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Review

Determinants of specificity at the protein–lipid interface in membranes

Andreas M. Ernst *, F. Xabier Contreras, Britta Brügger, Felix Wieland *

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

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ABSTRACT

The complexity of pro- and eukaryotic lipidomes is increasingly appreciated mainly owing to the advance of mass spectrometric methods. Biophysical approaches have revealed that the large number of lipid classes and molecular species detected have implications for the self-organizing potential of biological membranes, resulting in the formation of lateral heterogeneous phases. How membrane proteins are able to adapt specifically to their surrounding heterogeneous matrix, and whether this environment affects protein targeting and function, is therefore a matter of particular interest. Here, we review specific protein–lipid interactions, focusing on the molecular mechanisms that determine specificity at the protein–lipid interface, and on membrane proteins that require lipids as cofactors for their architecture and function.

1. Introduction

The high variety of lipid classes and species that make up biological membranes is receiving increasing attention, yet is far from being characterized in such detail as the functions of protein–protein interactions. The lipidome is complex, with thousands of different lipid classes and species [1], asymmetrically distributed within the bilayer. This complexity is reflected by the various tasks biological membranes have to fulfil, such as their function as a physical barrier of the cell and controlling the traffic in and out of cells and cellular compartments. These endomembrane systems mould distinct biochemical reaction centres within the cell, notably displaying distinct lipid compositions. In addition to their role as structural components of cellular membranes, membrane lipids also participate in signalling pathways.

Lipids display a rather simple architecture, being hydrophobic or amphipathic molecules, which are made from two types of basic building blocks: fatty acyl (phosphoglycero- and sphingolipids) and isoprene groups (sterols) [2,3].

2. Self-organization potential of membrane lipids

The way membranes were viewed was significantly influenced by the introduction of the fluid-mosaic model by Singer and Nicolson [4] that assumed an overall fluidity with random distribution of molecular components within the membrane, a strong simplification of actual biophysical membrane properties, as we are aware today. Only in the last decade, the presence of lateral heterogeneities was emphasized by the membrane *raft* hypothesis (for review see [5]). Membrane *rafts* were found to be highly complex domains that are characterized by a significant reduction in the lateral mobility of its composing lipids, forming a liquid ordered (l_o) phase, which is able to compartmentalize processes within the bilayer. They are dominated by the presence of two distinct lipid classes that are (together with a distinct set of membrane proteins) held responsible for the self-organization potential of membrane microdomains: sterols and sphingolipids.

Molecular distinctions between glycerophospholipids and sphingolipids become apparent when comparing phosphatidylcholine (PC) and sphingomyelin (SM). Despite their overall high degree of resemblance, the presence of the hydroxyl- and amide-moieties within SM are responsible for both a higher degree of acyl chain ordering and the pronunciation of intra- and intermolecular hydrogen bonds [6,7]. These structural characteristics of SM have substantial effects on its interactions with both sterols and membrane proteins [8].

Other types of sphingolipids, glycosphingolipids, are crucial for the self-organization potential of membrane *rafts* as well, in particular for the asymmetric distribution of lipids across the exoplasmic and cytoplasmic leaflets of the membrane and the recruitment of specific proteins to these platforms. Glycosphingolipids are characterized by a pronounced cone-shape, which is based on a large mismatch in the volume of the bulk and polar carbohydrate head groups, and the presence of long and saturated amide-linked fatty acids in their hydrophobic moieties. By providing additional weak interactions between the carbohydrate moieties, the sphingolipid network is further strengthened (for review see [5]).

Sphingolipids, however, require the presence of another lipid class in order to be able to phase-separate to a l_o phase. These other

* Corresponding authors.
E-mail address: felix.wieland@bzh.uni-heidelberg.de (F. Wieland).

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crucial players are sterol lipids, which are characterized by a rigid and hydrophobic ring structure, a flexible short acyl tail and a small polar hydroxyl moiety (head group). The most prominent sterols are cholesterol (Chol) in mammals and ergosterol (Ergo) in *fungi*, the latter differing from Chol by an additional methyl group in the ring structures, and additional double bonds in ring B and in the short tail. There are two distinct "faces" present in the ring structure of cholesterol, the "smooth" (steran) α-side and the "rough" β-side bearing the methyl groups. An important feature of sterols is their ability to alter physical properties of a membrane [9]. In a fluid membrane phase, cholesterol is able to induce local ordering and an increase in packing density of acyl chains. In addition, cholesterol is able to fluidize lipids in a gel phase and therefore acts as a universal regulator of membrane fluidity in cellular membranes. A significant reduction in membrane permeability for small solutes in presence of Chol in the bilayer is an additional important effect [10]. Chol is able to fit in between the hydrophobic moieties of other lipids, thus leading to a reduction of the free volume in the core of the bilayer. The α-side is likely to favour contacts with saturated acyl chains, whereas the rough β-side favours van der Waals contacts with unsaturated acyl chains [8]. Concerning the interaction of Chol with SM within lipid *rafts*, it is notable that molecular dynamics (MD) simulations revealed that no direct hydrogen-bonding between the two molecules occurs [8]. The molecular mechanism of the interaction between Chol and SM apparently has three main features: (i) van der Waals contacts between the sterol ring system of Chol and the amide-linked fatty acid of SM, (ii) an altered pattern of intramolecular hydrogen-bonding within SM, (iii) an electrostatic attraction between the polar oxygen of the hydroxyl moiety of Chol and the charged nitrogen of the choline head group of SM. This leads to an angular tilt of the choline head group of SM and an orientation of Chol perpendicular towards the bilayer plane. The latter effect also leads to the abolishment of hydrogen bonds of Chol to polar molecules in the interfacial region ("umbrella effect"; [8,11]), which is suspected to be the most important factor in the formation of SM–Chol networks, and responsible for the transition from a fluid to a gel phase. Interestingly, the angular tilt of Chol with regard to the bilayer plane appears to be a sensitive indicator for the local phase of the membrane [8], since it correlates with local acyl chain ordering. It is highly likely that even in liquid disordered (l_d) phases the principles of self-organization by the molecular distinctions of sphingolipids apply, as recent data indicate the presence of distinct SM-subdomains of defined molecular species in l_d phases (Ernst and Contreras et al., unpublished). This was predicted by the introduction of the term "critical domain concentration (CDC)" with regard to sphingolipids ([12]; reminiscent of the term CMC for the ability of detergents to produce micelles). A concentration dependent self-organization potential of sphingolipids and sterols in a local lipid environment and an exclusively lipid-dependent nucleation process of l_o phases can therefore be envisaged. However, there is only few experimental data available in vitro confirming the presence of such distinct phases. In vivo approaches only indirectly suggest the existence of separate phases, such as altered lateral diffusion coefficients of membrane proteins [13]. Therefore, important biophysical characteristics of membrane *rafts*, such as their lifetime, dynamic size, and exact lipid composition are still being investigated. Evidence for the coexistence of lateral heterogeneities in biological membranes due to self-organization principles prompted the need to revise the fluid-mosaic model. The local environment of membrane proteins is rather a defined network of distinct structures, as described by the title "Membranes are more mosaic than fluid" by Engelman [14]. This would further imply that membrane proteins must select either "passively" for distinct microenvironments by hydrophobic matching [15] or "actively" by specific interaction with membrane lipids.

3. The protein–lipid interface

In the current review, a selection of reports will be presented that suggest binding selectivity for lipids of proteins in biological membranes. The bulk of the lipid molecules in biological membranes acts as "solvent" for integral membrane proteins and do not display any specific interaction with hydrophobic surfaces of proteins. Others form a shell or annulus of distinct lipid classes and species around the protein, which display the highest degree of hydrophobic "compatibility". These lipids are referred to as "annular lipids" [16] as opposed to the "solvent" or bulk lipids. It is established that exchange between bulk and annular lipids is in general fast [17–19]. Interactions with the annular shell can therefore be non-specific, as expected for hydrophobic matching of a protein with solvent lipids, or specific in terms of a selection of the protein for distinct lipid classes and molecular species in its inner lipid shell.

In another type of membrane protein interaction with lipids, functional complexes are formed. Here, the lipids are neither bulk nor annular lipids but rather (internal) structural components of these complexes required for membrane protein function. These so-called non-annular lipids were identified in various crystal structures of membrane proteins, of which a selection will be discussed below.

Further, interaction of soluble proteins with lipids will be discussed. A variety of different protein motifs are known to be involved in membrane targeting and binding by the rather well described recognition of the polar head group moieties of lipids via C2, PDZ, PH domains and others. This targeting of distinct proteins to specific endomembranes is of great functional importance in cellular signalling processes. Other binding domains appear not to be specific for lipid head groups, yet enable the firm binding of proteins by exposable hydrophobic anchors (such as viral fusion peptides and SH4-domains) to membranes [20–22]. There are a few examples of soluble proteins, however, which appear to exceed the specificity for recognizing polar head groups by simple electrostatic attraction that will be discussed.

4. Specific interactions of membrane proteins with annular lipids

The 1.55 Å structure of bacteriorhodopsin, a light-driven ion pump, including its annular lipids was determined by X-ray diffraction of crystals grown in cubic lipid phase [23]. A bilayer of 18 tightly bound lipid chains was found to form an annulus around the protein (Fig. 1), and interestingly, lipids were found to exclusively mediate the contacts within the trimeric complex in the bilayer plane. Among those lipids, defined lipid classes such as phosphatidyl glycerophosphate methyl ester, glycolipid sulfates, phosphatidyl glycerol, phosphatidyl glycerolsulfates and a molecule of squalene (SQU) were identified. The latter was associated to the protein surface by a complementary groove, formed by three leucine and three valine residues (Leu19, Leu22, Val210, Val213, Val217 and Leu221). SQU, which is present in equimolar amounts to bacteriorhodopsin, strongly affects the reprotonation of the retinal Schiff base (Asp96) during the photocycle. The lipids forming the annulus around the trimeric complex are tightly bound by van der Waals-London forces in grooves formed by specific arrangements of amino acid side-chains on the hydrophobic protein surface. It could not be excluded, however, that the annular lipids were also fixed at the protein–lipid interface by interactions with their polar head group moieties. It is remarkable that a crystal structure of a membrane protein could actually "trap" the inner lipid shell, implying intimate and in total strong contacts at the interface. In contrast to bacteriorhodopsin, rhodopsin appears to

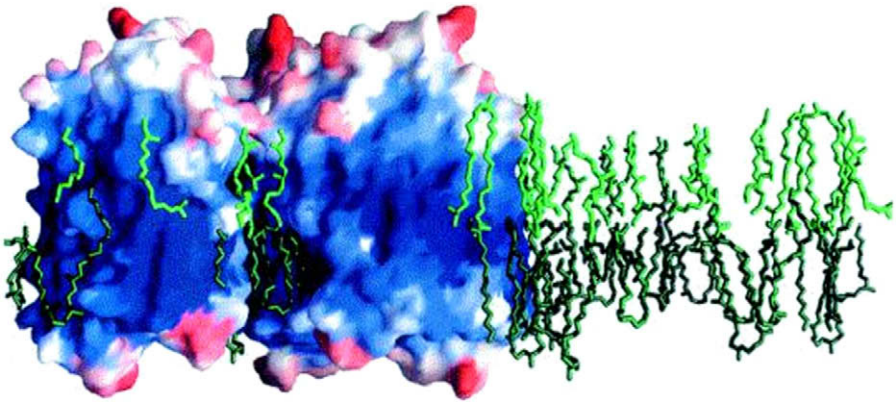

Fig. 1. Crystal structure of bacteriorhodopsin (PDB file 1C3W). Colouring of the protein according to temperature factors (blue: lower; red: higher). Annular lipids are arranged in a bilayer and form a perfect shell around the protein (ordered lipid tails are depicted in green) [23].

be a different case. Rhodopsin was extensively studied by NMR approaches using spin-labelled sterols and phospholipids [24]. An increasingly immobilized first shell of boundary lipids was detected, however, despite the fact that the mobility of this inner shell was significantly reduced compared to the bulk lipids, there was no evidence for specific and strong interactions at the protein–lipid interface and selectivity for molecular lipid species.

In order to test for potential structural responses to the hydrophobic thickness of the lipid bilayer surrounding a membrane protein, the potassium channel KcsA from *Streptomyces lividans* has been analyzed in vitro [25]. The theory of hydrophobic mismatch predicts that the boundary lipids of a membrane protein can compensate for a hydrophobic mismatch by stretching their hydrocarbon chains to match the hydrophobic protein surface. The relative binding constants of lipids to a membrane protein can be determined by Förster resonance energy transfer (FRET)-based approaches. The protein of interest is reconstituted into bilayers containing mixtures of non-brominated lipid and the corresponding brominated lipid, and comparisons of relative fluorescence intensities by the equations of Dewey and Hammes [26] and Koppel et al. [27] allow the deduction of relative lipid binding constants. Since no significant changes in lipid binding constants were detected for increasing chain lengths from C10 to C24, it was concluded that the hydrophobic surface of KcsA had to be distorted to match to that of the surrounding lipids, not vice versa. As observed for bacteriorhodopsin, Valiyaveetil et al. [28] additionally identified a non-annular phospholipid molecule bound within the tetrameric KcsA complex. Although the head group of this particular lipid could not be resolved, it was shown that KcsA function depends on the presence of negatively charged phospholipids.

Extreme examples of the impact of annular lipids on membrane protein function are mechanosensitive channels, which are regulated by alterations in their local lipid environment. As studied both by patch clamp and spectroscopic EPR analyses of the channel, they can be shifted from an open to a closed state by alterations in the lateral pressure profiles of the surrounding lipid bilayer, which is in turn influenced by asymmetries at the lipid–protein interface and between the leaflets [29]. In PC bilayers, the channels remain in a closed state. As soon as "cone-shaped", non-bilayer forming lysophospholipids were added to the lipid mixture, the activation thresholds for both prokaryotic (MscL) and eukaryotic (TREK-1, TRAAK) mechanosensitive channels were drastically lowered and finally shifted to an open state. Hence it was speculated that the release of intra-bilayer lateral pressure is the consequence of the structural inequality between cylindrical, bilayer forming lipids such as PC and conical, micelle-forming lysophospholipids. This mechanism is suggested to provide the mechanical force to shift the channel to an open conformation. The peripheral antenna complex LHII of *Rhodobacter sphaeroides* is another example for membrane proteins that specifically select for distinct phospholipids in their annular shell [30]. A high enrichment of phosphatidyl ethanolamine (PE) in the boundary lipid phase of LHII was detected, and sequence alignments confirmed the presence of a putative spectrin-like PE-binding site (IAEWKDGL). Hence, mutational analysis of the putative PE-binding domain was performed. Mutation of a critical glutamate residue (E20) to alanine leads to a loss of specific enrichment of PE and a gain of PC in the boundary lipids of the complex [31].

Another example for annular lipid selectivity is OmpF, a homotrimeric outer membrane porin of *Escherichia coli*. As described above, relative lipid binding constants were determined for reconstituted OmpF [32]. The highest lipid binding constant was found for di(14:1)PC, whereas shorter or longer fatty acids interact significantly less specific. In contrast to this apparent selectivity for molecular lipid species, OmpF did not display a significant selectivity for PE over PC, indicating a lack of lipid class specificity. In contrast to KcsA, however, the findings suggest a selection for distinct boundary lipids to compensate for hydrophobic mismatch at the protein–lipid interface (and not a structural rearrangement by the protein). Additionally, the authors were able to deduce the relative architecture of the homotrimeric OmpF complex.

Another interesting example of the effect of annular lipids on membrane protein function, in particular their effect on folding, is the peptide antibiotic gramicidine (gA; *Bacillus brevis*). Gramicidine peptides associate to form potassium-conducting channels in membranes, leading to cell death. These channels are formed when gA molecules associate by interaction of single-stranded $\beta^{6.3}$-helical monomers [33]. They can, however, adopt a double-stranded helical dimer form, impermeable for potassium. If the hydrophobic length of the surrounding acyl chains is not compatible with the length of the gA monomer [34], a loss of potassium conductance is observed. This simple model stresses the overall importance and magnitude of membrane deformation energy as a consequence of hydrophobic mismatch on the structure and function of a membrane protein.

The selectivity of membrane ATPases has been addressed in various reports as well. In the case of the Na,K-ATPase, spin-labelled lipids were utilized to test for selectivity at the protein–lipid interface, with the result of a clear specificity for cardiolipin [35]. In the case of the sarcoplasmatic Ca-ATPase, Starling el al. [36] analyzed the kinetics of the purified protein reconstituted into bilayers of varying PC molecular species. They found differences in the rate of dephosphorylation of the ATPase in response to the thickness of the bilayer, with the slowest rate found in a di(C24:1)PC bilayer.

Here, the rate of dephosphorylation was slow enough to be rate-limiting for ATP hydrolysis. In a study by East and Lee [37], however, there were no significant differences detectable in the relative lipid binding constants for different molecular species. Interestingly, this again suggests conformational changes by the protein in its attempt to adapt to different bilayer thicknesses to achieve hydrophobic matching. This protein-based adaptation opposes the effects observed for the bacterial porin OmpF and could account for the altered dephosphorylation–phosphorylation kinetics observed. There are also reports available on protein–lipid interactions of the Ca-ATPase of the plasma membrane (PMCA), which additionally point to a role of both ceramide (Cer) and diacylglycerol (DAG) as potent allosteric effectors [38].

5. Roles of non-annular lipids in protein function

Resolutions of crystal structures of proteins are often not sufficient to resolve lipid structures, in particular the positions of unsaturated bonds and acyl chain lengths of the hydrophobic moieties. In crystalline bovine heart cytochrome *c* oxidase (CcO), at 1.8 Å resolution 13 lipids were found specifically bound to the complex [39]. The fatty acyl compositions of the lipids at the inner mitochondrial membrane were analyzed by mass spectrometry. Interestingly, the structure of the CcO indicates that most of these lipids have a role in the overall protein architecture. The major mechanism of protein–lipid interaction for CcO is via specific interactions with head group moieties for all 13 lipids detected. The polar head groups are tightly fixed by many protein-derived hydrogen bonds. Notably, the hydrophobic moieties of the bound lipids appeared to be fixed to the surface of the protein as well, giving rise to clear electron density maps, however, without implying selectivity for a particular molecular species. In subunit III, two PG molecules were found in close proximity that apparently moulded a section of the oxygen transfer pathway with their fatty acyl tails. In silico, an alteration of the fatty acid species led to blockage of the oxygen channel (by replacing the palmitate in PG-1 for a stearate), implying that this particular section of the complex selects for the fatty acyl chain length. Additionally, Shinzawa-Itoh et al. describe an influence of a particular set of four non-annular phospholipids in mediating the homodimerization of the CcO-complex: two PE, one CL and a PG molecule.

The cytochrome bc_1 complex (QCR), another essential component of the respiratory and photosynthetic electron transfer chains, catalyzes electron transfer between ubiquinol and cytochrome *c*, a process coupled to the translocation of protons across the inner mitochondrial membrane [40,44]. It was shown that the enzymatic activity of QCR (and consequently the mitochondrial membrane potential) strongly depends on the presence of the phospholipid cardiolipin (CL): enzymatic digestion of its tightly bound phospholipids resulted in an inactivation of the cytochrome bc_1 complex, and addition of CL resulted in a reactivation of its enzymatic activity [41]. A total of five phospholipid molecules could be identified in the 2.3 Å resolution X-ray structure of yeast QCR [42]: two molecules of PE, one PC, one phosphatidylinositol (PI), and a CL molecule. As for the CcO, distinct amino acids seem to be involved in the firm attachment of the polar lipid head group moieties to the complex by a combination of specific hydrogen-bonding and ionic pairing. The PI molecule appeared to be essential for the architecture of the complex and its oligomeric state [43]. PI was specifically bound by four out of five transmembrane domains of the complex in an interhelical position, a hydrophobic cleft formed at the interface of the dimer. The CL molecule, essential for enzymatic activity of QCR, specifically interacts with the complex via hydrogen bonds to its two individual phophodiester head groups: one phosphodiester group interacts directly with a lysyl- and a tyrosyl-residue, and the other interacts with a second tyrosine and, via a water molecule, with a second lysine. One tryptophan residue apparently has a special role in the architecture of the complex. It is located between the bound CL and PE molecules, close to the ester carbonyls of these phospholipids. The tryptophane's indole ring is oriented in parallel to the dimer axis, resulting in a lamellar separation and restriction of lateral mobility of CL and PE, and stabilization of their binding to the protein [43].

In various reports, a dependence of the activity of membrane receptors is described on specific interactions with lipids, in particular sterol lipids (reviewed in [45]). In the oxytocin receptor, an allosteric modulation by cholesterol induces a shift to a high-affinity agonist binding state [46]. Additionally, sterols were found to act as positive allosteric regulators of the metabotropic glutamate receptor of *Drosophila melanogaster* (DMGluRA; [47]). In particular, it was shown that ligand binding of DmGluRa reconstituted in liposomes required the presence of ergosterol, which shifted the receptor to a high-affinity state and into sterol-rich microdomains. Importantly, in a recent publication of the 2.8 Å crystalline structure of the β2-adrenergic receptor (a G-protein coupled receptor (GPCR); [48]), two cholesterol molecules were apparently bound in a shallow surface groove formed by the transmembrane

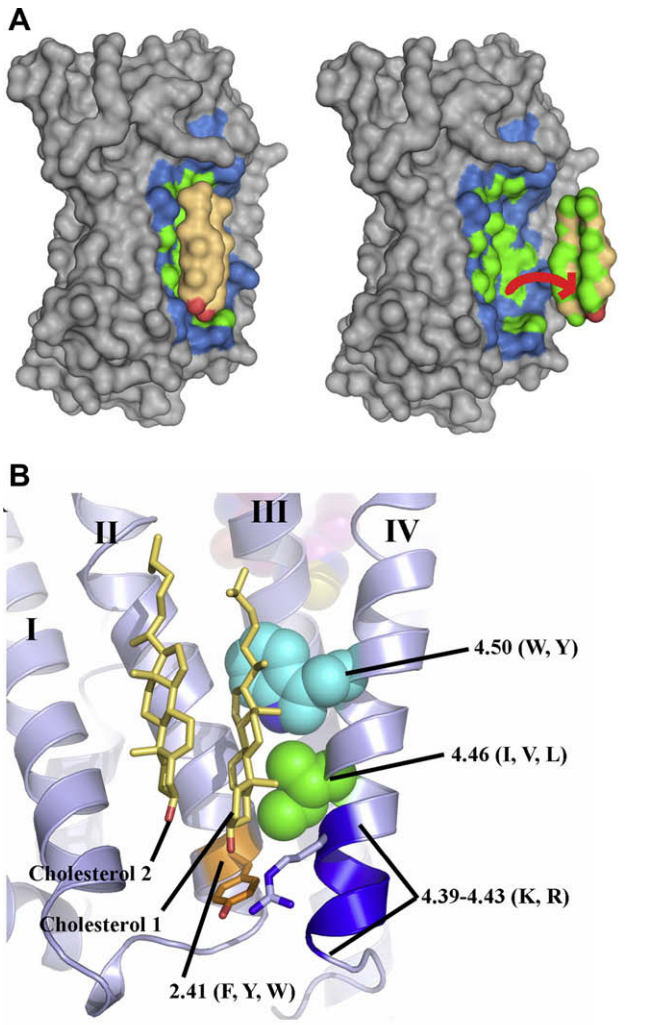

Fig. 2. (A) Molecular surface representation of the beta-adrenergic receptor. Contact surfaces are coloured according to relative distances (green: 4 Å and blue: 4–5 Å). In the right panel, the cholesterol molecules have been lifted out to give a better view of the binding pocket. (B) Critical residues and forming the cholesterol consensus motif (CCM), found in 70% of all class A receptors [48].

domains I–IV of the type III protein (Fig. 2A). Although the groove formed by the four helices accommodates two sterol molecules, one specifically bound by the protein (Chol 1), and the other by Chol 1. Specific interactions with helix II and IV, by four spatially distributed amino acids, form a postulated cholesterol consensus motif (CCM) for the interaction with Chol 1 (see below). These four residues consist of a tryptophan residue (interaction with Chol 1 ring D by a CH-π interaction), an isoleucine residue (van der Waals interaction of this beta-branched amino acid with rings A and B of Chol 1), a tyrosine residue (via van der Waals interactions with Chol 1 ring A), and an arginine residue (electrostatic interaction with the hydroxyl group of Chol 1). A screen of all class A receptors for the CCM resulted in the signature pattern presented in Fig. 2B that is conserved in over 70% of the GPCR superfamily.

Additional examples for specific interactions of membrane proteins with sterols can be found in the literature. Mutational analysis of the peripheral-type benzodiazepine receptor (PBR; [49]) lead to the identification of an amino acid consensus pattern responsible for the specific interaction with cholesterol: V/L X_{1-5} Y X_{1-5} K/R (X = any amino acid). The motif was also detected in other membrane proteins, such as caveolin-1 [50]. Notably, the two signatures defined by two groups in two different proteins are highly similar [48,49].

Various reports emphasize a role of non-annular lipids in regulating the oligomeric state of membrane proteins. The crystalline bovine mitochondrial ADP/ATP carrier, e.g., indicates a homodimerization mediated by protein–lipid interactions. Two cardiolipins were found at the dimerization interface, stabilizing the dimer by a combination of van der Waals forces and electrostatic interactions of the CLs head groups with a phenylalanine, two lysine and two isoleucine residues on the protein surface [51].

Another example is found in prokaryotes: under anaerobic conditions, the facultative anaerobe *E. coli* synthesizes quinol-nitrate oxidoreductase (nitrate reductase A) in the presence of nitrate, leading to the reduction of nitrate to nitrite and the generation of a proton-motive force. The 1.9 Å crystal structure of nitrate reductase A reveals the presence of a PG molecule within the heterotrimeric complex [52]. The polar head group of PG is specifically bound by electrostatic interactions with two arginines and a lysyl residue, underlining a function of non-annular lipids that act as structural building blocks of protein complexes. These building blocks can be simplified as "lipid bridges" that contribute unique features to protein folding and assembly, as also identified in the vacuolar-type (V-type) sodium ion-pumping ATPase from *Enterococcus hirae*. The pump is a decameric assembly, with each monomer contributing four transmembrane domains. A sodium ion is bound between two of these helices, trapped by a critical glutamate residue. In the crystal structure of the Na-ATPase at 2.1 Å resolution [53], electron densities at the inner surface of the complex were interpreted as 10 molecules dipalmitoyl-phophatidylglycerol (DPPG) and 10 1,2-dipalmitoyl-glycerol (DPG) units. The polar head group of each DPPG appeared to be specifically bound via electrostatic interaction to a lysine residue, and its hydrophobic moiety by a set of five amino acids: a leucine, a threonine and three phenylalanine residues. Each DPG interacts with the complex via two methionine, a phenylalanine and four leucine residues. The lipid shell within the complex most likely contributes to the increased packaging of the monomeric subunits [53].

6. Highly specific protein–lipid interactions of soluble proteins

This review deals with protein–lipid interactions of integral membrane proteins. Therefore, interactions of soluble proteins based on mere recognition of a head group are not discussed. There are soluble proteins however, with specificity above detecting the charges in the polar head group of a lipid. A very interesting report stresses the role of "quasi-non-annular lipids" in functioning as allosteric regulators of soluble proteins.

Differentiation and function of endocrine glands is regulated by the constitutively active orphan nuclear receptor steroidogenic factor 1 (SF-1). Surprisingly, in a 1.5 Å crystal structure of the SF-1 ligand binding domain (in complex with an LXXLL motif from a co-regulator protein), the presence of a phospholipid ligand in a large pocket was identified [54]. Apparently, the phospholipid is able to modulate SF-1 interactions with co-activators, and fits in the canonical active conformation of the receptor. The SF-1 pocket is vastly hydrophobic and contains two small hydrophilic patches of amino acids. The first patch moulds the entrance to the pocket and is formed by residues Y437, K441, E446 and Q340. The other polar patch of amino acids consists of H311, R314 and the backbone amide of V327. The latter residues are conserved in other nuclear receptors (RXR, RAR and TR), and mediate the interaction with carboxylate groups of retinoids and thyroid hormones. Due to the high resolution of the crystal structure, the bound phospholipid molecular species could be identified as a phosphatidylethanolamine (C32:1), which was also confirmed by mass spectrometry. The head group of the lipid is oriented with its phosphate to the polar patch of amino acids located at the entry of the pocket. The ethanolamine moiety apparently protrudes out of the pocket and interacts both with the solvent and a glutamate residue at the pocket entry. Strikingly, various interactions of the lipid to the AF-2 helix and other structural elements of SF-1 were observed, which stabilize the receptor in its active conformation. This is very important, since the interaction of co-activator proteins with the AF-2 helices of nuclear receptors leads to the stabilization of the active conformation (ligand-dependent nuclear receptors). In the absence, but also in the presence of PE molecular species with longer fatty acids, the interaction of SF-1 with co-activators was significantly reduced, which is explained by a conformational change to an inactive fold in SF-1 in absence of PE (32:1). Interestingly, the pocket can select for PE molecular species ranging from C12–C18 fatty acid chains. The shape of the pocket explains this promiscuity: it represents an elliptic, extended structure that can accommodate different molecular species. It was emphasized that due to the orientation of the ethanolamine moiety outside the pocket, SF-1 might allow binding of other phospholipids than PI and PC, as well. Mutational analyses of the polar patch of amino acids at the pocket entry (in particular a K441E mutation) further confirmed the strong correlation between phospholipid binding and the transcriptional activity of SF-1. Notably, mutations that lowered the volume of the pocket (A266W, A270W and L348W) and triggered the inactive conformation of SF-1 could be rescued and transformed to an active conformation by addition of lipids with shorter acyl chains. These findings lead the authors to speculate that SF-1 functions as a conventional nuclear receptor, which is able to sense fluxes in phospholipid concentrations, and therefore is not orphan any more. Some target genes of SF-1 encode proteins for sterol biosynthesis and homeostasis. In this way, phospholipids may well help regulating the balance between phospholipids and sterols.

Another example for highly specific interactions of soluble protein with lipid is the specific recognition of a single molecular diacylglycerol (DAG) species by a DAG-kinase in pig testes [55]. Its human homologue, DGK, was shown to be highly selective for arachidonate-containing species of DAG, providing a mechanism by which the cell can inactivate the lipid branch of the phosphoinositide-signalling pathway. Unlike other human isoforms of DAG-kinases, this particular enzyme contains two unique hydrophobic stretches, which are suspected to be involved in its anchoring to membranes [56]. These membrane contact sites imply that molecular distinctions in the hydrophobic moieties of DAGs are distin-

guishable by DGKε, and that this substrate-specificity arises from specific interactions of its hydrophobic stretches with the target lipid, and in particular with the C20:4 arachidonic acid.

A variety of reports also indicate high specificities of soluble proteins for sphingolipids. The V3-loop of the HIV-1 envelope glycoprotein gp120 is a prominent example. It is a sphingolipid-binding domain (SBD; [57]) that mediates adhesion of HIV-1 particles to membrane "rafts" by arginine, tyrosine and phenylalanine residues at the tip of a hairpin-like fold. Sphingolipids apparently induce lateral assembly of the HIV-1 fusion complex and stimulate conformational changes in gp120 that subsequently lead to fusion of the virus with host membranes [58]. There is evidence for interaction of the V3-loop predominantly with galactosylceramide and sphingomyelin [59–62]. Interestingly, a V3-like glycolipid-binding domain could also be identified in both the prion protein (PrP^c) and the Alzheimer β-amyloid peptide (Aβ; [63,64]; Fig. 3). Using the Langmuir film balance technology, a specific interaction with GalCer and SM was confirmed. Additionally, the V3-like domain of PrP bears a mutation site (E200K) that corresponds to the most common familial form of Creutzfeldt–Jakob disease [65]. Although this mutation did not appear to have an effect on the interaction with GalCer, a specific interaction with SM was lost. The sphingolipid-binding domains in PrP, the β-amyloid peptide Aβ and in gp120 of HIV-1 corroborate roles of sphingolipids in the pathogenesis of the respective diseases.

A V3-like loop was also detected on the Shiga-like toxin B and on the pancreatic bile salt-dependent lipase (BSDL; [66]). The latter is speculated to be transiently associated with membrane microdomains in the Golgi compartment up to the *trans*-Golgi network, where it finally, upon completion of *N*- and *O*-glycosylation, is phosphorylated on a threonine residue [67]. In this particular case, the interaction with *raft*-sphingolipids is understood as a mechanism of quality control, preventing unfolded BSDL to be released before completion of it post-translational modifications.

How specific are these SBDs? A few reports suggest lipid-binding domains on soluble proteins that do not seem to involve electrostatic attractions to the polar head groups of sphingolipids. One example is the sphingomyelin-specific, membrane-binding eukaryotic cytolysin equinatoxin II (EqtII; [68]). As shown by different experimental approaches, such as site-directed mutagenesis, surface plasmon resonance spectroscopy, lipid monolayer and liposome permeabilization assays, the toxin displays strict and sole specificity for SM.

In contrast to the above-mentioned SBDs these proteins select for SM and recognition does not include any specific protein-head group interaction. Rather, the recognition motif involves a tryptophan and a tyrosine residue, exposed on a large loop of EqtII and sufficient for the interaction with SM. Other examples for SM-binding specificity of proteins are found in bacteria, e.g. neutral sphingomyelinases of Gram-positive bacteria [69] that contain β-hairpin structures with exposed aromatic residues and a variety of toxins, e.g. the vacuolating cytotoxin (VacA) of *Helicobacter pylori* [70]. SM-binding specificity is also encountered in fungi, e.g. the cytolysin ostreolysin [71], and animals, e.g. the pore-forming toxin lysenin of *Eisenia foetida* [72]. The SBD of the Aβ peptide has recently been employed as a probe, which allows tracking of the movements of sphingolipids at the plasma membrane of neuronal cells [73]. Similar strategies have been employed for lysenin in order to track sphingomyelin specifically, e.g. in immunofluorescence applications (reviewed in [74]). Taken together, the reports strongly suggest a common mechanism for the specific interaction of soluble proteins with sphingolipids: a combination of specific NH–π interactions between the amide nitrogen and an aromatic amino acid, specific NH–π interaction of a second aromate with the conformationally restricted phosphodiester moiety (due to its strong intra-molecular hydrogen-bond), and additional specific hydrogen-bonding between the lipid and the protein molecules. Charged residues in the sphingolipid-binding domains, as present in the V3- and V3-like loops presented above, are therefore more likely to play a role in (additionally) detecting specific types of sphingolipid head groups.

7. Common patterns in the detection of the polar moiety of lipids

Notably, the asymmetry of positively charged amino acids within the bilayer correlates with the asymmetric distribution of anionic phospholipids [75] and the presence of high affinity lipid-

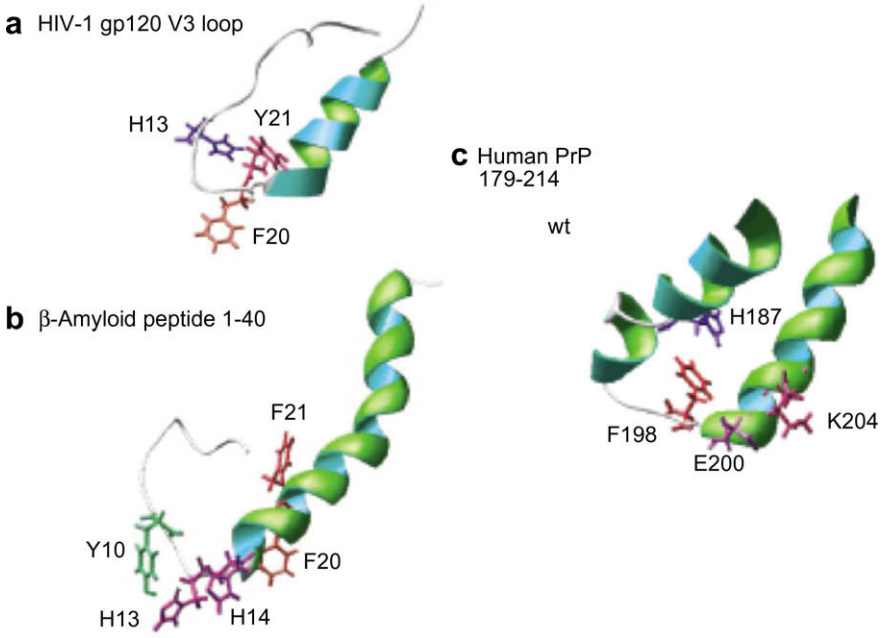

Fig. 3. Sphingolipid binding domains (SBDs; Fantini et al. [57]) of (a) the V3-loop of the gp120 protein of HIV; (b) the amyloid beta peptide; (c) the human prion protein PrP^c [79]. Expert Reviews in Molecular Medicine.

binding sites [76]. On the electronegative side of the membrane (*n* side), a defined subset of amino acids was identified that preferentially interact with phosphodiester moieties, with Arg > Lys > Tyr > His > Trp, Ser, Asn in combination with stabilizing interactions by Thr and Gln [76]. On the electropositive side (*p* side), positively charged amino acids are less frequently observed – Tyr, Thr, Asn, Gln, His and Arg act here as primary ligands. Phosphodiester moieties are often stabilized by a duet of a positively charged and a polar residue: KT, KW, KY, RS, RW, RY, RN, RS, HW and HY [76]. For PC and CL the modes of interaction appear to differ. The positively charged phosphocholine head group is stabilized by residues that are less charged, in fact, an absence of positively charged moieties in close proximity to PC binding sites was observed [76]. In particular, single interactions with His, Thr and Ser or pairs of His/Ser residues were identified. The head group of CL is frequently stabilized by triplets of KKY, RKY and HRN residues, leading to the definition of XXY as a CL-binding-motif (X = positively charged residue; Y = polar residue) [76]. Prediction of potential lipid head group-binding sites is difficult, as these assemblies are non-linear. Additionally, backbone nitrogen and oxygen atoms were found to contribute to specific head-group-binding sites via hydrogen-bonding and hence, the entire contributions at the protein–lipid head group interface might be only resolved in structure-based approaches.

8. Common patterns in the detection of the hydrophobic moiety of lipids

Various crystal structures of membrane proteins discussed above display distinct hydrophobic grooves and cavities with specifically bound lipids. In order to dissect the interactions at the protein–lipid interface in the core of the bilayer, the molecular mechanisms of intra-membrane protein–protein interactions should be revisited. For example, determinants for the pronunciation of van der Waals contacts have been well studied for a GXXXG helix–helix dimerization motif [77,78]. It was shown that the actual contacts between the helices were entirely dependent on the presence and conjugation of beta-branched amino acids like V, I and T in position (i + 1) or (i + 2) ("i" being a G, A or S residue). This is explained by the fact that the entropic cost or the energy penalty for the formation of van der Waals contacts between beta-branched amino acids, due to their conformational restriction and rigidity, appears to be significantly lower than for contacts formed by alpha-branched residues. Interestingly, beta-branched amino acids are also found in the cholesterol consensus motif (CCM) and other binding sites for the hydrophobic moieties of annular- and non-annular lipids in the examples discussed above. In some cases, those hydrophobic cavities are even sensitive enough to select for distinct molecular species. It can be envisaged that the presence of such molecular species-specific lipid-binding domains on membrane proteins could in general affect the hydrophobic mismatch at the protein–lipid interface and therefore have implications for the balance of protein–protein and protein–lipid contacts. In membranes low of the interacting (target) lipid, as encountered during secretion of a membrane protein, a situation of hydrophobic mismatch might be generated, leading to at least three possible scenarios. In the first scenario, the protein aggregates due to hydrophobic mismatch with the bulk lipid before encountering its target lipid and is "solubilised" upon specific interaction (monomerization). A second option would be that the protein is in hydrophobic match with the bulk lipid, yet trapped in hydrophobic mismatch upon encounter with its target lipid, which it firmly binds. The consequence would be the oligomerization of the protein (a reduction of unfavourable protein–lipid contacts). As a third possibility the protein, due to conformational flexibility, can adapt to the hydrophobic environment of both the bulk and the target lipid, respectively. This latter scenario has implications for both the targeting of membrane proteins to distinct local lipid environments (e.g. membrane *rafts*) and regulation of protein activity, which might both contribute to restricting protein function to specific subdomains in cellular membranes.

In summary, we are beginning to appreciate that interactions at protein–lipid interfaces in membranes underlie a degree of specificity of structural recognition likely comparable to the well-established interactions of biomolecules in the aqueous environment. These interactions emerge as determinants for most membrane-linked processes including assembly, targeting, and function of membrane proteins.

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