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Review

Specific lipid requirements of membrane proteins—a putative bottleneck in heterologous expression

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Abstract

Membrane proteins are mostly protein–lipid complexes. For more than 30 examples of membrane proteins from prokaryotes, yeast, plant and mammals, the importance of phospolipids and sterols for optimal activity is documented. All crystallized membrane protein complexes show defined lipid–protein contacts. In addition, lipid requirements may also be transitory and necessary only for correct folding and intercellular transport. With respect to specific lipid requirements of membrane proteins, the phospholipid and glycolipid as well as the sterol content of the host cell chosen for heterologous expression should be carefully considered. The lipid composition of bacteria, archaea, yeasts, insects, *Xenopus* oocytes, and typical plant and mammalian cells are given in this review. A few examples of heterologous expression of membrane proteins, where problems of speific lipid requirements have been noticed or should be thought of, have been chosen.

Keywords: Lipid requirement; Membrane protein; Heterologous expression

1. Introduction

Biological membranes in general consist of various phospho- and glycolipids and sterols, which amount to approximately 50% by mass, the other half being constituted by membrane proteins. Major deviations from this general lipid composition are found in the archaea, which contain ether lipids with phytanyl residues instead of ester-linked fatty acids, and in all prokaryotes which lack sterols altogether. An important open question has long been, whether membrane proteins are associated with specific lipids and whether they are dependent on these for structural integrity and function. Considerable evidence confirming that this is indeed the case has been published within the last few years. The idea that membrane proteins in reality are protein–lipid complexes has been commonly accepted. The old puzzle, namely, why are there so many lipid species, although very few would suffice to account for the barrier function of membranes, may at least in part be related to the specific requirement of fitting partners for membrane proteins.

It is evident that membrane proteins do require specific lipids, be it as cofactors for their functions or as ''co-structures'' for their correct folding and stability. This should be taken into account when one attempts to perform heterologous expression of a membrane protein. The requirement for specific lipids may pose problems, even if one uses the right promoters and terminators, the correct targeting signals and posttranslational processing.

In this review, we first present conclusive examples showing that membrane proteins and membrane protein complexes do depend on phospholipids and sterols for their integrity and activity. For the sake of brevity, we did not include bulk effects of lipid composition on the physical properties of membranes, although they definitely do affect membrane proteins. We tried to collect the available data concerning the lipid composition of various organisms, concentrating on those most frequently used for heterolo-

Abbreviations: CL, cardiolipin; GPCR, G protein-coupled receptor; LBPA, lysobiphosphatidic acid; PA, phosphatidic acid(s); PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PG, phosphatidyl glycerol; PGP, phosphatidyl glycerophosphate; PGS, phosphatidyl glycerosulfate; PI, phosphatidyl inositol; PL, phospholipid(s); PS, phosphatidyl serine; Pgp, P-glycoprotein (multidrug resistance protein); S-TGA-1, sulfated triglyceride lipid (=3-HSO_3-Galpβ1→6Manα1→2Glcα1→archaeol)

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gous expression. Finally, we briefly discuss a few cases of heterologous expression of membrane proteins.

2. Effect of phospholipids and sterols on activities of membrane proteins

Evidence establishing functions of lipids in membrane processes derived from studies of in vitro systems is based predominantly on analysis of kinetic parameters of substrate binding and/or ATP hydrolysis performed by a protein either in its native membrane or in an artificial lipid bilayer. Reconstitution of a purified membrane protein into sealed proteoliposomes of a defined composition has made it possible to assess the effects of lipids also on processes of vectorial transport. This approach includes protein solubilization and purification employing various detergents. The choice of a detergent and purification conditions determines, to a large degree, the membrane protein activity after its reconstitution. Complete membrane protein delipidation usually leads to protein inactivation, which, however, in some instances can be reversed by readdition of external lipids. The selectivity for the lipids that are able to restore the activity of the membrane protein represents another tool for studies of specific lipid requirements [1].

To understand the real situation in a living cell, the in vitro observations need to be validated by in vivo approaches. For this purpose, a genetic approach of disrupting a pathway responsible for the synthesis of a specific phospholipid has been widely employed. Utilizing a set of mutations in the phospholipid metabolism in *Escherichia coli* and *Saccharomyces cerevisiae*, it has recently been possible both to validate the knowledge from in vitro experiments and to uncover novel previously undocumented functions of phospholipids (for *E. coli* review, see Ref. [2]). In Table 1, examples of membrane proteins affected by specific lipids have been collected.

Among membrane proteins of mammalian cells, the multidrug resistance P-glycoprotein (Pgp) and the Ca^{2+} ATPase from sarcoplasmic reticulum are two proteins for which the effects of lipid environment have been intensively studied. Specific stimulatory and/or inhibitory effects on drug binding affinity and/or ATPase activity were reported for Pgp. However, a comprehensive study of Romsicki and Sharom [49] points to a modulation of the drug expulsion from cells by the lipid environment immediately adjacent to the transporter. Both the nature of the head group and the acyl chain composition as well as the state of lipid phase modulate Pgp activity. Binding affinity differs for different drugs. The ATPase activity was found to correlate with drug partitioning into the lipid phase of the membrane [49].

Direct and indirect influence of cholesterol on membrane protein function has been documented for a number of membrane receptors. Mild techniques of a reversible cholesterol depletion or its modification in membranes [13] accompanied by monitoring the membrane fluidity [14] distinguished two mechanisms how cholesterol affects the ligand binding function, either by changing membrane fluidity and/or by a specific cholesterol–receptor interactions [14,15]. Highly specific molecular interactions result either in the receptor stabilization until it reaches its place of function (e.g. rhodopsin—Ref. [50]) or stabilization of the receptor in a high-affinity state. Some of the receptors exhibit a very stringent and unique requirement for the exact sterol structure [10].

A large number of mitochondrial proteins interact with cardiolipin (CL) which was found to be strongly immobilized on the protein surface. Dissociation of CL from the ADP/ATP carrier can only be achieved under denaturing conditions [22]. On the other hand, the loss of tightly bound CL from cytochrome *c* oxidase does not lead to its complete inactivation [20]. More examples documenting the significance of CL in mitochondrial processes and its involvement in human diseases have recently been reviewed [19].

The role of phosphatidyl glycerol (PG) and CL in bacterial membranes and in mitochondria is fundamental; however, in some cases, it appeared that they can substitute for one another in certain essential biological functions [51]. Negatively charged lipids such as PG and CL appear to be indispensable for membrane insertion and protein translocation via the translocase [32,33], and for membrane targeting [52]. A specific function of CL was postulated in the formation of a pore in connection with hyaluronan synthase from *Streptococcus* [34].

In yeast, a general requirement for CL of the ATP/ADP carrier in mitochondrial membrane was unmasked in a mutant affected in CL binding. The addition of an excess of CL to the in vitro system restored nucleotide translocation by the mutated protein [23]. Reconstitution experiments with the Can1p permease from *S. cerevisiae* incorporated either into ergosterol-containing or ergosterol-free vesicles point to a direct interaction of the protein with ergosterol [39].

A crucial role of phosphatidyl ethanolamine (PE) in membrane processes of both eukaryotic and prokaryotic cells has recently been documented. Due to its headgroup, which is relatively small compared to the bulky acyl chains, PE has a tendency to form nonbilayer structures. This property determines PE localization in close vicinity of membrane proteins (it often copurifies with a protein) where it stabilizes the protein and compensates for a putative disturbance of the bilayer caused by polytopic protein insertion [53]. It was found to be indispensable or stimulatory for activities of a number of membrane proteins (Table 1). As early as 1984, a strict requirement for PE was reported for membrane potential-driven uptake of lactose by lactose permease (LacY) [25,54]. An energy-independent counterflow mediated by LacY showed a broad tolerance for various phospholipids. Using *E. coli* mutants lacking PE, Bogdanov et al. have documented in a series of excellent studies a novel and very specific role of PE. A combination of in vitro and in vivo studies has established the function of PE as a molec-

Table 1
Membrane proteins affected in their activities by specific lipids and/or sterols

Membrane protein	Lipid	Effect	Reference
P-glycoprotein	PC, PE	Restore activity of delipidated ATPase Headgroup and acyl chains affect drug binding affinity	[1]
	Cholesterol	Increases ATPase activity; alters Pgp function	[3]
Ca^{2+} ATPase	PI-4 phosphate	2- to 4-fold increase in ATPase activity	[4,5]
	PE	Stimulates catalytic activity	[6]
	Cholesterol	Specific binding sites at the lipid–protein interface	[7]
Na^+/K^+-ATPase	Cholesterol	Increases maximum specific activity	[8]
γ-Aminobutyric acid transporter	Cholesterol	20-fold stimulation of activity	[9]
L-Glutamic acid transporter	Cholesterol	5-fold stimulation of activity	[9]
Serotonin transporter	Cholesterol	Requirement for citalopram binding	[10]
Nicotinic acetylcholine receptor—AcChR	Cholesterol	Alters gating function via changes in the secondary structure	[11]
Rhodopsin	Cholesterol	Stabilization of the molecule	[12]
Oxytocin receptor	Cholesterol	Stringent requirement for the high-affinity receptor state	[13–15]
Galanin receptor GalR2	Cholesterol	Specific requirement for ligand binding	[16]
Human adenosine A_{2a} receptor	Cholesteryl hemisuccinate	Cholesteryl hemisuccinate increases receptor stability in detergent	[17]
Phosphate carrier Mitochondria	CL	30-fold increase of specific activity	[18,19]
Cytochrome *c* oxidase Mitochondria	see Table 2	2 CL per monomer required for activity	[19,20]
Pyruvate carrier Mitochondria	CL	Requirement for activity and stability	[19,21]
ADP/ATP carrier Mitochondria mammals	CL	Strong effect on conformational transition and ADP binding	[19,22]
Mitochondria *S. cerevisiae*		Strong stimulation of activity in a mutant with reduced protein-associated CL	[23]
Metabotropic glutamate receptor (DmGluRA) *D. melanogaster*	Ergosterol	Glutamate binding by DmGluRA overexpressed in photoreceptor cells is strictly dependent on the presence of ergosterol	[24]
Lac permease *E. coli*	PE	Required for H^+-coupled transport, not for energy-independent translocation	[25–28]
		Acts as a molecular chaperone for correct folding and membrane topology	[29]
ABC-transporter OpuA *L. lactis*	PG/PS	Osmotic stress sensed via alterations in ionic interaction with lipids	[30]
Pore protein PhoE *E. coli*	PE	Required for trimerization of PhoE in vitro	[31]
SecYEG translocase	PG	Essential for preprotein translocation	[32,33]
E. coli; *B. subtilis*	PE	Stimulatory in *E. coli*; essential in *B. subtilis*	

(continued on next page)

Table 1 (*continued*)

Membrane protein	Lipid	Effect	Reference
Hyaluronan synthase *Streptococcus*	CL	Pore formation together with the protein postulated	[34]
Monoglucosyl-diacylglycerol synthase *A. laidlawii*	PG, CL	Strong activation	[35]
Chitin synthase *S. cerevisiae*	PS	Required for activity in vitro	[36]
Plasma membrane ATPase *S. cerevisiae*	PI, PG	Required for activity in reconstituted system	[37]
Pdr5 *S. cerevisiae*	Ergosterol	*erg* mutants have a reduced Pdr5 efflux activity	[38]
Arginine permease Can1p	Ergosterol	Ergosterol–nystatin interaction uncouples the permease from proton motive force	[39]
S. cerevisiae	PE depletion in whole cells	Strong effect on Can1p activity and several other protonmotive force-driven permeases	[40]
		Affects targeting to the plasma membrane	[41]
Tryptophan permease	PS depletion in whole cells	Strong effect on tryptophan uptake	[42]
S. cerevisiae	Ergosterol	Low tryptophan transport in *Erg6* disruptant	[43]
Glucose/H^+ symporter Hup1p	Ergosterol	Stimulation of protein expressed in *E. coli* and reconstituted in vitro	[44]
Chlorella	PC	Stringent requirement for protein stabilization during solubilization	[45]
H^+-ATPase Corn roots	Sterols	H^+ pumping selectively stimulated by cholesterol and stigmasterol	[46]
Photosystem II Spinach	PG	Involved in dimer–monomer interconversions	[47]
Synechocystis		Essential for photosynthetic activity in vivo	[48]

ular chaperone directing the proper assembly of the permease in the membrane bilayer [55]. The same authors showed that the critical folding steps occur after LacY membrane insertion [27]. In the most recent study, they demonstrated that the topology of the permease in the membrane can be changed in a reversible manner in response to the presence or absence of PE. Thus, the N-terminal half adopts an inverted topology in PE-less cells; domains normally non-translocated are translocated and vice versa [29].

In *S. cerevisiae*, an essential role of PE that is independent of its ability to form nonbilayer structures, has recently been demonstrated in the function of mitochondria-related processes [56,57]. PE in yeast can arise via three independent pathways. Hence, for obtaining a mutant completely depleted of PE, three genes have to be disrupted (*PSD1*, *PSD2*, *DPL1*). The triple mutant can grow only in the presence of ethanolamine [58]. Its specific requirement for PE could not be satisfied by phosphatidyl propanolamine—a structurally related lipid capable, as PE, of forming the hexagonal phase [57].

When ethanolamine in the medium is replaced by choline, growth of the yeast triple mutant (*psd1Δ*, *psd2Δ*, *dpl1Δ*) ceased after two or three generations. For a limited time period, however, the choline-grown cells retain the same viability as those grown on ethanolamine. This fact made it possible to identify a specific effect of PE on transport processes coupled to the proton motive force [40]. In the case of the arginine permease Can1p, it was shown that severe PE depletion of the cells affects primarily the delivery of the permease through the secretory pathway to the plasma membrane [41]. This may very well be due to a wrong membrane topology as described for the lac permease of *E. coli* [29].

3. Lipids as components of membrane protein complexes

For most of the examples given in Table 1, the evidence that individual lipid species exert a specific effect on a membrane protein is not really compelling. Indirect effects like changes in fluidity may still, at least in part, be involved in the phenomena described. Large bulk effects, especially due to cholesterol content, on the physical state of the phospholipid bilayer have been well documented. It is

generally not easy to distinguish unambiguously bulk effects from specific ones. Even within related groups of membrane proteins, like the seven-transmembrane-helix G protein-coupled receptors (GPCRs), the activities of individual receptors exhibit different types of dependency on cholesterol. For example, the cholecystokinin receptor responds to fluidity effects caused by cholesterol, whereas the activity of the oxytocin receptor is affected mainly by highly specific interactions with cholesterol [14].

The strongest evidence for highly specific protein–phospholipid and/or sterol interactions comes from 3D structures of membrane proteins. Examples are briefly summarized in Table 2.

A specific CL requirement for the activity of cytochrome *c* oxidase has been studied since the 1970s [70]. Of 14 phospholipid molecules associated with bovine cytochrome *c* oxidase crystals, three are CL [59]. Whether all three have to be present and what their exact function is, can now—after 30 years—be asked. The question can possibly be answered with the employment of site-directed mutagenesis leading to the loss of binding of individual CL molecules. The electron density resolution of crystals of cytochrome *c* oxidase from *Paracoccus denitrificans* achieved so far allowed the modeling of one phosphatidyl choline (PC) molecule in subunit III. The lipid forms two ion pairs via its phosphate and its quaternary ammonium [61].

The most detailed analysis of a membrane protein concerning its lipid content has been reported for the cytochrome *bc*1 complex (ubiquinol:cytochrome *c* oxidoreductase, QCR) from yeast [62]. Five phospholipid molecules associated with the protein were identified. Amino acids interacting with phosphatidyl inositol (PI) and CL were changed by site-directed mutagenesis. The phosphodiester of PI interacts with Lys272. The mutant K272A is active in vivo, but shows only 1% of the activity when tested in vitro. This is due to the loss of one of the three essential subunits of the multisubunit protein complex, the Rieske subunit. It is concluded, therefore, that PI stabilizes this subunit within the complex. No stable complex at all could be isolated when the three lysyl residues interacting with CL were exchanged.

Table 2
Lipids as components of crystallized membrane proteins

Protein	Lipids	Remarks	Function	Reference
Cytochrome *c* oxidase Bovine	3 PE, 7 PG, 1 PC, 3 CL	5 molecules at the outer leaflet of the inner mitochondrial membrane, 9 at the matrix side	CL essential for activity	[59,60]
Cytochrome *c* oxidase *Paracoccus denitrificans*	1 PC	Forms two ion pairs with Arg233 and Asp74 of subunit III		[61]
Cytochrome *bc*1 *Saccharomyces cerevisiae*	2 PE[a], 1 PI[a], 1 PC[a], 1 CL[a]	1 PE interacts with both monomers PI is in interhelical position All lipids except PI are on the matrix side of the mitochondrial membrane	Dimer stabilisation Stabilizes complex via interaction with Lys272 One of the CL phosphodiesters may be part of the proton translocation path	[62]
Photosystem I *Synechococcus elongatus*	3 PG, 1 MGD	All located at the stromal side of the membrane Phosphodiester of one PG binds one antenna chorophyll *a*		[63]
Reaction center *Rhodobacter sphaeroides*	1 CL	One phosphodiester of CL interacts with His145 and Arg267 of subunit M		[64]
K^+ channel KcsA *Streptomyces lividans*	2 PG			[65]
Bacteriorhodopsin *Halobacterium salinarum*	6 diether lipids/trimer [sulfated triglyceride lipid (S-TGA-1)]		Stabilization of BR-trimer	[66]
	1 squalene and 5 PGP/monomer		1 squalene and 1 PGP essential for normal photocycle characteristics	[67]
	18 phytanyl lipids/trimer		Form annulus around trimer In part fill grooves of the proteins	[68,69]

[a] Per monomer.

In addition to the stabilizing function of lipids, the authors suggest that one of the phosphodiester groups of CL and the phosphodiester of PE might take part in proton conduction leading to the reduction of ubiquinone.

A specific interaction of CL with an integral membrane protein has been documented also for the reaction center of the photosynthetic bacterium *Rhodobacter sphaeroides* [64]: one phosphodiester group interacts with two basic amino acids of subunit M. An exchange of the corresponding amino acids has not been published so far. A resolution of 2.5 Å of another crystallized photosynthetic reaction center, Photosystem I of the cyanobacterium *Synechococcus elongatus*, allowed the detection of three molecules of PG and one monogalactosyl diglyceride within subunit PsaA/PsaB [63]. Functional analysis of the lipids has not been carried out. The same holds for two molecules of PG present in the K^+-channel (KcsA) of the bacterium *Streptomyces lividans* (Ref. [65] and pers. comm.).

Lipid components of the bacteriorhodopsin complex have been analysed in detail [66–69]. Two glycolipid sulfates, five phosphatidyl glycerophosphates (PGPs), one-half PG and one-half phosphatidyl glycerosulfate (PGS) (all containing phytanyl chains) accrue to one monomer. One PG as well as a squalene are essential for activity and are most likely required for reprotonation of retinal via Asp96 [67]. Several lipid molecules exactly fill corresponding grooves in the protein "displaying a surprising extent of structural complementarity" [68].

To fully understand the importance of various lipids comprised within membrane protein complexes, directed mutagenesis is used for exchanging the amino acids responsible for the lipid/protein interactions. Three-dimensional structures of the proteins are required to identify these amino acids, and therefore large quantities of purified active proteins have to be available. The activity and stability of these proteins, however, depend on specific lipids. This in turn has to be considered, especially if heterologous expression systems are used for protein overproduction (see below).

4. Lipid composition of different organisms

Among the various phospholipids, PC is generally considered a bilayer-forming lipid and is found in the majority of membranes. Every biological membrane has at least one nonbilayer-forming lipid component. Under physiological conditions, it is represented by PE and/or monogalactosyl/monoglucosyl diacylglycerol. The latter neutral lipids are found in high concentrations in chloroplasts, Gram-positive bacteria that lack PE [71] and in organisms like *Acholeplasma laidlawii* [35]. Negative charge is brought to the membrane by anionic lipids phosphatidyl serine (PS), PG, CL and phosphatidic acids (PA). While PS represents the major anionic lipid in plasma membranes of eukaryotes, PG and CL fulfill this function in prokaryotes and mitochondrial membranes. CL and PA can form nonbilayer structures in the presence of special divalent cations.

Organisms have a tendency to maintain the physicochemical properties of their membranes within defined limits. Even simple prokaryotic cells regulate their lipid composition to be optimal in response to environmental conditions. For instance, to maintain a proper balance between bilayer and nonbilayer lipids, the acyl chain composition of the lipid envelope of *E. coli* varies with growth temperature [72].

All eukaryotic cells are characterized by the presence of a large number of membrane-bounded organelles. The plasma membrane typically contains sterols and sphingolipids which are lacking in prokaryotic and in subcellular membranes. The subcellular membranes of eukaryotes resemble those of prokaryotes.

The lipid composition of membranes is not constant. In the simple eukaryotic cell of *S. cerevisiae*, the ratios of individual phospholipids differ not only among the different wild-type strains, but they also change depending on the carbon source and cultivation conditions [73]. The major sterol in yeast is represented by ergosterol. Sphingolipids in this organism are characterized by their inositol moiety and are located primarily in the plasma membrane where they account for 7–8% of the total mass of the membrane (30% of the plasma membrane phospholipids) [74].

Like in yeast, ergosterol is also the main sterol of *Drosophila*, when the flies are fed with a diet containing yeast [82].

In mammalian cells, the major sterol is cholesterol. Its cellular levels are highly regulated. To maintain the optimal concentration of cholesterol in the cell, its distribution is regulated between the different membranes. Sphingolipids, particularly sphingomyelin and glycosphingolipids, have been well established as essential components of mammalian cells, where they are predominantly found in the outer leaflet of the plasma membrane [91]. There is a selective confinement of cholesterol, sphingolipids and certain proteins in discrete regions of the membrane. These domains, named lipid rafts, appear to be an ubiquitous feature of mammalian cells [92]. Lipid rafts are likely to contribute to the structure and function of caveolae, plasma membrane invaginations, that are implicated in membrane traffic and signaling events. Similar domains were also described in yeast where they function in biosynthetic delivery of proteins to plasma membrane [93]. Generally, membrane proteins requiring high concentrations of cholesterol are located exclusively in rafts.

The lipid composition of plasma membranes of cultured cell lines (e.g. BHK cell) is usually similar to that found in plasma membranes of mammalian cells in general. Sphingomyelin, sphingolipids and free cholesterol are enriched in this membrane type. Lysosomal membranes in BHK cells contain a large amount of lysobiphosphatidic acid (LBPA) and the phospholipids characteristic for mammalian cells. LBPA is enriched in cultured BHK, human liver and rabbit alveolar macrophages [88].

5. Heterologous expression of membrane proteins

Excellent reviews about heterologous expression of membrane proteins have been published [94–98] and altogether, a myriad of papers concerning individual membrane proteins and membrane protein complexes have appeared, which obviously cannot be the topic of this review. A very detailed and critical analysis of the overexpression problems met with the serotonin transporter has been written by Tate [99]; a large number of different expression systems have been compared. In the following, we therefore have picked only a few examples of heterologous membrane protein expression, where problems of specific lipid requirement have been noticed or should at least be thought of.

5.1. Prokaryotes as hosts

As summarized in Table 1, for 10 mammalian membrane proteins, positive effects were shown to be caused by the presence of cholesterol. Expression of these proteins in bacteria, therefore, would be expected not to yield fully functional proteins. Successful expression of the majority of fully functional GPCR proteins has been achieved in *E. coli*, however, indicating that cholesterol is unimportant for the function of these particular receptors. For reconstitution of *E. coli* expressed receptors, however, it has been shown that the presence of cholesteryl hemisuccinate during the solubilization and purification steps was required. Only then a 100% functional neurotensin receptor from rat [100] and an adenosine A2a receptor from human [17] was obtained in good yield. These examples point to a non-specific bulk effect of sterols required for stabilization of the receptor molecules in their native conformation before they are embedded in a lipid bilayer. Also for the olfactory receptor expressed in bacteria, cholesterol was not essential for activity [101].

The failure to express functional serotonin transporter in *E. coli* is thought to be due to the lack of cholesterol [99]. On the other hand, mouse mdr1 protein has been functionally expressed in *E. coli* [102], although it has been shown that in vitro mammalian Pgp does require PC and cholesterol for its optimal activity [1,3]. Because *E. coli* lacks both these membrane components (Table 3), this discrepancy could be due to the fact that generally qualitative but rarely quantitative comparisons (specific activities) are made [102]. Another possible explanation could be that the positive effects of cholesterol and PC on Pgp activity observed in vitro may not be observable to the same extent in vivo. Other phospholipids of *E. coli* might replace, at least partially, the eukaryotic lipid components in vivo.

A plant protein—the *Chlorella* hexose/proton symporter (HUP1 gene product)—with 12 transmembrane helices, has also been functionally expressed in *E. coli* [44]. The activity of the solubilized and reconstituted protein was stimulated in the presence of ergosterol. However, the activity of the same transporter expressed in and solubilized from *Schizosaccharomyces pombe* (which contains ergosterol) showed a five-fold higher in vitro activity than that expressed in *E. coli* and reconstituted in the presence of ergosterol. Later it was found that the HUP1 protein obligatorily requires PC for stabilization [45]. The low activity of the protein produced in *E. coli* might be due to the lack of PC during the preparation. Again, it is not sure to what extent the HUP1 protein requires PC and sterols in vivo. However, the finding that the transporter expressed in yeast and purified to homogeneity contains one to three molecules each of ergosterol, PC and PE per HUP1 molecule, certainly is in line with the positive effects of these components on the transporter activity [44,45]. The in vivo PE requirement of a number of H^+-symporters [41] has been discussed above.

A promising study with the aim to yield large amounts of heterologously expressed membrane proteins was started by Turner et al. [103]. It was initiated by the observation that up to 30 mg of bacteriorhodopsin can be obtained per liter of *Halobacterium salinarum* culture, and by the idea that similar amounts of various heterologous membrane proteins might be obtained by overexpression in *H. salinarum*. From three receptors tested, only the Ste 2 pheromone receptor from yeast was found in the membrane fraction while two human GPCRs could not be detected [103]. Whether this was solely a transcriptional problem or whether the cholesterol requirement shown for some of GPCRs (see Table 1) caused an additional problem remains an open question. Certainly, the unusual lipid composition of the archaea is expected to aggravate the situation whenever specific lipid components are required for proper function of heterologously expressed proteins.

It has been stated that for overexpression of proteins, it "generally does not matter in which particular cellular membrane they are located, as long as they are correctly folded and are processed to their active form" [94]. But can, for example, cytochrome *bc*1 requiring CL (Table 2), be correctly folded in a membrane lacking this phospholipid? In this context, it is of interest that *E. coli* mutants were selected that proliferated a large amount of internal membranes when overproducing subunit b of F_1F_0 ATP synthase [104]. The lipid composition of these intracellular membranes differs from those of the cytoplasmic membrane; they are enriched in CL and contain almost half of the normal amount of PG. [104].

5.2. Insect cells as hosts

Insect cell lines contain a rather low quantity of cholesterol and no PS in their plasma membrane, whereas the PI content is comparatively high [81]. The very low content of sterols as compared to phospholipids (a ratio of 0.04; for mammalian and yeast plasma membranes, it is >0.5; see Table 3) could potentially be a bottleneck for the overexpression of sterol-requiring membrane proteins. Indeed,

Table 3
Lipid composition of biological membranes

Organism	Lipid	Reference
PROKARYOTES		
Escherichia coli	Inner membrane:	[2,75]
Gram-negative	PE 70–80%; PG 15–20%; CL 5%	
Bacillus megaterium	PE—35%, PG—48%, CL—11%, glucosoaminyl PG—6%	[75,76]
Gram positive		
Archaea:		
Halobacterium	Analogs of archaeol (=diphytanylglycerolether): PG, PGP, PGS, PA, methyl-PGP (main phospholipid); S-TGA1 and other glycolipids	[71,77]
Thermoacidophiles[a]	Analogs of caldarchaeol (=dibiphytanyldiglycerol tetraether) with inositol phosphate and various saccharides, respectively, attached; other glyco- and phosphoglycolipids	
EUKARYOTES		
Yeasts		
Saccharomyces cerevisiae[b]	Percentage of plasma membrane PL	
Plasma membrane	PC 17%; PE 20%; PI 18%; PS 34%; PA 4%; CL 0.2%	[74,78,79]
	(Sphingolipids ~ 30%)	
	Ergosterol/PL (mol/mol) ~ 0.9	
Mitochondria	Percentage of mitochondrial PL	
	PC 40%; PE 26%; PI 15%; PS 3%; PA 2%; CL 13%	[78]
	Ergosterol/PL (mol/mol) 0.2	
Pichia pastoris[b]	Percentage of total lipids	
Whole cell extract	PL 48%; ceramides 2%; sterol (free) 31%; sterol derivatives 16%	[80]
	Percentage of total PL recovered from whole cells	
	PC 38%; PS 28%; PE 18%; PI 11%; PA 3%; CL 2%	
Insect cell lines		
Spodoptera frugiperda Sf9	Percentage of total phospholipid recovered from whole cells	[81]
	PC 35 (43)%; PI 23 (17)%, PE 36 (36)%; CL 4.6 (4.7)%	
(*Trichoplusia ni*)	PS, glyco- and sphingolipids—not detected	
Whole cell extract	Cholesterol/PL (mol/mol) 0.04	
Drosophila melanogaster[c]	PC, PE, PS, PI—not quantified	[82]
Embryonic membranes	Glycosphingolipids: two ceramides—not quantified	
	Sterols: ergosterol 69%, cholesterol+dehydrocholesterol 14%, campesterol+ sitosterol 9%, others 8%	
Xenopus oocytes	Percentage of total PL recovered from whole cells	[83,84]
	PE 19%; PC 65%; PI 10%; PS 2%; sphingomyelin 5%	
	Cholesterol/PL (mol/mol) 0.6–0.7	
Plants		
Plasma membrane (oat coleoptile and root)	Percentage of total lipids	[85–87]
	Phospholipids 42–50%	
	PA 11–15%; PE 9–15%; PC 9–14%; PS 3–4%; PI 2%; PG 1–2%	
	Glycolipids 25–39%[d]	
	sterylglycoside 13–15%; glycerocerebroside 10–26%[d]	
	Sterols (free) 19–25%[e]	
	sitosterol 5–9%; campesterol 2%; stigmasterol 2–12%[e]	
	others 4–8%	
Choloroplasts	Percentage of total chloroplast lipids	
	Galactolipids 60–70%	
	monogalactosyldiglycerides 40–50%	
	digalactosyldiglycerides 15–25%	
	Sulfolipids 1–5%	
	Phospholipids 16%	
	mainly PG	

Table 3 (*continued*)

Organism	Lipid	Reference
Mammalian cells		
BHK21 cell line	Percentage of plasma membrane phospholipids	[88,89]
Plasma membrane	PE 29%; PC 26%; sphingomyelin 24%; PS 18%; PI 3% Cholesterol/PL (mol/mol) ~ 0.9	
Rat hepatocytes	PC 32–47%; PE 14–20%; sphingomyelin 13–24%; PS 4–8%, PI 7–10%; cerebrosides 1–3%; PA 2–3%	[90]
Plasma membrane	Cholesterol/PL (mol/mol) 0.6–0.7	

[a] Surprisingly, some of the most extreme thermophiles possess lipids exclusively of the archaeol type [77].
[b] Cells grown on glucose.
[c] Reared on yeast-based medium.
[d] Low value for roots.
[e] High value for roots.

an addition of cholesterol to the medium of infected Sf9 insect cells positively affected the properties of the heterologously expressed mammalian oxytocin receptor [105]. The number of the high-affinity receptor binding sites increased by a factor of 3. The low cholesterol level in Sf9 may be the cause of the low activity of the GPCRs expressed in this insect cell line [106]. However, the same low level of cholesterol is obviously sufficient for expressing functional Na^+/K^+-ATPase [107].

An interesting alternative to the conventional baculovirus expression system has been reported recently [24]. Photoreceptor cells of transgenic *Drosophila melanogaster* were used for an expression of GPCRs. Two homologous proteins were obtained in high yield. The expression level of the human vasopressin 1A receptor (V1A) was claimed to be better than when expressed in *E. coli* and Sf9 cells [24]. A homologous receptor expressed in photoreceptor cells requires obligatorily ergosterol, which indeed is present in *D. melanogaster* reared on yeast-based diet (see Tables 1 and 3) [82].

5.3. Yeast cells as hosts

The yeasts *S. cerevisiae*, *S. pombe* and *Pichia pastoris* have frequently been used as host organisms for heterologous expression, mainly also because, in contrast to mammalian or insect cell lines, these eukaryotic cells can be grown in large amounts in inexpensive media [94]. *S. cerevisiae* has also been used to identify a large number of membrane transport proteins from plants by complementing defects in the uptake of specific substrates [96,108]. Complementation studies, however, often do not reflect quantitative aspects of functional expression and thus successful complementation is no evidence that the corresponding proteins are optimally furnished with their required lipids.

The bottleneck in the expression could again be due to a shortage of cholesterol in the case of mammalian membrane proteins, and of specific sterols (sito-, stigma- and campesterol) in the case of plant proteins. The main fungal sterol, ergosterol (a 24 methyl, 7, 8/22, 23 dehydro cholesterol) might replace the plant and animal sterols, but this often does not result in fully functional heterologous proteins. Human MDR1 expressed in *S. cerevisiae* showed greatly decreased drug binding as compared to that of the authentic protein. It was found that, indeed, ergosterol inhibits azidopine binding [109]. On the other hand, in other studies, no significant differences between MDR proteins expressed in *S. cerevisiae* and mammalian cells were observed [110,111]. However, it has also been documented that MDR1 protein expressed in *S. pombe* [112] although reacting with peptide substrates (valinomycin and actinomycin D) did not accept adriamycin, which normally is one of its substrates, too. This may indicate that the "correct" membrane environment (cholesterol?) influences the substrate specificity of this protein. Ergosterol also is not able to compensate for the cholesterol requirement of the serotonin transporter [99].

The multidrug resistance-associated protein (MRP1) transporting glutathione, glucuronide, and sulfate-conjugated organic anions out of cells, was overexpressed in *P. pastoris* [113] at a level 30-fold higher than in HeLa/MRP1 transfectants. No functional difference between the two heterologous proteins was observed [110]. No specific lipid requirements have so far been reported for this transporter subgroup of the ABC family.

Cholesterol was shown to accelerate the phosphorylation/dephosphorylation reaction of the Na^+/K^+-ATPase in vitro (Table 1). However, the enzyme was also functionally expressed in *S. cerevisiae* [114,115] indicating thus that cholesterol might have been replaced by ergosterol.

The properties of two human GPCR proteins, the μ-opioid receptor and the D2S dopamine receptor, were shown to be influenced by membrane components. The ligand binding to μ-opioid receptor was increased in yeast membranes when ergosterol was removed and replaced by cholesterol [116]. Ligand binding affinity of the dopamine receptor expressed in yeast differed significantly from the affinity of the receptor embedded in its natural environment. Moreover, the receptor affinities were different when the proteins were expressed in *S. pombe* and *S. cerevisiae* and compared to each other [117]. The component responsible for these differences has not been identified.

To date, only a few plant membrane proteins heterologously expressed in *S. pombe* and in *S. cerevisiae* have been

purified and studied in vitro [118–121]. Quantitative comparative studies of the heterologous proteins with the proteins in their native membranes are almost completely missing. The substrate spectrum and kinetic parameters for the *Chlorella* HUP1 protein expressed in *S. pombe* were found to be identical with that from *Chlorella* [122]. The K_m values for ATP hydrolysis, as well as the specific molecular activity of the heterologous plant H^+-ATPase purified from *S. cerevisiae* agree with the data reported for ATPase obtained from the native membrane [121]. These observations indicate that ergosterol can, to a large extent, replace the specific plant sterols.

Finally, it should be mentioned that the first successful heterologous expression of a membrane protein in yeast (bacterio opsin from *H. salinarum*) was achieved by Hildebrandt et al. [123]. Today, we know about the specific lipid components visualized within the bacteriorhodopsin crystals (Table 2). These lipid components are not present in yeast and it is therefore surprising that the authors obtained immunopositive material at all and achieved an incorporation of retinal supplied to the medium.

6. Conclusion and summary

Biologically active membrane proteins frequently occur as protein–lipid complexes. We gathered here 30 examples of membrane proteins from prokaryotes, yeast, plants and mammals, in which specific phospholipids and sterols were shown to be important for optimal activities. Although bulk effects of lipid components can rarely be fully excluded, the collective evidence clearly supports the essential role of specific phospholipid headgroups and/or specific structural features of sterols in functional protein–lipid interactions.

Direct evidence for defined lipid–protein contacts is corroborated by six crystallized membrane protein complexes discussed in this survey. A definition of the actual function of the associated lipid molecules will require studies involving an exchange of the interacting amino acids by site-directed mutagenesis. For the yeast cytochrome *bc*1 complex, this has partly been conducted; the amino acid changes resulted in pronounced instabilities of the protein complex (demonstrated mainly in vitro) [62].

In addition to their stabilization function, specific phospholipids were shown to be required for the correct orientation of certain transmembrane helices within the membrane [29] or for correct transport through the secretory pathway [41]. These lipids may play a transitory role, therefore, and may not be necessary for the function of the final product.

Heterologous expression of membrane proteins is usually designed with the aim to obtain large amounts of the fully active protein of interest. With respect to the specific lipid requirements of membrane proteins, it is desirable that the phospho- and glycolipid, as well as the sterol content of the host cell chosen for the heterologous expression, are carefully considered. The lipid composition of bacteria, archaea, yeasts, insects, *Xenopus* oocytes, typical plant and mammalian cells are given in Table 3 of this review.

Problems in heterologous expression of membrane proteins due to different lipid environments within host cells have been observed at times. Often they may have escaped notice and may have been comprised under "inefficient expression system". Finally, it should be pointed out that the presence of specific lipid components during crystallisation trials may be of crucial importance [124].

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