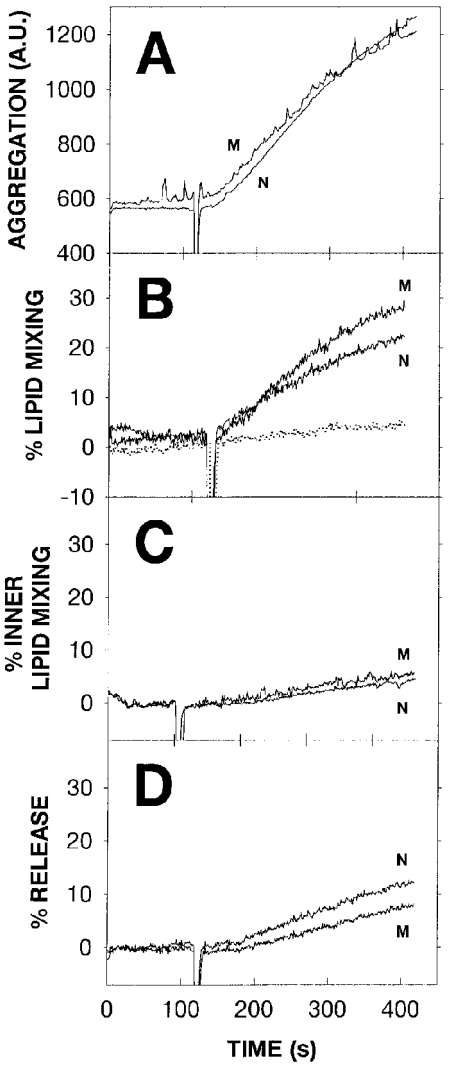

Figure 1. Effects of TrwD on LUV stability. (*a*) Vesicle aggregation measured as light scattering (520 nm). (*b*) Intervesicular lipid mixing measured by the R18 method (continuous line) and intervesicular mixing of aqueous contents (dotted line). (*c*) Intervesicular mixing of inner monolayer lipids measured with NBD-PE and rhodamine-PE. (*d*) Release of vesicular aqueous contents measured with the ANTS/DPX system. Phospholipid and protein concentrations were respectively of 0.2 and 17 nM. N and M refer to the native and mutant (ATPase-inactive) forms of TrwD, respectively (from Machón *et al*. 2002).

mechanism proposed by Yeo *et al*. (2000) is applicable to TrwD. Moreover, a transmembrane protein pore, as proposed by those authors, would hardly be compatible with a lack of mixing of vesicle inner monolayers (figure 1(*c*)). In general, the data by Machón *et al*. (2002) point more toward a kind of TrwD organization in the membrane that affects primarily the outer lipid monolayer in the vesicles.

α-Haemolysin from E.coli, a member of the RTX toxin family

α-Haemolysin from *Escherichia coli* is a typical representative of the group of non-permanent proteins with very long-

lived (in practice irreversible) membrane contacts that do not cause covalent modifications of the lipids. It is a protein toxin ($\approx$107 kDa) with a wide target cell specificity that has been associated with urinary tract infections and septicaemia (Stanley *et al*. 1998). α-Haemolysin (HlyA) belongs to the so-called RTX (repeats in toxin) family, a series of protein toxins that contain a number (16 in HlyA) of glycine- and aspartate-rich nonapeptide tandem repeats near their C-terminal ends (Welch 2001). Like other members of the family, HlyA is secreted extracellularly as a soluble protein, then it binds the membranes of eukaryotic cells to produce its pathogenic effects.

Soloaga *et al*. (1999) examined in detail the mode of irreversible insertion of HlyA into liposomal membranes using both prediction and experimental methods, under conditions in which the toxin has been shown to display its lytic activity in those model systems (Ostolaza *et al*. 1993). The prediction studies and experimental results described by Soloaga *et al*. (1999) are best interpreted in terms of α-haemolysin being embedded in the host lipid bilayer as an intrinsic protein, not traversing the membrane but rather occupying only the outer monolayer.

The fact that α-haemolysin widens the gel–fluid transition of saturated phosphatidylcholines, decreasing their associated enthalpy with little change in T_m, has long been recognized as typical of intrinsic proteins, as is the effect of HlyA increasing DPH polarization in fluid bilayers. Equally, Triton X-114 has been shown to solubilize membranes at 0°C, and separate into a detergent-rich phase, containing the integral membrane proteins, at 30°C. This is the way in which α-haemolysin behaves (Soloaga *et al*. 1999).

The simplest and clearest suggestion that HlyA is not a transmembrane protein comes from the lack of 'particles' on the fracture faces of vesicles containing inserted α-haemolysin under conditions that would give rise to vesicle leakage, as seen in Soloaga *et al*. (1999). In addition to this, quantitative results arising from differential scanning calorimetry and fluorescence polarization studies provide strong support to the hypothesis of the non-transmembrane character of HlyA, only one of the lipid monolayers being occupied by the protein. In fact, both techniques can provide a figure of the average number of lipids that are perturbed by the presence of the protein, so that they appear to be removed from the gel–fluid phospholipid transition (Chapman *et al*. 1979). In the present case, the figure is of ~400–500 phospholipids per protein molecule, a very large number compared with transmembrane proteins of equal or even higher molecular mass (table 2). However, two proteins associated with the lung surfactant, SP-B and SP-C, that are expected to lie down along the surfactant monlayer, rather than traversing any membrane, are also found to perturb a disproportionately high number of lipids (table 2). It can also be intuitively understood that the same mass of peptide, spread over a membrane or crossing it over more or less perpendicularly, may cause a very different perturbation, affecting a much larger number of lipids in the former case.

There are several lytic peptides with helical structures that have been proposed in recent years to lie oriented parallel to the surface of the membrane. This is the case of magainins 1 and 2 (Bechinger *et al*. 1993, Matsuzaki *et al*. 1994, Bechinger 1997), as well as the model peptide 18L (Polozov *et al*. 1997). It is interesting in this respect that 18L-lytic activity is modulated by the propensity of the membrane lipids to give rise to non-lamellar aggregates (Polozov *et al*. 1997), as was the case for α-haemolysin (Ostolaza *et al*. 1993). Thus, the interaction of HlyA with the lipid bilayer, through its amphipathic helices, would follow the same pattern as the above lytic peptides.

Sphingomyelinase and its effects on membrane permeability

Sphingomyelinase is an enzyme that cleaves sphingomyelin, giving rise to ceramide and water-soluble phosphorylcholine (Schneider and Kennedy 1967). Within this classification of non-permanent membrane proteins (table 1), it belongs to the group of proteins that interact strongly with the membrane, producing chemical modification of the lipids. This enzyme interacts reversibly with the bilayer, but it can remain membrane-bound for prolonged periods of time. Sphingomyelin has long been known as a lipid component of cell membranes, but only recently the attention of biologists has focused on this sphingophospholipid, as a result of a series of discoveries. Chief among these are the role of ceramides and other sphingomyelin derivatives in cell signalling (see Kolesnick *et al*. (2000) for review). Also, the recent discovery that human serum low-density lipoprotein

Table 2. Average number of phospholipid molecules perturbed per protein molecule in model systems containing phospholipids and reconstituted intrinsic proteins (from Soloaga *et al*. 1999).

Protein	*M* (kDa)	No. of lipids	Technique
Cytochrome c oxidase	200	55±5	EPR
Cytochrome c reductase	270	100	DSC
Na^+, K^+-ATPase	265	57–72	EPR
Ca^{2+}-ATPase	115	30	DSC, fluorescence
i.d.	115	30	EPR
Rhodopsin	40	21–24	EPR
Myelin proteolipid	25	37	Fluorescence
Bacteriorhodopsin	26	25	DSC, fluorescence
Melittin	3	10	DSC
α-Haemolysin	107	400–500	DSC, fluorescence
Lung surfactant SP-B	9	51	DSC
Lung surfactant SP-C	4	35	DSC

has a sphingomyelinase activity (Holopainen *et al*. 2000) may link ceramides to the pathogenesis of atherosclerosis. Sphingomyelin, and sphingolipids in general, have rather unique thermotropic properties, in that they usually have gel–fluid phase transitions in the 30–45°C range, i.e. very near the physiological temperature of mammals, but much above the transition temperatures of other membrane lipids. Thus, sphingolipids may exist under certain physiological conditions in the ordered or gel state, and in turn this may be related to their capacity to form separate lipid domains.

One striking property of sphingomyelinase that may be linked to its physiological effect is its capacity to restructure, via ceramide formation, the permeability barrier of model and cell membranes, thus giving rise to vesicle or cell efflux. Sphingomyelinase-induced release of aqueous contents from liposomes and resealed erythrocyte ghosts was first observed by Ruiz-Argüello *et al*. (1996), who induced *in situ* generation of ceramide by treating the sphingomyelin-containing model or cell membranes with bacterial sphingomyelinase. Ceramide-induced membrane efflux may be important physiologically, e.g. in generating local ion fluxes, or even in the release of large molecules (like cytochrome c) whose efflux from mitochondria is crucial for the activation of apoptosis.

In order to study in more detail the phenomenon of sphingomyelinase-induced membrane efflux, Montes *et al*. (2002) prepared large unilamellar vesicles with varying lipid compositions, sphingomyelin ranging between 0–50 mol % of the total lipid, and loaded them with water-soluble fluorescent molecules. Ceramides have been generated *in situ* by sphingomyelinase, or added to the preformed vesicles, or mixed with the other lipids in the process of liposome preparation. Their results show that the presence of ceramides can indeed allow the efflux of large molecules (molecular mass $\approx$20 kDa) through membranes, and that both *in situ* generation and external addition of ceramides can induce release of aqueous contents (Montes *et al*. 2002).

When large unilamellar vesicles composed of SM:PE:Ch (2:1:1 mole ratio) were treated with sphingomyelinase under the conditions described by Montes *et al*. (2002), ceramide was generated within the lipid bilayers as a result of sphingomyelin cleavage (figure 2(*a*)). When the vesicles were loaded, under isotonic conditions, with water-soluble fluorescent probes, efflux could be observed concomitantly with ceramide production (figure 2(*b*)). In a previous study, Ruiz-Argüello *et al*. (1996) described the release of low molecular weight markers, i.e. ANTS, DPX, from the vesicles. Montes *et al*. (2002) extended these observations to include the release of larger molecules, of molecular masses up to 20 kDa. As seen in figure 2(*b*), fluorescent dextrans the size of small proteins, e.g. cytochrome c, could be released through the activity of sphingomyelinase on SM-containing bilayers.

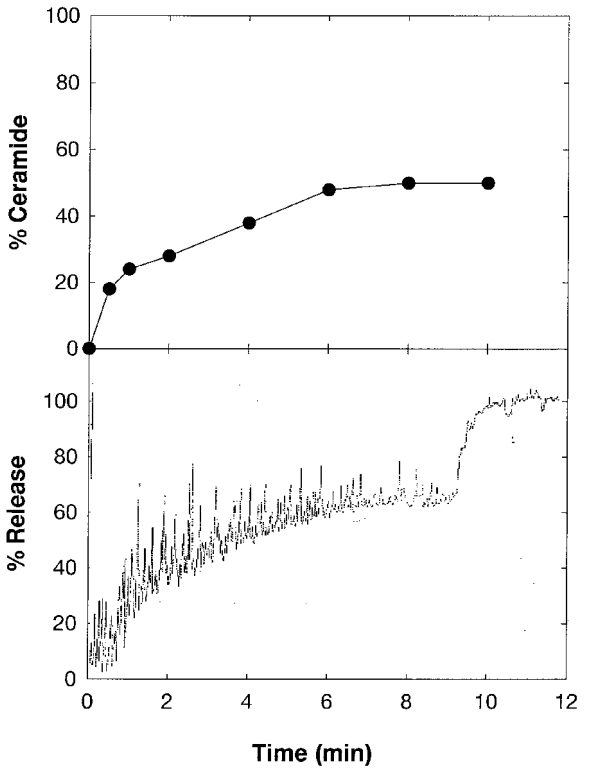

Figure 2. Ceramide-induced efflux of FITC-dextran 20000. Ceramide was generated by sphingomyelinase action on SM:PE:Ch (2:1:1) LUV. Lipid concentration: 0.3 mM. Enzyme concentration: 1.6 units/ml. (*a*) Ceramide production, expressed as mol% of total lipid. (*b*) Release of entrapped FITC-dextran 20000. 100% release was obtained after addition of 5 mM Triton X-100 (from Montes *et al*. 2002).

Concluding remarks

From a thermodynamic point of view, cell membranes constitute an open system, far from equilibrium, in constant exchange of matter and energy with their environment. Molecules as important (qualitatively ad quantitatively) as proteins may become in contact with the membranes, either for short or long periods. The term 'non-permanent proteins' has been suggested to encompass the variety of such molecules that, at some stage, come to interact with any of the cell membranes. Some of them are originated by the own cell genome, others arise from a foreign genome. A proposed classification divides them either according to the reversibility of the membrane contact or according to the strength of the interaction. The proposed nomenclature and classification will, of necessity, be the object of criticism and review. New methods to study the interaction of the membrane with these visitors, particularly with those that favour very short visits, will have to be developed. And, most important of all, one's view of the structure and dynamics of cell membranes will have to be gradually broadened, to encompass the increasing number of proteins that, being only transitorily part of the membrane, are not less worthy of attention by the membranologist than the more permanent ones.

References

Alonso, A., Goñi, F. M. and Buckley, J. T., 2000, Lipids favoring inverted phase enhance the ability of aerolysin to permeabilize liposome bilayers. *Biochemistry*, **39**, 14019–14024.

Arnold, R. S., De Paoli-Roach, A. A. and Cornell R. B., 1997, Binding of CTP: phosphocholine cytidylytransferase to lipid vesicles: diacylglycerol and enzyme dephosphorylation increase the affinity for negative charged membranes. *Biochemistry*, **36**, 6149–6156.

Bakás, L., Ostolaza, H., Vaz, W. L. C. and Goñi, F. M., 1996, Reversible adsorption and nonreversible insertion of *Escherichia coli* α-hemolysin into lipid bilayers. *Biophysical Journal*, **71**, 1869–1876.

Basañez, G., Nieva, J. L., Goñi, F. M. and Alonso, A., 1996, Origin of the lag period in the phospholipase C cleavage of phospholipids in membranes. Concomitant vesicle aggregation and enzyme activation. *Biochemistry*, **35**, 15183–15187.

Bazzi, M. D. and Nelsestuen, G. L., 1993, Protein kinase C and annexins: unusual calcium response elements in the cell. *Cell Signal*, **5**, 357–365.

Bechinger, B., 1997, Structure and functions of channel-forming peptides: magainins, cecropins, melittin and alamethicin. *Journal of Membrane Biology*, **156**, 197–211.

Bechinger, B., Zasloff, M. and Opella, S. J., 1993, Structure and orientation of the antibiotic peptide magainin in membranes by solid-state nuclear magnetic resonance spectroscopy. *Protein Science*, **2**, 2077–2084.

Bolland, S., Llosa, M., Avila, P. and de la Cruz, F., 1990, General organization of the conjugal transfer genes of the IncW plasmid R388 and interactions between R388 and IncN and IncP plasmid. *Journal of Bacteriology*, **172**, 5795–5802.

Bolton, A. E., 1986, Comparative methods for the radiolabeling of peptides. *Methods in Enzymology*, **124**, 18–29.

Brinkman , H. J., Mertens, K. and van Mourik, J. A., 2002, Phospholipid-binding domain of factor VIII is involved in endothelial cell-mediated activation of factor X by factor IXa. *Arteriosclerosis and Thrombosis Vascular Biology*, **22**, 511–516.

Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C. and Sternweis, C., 1993, ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell*, **75**, 1137–1144.

Buckley, J. T., 1999, *Comprehensive Sourcebook Bacterial Protein Toxins* (New York: Academic Press), pp. 362–372.

Burn, P., 1988, Amphitropic proteins: a new class of membrane proteins. *Trends in Biochemical Sciences*, **13**, 79–83.

Caaveiro, J. M. M., Echabe, I., Gutiérrez-Aguirre, I., Nieva, J. L., Arrondo, J. L. R. and González-Mañas, J. M., 2001, Differential interaction of equinatoxin II with model membranes in response to lipid composition. *Biophysical Journal*, **80**, 1343–1353.

Chalfant, C. E, Ogretmen, B., Galadari, S., Kroesen, B. J., Pettus, B. J. and Hannun, Y. A., 2001, FAS activation induces dephosphorylation of SR proteins; dependence on the novo generation of ceramide and activation of protein phosphatase 1. *Journal of Biological Chemistry*, **276**, 44848–44855.

Chapman, D., Gómez-Fernández, J. C. and Goñi, F. M., 1979, Intrinsic protein–lipid interactions. Physical and biochemical evidence. *FEBS Letters*, **98**, 211–223.

Chernomordik, L., Chanturiya, A. and Green, J., 1995, The hemifusion intermediate and its conversion to complete fusion. Regulation by lipid composition. *Biophysical Journal*, **69**, 922–929.

Coote, J. G., 1996, The RTX toxins of Gram-negative bacterial pathogens: modulators of the host immune system. *Reviews in Medical and Microbiology*, **7**, 53–62.

Cortajarena, A. L., Goñi, F. M. and Ostolaza, H., 2001, Glycophorin as a receptor for *Escherichia coli* α-hemolysin in erythrocytes. *Journal of Biological Chemistry*, **276**, 12513–12519.

Davies, S. M., Epand, R. M., Kraayenhof, R. and Cornell, R. B., 2001, Regulation of CTP: phosphocholine cytidylyltransferase activity by the physical properties of lipid membranes: an important role for stored curvature strain energy. *Biochemistry*, **40**, 10522–10531.

Díaz-Meco, M. T., Municio, M. M., Frutos, S., Sánchez, P., Lozano, J., Sanz, L. and Moscat, J., 1996, The products of par-4, a gene induced during apoptosis, interacts selectively with the atypical isoforms of protein kinase C. *Cell*, **86**, 777–786.

Garda, H. A., Arrese, E. L. and Soulages, J. L., 2002, Structure of apolipophorin-III in discoidal lipoproteins: interhelical distances in the lipid bound state and conformational change of ApoLp-III upon binding to lipid. *Journal of Biological Chemistry*, **277,** 19773–19782.

Goñi, F. M. and Alonso, A., 1999, Structure and functional properties of diacylglycerols in membranes. *Progress in Lipid Research*, **38**, 1–48.

Gutfreund, H., 1972, *Enzymes: Physical Principles* (London: Wiley-Interscience), pp. 71–73.

Heinrich, M., Wickel, M., Schenider-Brachert, W., Sandberg, C., Gahr, J., Schwandner, R., Weber, T., Saftig, P., Peters, C., Brunner, J., Kronke, M. and Schutze, S., 1999, Cathepsin D targeted by acid sphingomyelinase-derived ceramide. *EMBO Journal*, **18**, 5252–5263.

Holopainen, J. M., Medina, O. P., Metso, A. J. and Kinnunen, P. K., 2000, Sphingomyelinase activity associated with human plasma low density lipoprotein.*Journal of Biological Chemistry*, **275**, 16484–16489.

Huwiler, A., Brunner, J., Hummel, R., Vervoordeldonk, M., Stabel, S. and van der Bosch, H., 1996, Ceramide-binding and activation defines protein kinase c-Raf as a ceramide-activated protein kinase. *Proceedings of the National Academy of Sciences (USA)*, **93**, 6959–6963.

Huwiler, A., Doriano, F. and Pfeilschifter, J., 1998, Selective ceramide binding to protein kinase C-a and -d isoenzymes in renal mesangial cells. *Biochemistry*, **37**, 14556–14562.

Jaken, S. and Parker, P. J., 2000, Protein kinase C binding partners. *Bioessays*, **22**, 245–254.

Kahn, R. A., Yucel, J. K. and Malhotra, V., 1993, ARF signaling: a potential role for phospholipase D in membrane traffic. *Cell,* **75**, 1045–1048.

Kalafatis, M. and Mann, K. G., 2001, The role of the membrane in the inactivation of factor Va by plasmin. Aminoacid region 307-348 of factor V plays a critical role in factor Va cofactor function. *Journal of Biological Chemistry*, **276**, 18614–18623.

Kolesnick, R. N., Goñi, F. M. and Alonso, A., 2000, Compartmentalization of ceramide signaling: physical foundations and biological effects. *Journal of Cellular Physiology*, **184**, 285–300.

Koppaka, V. and Lentz, B. R., 1996, Binding of bovine factor Va to phosphatidylcholine membranes. *Biophysical Journal*, **70**, 2930–2937.

Macek, P. and Lebez, D., 1988, Isolation and characterization of three lethal and hemolytic toxins from the sea anemone *Actinia equina* L. *Toxicon*, **26**, 441–451.

Machón, C., Rivas, S., Albert, A., Goñi, F. M. and de la Cruz, F., 2002, TrwD, the hexameric traffic ATPase encoded by plasmid R388, induces membrane destabilization and hemifusion of lipid vesicles. *Journal of Bacteriology*, **184**, 1661–1668.

Matsuzaki, K., 1998, Magainins as paradigm for the mode of action of pore forming polypeptides. *Biochimica et Biophysica Acta*, **1376**, 391–400.

Matsuzaki, K., Murase, O., Tokuda, H., Funakoshi, S., Fujii, N. and Miyajima, K., 1994, Orientational and aggregational states of magainin 2 in phospholipid bilayers. *Biochemistry*, **33**, 3342–3349.

Montes, L. R., Ruiz-Argüello, M. B., Goñi, F. M. and Alonso, A., 2002, Membrane restructuring via ceramide results in enhanced solute efflux. *Journal of Biological Chemistry*, **277**, 11788–11794.

Morero, R. D., Vinals, A. L., Bloj, B. and Farias, R. N., 1985, Fusion of phospholipid vesicles induced by muscle glyceraldehyde-3-phosphate dehydrogenase in the absence of calcium. *Biochemistry*, **24**, 1904–1909.

Nir, S. and Nieva, J. L., 2000, Interactions of peptides with liposomes: pore formation and fusion. *Progress in Lipid Research*, **39**, 181–206.

Ostolaza, H. and Goñi, F. M., 1995, Interaction of the bacterial protein toxin α-haemolysin with model membranes: protein binding does not always lead to lytic activity. *FEBS Letters*, **371**, 303–306.

Ostolaza, H., Bartolomé, B., Ortiz de Zárate, I., de la Cruz, F. and Goñi, F. M., 1993, Release of lipid vesicle contents by the bacterial protein toxin α-haemolysin. *Biochimica et Biophysica Acta*, **1141**, 81–88.

Pereira, F. B., Goñi, F. M., Muga, A. and Nieva J. L., 1997, Permeabilization and fusion of uncharged lipid vesicles induced by the HIV-1 fusion peptide adopting an extended conformation: dose and sequence effects. *Biophysical Journal*, **73**, 1977–1986.

Polozov, I. V., Polozova, A. I., Tytler, E. M., Anantharamaiah, G. M., Segrest, J. P., Woolley, G. A. and Epand, R. M., 1997, Role of lipids in the permeabilization of membranes by class L amphipathic helical peptides. *Biochemistry*, **36**, 9237–9245.

Rivas, S., Bolland, S., Cabezón, E., Goñi, F. M. and de la Cruz, F., 1997, TrwD, a protein encoded by the IncW plasmid R388, displays an ATP hydrolase activity essential for bacterial conjugation. *Journal of Biological Chemistry*, **272**, 25583–25590.

Ruiz-Argüello, M. B., Basañez, G., Goñi, F. M. and Alonso, A., 1996, Different effects of enzyme-generated ceramides and diacylglycerols in phospholipid membrane fusion and leakage. *Journal of Biological Chemistry*, **271**, 26616–26621.

Schneider, P. B. and Kennedy, E. P., 1967, Sphingomyelinase in normal human spleens and in spleens form subjects with Niemann-Pick disease. *Journal of Lipid Research*, **8**, 202–209.

Soloaga, A., Ostolaza, H., Goñi, F. M. and de la Cruz, F., 1996, Purification of *Escherichia coli* prohaemolysin and a comparison with the properties of mature α-haemolysin *European Journal of Biochemistry*, **238**, 418–422.

Soloaga, A., Veiga, M. P., García-Segura, L. M., Ostolaza, H., Brasseur, R. and Goñi, F. M., 1999, Insertion of *E. coli* α-haemolysin in lipid bilayers as a non-transmembrane integral protein: prediction and experiment. *Molecular Microbiology*, **31**, 1013–1024.

Song, L., Hobaugh, M. R., Shustak, C., Cheley, S., Bayley, H. and Gouaux, J. E., 1996, Structure of staphylococcal α-hemolysin, a heptameric transmembrane pore. *Science*, **274**, 1859–1866.

Stanley, P., Koronakis, V. and Hughes, C., 1998, Acylation of *Escherichia coli* hemolysin: a unique protein lipidation mechanism underlying toxin function. *Microbiology and Molecular Biology Reviews*, **62**, 309–333.

Stopar, D., Spruijt, R. B., Wolfs, C. J. and Hemminga, M. A., 2002, Structural characterization of bacteriophage M13 solubilization by amphiphile. *Biochimica et Biophysica Acta*, **1594**, 54–63.

Van Blitterswijk, W. J., 1998, Hypothesis: ceramide conditionally activates atypical protein kinases C, Raf-1 and KSR through binding to their cysteine-rich domains. *Biochemistry Journal*, **331**, 679–680.

Viguera, A. R., Mencía, M. and Goñi, F. M., 1993, Time-resolved and equilibrium studies of the effects of poly(ethylene glycol) on small unilamellar phospholipid vesicles. *Biochemistry*, **32**, 3708–3713.

Wang, Y., Bjes, E. S. and Esser, A. F., 2000, Molecular aspects of complement-mediated bacterial killing. Periplasmic conversion of C9 from a protoxin to a toxin. *Journal of Biological Chemistry*, **275**, 4687–4692.

Welch, R. A., 2001, RTX toxin structure and function: a story of numerous anomalies and few analogies in toxin biology. *Microbiology and Molecular Biology Reviews*, **62**, 309–333.

Wickel, M., Heinrich, M., Weber, T., Brunner, J., Krönke, M. and Schütze, S., 1999, Identification of intracelullar ceramide target proteins by affinity chromatography and TID-ceramide photoaffinity labelling. *Biochemistry Society Transactions*, **27**, 393–398.

Wilson, J. E., 1978. Ambiquitous enzymes: variation in intracellular distribution as a regulatory mechanism. *Trends in Biochemical Science*, **3**, 124–125.

Wimley, W. C. and White, S. H., 1996, Experimentally determined hydrophobicity scale for proteins at membrane interfaces. *Nature Structural Biology*, **3**, 842–848.

Wirtz, K. W. A., 1997, Phospholipid transfer proteins revisited, *Biochemical Journal*, **324**, 353–360.

Wu, J. R., Zhou, C., Majumder, R., Powers, D. D. Weinreb, G. and Lentz, B. R., 2002, Role of procoagulant lipids in human prothrombin activation. 1. Prothrombin activation by factor X (a) in the absence of factor V (a) and in the absence and presence of membranes. *Biochemistry*, **41**, 935–949.

Yeo, H. J., Savvides, S. N., Herr, A. B., Lanka, E. and Waksman, G., 2000, Crystal structure of the hexameric traffic ATPase of the *Helicobacter pylori* type IV secretion system.*Molecular Cell*, **6**, 1461–1472.

Zhang, Z., Yao, B., Delika, S., Bayoumy, S., Lin, X.-H., Basu, S., McGinley, M., Chan-Hui, P.-Y., Lichenstein, H. and Kolesnick, R., 1997, Kinase suppressor of Ras is ceramide-activated protein kinase. *Cell*, **89**, 63–72.

Received 10 June 2002, and in revised form 22 July 2002.