



# Specificity of Intramembrane Protein–Lipid Interactions

Francesc-Xabier Contreras, Andreas Max Ernst, Felix Wieland, and Britta Brügger

Heidelberg University Biochemistry Center, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany

Correspondence: [britta.bruegger@bzh.uni-heidelberg.de](mailto:britta.bruegger@bzh.uni-heidelberg.de)

Our concept of biological membranes has markedly changed, from the fluid mosaic model to the current model that lipids and proteins have the ability to separate into microdomains, differing in their protein and lipid compositions. Since the breakthrough in crystallizing membrane proteins, the most powerful method to define lipid-binding sites on proteins has been X-ray and electron crystallography. More recently, chemical biology approaches have been developed to analyze protein–lipid interactions. Such methods have the advantage of providing highly specific cellular probes. With the advent of novel tools to study functions of individual lipid species in membranes together with structural analysis and simulations at the atomistic resolution, a growing number of specific protein–lipid complexes are defined and their functions explored. In the present article, we discuss the various modes of intramembrane protein–lipid interactions in cellular membranes, including examples for both annular and nonannular bound lipids. Furthermore, we will discuss possible functional roles of such specific protein–lipid interactions as well as roles of lipids as chaperones in protein folding and transport.

Our concept of biological membranes has markedly changed in the last two decades, from the fluid mosaic model (Singer and Nicolson 1972), in which the membrane was thought to be formed by a homogenous lipid fluid phase with proteins embedded, to the current model that lipids and proteins are not homogeneously distributed, but have the ability to separate into microdomains, differing in their protein and lipid compositions. A well established example of domains are lipid rafts (see Box 1 for definitions). Raft domains are described as dynamic domain structures enriched in cholesterol, sphingolipids, and membrane proteins

(Brown and London 1998; Simons and Ikonen 1997) that have an important role in different cellular processes (Lingwood and Simons 2010). Formation of domains within cellular membranes has been extensively investigated over the past years leading to various models that differ in the primary forces involved in the formation and the recruitment of surrounding membrane components into such domains.

According to one model, membrane domains can form by specific protein–protein interactions (Douglass and Vale 2005). This model is based on single-molecule microscopy experiments. In these studies, single fluorophores

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### BOX 1. Definitions

#### Annular Lipids/Lipid Shell

An annular lipid shell is formed when selected lipid classes or molecular species bind preferentially to the hydrophobic and/or hydrophilic surfaces of a membrane protein. Per definition these lipids show markedly reduced residence times at the protein–lipid interface as compared to bulk lipids.

#### Bulk Lipids

Lipids within the membrane that diffuse rapidly in the bilayer plane and show a low residence time at the protein–lipid interface following random collisions. Typical diffusion coefficients for bulk lipids in a liquid disordered phase are in the range of  $D_L = 7 \times 10^{-12} \text{ m}^2/\text{sec}$  (DOPC) (Filippov et al. 2003).

#### Hydrophobic Mismatch

A term to describe any deviation from the compatibility of the hydrophobic surface of membrane proteins (their TMDs) to the vertically and laterally encountered hydrophobic surfaces of the lipid bilayer in biological membranes. In the case of a hydrophobic mismatch, the resulting energy penalty may cause the recruitment of a suitable local lipid environment, the deformation of the membrane and/or in conformational changes of the protein to achieve a status of hydrophobic match (for advanced reading, see Killian 1998).

#### Lateral Pressure Field/Profile of Membranes

Biological membranes can be considered as the “solvent” for membrane proteins that are embedded in them. The lateral pressure profile ( $\Omega(z)$ ) describes the force or pressure that is exerted by the membrane on the matter residing inside it. This pressure is modulated by different extents of lipid–lipid interactions and asymmetries across and within the bilayer, which in turn results in varying lateral pressures that may locally correspond to several hundreds of atmospheres.

#### Lipid Rafts

Sterol and sphingolipid-dependent microdomains that form a network of lipid–lipid, protein–protein, and protein–lipid interactions; involved in the compartmentalization of processes such as signaling within biological membranes.

#### Liquid-Disordered Phase ( $L_d$ )

A predominantly fluid phase of lipids, characterized by a high degree of mobility (*cis*-gauche flexibility of acyl chains; lateral diffusion) and a high content of short and/or unsaturated fatty acyl chains.

#### Liquid-Ordered Phase ( $L_o$ )

A liquid crystalline phase (that displays physical properties of both liquids and of solid crystals), characterized by a high degree of acyl chain order (“packing”), a reduced lateral mobility of lipid and protein molecules, and a reduction in the elasticity of the membrane as a result of specific interactions between sterols and phospholipids containing long, saturated acyl chains and/or glycosphingolipids.

#### Microdomains

Membrane compartments of distinct lipid and protein composition that may modulate the enzymatic functions of membrane proteins.

#### Molecular Lipid Species

Individual members of a lipid class that differ in their fatty acid composition.

**Nonannular Lipids**

Lipids that specifically interact with membrane proteins are neither bulk lipids, nor do they belong to the shell/annulus of lipids that surround the membrane protein. These nonannular lipids often reside within membrane protein complexes, in which they may fulfill diverse functions ranging from structural building blocks to allosteric effectors of enzymatic activity (see text). Nonannular lipids bind to distinct hydrophobic sites of membrane proteins or membrane protein complexes.

were chemically attached to specific proteins, and the dynamics of individual proteins was tracked by monitoring the fluorescent probe. In this kind of set up, a dynamic behavior of lipids is not assessed. Here, proteins involved in signaling processes are trapped within interconnected microdomains created by specific protein–protein interactions, probably involving additional scaffolding proteins. The proteins of such domains can exchange with the surrounding membrane area at individual kinetics, some components are immobile over minutes, and others can diffuse rapidly.

Another model emphasizes the importance of lipid–lipid interactions, initiating the formation of subdomains of defined lipid compositions. Transmembrane proteins then can be attracted to such subdomains via various specific interactions with lipids. The resulting lipid–protein complexes then eventually coalesce to form larger lipid–protein assemblies (Anderson and Jacobson 2002).

The idea of lipid-dependent domain formation is inherent to the biophysical properties and therefore to the complex lipid composition of cellular membranes that include up to a thousand lipids that vary in structure (van Meer et al. 2008). This wide range of lipid species has been proposed to facilitate the “solvation” of membrane proteins. Taken into account the sum of lipid species present in a cellular membrane, it is important to understand the different interactions and affinities within the bilayer between different lipids. Molecular dynamics simulations have been successfully employed to investigate lipid interactions between different lipid species and found specific interactions of various lipid classes and molecular species (Hofsass et al. 2003; Niemela et al. 2004, 2006, 2009; Pandit et al. 2004; Zarskaya and Jeffrey 2005; Bhide et al. 2007).

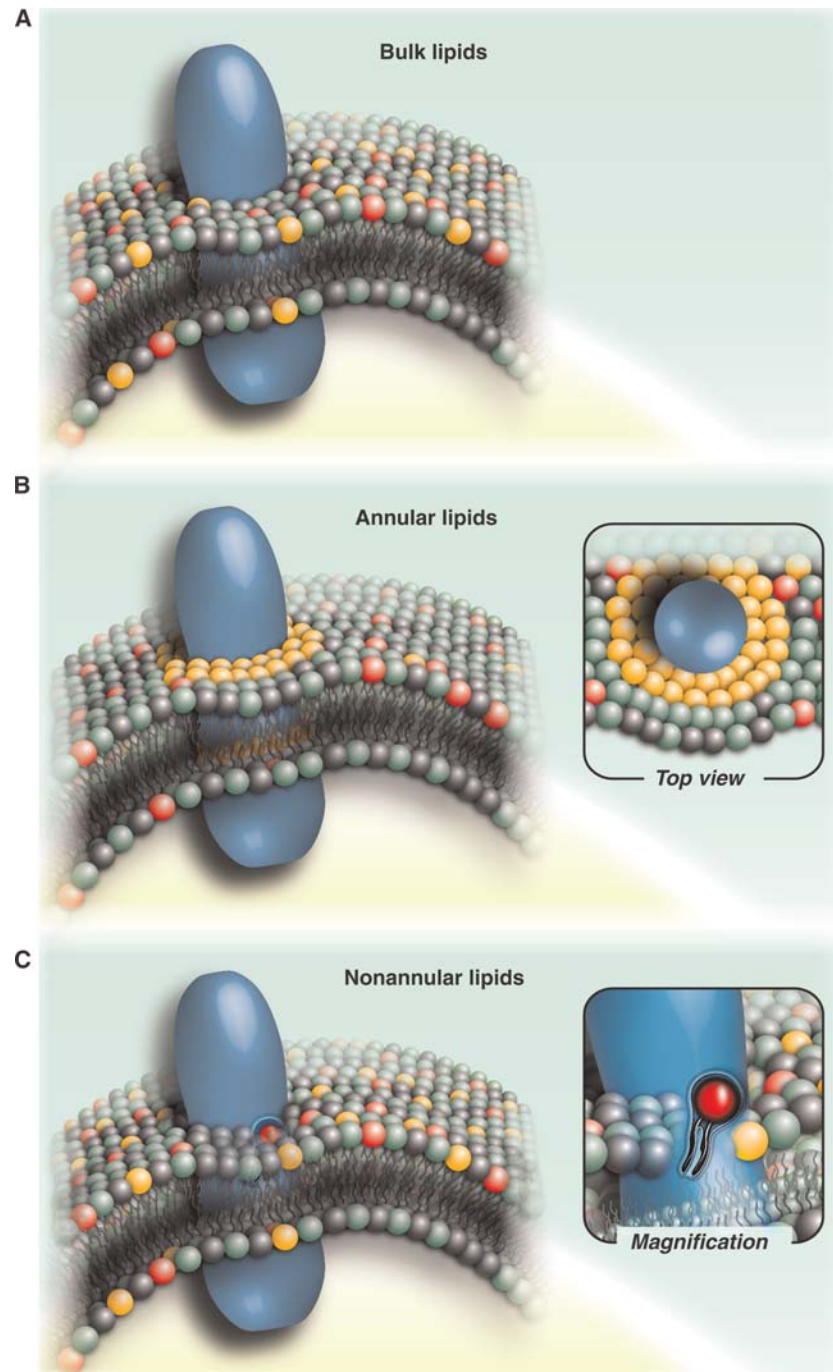
These results are supported and expanded by recent data from our group that suggest a specific order of interactions of sphingomyelin species with cholesterol in membranes (A.M. Ernst, F. Wieland, and B. Brügger, unpubl.). At low cholesterol concentrations, some sphingomyelin species preferentially interact with cholesterol, whereas others prefer their kin. At higher cholesterol concentrations, all sphingomyelin species investigated display an increased affinity for the sterol. These findings open the possibility of differentiated pathways of self-assembly of microdomains, dependent on molecular lipid species.

In the present article the various modes of intramembrane protein–lipid interactions in cellular membranes (Fig. 1) will be discussed. This includes possible functional roles of such specific protein–lipid interactions.

**METHODS USED TO ANALYZE PROTEIN–LIPID INTERACTIONS**

Crystallization of membrane proteins (Knapp et al. 1985) is one of the most powerful methods to define lipid-binding sites on proteins (Hunte and Richers 2008). Crystallography has the potential to revealing complete structural insight at atomic resolution, although in many cases lipid classes and molecular species cannot be determined unequivocally.

For structures in which crystals are not available, nuclear magnetic resonance (NMR) methods come into play, specifically solid state NMR of multiple protein/bilayer stacks that provides various modes to assess the orientation of a TMD toward lipids within the membrane. Current analytical methods to assess protein–lipid interactions are listed in Box 2, and explained below.



**Figure 1.** Intramembrane protein–lipid interactions within a cell membrane. (A) Bulk lipids; (B) annular lipids; (C) nonannular lipids/lipid ligands. For details see text.

## BOX 2. Selected Methods to Study Protein–Lipid Interactions

### X-Ray Crystallography

X-ray crystallography is the method of choice to obtain high-resolution structures of membrane proteins, with the number of protein structures at high resolution ( $<3 \text{ \AA}$ ) rapidly increasing. However, crystals of membrane proteins are always obtained from detergent solutions, which do not reflect their native lipid environment. Lipid molecules present in protein–lipid assemblies might not survive the purification procedure, or if copurified and crystallized appear not sufficiently ordered in the crystal to allow for their unambiguous characterization. In many cases lipid polar groups are not resolved and the acyl chains show unusual conformations, raising the possibility of inadequate refinement.

### Electron Crystallization

Electron crystallization is increasingly used to solve membrane protein structures with atomic resolution from two-dimensional (2D) crystals in a lipid environment (for recent reviews see (Raunser and Walz 2009; Reichow and Gonen 2009). 2D crystals (typically with a thickness of only one protein per array) can be produced of the purified protein in detergent by dialysis in the presence of lipids in a specific ratio to the protein, to allow membrane formation and 2D crystallization of the stabilized protein. This technique was successfully employed to solve the structure of bacteriorhodopsin at  $3 \text{ \AA}$  resolution (Kimura et al. 1997; Mitsuoka et al. 1999) and of aquaporin at  $1.9 \text{ \AA}$  resolution (Gonen et al. 2005), including individual lipid molecules. In the case of aquaporin a complete lipid shell around one aquaporin tetramer was resolved.

### NMR Spectroscopy

NMR spectroscopy is an important analytical technique to obtain molecular structures in solution. However, transmembrane proteins in bilayers are not amenable to solution NMR. Technical improvements like magic-angle spinning (MAS) and cross polarization (CP) approaches allow to analyze transmembrane proteins by solid-state NMR with a resolution close to that obtained in solution NMR experiments. In contrast to solution NMR, solid state NMR can be employed to study molecules larger than 100 kDa (Tycko 2001).

### Atomic Force Microscopy (AFM)

AFM provides a three-dimensional surface profile of the biological sample. Samples do not require any specific treatment. Notably, AFM can be performed conveniently in ambient air or in a liquid environment. Only few micrograms of sample are necessary. The lateral resolution is several nanometers, and the height resolution about  $0.1 \text{ nm}$ . High quality AFM profiles are taken at low time resolution (min), making dynamic processes less tractable. Unlike NMR or X-ray crystallography, AFM does not provide insight into changes down to the level of secondary protein structure.

### IR Spectroscopy

The technique of FT-IR (Fourier Transform-Infrared spectroscopy) has the potential to measure lipid acyl chain configuration, phospholipid head group-ion interactions (Dluhy et al. 1983), and protein secondary structure in a single experiment. No extrinsic probe molecules are required that could perturb the properties of the system under investigation (Taylor and Smith 1981). Relatively small amounts of material (in the microgram range) may be examined in a variety of physical states, such as bilayer vesicles or monolayer films on an IR substrate or aqueous surface (Dluhy and Cornell 1985). Interpretation of IR data is often difficult because of water vapor that interferes with protein amide I and II bands, and hampered by the complexity of most biological samples.

### Electron Spin Resonance (ESR) and Electron Paramagnetic Resonance (EPR)

With these methods one can, at high sensitivity and speed, estimate the number of lipids bound to the protein, and, for example, determine the residence time of one lipid in a protein–lipid complex. The



method depends on large amounts of protein and on the introduction into the sample molecule of reporter groups (spin labels) that cannot report fine details of order and mobility gradients.

#### Differential Scanning Calorimetry (DSC)

DSC is a useful tool to study molecular interactions of membrane proteins, including formation of membrane domains and lipid hydrocarbon chain order. Basically, this technique monitors differences in energy required to maintain the sample and the reference at the same temperature. The protein of interest can be reconstituted at various molar concentrations into liposomes of a defined lipid composition, to monitor (e.g., phase transitions within the membrane or heat-denaturation of the protein). High amounts of material (lipid and protein), and long analysis times (~30 min) are required.

#### Molecular Dynamics Simulations

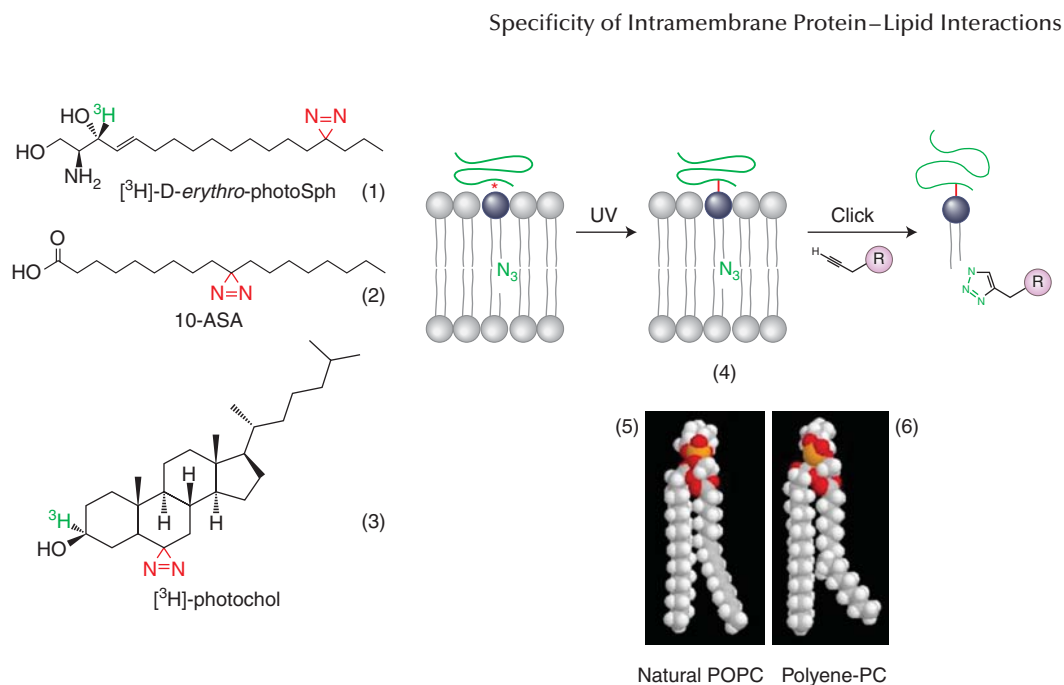
In silico approaches are of increasing importance for analyzing the molecular mechanisms of protein–lipid interactions (Lindahl and Sansom 2008). The atomistic simulations of molecular mechanics used to be limited by the calculation times on the available computer systems. This has been partially overcome by the use of parallelized super computers and a variety of optimized simulation software, which enable simulations in atomistic details for systems. The simulation of molecular dynamics (MD) allows the analyses of the interactions of atoms and macromolecules for a short period of time by the established laws of physics, and can hence be considered as an animation of Newtonian mechanics. Unlike NMR and X-ray based approaches, the motions and interactions of proteins and lipids can be monitored in atomistic detail with high temporal and spatial resolution. To model even larger systems or to analyze longer simulation times, systems can be simplified and simulated at “coarse-grained” resolution. Coarse-graining refers to combining neighboring atoms into a single interaction site, leading to a significant reduction in the number of particles and interactions in the MD simulation, enabling the simulations of processes beyond 1 microsecond, although with a loss in molecular detail (Ding et al. 2003; Paci et al. 2002; Smith and Hall 2001). The major limitation in simulating the MD of macromolecules is the number of particles that need to be analyzed, with an increasing particle number resulting in exponentially increasing computational times. Although MD simulations have been successfully employed to study lipid–lipid and protein–lipid interactions (reviewed in Niemela et al. 2009), further improvements in the hardware of computer systems might be necessary to broaden the access to this approach.

More recently, chemical biology approaches are being developed to analyze protein–lipid interactions. Such methods have the advantage of providing highly specific cellular probes.

One method to analyze specificity of cellular intramembrane protein–lipid interactions is based on a combination of radioactive labeling with covalent photo-crosslinking. Tritium-labeled membrane lipids or precursors thereof that carry a photoactivatable group (e.g., see structures 1–3 in Fig. 2) are fed to cells, and their conversion into membrane lipids is monitored by thin layer chromatography and autoradiography. Lipids are photo-activated by irradiation of the cells, and after immunoprecipitation of the protein of interest, covalently cross-linked

lipids are visualized by autoradiography after gel electrophoresis (Thiele et al. 2000; Haberkant et al. 2008).

A combination of covalent cross-linking by photo-labeling of one site of a lipid interacting with a protein, and labeling at another site for a sensitive readout of this interaction, has proven powerful. Here readout is based on introducing a click chemistry group that can then react with a second molecule, that is, biotin for detection with avidin. Such approaches are reviewed in Haberkant and van Meer (2009) and Moses and Moorhouse (2007), and typical chemistry is depicted in Figure 2 (scheme 4). For cellular labeling, such a bifunctional lipid is fed to cells, and, after photo-activation by



**Figure 2.** Novel lipid tools to study protein–lipid interactions. For details see text. (Structure 4 is adapted from Haberkant and van Meer [2009] and reprinted with permission from de Gruyter © 2009.)

irradiation to trigger covalent cross-linking, the cells are broken to perform the click reaction, with subsequent quantifying of the read-out molecule.

Fluorescence quenching has been successfully used to assess *in vitro* intramembrane protein–lipid interactions (Leto et al. 1980; East and Lee 1982; Markello et al. 1985; Gonzalez-Manas et al. 1992; Powl et al. 2005). Halogenated (mainly bromylated) membrane phospholipids together with nonlabeled lipids are reconstituted with membrane proteins into proteoliposomes. Binding constants for transmembrane–lipid complexes are then deduced by fluorometrically monitoring resonance energy transfer from aromatic intramembrane amino acid residues (usually tryptophan residues) to the dark acceptor halogenide.

Another approach for *in vitro* and live cell analysis of protein–lipid interactions is based on a novel class of fluorescent lipids with a conjugated pentanyl-group (Kuerschner et al. 2005). These fatty acyl analogs have a structure highly similar to their endogenous counterparts (Fig. 2, structures 5 and 6), and, unlike conventional fluorescently tagged lipids

(e.g., NBD- and BODIPY-derivatives) their lipid derivatives distribute within the cell together with their physiological kins. Pentanyl lipids have been employed to localize various lipid classes to intracellular compartments in live cells (Kuerschner et al. 2005). Notably, these fluorophores are able to serve as Förster resonance energy transfer (FRET) acceptors from protein-tryptophanyl residues exclusively in a hydrophobic environment as donor. Therefore, they are highly suited as probes for intramembrane protein–lipid interactions in reconstituted liposomal systems, and have been used *in vitro* to confirm a specific lipid molecular species–protein interaction found in live cells (E-X. Contreras, F. Wieland, and B. Brügger, unpubl.).

#### DEFINING PROTEIN–LIPID INTERACTIONS

For membrane proteins, different types of interactions with lipid molecules can be distinguished by the relative “residence” time of a particular lipid at the protein–lipid interface (Lee 2003). If a lipid displays a low degree of interaction with the transmembrane domain (TMD) of the protein, it is considered a “bulk”

lipid, defined by its fast exchange rate with lipids in close proximity (Fig. 1).

The residence time can be mediated by specific interactions with the lipid polar head group, by hydrophobic matching to the lipids' hydrocarbon chains, or both (discussed below). Such interactions lead to a significant reduction of exchange rates with the “bulk” lipids and the formation of an annulus or shell of (“annular”) lipids that surround the membrane protein. For large, multiple transmembrane-spanning proteins, the composition of this shell is not necessarily homogenous, because the interactions depend on the local architecture of the membrane protein and its compatibility with the various lipids. Individual lipids of a shell may vary in their residence time (for a review see Anderson and Jacobson 2002).

Lipids with even lower exchange rates are denominated as nonannular lipids (Fig. 1). These lipids often reside within large membrane protein complexes with a large number of subunits or within proteins that contain multiple transmembrane domains.

### ANNULAR PROTEIN–LIPID INTERACTIONS

As described above, the major distinction between annular and bulk lipids is based on their exchange rates at the protein–lipid interface of membrane proteins. The composition of a lipid annulus or shell around TMDs is dictated by their local architecture. This may result in various specificities for lipid classes (and molecular species), at various interfaces with the same membrane protein complex. Atomistic molecular dynamics simulations suggest that membrane proteins, together with their adjacent lipids, form a dynamic protein–lipid complex with up to 50–100 lipids (Niemela et al. 2010). Within this lipid shell, the diffusion rates, and hence the exchange rates with the bulk lipids, are found to be significantly reduced. Accepting that membranes are “more mosaic than fluid” (Engelman 2005), it becomes difficult to tell apart an actively recruited annulus from lipids in preexisting liquid-ordered microdomains, in which the lateral mobility of lipids is reduced.

### Impact of Membrane Properties and Membrane Protein Architecture on Interactions at the Protein–Lipid Interface

Several mechanisms rule the interactions of membrane proteins with distinct lipids: (1) hydrophobic thickness of the lipids, (2) the lateral pressure field of the membrane, (3) the distribution of charges at the protein–lipid interface, and (4) from the protein side the presence of specifically localized amino acid side chains.

The hydrophobic thickness of a lipid bilayer defines the distance between opposing head groups of the inner and outer leaflet, typically between 35 and 55 Å. The hydrophobic thickness is determined mainly by the lipid composition of the bilayer.

An important factor that influences the structure and dynamics of membrane proteins is the lateral pressure profile of membranes. It describes, similar to the buoyant force that is effective on matter embedded in fluids, the influence of a membrane as a solvent on a membrane protein as the matter dissolved in it. The highest pressure is at the interfacial region between hydrophobic and hydrophilic areas, at this location reaching peak values that correspond to pressures of hundreds of atmospheres. This force is further modulated by the degree of order of the surrounding lipids as well as the degree of hydrophobic mismatch at the protein–lipid interface and across the bilayer (Cantor 1999a,b; Gaines 1966).

Hydrophobic mismatch describes any deviation from an ideal hydrophobic compatibility of a transmembrane domain with its surrounding lipids. The above mechanisms are illustrated with the example of mechanosensitive channels. Mechanosensitive channels are found in both prokaryotes (MscL) and eukaryotes (TREK-1, TRAAK), and belong to a class of membrane proteins that are regulated by their local membrane environment, and for which the importance of binding of anionic annular lipids to a “hot spot” of positively charged amino acid residues was shown (Powl et al. 2008a,b). Alterations of the local lateral pressure fields were proposed as the molecular mechanism that



provides the mechanical force to shift the channels from an open to a closed state. This was shown by activation of the channels on addition of nonbilayer-forming phospholipids to cylindrical, bilayer-forming ones (Perozo et al. 2002).

As another example, the peptide antibiotic gramicidin (gA) of *Bacillus brevis* can only form channels if the hydrophobic length of the surrounding lipid acyl chains is exactly (hydrophobically) matched to its potassium-conducting conformation (Koeppel and Anderson 1996; Girshman et al. 1997; Mobashery et al. 1997).

An example of how the distribution of charges at the protein–lipid interface gives rise to the selectivity of membrane proteins for distinct polar moieties of lipids is given with the peripheral antenna complex LHII of *Rhodospira rubra*. Here, the enrichment of phosphatidylethanolamine (PE) in the boundary phase is thought to be mediated by specific spectrin-like PE-binding sites (Liu et al. 2004; Kwa et al. 2008).

The availability of an increasing number of membrane protein structures solved by X-ray crystallography (Hunte and Richers 2008; White 2009; Byrne and Iwata 2002) has enormously contributed to our understanding of protein–lipid interactions. In some of these structures, lipids tightly bound to the transmembrane domains have been observed (see Tables 1 and 2). These lipids appear in the electron density map as elongated structures mainly oriented perpendicular to the membrane plane. Correspondingly, the majority of the bound lipids are reproducibly copurified with the protein. Only few crystal structures containing an inner shell of annular lipids have been completely characterized. These annular lipids, bound to the surface of the protein, mediate between the protein and the bulk lipids, and seem to play a major role in the orientation of the membrane-spanning domain within the bilayer. In the yeast cytochrome *bc*<sub>1</sub> complex, phospholipids of the matrix leaflet (Lange et al. 2001) and phospholipids present in the intermembrane leaflet (Palsdottir et al. 2003) have been used to determine the hydrophobic thickness of the annulus surrounding the

protein complex. Here, the distance between the phosphodiester groups of two oppositely oriented phospholipids (36 Å) is in good agreement with the published thickness measure for a dioleoyl-phosphatidylcholine (DOPC) bilayer (38 Å) (Lewis and Engelman 1983). Another example of an annular shell is a bilayer of up to 18 tightly bound lipids that covers 80% of the surface of the trimeric seven-transmembrane-span protein bacteriorhodopsin (Belrhali et al. 1999; Luecke et al. 1999).

Finally, a well-established example of an annular shell surrounding a transmembrane domain is present in the X-ray structure of the membrane rotor ring of the Na<sup>+</sup>-ATPase from *Enterococcus hirae*, in which the internal hydrophobic ring surface is covered in both leaflets by 10 molecules of 1,2-dipalmitoyl-phosphatidylglycerol (DPPG) and 10 molecules of 1,2-dipalmitoyl-glycerol (DPG), respectively (Murata et al. 2005). This highly defined structure exemplifies a problem in classifying protein–lipid interactions: Although the lipid molecules form a ring around the transmembrane domains, and therefore represent annuli, the shell is tightly bound to the protein by specific interfaces with the individual lipids on the inner hydrophobic surface of the oligomeric assembly, causing very low exchange rates. Such ring-shaped assemblies are usually characteristic of annular lipids.

In most of the X-ray structures published, lipid–protein interactions are mainly stabilized by polar interactions between the lipid head group and specific amino acids. The majority of the tightly bound lipids are generally stabilized by at least two polar interactions between the phosphodiester group and a set of molecules generally combining a positive charge and a polar amino acid. Nevertheless, the observed binding domains are nonlinear and can even consist of a set of amino acids localized to different subunits, as observed for the yeast cytochrome *bc*<sub>1</sub> complex (Palsdottir et al. 2003). Aromatic amino acids are very often involved in lipid stabilization. Tyrosine residues, present in the interfacial region, interact with the lipid phosphodiester group either alone (via ion pair or hydrogen bond) or in combination

**Table 1.** Examples of nonannular lipids

Protein	Method	Lipid	Function	Selected references
β2-adrenergic receptor)	X-ray crystallography	Chol	Protein fold (putative)	Hanson et al. 2008
Caveolin-1	SDS-PAGE, in vitro oligomerization	Chol	oligomeric state	Monier et al. 1995; Murata et al. 1995
Cytochrome <i>bc</i> <sub>1</sub> complex	X-ray crystallography	PI, CL	Stability and integrity of the complex, enzymatic activity	Gomez and Robinson 1999; Lange et al. 2001
Cytochrome <i>c</i> oxidase	X-ray crystallography	PG	Oxygen transfer (putative)	Shinzawa-Itoh et al. 2007
K <sup>+</sup> -channel	X-ray crystallography	anionic PLs	Potassium conductance	Valiyaveetil et al. 2002; Marius et al. 2008
Metabotropic glutamate receptor	Agonist binding kinetics, lipid mass spectrometry	Erg	Allosteric effector, targeting to sterol-rich microdomains	Eroglu et al. 2003
Mitochondrial ADP/ATP carrier	X-ray crystallography	CL	Oligomeric state	Nury et al. 2005
Nitrate reductase A	X-ray crystallography	PG	Oligomeric state/protein fold (“building blocks”)	Bertero et al. 2003
Oxytocin receptor	Agonist binding kinetics	Chol	Allosteric effector	Gimpl et al. 2002a
Peripheral-type benzodiazepine receptor	Ligand-dependent cholesterol uptake and release kinetics, mutational analyses	Chol	Cholesterol transport and compartmentalization (putative)	Li and Papadopoulos 1998; Jamin et al. 2005
Plasma membrane Ca <sup>2+</sup> -ATPase	Transport & fluorescence assays	Cer DAG	Allosteric effector	Perez-Gordon et al. 2009
Rhodopsin	In vitro photolysis assays, differential scanning calorimetry (DSC)	Chol	Metarhodopsin formation, reprotonation (photocycle)	Mitchell et al. 1990; Bennet et al. 2008
Serotonin1a receptor	Agonist binding kinetics	Chol	Allosteric effector	Pucadyil and Chattopadhyay 2004
Vacuolar-type Na <sup>+</sup> -ATPase	X-ray crystallography	DPPG, DPG	Oligomeric state/protein fold (“building blocks”)	Murata et al. 2005

For details, see text and Ernst et al. 2010.

with positively charged amino acids. Likewise, tryptophan residues are mainly localized in the interfacial region, with the indole group pointing toward the center of the bilayer (Deisenhofer and Michel 1989). Hydrogen bonds are frequently observed between the indole

nitrogen atom and the lipid phosphodiester group (Ridder et al. 2000). Furthermore, a stabilization of the lipid acyl chains has been observed by a lamellar orientation of the indole ring, providing a mechanism for the anchoring of the transmembrane domain to the first shell

**Table 2.** Examples of annular lipids

Protein	Method	Lipid	Function	Selected references
Aquaporin	Electron crystallography, X-ray crystallography	various	Protein structure	Recently reviewed in Andrews et al. 2008
Bacteriorhodopsin	X-ray crystallography, electron crystallography	various	Protein fold (“building blocks”), reprotonation (photocycle)	Luecke et al. 1999; recently reviewed in Raunser and Walz 2009
Gramicidin (gA)	Capacitance measurements, solid-state NMR	di-18:2-PC, lyso-PLs	Conformational state (conductance)	Koeppel and Anderson 1996; Mobashery et al. 1997; Martinac and Hamill 2002
Large-conductance mechanosensitive channel ( <i>E. coli</i> ; MscL)	Patch clamp analyses, electron paramagnetic resonance (EPR)	lyso-PLs	Conformational state (conductance)	Vasquez et al. 2008; for a review see Powl et al. 2005
Light-harvesting complex II (LHII)	X-ray crystallography, lipid mass spectrometry	PE, PG	Photosynthetic membrane biogenesis	Liu et al. 2004; Kwa et al. 2008
Na,K-ATPase	Electron spin resonance spectroscopy (ESR)	PS, CL	Enzymatic activity (antiport of Na <sup>+</sup> and K <sup>+</sup> )	Reviewed in Esmann et al. 2006
Outer membrane protein F ( <i>E. coli</i> ; OmpF)	Förster resonance Energy transfer (FRET)	di-14:1-PLs	oligomeric state (trimeric complex)	O’Keeffe et al. 2000
Sarcoplasmic Ca <sup>2+</sup> -ATPase (SERCA)	Transport and fluorescence assays, phosphorescence anisotropy	di-18:1-PC, PE	rate of dephosphorylation, modulation of the phosphorylation domain	Starling et al. 1995; Hunter et al. 1999a,b

For details, see text and Ernst et al. 2010.

of annular lipids. In addition to the previous statement, lipid head groups are also stabilized by multiple interactions within the protein (Lange et al. 2001). Nonpolar interactions between the hydrophobic lipid acyl chains and the transmembrane domain stabilize the binding (Luecke et al. 1999; Lange et al. 2001).

### NONANNULAR PROTEIN–LIPID INTERACTIONS

#### Lipids as Structural Building Blocks and Allosteric Effectors of Membrane Proteins

Most of the nonannular protein–lipid interactions have been identified by X-ray-crystallographic approaches (see the following examples).

Frequently, as identified in the vacuolar-type (V-type) sodium ATPase of *Enterococcus hirae* (Murata et al. 2005), lipids that reside within oligomeric membrane protein assemblies act as “molecular glue,” strengthening the contacts of the subunits. Another example, in which lipids were identified as structural building blocks of protein assemblies, are caveolae. Caveolae and caveolae-like domains are found at plasma membranes of higher eukaryotes and are morphologically heterogeneous (Parton et al. 1994; Scherer et al. 1997). They have been suggested to play crucial roles in signal transduction acting as signaling platforms. Today, the role of caveolin in signaling is far from being completely resolved because in cells lacking

caveolin the distribution of signaling proteins is not affected (Parton and Simons 2007). The major caveolae proteins are members of the caveolin protein family. Caveolae show a unique lipid composition, predominantly consisting of cholesterol, sphingomyelin, and the ganglioside GM1 (Smart et al. 1995; Schnitzer et al. 1996). Regulation of caveolin-1 expression was shown to be tightly connected to cellular cholesterol levels (Bist et al. 1997). Caveolin-1 binds to cholesterol with a 1:1 stoichiometry, resulting in a complex that even resists treatment with SDS (Murata et al. 1995). Cholesterol is able to promote the oligomerization of caveolin-1 subunits in microsomes (Monier et al. 1995), underlining its role as a structural building block of caveolae. Another example for a nonannular lipid-binding site is found in KcsA, a potassium channel of *Streptomyces lividans*. This tetrameric complex selects for anionic phospholipids in its core, in which they strongly influence the ability of the protein complex to conduct potassium (Valiyaveetil et al. 2002). Li and Papadopoulos identified a cholesterol-binding motif in the peripheral-type benzodiazepine receptor (PBR) (Li and Papadopoulos 1998), a motif also found in caveolin-1. This consensus motif consists of hydrophobic, aromatic and positively charged amino acids: L/V-(X)<sub>1-5</sub>-Y-(X)<sub>1-5</sub>-R/K (cholesterol recognition/interaction amino acid consensus [CRAC]).

A similar motif was found responsible for the tight interaction of nonannular cholesterol molecules within the  $\beta$ 2-adrenergic receptor (Hanson et al. 2008), a seven transmembrane-containing G-protein-coupled receptor (GPCR). Here two cholesterol molecules are bound in a shallow surface groove, with four out of the seven TMDs contributing to this lipid-binding structure. Strikingly, a relaxed version of this motif, W/Y-(X)<sub>1-3</sub>-I/V/L-(X)<sub>1-7</sub>-K/R, termed the cholesterol-consensus motif (CCM), was identified in a large number of GPCRs. The precise function of cholesterol as a nonannular lipid in these receptors is controversial, however. Two models have been suggested how the presence of the lipid could modulate the receptor's structure and function: (1) directly, via a conformational change following interaction with cholesterol,

and (2) indirectly, because of an alteration of membrane biophysical properties (Gimpl et al. 2002a,b). Either one (or a combination of both) might mediate targeting of these receptors to distinct membrane domains and/or modulate their affinity for ligands. Other GPCRs, such as rhodopsin, a photoreceptor present in retinal rod cells, are modulated by cholesterol as well. The ordering effect of cholesterol on acyl chains of lipids adjacent to transmembrane domains was shown to affect the formation of metarhodopsin, an intermediate state that is in equilibrium with the light-activated receptor (Mitchell et al. 1990; Bennett and Mitchell 2008). In addition to their role as "nonannular" structural building blocks within GPCRs, sterols are discussed to function as allosteric effectors. For example, Pucadyil and Chattopadhyay showed for the hippocampal serotonin1A receptor (another GPCR with a CCM) that ligand-binding affinity and G-protein coupling was affected by cholesterol (Pucadyil and Chattopadhyay 2004). This was also observed for other GPCRs, for example, the metabotropic glutamate receptor of *Drosophila melanogaster* (DMGluRA) (Eroglu et al. 2003) and the oxytocin receptor (Gimpl et al. 2002b), in which the presence of sterols was shown to shift these receptors to a high-affinity agonist-binding state. In the case of DMGluRa, this protein-lipid interaction was further shown to target the receptor to sterol-rich microdomains.

In addition, other lipid classes were reported to regulate as nonannular lipids enzymatic functions of membrane proteins, such as diacylglycerol that activates the Ca<sup>2+</sup>-ATPase (PMCA) (Perez-Gordones et al. 2009). In summary, these and other examples (see Tables 1 and 2) emphasize the importance of nonannular lipids within membrane protein complexes in modulating their architecture (structure/conformation), localization (targeting to distinct membrane domains), and enzymatic functions.

### ROLE OF LIPIDS AS CHAPERONES

It has been postulated that protein-lipid interactions can be critical for correct insertion, folding, and topology of membrane proteins



in a way similar to protein chaperones (van Klompenburg et al. 1997; Dowhan and Bogdanov 2009). Here, lipids assuming protein chaperone-like functions are defined as lipochaperones. This term must not be confused with the expression lipid chaperones, defining proteins that keep soluble or transport individual lipid molecules, such as lipid transport proteins (Hanada et al. 2003; Ile et al. 2006; Wirtz 2006) and fatty acid binding proteins (Storch and McDermott 2009). As a prominent lipochaperone function, membrane lipids can rule the topology of membrane proteins. X-ray data show that the majority of lipids tightly bound to membrane proteins via head groups are localized in the electronegative side of the membrane. In addition to sequential protein–protein interactions within the translocon, specific interactions between negatively charged phospholipids and positively charged amino acids help guiding membrane protein orientation (von Heijne 2006; Rapoport 2007). This is in agreement with the asymmetric lipid distribution of biological membranes, in which anionic lipids are localized in the cytoplasmic leaflet. This preferential localization is consistent with the “positive-inside” rule described previously (von Heijne and Gavel 1988; von Heijne 1989), in which membrane proteins facing the negative side of the membrane are generally enriched in arginine and lysine residues. For example, addition or removal of positively charged amino acids in *E. coli* leader peptidase Lep completely changed the membrane proteins’ orientation, which is influenced by the extent of anionic lipids (van Klompenburg et al. 1997). In the case of the secondary transporter LacY (lactose permease) it has been shown that PE is necessary for its cellular function, assembly, and folding (Bogdanov and Dowhan 1998, 1999; Bogdanov et al. 1999). This zwitterionic phospholipid is not required for membrane insertion because LacY, a 12 TMD protein, can be inserted in *E. coli* cells lacking PE, but its active transport function is disturbed. This lack of activity in the absence of PE is because of a different membrane topological organization. Under these conditions, some transmembrane domains and hydrophilic

domains are topologically inverted with respect to the bilayer. However, addition of PE after membrane insertion restores the topological orientation, facilitates the LacY structural maturation, and reestablishes its transport activity. Therefore, PE seems to play an active role in controlling membrane protein topology and assembly (Bogdanov et al. 2002). Further indication that the topological organization of lacY is regulated by the membrane phospholipid composition comes from reconstitution of lacY in liposomes of different lipid composition, in which only in presence of PE active transport of lactose by LacY was achieved. Interestingly, in liposomes containing another zwitterionic lipid (PC), the membrane topology of the C6 domain was the same as that observed in presence of PE, however, no active transport was detected. In these in vitro studies, the presence of zwitterionic lipids is sufficient to drive and facilitate membrane topology. In addition, other properties of PE are required to sustain active transport (Wang et al. 2002). One important difference between PC and PE is that the latter can form hydrogen bonds or exchange protons, which will help the cotransport of a proton along with lactose to couple substrate uptake with the proton electrochemical potential across the bilayer. Likewise, a requirement of PE for proper topological organization and function has been described for other secondary transporters (e.g., phenylalanine permease [PheP] or  $\gamma$ -aminobutyric acid [GABA]) (Zhang et al. 2003, 2005).

The number of diseases related to lipids that interfere with protein folding is growing over the years (Kuznetsov and Nigam 1998). In Alzheimer’s disease the specific molecular initiator of the disease is still unresolved. However, biochemical studies indicate that soluble amyloid- $\beta$  ( $A\beta$ ) oligomers, mainly dimers, are the key intermediates in the manifestation of the disease (for a recent review see Li et al. 2010). The formation of  $A\beta$  aggregates in the brain of patients with Alzheimer disease correlates with a specific peptide–lipid interaction. Several studies showed that  $A\beta$  peptides strongly interact with the monosialoganglioside GM1 (Yanagisawa et al. 1995; Wakabayashi et al. 2005).

A $\beta$  specifically recognizes GM1 clusters at the cell surface, and following GM1-A $\beta$  interaction, A $\beta$  undergoes a conformational change from a  $\alpha$ -helix-rich structure to a  $\beta$ -sheet-rich structure and serve as a seed for A $\beta$  fibrillogenesis (Matsuzaki et al. 2010). In this case, GM1 functions as an “anti”-lipochaperone activity in facilitating aggregation of A $\beta$  peptides.

Expanding the term lipochaperones beyond roles in the folding and topology of transmembrane proteins, lipids might assist the transport of proteins to their target membrane. Within its transport pathway a newly synthesized or cycling TMD is faced with membranes composed of varying lipid classes and molecular species. These compositions determine the physicochemical characteristics of a membrane as well as its bilayer thickness. As a consequence, a given TMD may be confronted with unfavorable membrane environments, mainly because of a lack of hydrophobic match. Specific binding of lipid molecular species to a transmembrane domain can minimize such hydrophobic mismatch (Hite et al. 2010), which will decrease the corresponding energy penalty. Varying local energy states of a TMD, because of locally different lipid compositions, would then allow efficient protein sorting. In eukaryotic cells, transmembrane domains of plasma membrane proteins in the average are five amino acids longer than proteins localized to the Golgi (Bretscher and Munro 1993; Sharpe et al. 2010). Thus, plasma membrane proteins that travel within the Golgi have TMDs too long to be accommodated by the membrane, and therefore their helices will either tilt within the bilayer, or their annular lipids expand to increase hydrophobic thickness. In both cases bilayer stress will be increased. Such unfavorable situation could be ameliorated by lipid molecular species tightly bound to the transmembrane domain to reduce the hydrophobic mismatch. Based on the fact that each organelle has a specific lipid composition, a state of minimal energy will contribute to a preferential distribution of the membrane protein to its target membrane.

Additional functions can be envisaged for interactions between specific lipid molecular species and TMDs. Employing novel methods

to analyze protein–lipid interactions, we have observed highly specific protein–lipid molecular species interactions that regulate the oligomerization of TMDs (F-X Contreras, F Wieland, and B Brügger, unpubl.). Furthermore, binding of specific lipids to a membrane receptor can regulate its activity directly as allosteric effectors (as outlined above), or by targeting to specific membrane domains, or both. Likewise, a membrane protein may be kept in its resting state by interaction with a specific lipid molecular species, therefore decreasing unspecific interactions with other proteins. Following ligand association, a conformational change will release the lipid, leading to the association of the protein with other proteins to form an active complex (U Coskun, M Grzybek, and K Simons, pers. commun.).

In summary, such mechanisms involving specific intramembrane protein–lipid interactions are reminiscent of chaperone functions.

## OPEN PROBLEMS

A major obstacle for rapid progress in our understanding of the physiology of protein–lipid interactions is the lack of molecular and cell biology-based tools to probe interactions at the level of lipid molecular species specificity. Unlike analyses of protein function by targeted knockdown or knockout of individual proteins, or introduction of mutations, no equivalent methods exist to manipulate lipid molecular species individually in live cells. Although qualitative and quantitative lipid analysis has seen rapid progress (Brügger et al. 1997; Shevchenko and Simons 2010), mass spectrometric analysis on the protein side, to determine TMDs or derivatives thereof, is still challenging, hampering the characterization of TMD–lipid complexes. The fundamental question is still open whether protein TMDs exist that are simply dissolved in the hydrophobic phase of the membrane, with no specificity for any lipid whatsoever, or if there is some specificity in each TMD for one or more of the manifold lipid species that make up a membrane. A systems biology approach may help to answer such questions: To this end, a high throughput system

would be needed to screen all physiological TMDs for their interactions with a comprehensive set of membrane lipid building blocks. Technically, such a screen may be assisted by the availability for read out of fluorescent detection methods as outlined above.

Protein–lipid interactions and their physiological relevance have now found the interest of a wide community of molecular cell biologists, chemists, physicists, and pharmacologists, and synergy derived from their combined activities will further help spurring this field in the molecular life sciences.

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