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Cell-free synthesis and folding of transmembrane OmpA reveals higher order structures and premature truncations

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ABSTRACT

We use a cell-free transcription–translation system to monitor the effect of different lipids on the synthesis and folding of the transmembrane domain of the outer membrane protein OmpA from *E. coli* under physiological conditions. Folding is consistent with previous observations made *in vitro* at high pH. Synthesis and folding yields are optimal in phosphocholine lipids, particularly in short chain lipids and small vesicles, while lipid rafts do not promote folding compared to the folding in the absence of lipids. Truncated species are observed during translation in the presence of the periplasmic chaperone Skp, which likely binds to the newly synthesized polypeptide chain during cell-free translation and thus prematurely terminate polypeptide chain synthesis. In contrast, folded and unfolded dimers of OmpA correlate negatively with folding yields. This suggests that dimer formation competes with folding and insertion of monomeric OmpA, though folded dimers slowly appear to convert to folded monomers.

1. Introduction

Membrane proteins (MPs) comprise 30% of the total genome and present important objects for both basic and applied research, due to their adaptation to a membrane environment and role in signal transduction [1,2]. However, studies are hampered by their low natural abundance and challenges in heterologous overexpression. These challenges arise from the insertion of large amounts of recombinant MPs into cellular membranes, channel- or pore forming activities, non-specific interaction with other MPs and the general overload of host transport mechanisms when the MP is targeted to the membrane for functional folding. This often results in significant stress or even toxicity for the host cells, leading to strong selection against high-expressing clones. In some cases this can be remedied if the MPs form aggregated inclusion bodies in the cytosol instead, but an efficient refolding protocol is then required, which is notoriously difficult [3,4]. An alternative is to use a cell-free expression system in which ribosomes, tRNAs and other translation factors are extracted from cells and assembled together with the vector encoding the protein of interest to be expressed, RNA polymerase, amino acids and other small molecules needed to drive the syntheses. This separates cell viability and protein expression [5–9]. Such a system allows us to approximate cellular conditions while at the same time controlling the exact composition of the membrane or membrane-mimicking environment into which the membrane protein will fold. Such an approach has recently produced sufficient amounts of the extremely hydrophobic membrane protein EmrE to allow crystallization and elucidation of its three-dimensional structure [10]. The mechanism by which membrane proteins are incorporated into synthetic phospholipid membranes in the absence of transport systems such as the signal recognition particle and the SecYEG translocon [11] is not fully clarified, but the successful reconstitution of bacteriorhodopsin required the lipid to be in the liquid disordered phase rather than the gel phase, and showed a dependence on bilayer thickness with DOPC (hydrophobic thickness 27.1 Å) leading to 20% higher levels of insertion than DMPC (thickness 26.2 Å) [12]. Overall yields of bacteriorhodopsin were comparable to those in the native host. In other studies, apocytochrome b_5 has been reconstituted directly into giant unilamellar phospholipids with comparable results in DOPC and DMPC [13], while liposomes gave higher and more reproducible yields of a human stearoyl–CoA desaturase complex than surfactants [14]. Nevertheless there have been few studies which use cell-free transcription–translation systems to directly elucidate aspects of membrane protein folding and insertion.

The 325-residue outer membrane protein A (OmpA) has long served as a model for model membrane protein folding studies. Residues 1–176 form an 8-stranded β-barrel in the outer membrane, while the C-terminal residues 177–325 form a periplasmic globular protein. OmpA's transmembrane β-barrel's atomic-level structure is known from both NMR [15] and X-ray studies [16]. OmpA spontaneously folds into both detergent micelles and lipid vesicles without the need for metabolic energy, and its membrane insertion and folding has been studied extensively both *in vitro* and *in vivo*

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[17–22]. OmpA is one of the major constituent of the outer membrane of Gram negative bacteria [23] and is involved in diverse biological phenomena, such as bacteriophage binding to *E. coli* [20], adhesion and invasion of meningitis strains of bacterium to brain microvascular endothelial cells [24,25] and *E. coli* biofilm formation [24,26]. In addition, OmpA also forms anion-selective ion channels [27], reflecting the polar interior of the barrel.

Here we use a cell-free coupled transcription–translation system to mimic the *in vivo* process by which the transmembrane domain of OmpA (TM-OmpA) inserts into the membrane. Previous *in vitro* studies of OmpA's membrane insertion process have typically started out with OmpA denatured in high concentrations of denaturant and have used high protein:lipid ratios and highly alkaline conditions to reduce aggregation [18,28]. In our studies we synthesize the TM-OmpA polypeptide chain *in situ* and monitor its folding in real time in the presence of different lipid vesicles. This mimics the actual folding step of TM-OmpA, *i.e.* the insertion in the periplasmic space of the prefolded state of TM-OmpA into the outer membrane, whose inner leaflet corresponds in composition to that of the plasma membrane. The cell-free system and absence of a cytosol-periplasm barrier allows us to dispense with an N-terminal signal sequence to target TM-OmpA to the periplasm via the translocon [21]. Furthermore, we do not include outer membrane components such as lipopolysaccharides (LPS) despite their demonstrated interaction with OmpA *in vitro* [22] since the LPS is not present in the inner leaflet of the outer membrane. We find expression levels to be ranked in the order PC>PS>PE>>PG, while the detergent octyl maltoside severely inhibits expression level. In terms of bilayer thickness, the short chain lengths C_9–C_{12} are optimal, while insertion into DOPC vesicles is best when vesicles are sonicated. Lipid rafts and PG severely reduced insertion into mixed vesicles. These results closely replicate results *in vitro* from the urea-denatured state, indicating that the folding pathway is independent of the initial state of denaturation. Additionally, the periplasmic chaperone Skp led to truncated OmpA bands while the other chaperones Fkp and SurA had no effect. Dimer bands were observed in competition with folded monomers, indicating that the two processes occur in parallel.

2. Materials and methods

2.1. Materials

The following lipids were used: DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), DOPS (1,2-dioleoyl-*sn*-glycero-3-phosphoserine), DOPG (1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol) (sodium salt)), DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine), DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), DMPC (1,2-di-myristeoyl-*sn*-glycero-3-phosphocholine), C_{10}PC (1,2-didecanoyl-*sn*-glycero-3-phosphocholine), C_9PC (1,2-dinonanoyl-*sn*-glycero-3-phosphocholine), C_8PC (1,2-dioctanoyl-*sn*-glycero-3-phosphocholine) and C_6PC (1,2-dihexanoyl-*sn*-glycero-3-phosphocholine). All lipids were from Avanti Polar Lipids (Alabaster, AL), octyl-β-D-maltopyranoside from Anatrace (Maumee, OH), porcine RNase inhibitor (32,200 units/ml) from Amersham Bioscience (Uppsala, Sweden), T7 RNA polymerase (20 units/μl) from New England Biolabs (Ipswich, MA), and S^{35}-labeled methionine (1175 Ci/mMol) from Perkin-Elmer (Boston, MA). All other chemicals were of analytical grade and purchased either from Sigma (St. Louis, MO) or Merck (Darmstadt, Germany). Transmembrane OmpA (TM-OmpA) was expressed from a pET22b plasmid containing the DNA sequence coding for the N-terminal membrane-bound β-barrel domain of TM-OmpA, comprising residues 1–176 without a signal sequence (a generous gift from Dr. J. Kleinschmidt). The plasmid was transformed into XL1 Blue cells by electroporation, cells were grown up in LB media containing 50 μg/ml ampicillin and the plasmid was isolated with standard procedures.

2.2. Purification of Skp, Fkp and SurA

Skp was expressed in the periplasmic fraction of *E. coli* CAG16037 containing plasmids pSkp (pQE60 from Qiagen carrying the Skp gene) and pPLT13 (mini-F carrying *lacIq*), generously provided by Dr. J. Kleinschmidt. Purification followed the published protocol [22]. Purified Skp was dialyzed against buffer containing 10 mM Tris-acetate, 14 mM Mg-acetate, 60 mM KCl, 1 mM DTT, pH 8.2. FkpA [29] and SurA [30] were purified according to published protocols and dialyzed against the same buffer as for Skp.

2.3. Cell-free expression of TM-OmpA

The S30 extract used for protein synthesis was prepared from *E. coli* strain A19 using a modification of established procedures [1,31]. Cells for the S30 extract were grown in Luria–Bertani (LB) broth and harvested in mid-log phase (A_{600}~0.6). The cells were lysed by passing twice through a French press (Thermo Electron Corporation, Waltham, MA) at 800 psi. The cell lysate was dialyzed (12–14 kDa MWCO) overnight against buffer containing 10 mM Tris-acetate, 14 mM Mg-acetate, 60 mM KCl, 1 mM DTT, pH 8.2. Coupled transcription-translation took place in the following buffer: 40 mM Tris-acetate (pH 8.2), 25 mM ammonium acetate, 51 mM potassium acetate, 7 mM magnesium-acetate, 23 μg/ml folinic acid, 0.3 mM cAMP, 27 mM phosphoenolpyruvate (50:50 mixture of trisodium and monopotassium salts), 350 μM each of 19 unlabeled amino acids, 1.5 mM dithiothreitol (DTT), 1.2 mM ATP, 0.8 mM each of CTP, GTP, and UTP, 0.23 mM glucose-6-phosphate, 13 μg/ml pyruvate kinase, 2.67 U/μl T7 RNA polymerase, 25–30 μg/ml plasmid DNA, 167 μg/ml rifampicin, 0.5 mM EDTA (pH 7.8), 0.016% sodium azide and 0.225 volume of S30 extract. Reactions for autoradiography analysis determination contained 0.25 μM ^{35}S-Met (1175 Ci/mmol). PEG-8000 was omitted, as it interferes with the migration of proteins below 30 kD [32]. The negative control (NC) for expression was a cell-free reaction with no added DNA template. All reactions (10 μl final volume) were incubated at 37 °C for 2 h. The reaction mixture was diluted with 1X gel loading buffer and run on a 15% SDS polyacrylamide gel. Subsequently the gel was dried, exposed to a storage phosphor screen from Molecular Dynamics for 12 h, scanned under the best resolution mode with 50 μm pixel size using a Typhoon Trio variable mode Imager from Amersham Bioscience (Sunnyvale CA) and band intensity was quantified using the ImageJ programme [32]. Protein yields for standard cell-free batch reactions were determined from gel runs using 3 μl aliquots of the cell-free reaction mixture. Yields were determined from the measured amount of incorporated radioactivity (^{35}S-Met) using a calibration gel with different amounts of ^{35}S-Met placed on the dried gel. The autoradiogram intensities of bands on both sample and calibration gel were measured under identical conditions. Standard errors were in the order of 10–15% and are omitted from the graphs for clarity. For pulse chase experiments, OmpA was expressed in the presence of 0.25 μM ^{35}S-Met for 20 min as previously described, followed by the addition of 100 μM (*i.e.* 400-fold excess) of cold Met. At different time points after adding cold Met, aliquots were taken out and mixed with SDS-PAGE loading buffer to stop further translation and folding.

2.4. Preparation of small unilamellar vesicle (SUVs)

SUVs of phospholipids were prepared by sonication as follows. Thin films of different phospholipids were deposited on the walls of glass tubes from a solution in chloroform and methanol (2:1 v/v) by evaporation under a gentle stream of dry nitrogen. The lipid films were dried in desiccators for at least 48 h and then hydrated in a buffer containing 10 mM Tris-acetate, pH 7.4. Phospholipids were suspended by vortexing and then sonicated using a Bandelin Sonopuls (Progen Scientific, Mexborough, UK) with 15% power and 50% cycles

until no further optical clearing of the suspension was observed. For DMPC SUVs (phase transition temperature 23 °C), the phospholipid film was suspended in the buffer at 37 °C and always kept at 37 °C. SUVs were centrifuged to remove titanium particles before use. Vesicles with different sizes were prepared by passing through the membrane of required size (50, 100 or 200 nm) on an extruder (Northern Lipids, Burnaby, Canada) following 10 cycles of freezing and thawing of the lipid suspension.

For cell-free expression studies, we use a large excess of lipid. Based on our calibrations, we estimate the total yield of monomeric OmpA in DOPC to be around 2.5 ng per 10 μl reaction mixture. This occurs in the presence of 5 mg/ml lipid, giving a protein:lipid ratio of 1:20,000 (w/w) or approximately 1:1000 (molar ratio).

2.5. Quantification of OmpA yields

We quantify the yield of folded TM-OmpA in our cell-free transcription-translation samples using SDS-PAGE in combination with autoradiography. In this way we only follow the synthesis and folding of TM-OmpA, since this is the only protein that will incorporate ^{35}S-Met during translation. We are able to distinguish between folded and unfolded TM-OmpA, since folded TM-OmpA will migrate slower on an SDS-PAGE gel than unfolded TM-OmpA, corresponding to apparent molecular weights of 21 and 18 kDa respectively. The reverse is the case for full-length OmpA containing both the β-domain and the periplasmic domain [28,33]. While folded TM-OmpA is resistant to SDS at room temperature, boiling the sample denatures it. Use of this simple switch allows us to confirm that we are monitoring different conformational states. Note however that in addition to folded and unfolded TM-OmpA, SDS-PAGE gels of TM-OmpA translation also reveal a number of other bands, which we ascribe to multimers and truncated translation products (see Results and discussion). These bands are not included in the quantification of TM-OmpA unless specifically stated.

We also investigated the possibility of using detergents to monitor TM-OmpA folding. However, the inclusion of 5 mM of the detergent octyl maltoside (OM), which is one of the most successful detergents for crystallization of membrane proteins [35], reduced yields of folded TM-OmpA by ~50% compared to DOPC at 37 °C and showed no folded protein at 20 °C (Fig. 1A). This appears to be a general phenomenon in cell-free transcription/translation, since expression of the globular protein S6 was also severely inhibited by increasing concentrations (0–50 mM) of OM, particularly above the cmc of 20 mM (data not shown). Similar observations have been made for octyl glucoside in cell-free bacteriorhodopsin expression [36]. The detergent most likely interferes with protein expression by interacting with cell-free expression components.

3. Results and discussion

3.1. Establishing optimal conditions for cell-free expression of TM-OmpA

TM-OmpA expresses and folds into vesicles consisting of the 5 mg/ml zwitterionic lipid DOPC both at 20 °C and 37 °C (Fig. 1A). While the expression yields are very similar at both temperatures, folding percentages are significantly higher at 37 °C. 66% and 45% of monomeric TM-OmpA fold at 37 °C and 20 °C, respectively, based on density scanning of the bands in Fig. 1A. This most likely reflects the high activation energy to folding of OmpA [34], which makes rising temperature significantly increase the rate of folding and thus the yield of native protein. Since TM-OmpA as a membrane protein has a high proportion of hydrophobic amino acids on the surface of the β-barrel, one might expect a higher tendency to aggregate at higher temperatures since the hydrophobic effect, which also promotes aggregation, increases with temperature. This would compete with folding. However, the increased folding yield indicates that this effect is more than compensated for by the lowering of the activation energy for unfolding, so that the protein has time to insert and fold into a state that is not susceptible to aggregation. Therefore, all subsequent experiments are carried out at 37 °C unless otherwise specified.

Both monomeric expression and folding levels increase around 3-fold as the lipid concentration increases from 0 up to a plateau at 1–5 mg/ml DOPC. This indicates that partitioning into the lipid vesicles enhances the protein translation mechanism. Most likely, unfolded (and non-lipid-bound) TM-OmpA inhibits protein synthesis by remaining associated with the ribosome and preventing access of other translational components such as tRNA and elongation and termination factors. Subsequently, expression levels drop 3–4 fold as the lipid concentration increases from 5 to 10 mg/ml DOPC (Fig. 1B). In all subsequent experiments, we use 5 mg/ml lipid because at this concentration higher multimers and truncated forms of TM-OmpA are observed to a significantly lower extent than at lower lipid concentrations (Fig. 1B). At all lipid concentrations, the amount of unfolded OmpA exceeds folded OmpA at 20 °C (Fig. 1C), but the situation is reversed at 37 °C (Fig. 1B), in good agreement with Fig. 1A.

Time-dependent expression assays (Fig. 1D and E) show that the total amount of protein (*i.e.* the sum of bands corresponding to native, denatured, higher molecular weight bands and truncated OmpA) as well as the amount of unfolded OmpA and lower molecular weight bands (which correspond to truncated protein) reach a plateau level after 1 h. After an initial quick rise in intensity, the amount of folded monomer continues to rise more slowly over the next 6 h. In parallel, there is a decline in the intensity of the higher molecular weight bands which most likely correspond to OmpA dimers, trimers and even higher order structures. We have also observed accumulation of these higher order structures during *in vitro* folding studies in the presence of limiting amounts of surfactants (K.K. Andersen and D.E.O., unpublished observations). The concomitant rise and decline in monomeric and multimeric species, respectively, suggests that the multimers slowly dissociate to the folded monomeric state.

3.2. Effect of lipid composition on TM-OmpA expression and folding

Having established appropriate conditions for expression and folding, we investigated the effect of head groups and chain lengths on overall yields and folding levels. For head groups we were able to establish the following ranking order for TM-OmpA expression: PC>PS~PE>>PG (Fig. 2A) using lipids with dioleoyl acyl chains. Notably, the decline in absolute expression levels also correlated with a decline in relative folding yields, indicating that efficient folding was a prerequisite for efficient ribosomal synthesis. PG lipids are known to retard OmpA folding, presumably because of the electrostatic repulsion between the negatively charged lipids and OmpA [37]. Although both PS and PG lipids are anionic lipids, there must be other differences between the two head groups which influence the expression and folding yield, and which make the expression in PG even lower than in the absence of lipid. It cannot be ruled out that the PG lipids have a generally deleterious effect on the ribosomal translational apparatus, since DOPG but not DOPS also inhibits expression of the helical membrane protein Mistic (D.D. and D.E.O., unpublished observations).

When we focus on the phosphocholine lipids, chain length also plays an important role (Fig. 2B), and both total synthesis and folding reach an optimum around 9–12 carbon atoms. A concentration dependent study showed that even at concentrations as low as 2.5 mg/ml, C_9PC and C_{10}PC lead to ~100% folding of the translated TM-OmpA (Figs. 2C and D), which is significantly better than any other vesicles. A possible explanation for the higher folding yield in these short chain SUVs is that the thinner bilayers are more flexible. This will facilitate both conformational changes on the surface and subsequent insertion of surface adsorbed OmpA. Interestingly, C_6-

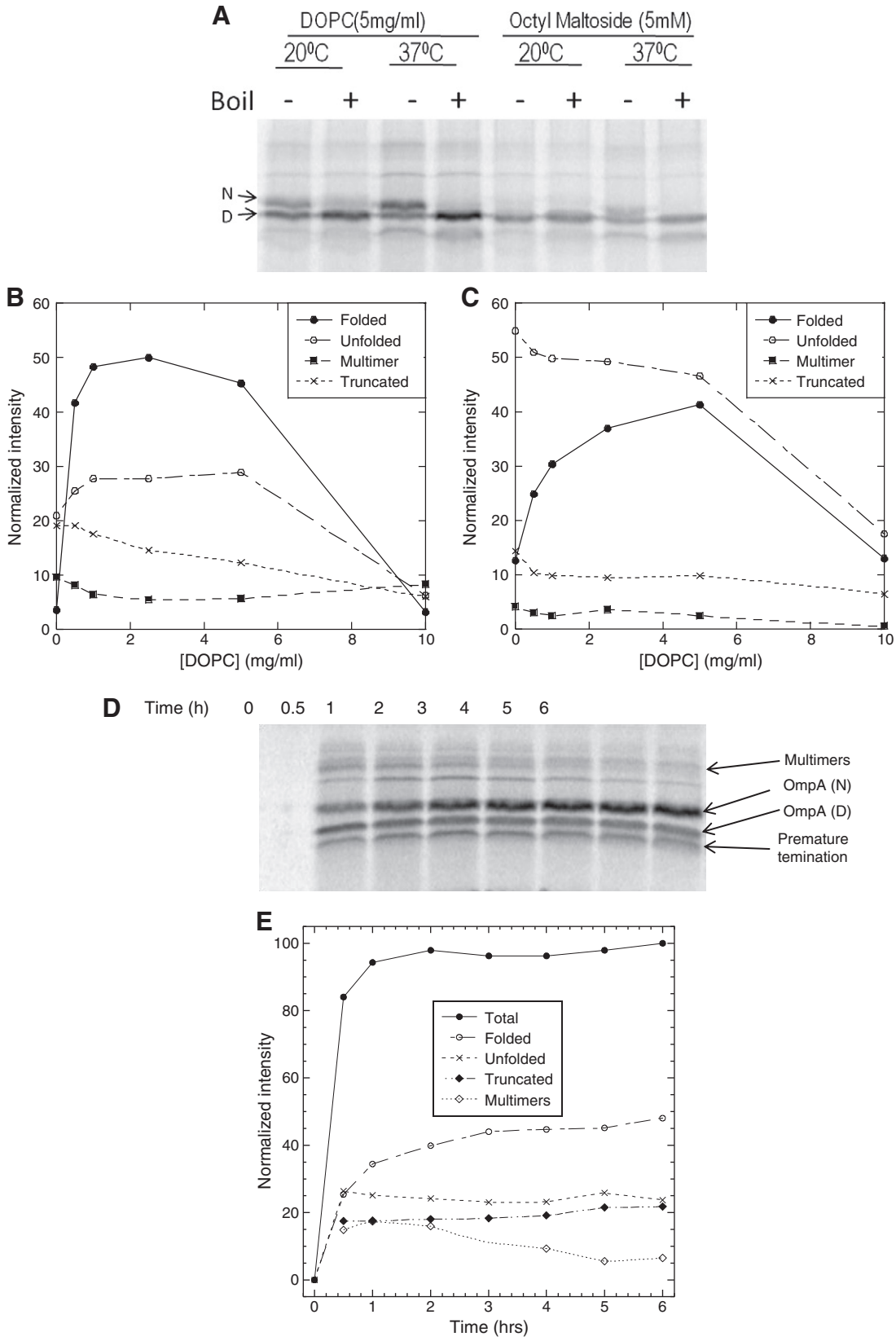

Fig. 1. (A) TM-OmpA expressed in presence of 5 mg/ml DOPC and 5 mM octyl-β-D-maltoside at 20 °C and 37 °C. Samples were run in 10% SDS-PAGE with and without boiling. (B) Populations of folded, unfolded, truncated and multimeric OmpA from cell-free expression at 37 °C at different DOPC concentrations. Data normalized to total intensity at 1 mg/ml DOPC (where maximal total intensity is reached). (C) As B with cell-free expression at 20 °C. Data normalized to total intensity at 5 mg/ml DOPC (where maximal total intensity is reached). (D) SDS-PAGE of the cell-free expressed TM-OmpA in 5 mg/ml DOPC at 37 °C as a function of time. (E) Time-dependent expression levels of the different species displayed in panel D, based on quantification of their band intensities. Data are normalized to total band intensity for all species at 6 hrs, including multimers and truncated forms.

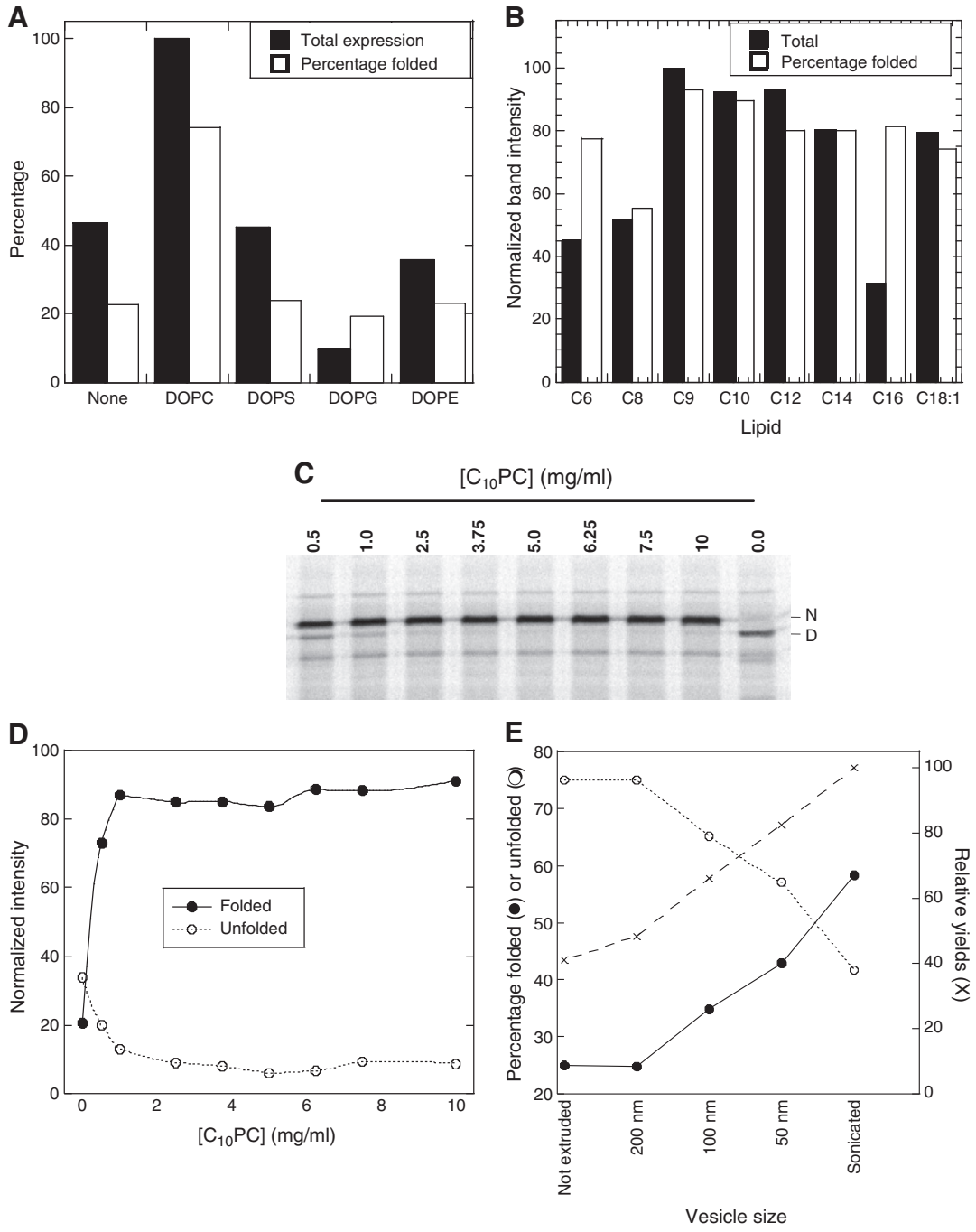

Fig. 2. (A) Relative expression of total monomeric and folded monomeric TM-OmpA in 5 mg/ml sonicated vesicles with different head groups at 37 °C. Data normalized to the highest total band intensity in DOPC. (B) Relative expression of total monomeric and folded monomeric TM-OmpA in 5 mg/ml sonicated vesicles with different chain lengths at 37 °C. Data normalized to the highest total band intensity (C_9PC). (C) SDS-PAGE of TM-OmpA translation in different concentrations of 5 mg/ml sonicated C_{10}PC vesicles at 37 °C. (D) Relative amounts of folded and unfolded monomeric OmpA in C_{10}PC based on data in panel C. (E) Relative amounts of folded and unfolded monomeric OmpA in DOPC vesicles of different sizes.

and C_8-PC led to significantly lower levels of expression and folding yields. These two lipids have such short chains that they form micelles rather than bicelles, with critical micelle concentrations around 12.5 and 0.1 mM, respectively. This is consistent with the adverse effects of octyl maltoside on folding (see Materials and methods) and emphasizes the superiority of using proper vesicles rather than micelles. However, small unilamellar vesicles of shorter chain length lipids (10–12 carbon chain lipids) lead to more folding of OmpA than longer chain lipids *in vitro* [38], probably due to a combination of hydrophobic matching and the bending fluctuations and surface curvature stress of the short chain vesicles. In agreement with this, translation studies in the presence of unilamellar DOPC vesicles of different sizes (non-extruded, 200, 100 and 50 nm) show that the folding fraction, as well as the overall synthesis, increases with decreasing vesicle size (Fig. 2E). This result is in good correspondence with previous *in vitro* observations on the need for SUVs to obtain good folding yields [17,18]. Because of their high curvature and intrinsic curvature stress, SUVs have more packing defects, facilitating protein insertion.

Expression yields fall dramatically as we go from DMPC to the longer chain length DPPC (Fig. 2B). This is most likely because the translation process occurs below DPPC's transition temperature

(42 °C), so that the nascent polypeptide chain is exposed to the gel phase which is more tightly packed than the liquid disordered phase. *In vitro* the gel phase effectively stalls folding of OmpA in a partially folded intermediate [39]. Nevertheless the folding percentage in DPPC in the cell-free system is essentially unaffected (Fig. 2B). This suggests that the gel phase of DPPC may temporarily trap the ribosome-nascent chain complex during the folding process and thus slow down the translation, while the actual folding proceeds at the same rate as for the liquid disordered phase. We speculate that the binding of the ribosome to the lipid, although slowing down the translation overall, may facilitate insertion by perturbing the lipid bilayer. Similar effects are seen for insertion of a small peptide into membranes, where folding is promoted by simple binding of the peptide to the membrane [40]. We subsequently analyzed the effect of mixing different lipids in the same vesicle. As previously mentioned, pure DOPG vesicles gave very poor OmpA yields (Fig. 2A). Even the inclusion of 7.5% DOPG in DOPC vesicles lowered protein expression by 45% and folding by 50% (data not shown). The effect of anionic lipids varies with chain length, since the addition of 7.5% of DMPG to DMPC (14 carbon atoms) did not affect yields and folding levels (Fig. 3A). However, mixed vesicles of 70% DMPC and 30% DMPG lowered the total protein expression by 47% and folding levels by 54%. Thus PG containing vesicle may not be suitable for *in vitro* coupled transcription-translation of TM-OmpA as seen for other membrane proteins [41]. Similarly, inclusion of 30% of the zwitterionic lipid phosphoethanolamine (PE) decreases expression yields both in DOPC and DMPC (Fig. 3A), while the folding level is essentially unaffected. DMPE is a non-bilayer-forming lipid which is known to have a complex effect on α-helical membrane protein insertion. It reduces the folding yield of bacteriorhodopsin [42], but has no direct effect on cell-free folding of the same protein [12]. It may in other cases act as a general folding chaperone, since it increases the stability of LacY [43] and promotes the tetramerization of α-helical membrane proteins such as KcsA [44] once it is folded into the membrane. PE lipids are reported to stabilize OmpA significantly in phospholipid vesicles *in vitro* against unfolding [45], but the effects of PE lipids on the actual insertion kinetics are not known and it is possible that the stabilization occurs at the expense of a high activation barrier for membrane insertion, leading to a lower folding yield.

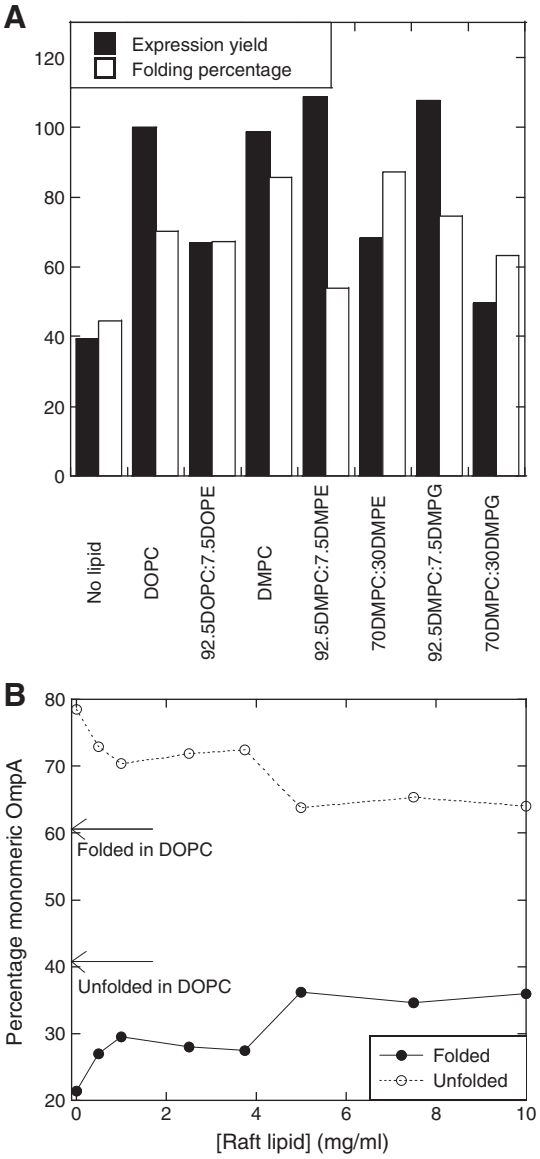

Fig. 3. (A) Total synthesis levels and folding percentages of TM-OmpA in different lipids normalized to the synthesis in DOPC. (B) Fraction of folded and unfolded TM-OmpA as function of lipid raft concentration.

3.3. Lipid rafts do not promote TM-OmpA expression and insertion

Having examined the effect of different chain lengths and head groups on TM-OmpA expression and folding, we now turn to the more complex lipid mixtures found naturally in lipid rafts. Lipid rafts are microdomains enriched in cholesterol and sphingolipids, and are thought to compartmentalize cellular process by serving as organising centers for the assembly of signaling molecule, influencing membrane fluidity and membrane protein trafficking and regulating neurotransmission and receptor trafficking [46]. Lipid rafts are more ordered and tightly packed than the surrounding bilayer but float freely in the membrane bilayer. Although lipid rafts do not occur in bacterial membranes, their ability to accommodate outer membrane proteins such as TM-OmpA may shed light on the effect of lipid organization on membrane protein insertion.

We therefore measured TM-OmpA membrane insertion in the presence of canonical synthetic lipid rafts composed of a 1:1:1 molar ratio of dipalmitoylphosphatidylcholine (DPPC), sphingomyelin and cholesterol (1:1:1 molar ratio) [47]. Lipid rafts are a very poor medium for TM-OmpA folding (Fig. 3B). The fraction of folded TM-OmpA is around 36%, which is the same as the degree of folding in the absence of lipids and significantly less than the 60% observed in 100% DOPC vesicles. While lipid raft formation may be important for the association of accessory membrane proteins to enhance membrane protein insertion, rafts clearly do not directly facilitate TM-OmpA protein insertion, presumably because of the tight packing that makes it difficult to insert membrane proteins into the bilayer.

3.4. Role of periplasmic chaperones

The periplasmic chaperone Skp stabilizes OmpA in an unfolded conformation, forming a complex which is unable to fold into the membrane [22]. Skp also has a dramatic effect on the cell-free translation of TM-OmpA. When TM-OmpA is translated in the presence of Skp, there is a marked increase in the intensity of a band (band C in Fig. 4A) migrating just below the denatured state (band B). A band of even smaller molecular weight (band D), which does not occur in the absence of Skp, also becomes very prominent. The intensity of the denatured state band is not affected, while the intensity of the folded state (band A) diminishes greatly. If we boil the samples, bands A (native state) and B (denatured state) merge to band B. This is expected because the native state is only resistant to SDS at room temperature and denatures at higher temperatures. However, bands C and D do not merge to one band upon boiling (data not shown), indicating that they do not represent differently folded conformers of the same sequence. Thus Skp facilitates the formation

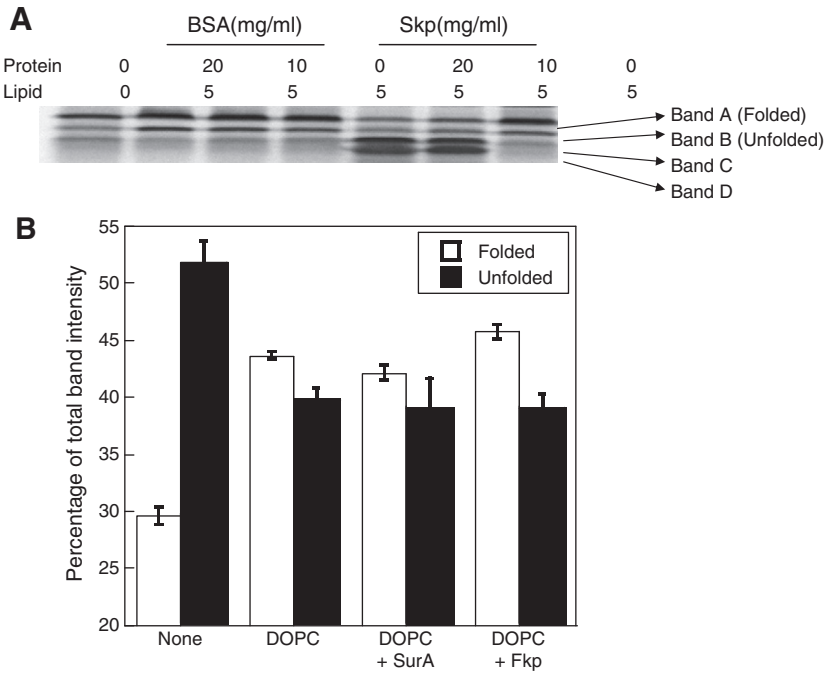

Fig. 4. (A) Expression and folding of TM-OmpA in the presence and absence of BSA and Skp. The lipid used is 5 mg/ml sonicated DOPC at 37 °C. (B) Expression and folding of TM-OmpA in the presence and absence of SurA and FkpA. Percentages are relative to total intensity of folded, unfolded and truncated bands.

of two lower molecular weight OmpA species. This phenomenon is not due to unspecific protein interference with the translation process, since BSA does not induce the formation of bands C and D (Fig. 4A), but must reflect a specific interaction of Skp with the ribosome-nascent polypeptide complex.

We speculate that Skp may bind to the newly synthesized polypeptide chain during cell-free translation and thus prematurely terminate polypeptide chain synthesis. Skp is localized in the periplasm *in vivo*, and thus cannot interfere directly with the biosynthesis of OmpA. Nevertheless, Skp is known to interact with the transmembrane N-terminal domain of OmpA, as well as other outer membrane proteins [48–50], during refolding *in vitro* [22]. In the presence of zwitterionic lipids such as DOPC, Skp actually retains OmpA in an unfolded form; it only releases OmpA in the presence of negatively charged lipids [37]. This is consistent with our observation that Skp binds strongly to OmpA at an early stage in the polypeptide chain synthesis. It is likely that the Skp-OmpA interaction prevents the membrane protein's hydrophobic collapse and misfolding, allowing it to be delivered to the outer membrane. The physical separation of Skp from the ribosome by the inner membrane may thus be important to avoid unwanted interference of Skp with the translation process. In addition, Skp is highly basic (pI = 9.5) and may modulate the surface properties of the periplasmic leaflet of the outer membrane [34]. Kleinschmidt and co-workers have shown that Skp in combination with LPS enhanced the rate of folding as well as yield of folded protein. Even at pH 10, Skp binds to TM-OmpA fragments and prevents their association [51], which indicates that positively charged Skp can bind negatively charged TM-OmpA fragments.

We also examined two other periplasmic chaperone proteins, namely Fkp and SurA, which function both as chaperone and folding catalyst (PPIases) [50]. However, Fkp showed a very modest increase in the yield of folded TM-OmpA (2% compared to the DOPC control), while SurA had no effect (Fig. 4B). Furthermore, there are no reports of FkpA interacting with outer membrane proteins [50]. There is more evidence for SurA's role in outer membrane protein folding and transport. A *surA* null mutant exhibits reduced levels of properly folded LamB, OmpA, OmpC and OmpF in the outer membrane, indicating that it functions in folding and assembly of OMPs [52]. SurA has also been reported to interact with some of the OMPs like immature PhoE, LamB and OmpF as well as mature PhoE [53]. Nevertheless, our data suggest that this interaction may be indirect and mediated by other components, rather than a direct interaction between SurA and the unfolded TM-OmpA chain.

3.5. Pulse chase experiments

To monitor the time-course of insertion of TM-OmpA synthesized within a short period, we performed pulse chase experiments in the presence of three different lipids, namely C_{10}PC, DMPC and DOPC. TM-OmpA behaved very differently in the three lipids. In the presence of C_{10}PC, TM-OmpA inserts into the membrane as soon as it is synthesized from the ribosome (Fig. 5A). In DMPC vesicles, TM-OmpA also folds into the vesicles rapidly but at a slightly lower overall folding yield, indicating that DMPC vesicle does not favor folding as efficiently as C_{10}PC. In DOPC, unfolded TM-OmpA is initially accumulated as unfolded protein and then gradually folds to a level of ~50%. Interestingly, some higher molecular weight bands of around 30 and 40 kDa, corresponding in weight to dimers of folded (band A) and unfolded (band B) TM-OmpA, accumulate at the beginning of the pulse labelling experiment (Fig. 5B). With time, band A disappears, while the intensity of band B remains constant throughout the 2 hour time of the pulse chase experiment. This indicates that initially both unfolded TM-OmpA and dimeric TM-OmpA accumulate; the folded dimer slowly dissociates and the unfolded monomer but not the unfolded dimer can slowly fold to the native state.

Interestingly, dimer formation is lipid dependent. In C_{10}PC and DMPC, dimer levels are negligible (they constitute 6–7% of total protein content at 20 min, and only 1.8–2.0% after 2 h). However, in DOPC dimers initially reach about 16% and only reduce to 10% after 2 h. Similar levels of higher order TM-OmpA in DOPC were observed in the time-dependent expression and folding followed without pulse chase experiments (Fig. 1E). We speculate that dimer formation competes with monomer folding and insertion. This means that the lipids that allow efficient insertion reduce dimerization. Based on this correlation, we suggest that translation may occur in concert with

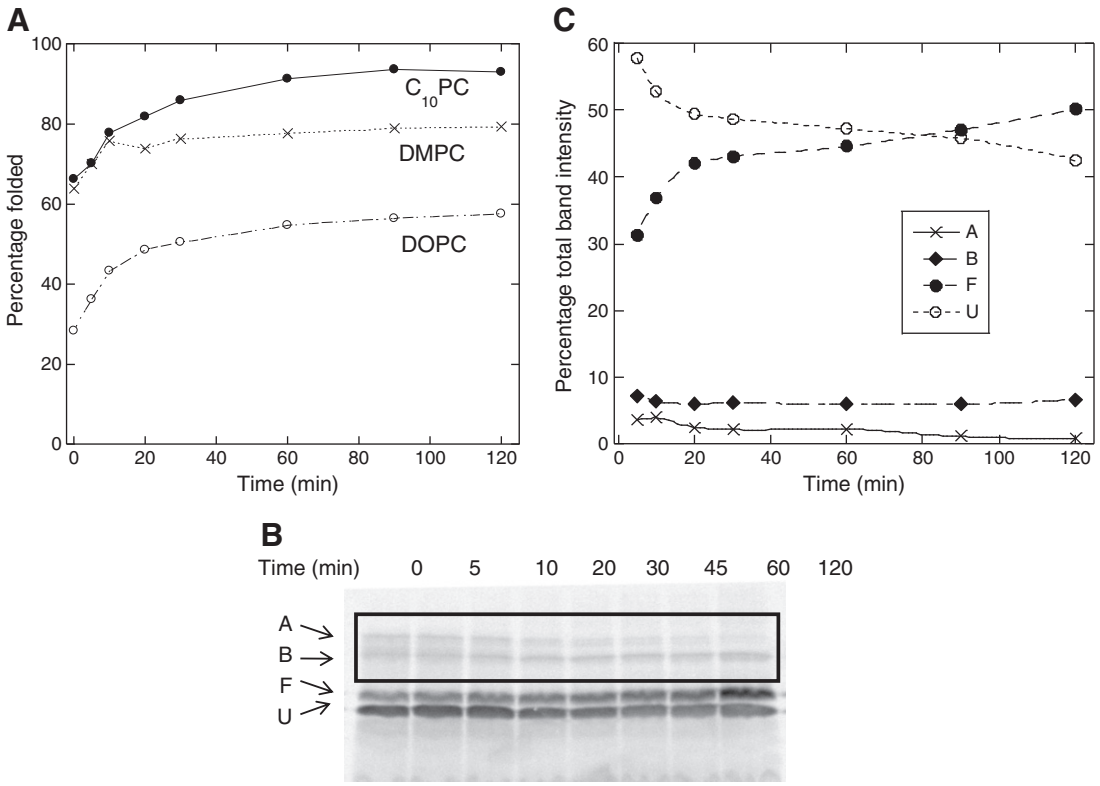

Fig. 5. Pulse chase experiments of TM-OmpA expression and folding. (A) Pulse chase expression and folding of TM-OmpA in the presence of three different lipid vesicles (C_{10}PC, DMPC and DOPC). After 20 min of expression, 100 mM cold methionine was added and at different time points mixture taken out, the reaction stopped with 1× SDS loading buffer and the fraction folded TM-OmpA quantified by SDS-PAGE. Folding percentages normalized relative to total amount of monomeric OmpA. (B) Formation of higher molecular weight OmpA bands during pulse chase expression and folding of TM-OmpA in 5 mg/ml DOPC.

membrane insertion and folding for the following reason: Less efficient folding means that insertion occurs more slowly, which again implies that the emerging polypeptide chain has time to form non-native interactions and dimerize. These dimers can continue to fold as separate domains and then slowly dissociate to native monomers. In addition, lack of efficient membrane insertion can possibly allow the nascent chain to fold back on itself in non-native interactions, block the orderly exit of protein from the membrane and thus stall ribosomal synthesis at a premature stage.

4. Conclusion

There are several advantages to the use of a cell-free transcription-translation system to study folding of membrane proteins. Firstly, we can monitor both overall folding yields as well as the ratio between folded and unfolded states. Secondly, the protein is produced *in situ* in the presence of a well-controlled amount of lipid of defined composition, and is unfolded from the start without the aid of denaturants which otherwise have to be diluted out to allow the protein to fold. This allows us to carry out the experiments at physiological pH, rather than the elevated pH required to avoid non-productive aggregation of OmpA in the aqueous phase [17,34]. Reassuringly, our results are consistent with *in vitro* studies carried out by Kleinschmidt, Tamm and others at elevated pH, indicating that it is possible to extrapolate from high pH to physiological conditions. Thirdly, although the yield of TM-OmpA in the present transcription-translation system (~0.25 μg/ml) is several orders of magnitude lower than that required for structural studies [54], the low concentrations mean that we minimize artifactual protein aggregation which may occur in other *in vitro* folding studies. We have used a transmembrane construct rather than full-length OmpA, which also has a C-terminal periplasmic domain (residues 177–325) in order to focus entirely on the membrane insertion process. The absence of separating membrane chambers means that we can dispense with signal sequences. Finally, we can monitor the formation of truncated forms of OmpA and interpret their significance within the context of protein folding. To our knowledge, no reports have been made of such truncated forms in a cellular context. We speculate that these forms are difficult to detect *in vivo* because they will be recognized as misfolds and rapidly degraded. Furthermore, the effect of Skp is unlikely to be replicated *in vivo* because the separation between the periplasmic and cytosolic compartments. Nevertheless, the cell-free transcription-translation set-up allows us to monitor whether periplasmic chaperones have an intrinsic preference for emerging parts of the polypeptide chain, and this only seems to be the case for Skp, consistent with its reported strong interaction with unfolded OmpA [37] and in contrast to the weak or non-existing interactions with SurA and Fkp.

Lipid head groups play a remarkable role in expression and insertion. Among the different head group lipids tested, PC containing vesicles are best both for expression and folding. Among the different PC vesicles, short chain vesicles are most effective in folding the TM-OmpA. Insertion and folding may depend on bilayer flexibility and surface curvature stress and therefore indirectly on lipid chain length. A more flexible bilayer might support conformational changes of surface adsorbed folding intermediates that lead to insertion and folding. In line with this, larger vesicles did not support the insertion of protein since they have fewer defects than the smaller vesicles and thus fewer openings for membrane proteins to insert into. Among the three different periplasmic chaperones tested, only Skp significantly affected synthesis and insertion. The emergence of two truncated species in the presence of Skp is an unexpected observation that indicates a very intimate interaction between Skp and the nascent chain of TM-OmpA.

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