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Phospholipid dependency of membrane protein insertion by the Sec translocon

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ABSTRACT

Membrane protein insertion into and translocation across the bacterial cytoplasmic membrane are essential processes facilitated by the Sec translocon. Membrane insertion occurs co-translationally whereby the ribosome nascent chain is targeted to the translocon via signal recognition particle and its receptor FtsY. The phospholipid dependence of membrane protein insertion has remained mostly unknown. Here we assessed in vitro the dependence of the SecA independent insertion of the mannitol permease MtlA into the membrane on the main phospholipid species present in *Escherichia coli*. We observed that insertion depends on the presence of phosphatidylglycerol and is due to the anionic nature of the polar headgroup, while insertion is stimulated by the zwitterionic phosphatidylethanolamine. We found an optimal insertion efficiency at about 30 mol% DOPG and 50 mol% DOPE which approaches the bulk membrane phospholipid composition of *E. coli*.

1. Introduction

Membrane protein biogenesis is a vital process in all forms of live. In bacteria, proteins are inserted into the membrane by the SecYEG translocon, which involves the co-translational targeting of the ribosome nascent chain complex (RNC) by signal recognition particle (SRP). SRP binds to the first transmembrane domain of the nascent membrane protein when it emerges from the ribosome [1]. Next, the RNC, in association with SRP, is targeted towards the membrane where it associates with the SRP receptor (FtsY) to form a heterodimeric SRP-FtsY complex [2]. The latter are released from the nascent chain upon GTP hydrolysis whereby the RNC is transferred to the SecYEG translocon [3,4]. SecA, a motor protein involved in the post-translational translocation of proteins across the membrane, may associate with the inserting nascent protein when there is a demand for the translocation of larger hydrophilic polypeptide segments across the membrane [5,6].

The membrane environment is critically important for the functioning of the Sec translocon. So far, the influence of the membrane phospholipid composition has mostly been studied for protein translocation. Here, anionic phospholipids are an essential component of the membrane [7–10]. The *Escherichia coli* inner membrane is composed of the zwitterionic phospholipid phosphatidyl-ethanolamine (PE, ~50–70 %) and the anionic phospholipids phosphatidylglycerol (PG, ~40–20 %) and cardiolipin (CL, 0–10 %), depending on the stage of growth [11,12]. PG provides a negative charge to the membrane, whereas highly unsaturated PE shows polymorphism and forms non-bilayer structures in isolation. Previous studies have shown that for viability, both elements, i.e., negative charge and phospholipid polymorphism are critical aspects of a functional membrane environment influencing many membrane-associated processes. Indeed, when the PG content is reduced, protein translocation is abolished [7,10]. Anionic phospholipids affect the protein translocation mechanism at different levels. First, anionic phospholipids induced alfa-helicity to the signal sequence of secretory proteins, and this is believed to be critical for the opening of the translocation channel [13,14]. Further, SecA associates with anionic phospholipids through its amphipathic N-terminus and this inflicts a conformational change to SecA which allows it to bind with high affinity to the SecYEG translocon activating its ATPase activity [15,16]. In the absence of PG and CL, or any other anionic phospholipids, these processes are abrogated. Interestingly, anionic phospholipids are enriched in the annular phospholipid shell of the SecYEG translocon further highlighting their importance [17]. Molecular dynamics simulations have identified three sites of interaction: i) at the interface of SecG and SecY; ii) at the interface of SecG and the amphipathic N-terminus of SecA; and iii) close to the lateral gate [18]. The exact roles of these three sites of PG binding are not known, but distinct roles have been proposed. The PG that interacts with the lateral gate, may induce the alfa-helicity of the signal sequence as suggested by studies with model signal

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sequence peptides, whereas the two other sites may facilitate SecA binding and further conformational transitions of the SecYEG translocon. Bulk concentrations of PE are also important for translocation but this effect is less pronounced as for anionic phospholipids [10]. It was suggested that unsaturated PE (dioleoyl PE) stimulates protein translocation through it non-bilayer forming ability as saturated PE variants that do not show this polymorphism do not stimulate translocation.

In contrast with translocation, the effect of phospholipids on membrane protein insertion has not been studied systematically. Since MD simulations indicated enrichment of PG near the SecY-SecG interface gate [18], which is not only important for SecA interaction but also for the ribosome interaction [19], a major influence of the phospholipid composition is also expected for membrane protein insertion. Structural studies with the SecYEG-RNC complex in lipid filled nanodiscs indicate that the ribosome approaches the membrane close to the lateral gate thereby creating a local membrane distortion enriched with zwitterionic phospholipids [19,20]. Depending on the study, these zwitterionic phospholipids were identified as PE or phosphatidylcholine (PC), although the latter is not present in the *E. coli* membrane. Further, FtsY associates with anionic phospholipids and potentially this could influence membrane protein insertion [21,22]. Lastly, as SecA is involved in the translocation of large periplasmic domains of membrane proteins, an anionic phospholipid dependence is expected for membrane proteins that harbor such domains [23].

Here, we have studied the phospholipid dependence of the insertion of mannitol permease A (MtlA), which is the membrane integrated domain of the mannitol phosphotransfer system. Since membrane insertion of MtlA is not dependent on SecA, the observations represent the isolated dependence of the insertion process on phospholipids [24]. The data indicates a strong dependence on both PG and PE, although the latter dependence seems not associated with its potential non-bilayer forming properties. Possible mechanisms underlying these dependencies are discussed.

2. Methods

2.1. Cloning

The expression vector for MtlA was obtained via amplification of the *mtlA* gene from *E. coli* BL21 using the primers 1-MtlA-F-NdeI and 2-MtlA-R-*Xho*I. The resulting PCR fragment was cloned into the overexpression vector pET-Duet1 forming pET-Duet-MtlA which was transformed into DH5α (Table 1). Plasmids were purified using NucleoBond Xtra Midi kit from Bioké.

2.2. Overexpression SecYEG, ffh and FtsY

All strains and plasmids are listed in Table 1. Overexpression of SecYEG/YidC [25], Ffh [26] and FtsY [27] was done as previously described. SecYEG, YidC and FtsY were overexpressed in *E. coli* BL21 (DE3) and Ffh in *E. coli* BL21 (DE3) PlysS. Cells were grown in LB media containing the appropriate antibiotics starting by inoculation from an overnight culture at an OD_{600} of 0.05 and induction of overexpression of the various cell cultures was done at an OD_{600} of 0.6 using 0.5 mM ITPG after which cells were grown for an additional 2 h. Cells were harvested by centrifugation (6000 rcf, 4 °C, 15 min) and resuspended in their respective buffer, SecYEG/YidC: 50 mM Tris-HCl, pH 8, 100 mM KCl and 15 % glycerol; FtsY: 20 mM HEPES, pH 7.5, 150 mM KCl and 10 % glycerol; ffh: 20 mM HEPES, pH 7.5, 60 mM NH_4Cl, 7 mM $MgCl_2$, 150 mM KCl and 10 % glycerol. Following addition of DNAse(Merck) and protease inhibitors (Roche), cell were disrupted by done by French press (Constant Cell Disruptor Systems, LA biosystem) at 12.5 kpsi. Unbroken debris was removed by centrifugation (6000 rcf, 15 min, °C). FtsY and Ffh were purified from the cell lysate. For SecYEG, total membranes were first isolated by ultra-centrifuge (280.000 rcf, 90 min, 4 °C) and resuspended in the same buffer.

2.3. Purification of SecYEG, YidC, Ffh and FtsY

Isolated total membrane fractions containing SecYEG or YidC were solubilized in 2 % n-dodecyl β-D-maltoside (DDM), incubated with Ni^+ NTA beads (Qiagen) for 30 min and subsequently transferred to a Bio-spin micro column (Bio-Rad). The beads were washed with 10 column volumes of washing buffer containing 50 mM tris-HCl, pH 8.0, 100 mM KCl, 0.05 % DDM, and 40 mM imidazole. SecYEG or YidC were eluded at an imidazole concentration of 200 or 300 mM respectively.

Ffh was purified with a similar method. Following a 1 h incubation of the cell lysate with Ni^+ NTA beads and transfer to a Bio-spin micro column, beads were washed using 10 column volumes of 20 mM Hepes, pH 7.5, 60 mM NH_4Cl, 7 mM $MgCl_2$, 1 M KCl, and 20 mM imidazole. Ffh was eluded with 250 mM imidazole. Likewise, FtsY in a cellular lysate was bound to Ni^+ NTA beads that were washed with 20 mM HEPES, pH 7.5, 1 M KCl, 10 % glycerol, and 20 mM imidazole and eluded with 150 mM KCl and 200 mM imidazole. Purified Ffh and FtsY were subjected to buffer exchange to 20 mM HEPES, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$ and 10 % glycerol on a Econo-Pac 10DG desalting column (BioRad). Concentrations of all purified proteins were estimated by SDS-PAGE and spectrophotometrically using the extinction coefficients of 71.000 M^{-1} cm^{-1}, 19.480 M^{-1} cm^{-1} and 2.980 M^{-1} cm^{-1} for SecYEG, FtsY and Ffh, respectively.

Table 1
Strains, plasmids and primers used. Underlined sequences indicate overlapping region between the two primers.

	Short description	Source
Strains		
E. coli DH5α	Strain used for cloning and plasmid production	Invitrogen
E. coli BL21 (DE3)	Overexpression strain	[28]
E. coli BL21 (DE3)PlysS	Overexpression strain for reduced background	[29]
E. coli BL21 Rosetta (DE3)	Used for the formation of cell lysate	Novagen
Plasmids		
pET-Duet-1	Expression vector (Amp^r), T7 promotor	Novagen
pET-Duet-MtlA	*E. coli mtlA* under T7 promotor	This paper
pET610	N-terminally his-tagged SecYEG from *E.coli* with ΔHincII in SecE under *trc* promotor	[30]
pET9-FtsY	C-terminally his-tagged *ftsY* from *E. coli* under T7 promotor	[31]
pETffh	C-terminally his-tagged *ffh* from *E. coli* under T7 promotor	[32]
pTrc99 SecA	*E. coli* SecA under *trc* promotor	[15]
pEH1hisYidC	N-terminally his-tagged *yidC* from *E.coli* under *lac* promotor	[33]
Primers	**Sequence**	**Restriction site**
1-MtlA-F-NdeI	GAAGGGGTGTTCATATGTCATCCGATATTAAG	*NdeI*
2-MtlA-R-XhoI	ACCTTCTCCATGTCTCGAGGGTGGGATTGGAT	*XhoI*
3-RNA-Template-F	TAATACGACTCACTATAGGGGGCTCTGTTGGTTCTCC-CGCAACGCTACTCTGTTTACCAGGTCAGGTCCGGAAGG	–
4-RNA-Template-R	GGGTGGGGGCCCTGCCAGCTACATCCCGGCACACGCG-TCATCTGCCTTGGCTGCTTCCTTCCGGACCTGACCTGGTA	–

2.4. SecYEG reconstitution

Protein reconstitution was performed in liposomes containing the desired lipid ratios. Chloroform stocks were mixed to the appropriate ratio, air dried and subsequently formed lipid film was resolubilized in Buffer A (50 mM Tris-HCl pH 8.0, 100 mM KCl, 2 mM DTT) to create multilamellar vesicles (MLVs). MLVs were diluted to 5 mM and made small unilamellar vesicles (SUVs) by sonication on ice (3 × 5 seconds, 4 μm). Liposomes containing no or a very limited amount of anionic lipids were sonicated more cycles (up to 40 × 5 seconds 4 μm when anionic lipids were completely absent) until the opacity decreased to a similar level compared to the other lipid mixtures.

SUVs were solubilized by 0.5 % triton X-100 and combined with purified SecYEG for a 1:500 SecYEG:phospholipid ratio. For co-reconstitution with YidC, a 1:1:500 SecYEG:YidC:phospholipid ratio was used. Triton was removed by several rounds of bio-beads (2 × 1 hour, 16 h and a final 30 min) where the solution was transferred to fresh bio-beads in each round. Proteoliposomes were harvested by ultra-centrifugation (TLA 110, 550.000 rcf, 30 min) and resuspended in halve the starting volume using Buffer A. LC-MS was used to determine the concentration of the obtained liposomes whereby samples were diluted to 5 mM phospholipid before used experimentally.

2.5. RNA synthesis

To acquire the 4.5S RNA required for SRP we created a dsDNA segment containing a T7 promotor prior to the 4.5S RNA segment which could subsequently be transcribed by T7 polymerase. The dsDNA template strand was formed by a short PCR (10 cycles: 15 s 98 °C, 30 s 50 °C, 20 s 72 °C) on primers 3 and 4. Transcription was performed using the MEGAscript kit (ThermoFisher) as described by the manufacturer, following the indications for short transcripts (<300 bp). The reaction was terminated by diluting 5 times and the addition of 600 mM ammonium acetate and 12 mM EDTA. RNA was isolated by the addition 1 volume of TRizol/chloroform followed by 1 volume chloroform. RNA was recovered by mixing the aqueous phase with 1 volume isopropanol, incubating at −20 °C for 15 min and centrifuging at 12.000 rcf at 4 °C for 15 min. The pellet was dissolved in RNAse free water. RNA concentrations were determined by with a NanoDrop.

2.6. Cell lysate preparation

E. coli Rosetta (DE3) were grown in 2× YT + P media (2× YP supplemented with 22 mM KH_2PO_4, 40 mM K_2HPO_4 and 2 % glucose) at containing 25 μM chloramphenicol 37 °C. To obtain exponentially growing cells, single isolated colonies were used for inoculation and growth for 12 h was started. This overnight culture was diluted 100-fold in fresh media, and grown for an additional 8 h. Larger scale cultures were started at an OD_{600} of 0.05 and grown until an OD_{600} of 0.6. Cells were harvested by centrifugation (4.000 rcf, 4 °C, 15 min), resuspended in Buffer B (20 mM Tris/HCl, pH 7.5, 60 mM KOAc, 14 mM KOAc) with 7 mM β-mercaptoethanol, centrifuged again and resuspended in 1.4 ml per gram of cell in Buffer B with 1 mM DTT. Cells were lysed by French press and unbroken cells were removed by centrifugation (4.000 rcf, 4 °C, 15 min). First round of membrane removal was done by 2 times centrifugation at 30.000 rcf (4 °C, 30 min). mRNA was removed by incubation in the dark at 37 °C for 90 min. Lastly, membranes were removed by an additional 2 rounds of centrifugation at 135.000 rcf (4 °C, 30 min). The lysate was dialyzed overnight against Buffer B (pH 8.2) and stored −80 °C for up to several months.

2.7. Protein synthesis and insertion assay

Isolated lysate (2/5 of total volume of final reaction) was mixed with 0.5 mM (proteo)liposomes, 40 units T7 RNA Polymerase (Thermo), 40 units RNase Inhibitor (Thermo), 19 amino acids (2 mM of each, no methionine), 25 pg/ul Plasmid DNA, 200 μg/ml creatine kinase and an energy mix containing: 55 mM Tris-HCl, pH 7.5, 35 μg/ml folinic acid, 28 mM NH_4OAc, 10 mM $Mg(OAc)_2$, 0.05 % NaN_3, 1.7 mM DTT, 1.2 mM ATP, 0.8 mM GTP, 0.8 mM CTP, 0.8 mM UTP, 0.65 cAMP, 80 mM creatine phosphate, 20 mM K-glutamate, 10 μg/μl tRNA (*E. coli*), 2 % PEG 8000, and 1 mM spermidine. For reaction with PURE (New England Biolabs), product specifications were followed with the addition of 40 units T7 RNA Polymerase and 40 units RNase Inhibitor. Reactions in lysate and the PURE system were started by the addition of 5.5 μCi [^{35}S]-methionine/[^{35}S]-cysteine from the Easytag Protein Labeling Mix (Perkin Elmer). Reaction was performed at 37 °C for 40 min (4 h for PURE reactions). To obtain a membrane protected fragment of MtlA, reactions were equally split in two and supplemented with 5 μg/μl proteinase K. Samples were incubated on ice for 30 min. Proteins were precipitated by addition of 5 volumes 10 % TCA, incubated on ice for 30 min, and centrifuged (16.000 rcf, 5 min). The obtained pellet was washed twice with ice cold acetone and resuspended in 2× SDS sample buffer. Samples were run on SDS-PAGE, dried and imaged via autoradiography. Raw images were analyzed on band intensity using ImageJ. For visibility, images were adjusted on brightness and contrast. Insertion experiments were reproduced at least twice, and typical results are shown.

2.8. LC-MS phospholipid analysis

To determine the phospholipid concentrations, LC-MS was performed as described earlier [34]. In short, lipids were isolated by 1-butanol extraction and airdried to form a lipid film. The films were dissolved in methanol and injected on a Waters ACQUITY Premier CSH C18 (1.7 μm, 2.1 × 150 mm) column. Separation was done by a gradient from eluant A: MQ:MeCN (40:60) containing 5 mM ammonium formate to eluent B: MQ:MeCN:1-BuOH (0.5, 10:90) also containing 5 mM ammonium formate. MS specifications and settings were described before [35]. Spectral data was analyzed using Scientific XCalibur processing software. 10:0 PG was used as an internal standard used for normalization. Total phospholipids leftover in cell lysate were estimated by summation of all known lipid species, and the normalized ion counts were calibrated to a lipid standard containing a known amount DOPG and DOPE [34].

3. Results

3.1. Reconstitution of MtlA membrane insertion

To study the phospholipid dependence of membrane protein insertion, the integral membrane subunit MtlA of the mannitol phosphotransferase system was chosen for two reasons. First, MtlA contains a predicted number of six or eight transmembrane segments followed by a large cytoplasmic domain [36,37]. However, the Alphafold2 models suggest a more complex membrane fold (Sup. Fig. 1) [38,39]. Importantly, both the N- and C-termini of MtlA face the cytosolic side of the membrane. For MtlA, a convenient in vitro membrane insertion assay has been developed where once the protein has been inserted into the membrane, the membrane-embedded domain becomes inaccessible to proteinase K (protK) digestion, whereas the exposed C-terminal domain can be degraded (Fig. 1) [6]. The membrane protected fragment can then be visualized by SDS-PAGE as a 30 kDa proteolytic fragment. Second, membrane insertion of MtlA occurs independently of SecA [6,24], which precludes that observations on phospholipid dependence are due SecA which shows a strict requirement for acidic phospholipids for activity. The *E. coli mtlA* gene was cloned into a pET-Duet-1 expression vector under control of the T7 promotor for use in an in vitro expression system by addition of T7 polymerase. Herein, a cell lysate was obtained from the *E. coli* BL21 Rosetta (DE3) strain isolated during the exponential growth phase. To prevent background activity, endogenous membranes were removed from the lysate by two consecutive ultra-centrifugation runs at 135.000 rcf followed by careful

transfer of the supernatant yielding an S135 lysate. To validate that there are no remaining membrane contamination in the S135 lysate, MtlA insertion assays were performed without the addition of SecYEG proteoliposomes followed by proteinase K digestion (Fig. 2a, lane 1–2). Under those conditions, no proteolytic 30 kDa fragment of MtlA could be detected. Furthermore, we used LC-MS analysis to assess the residual concentration of phospholipid present in the lysate which was estimated at 63 μM per reaction. This background level is small compared to the 500 μM of proteoliposomes used in each further reaction, is low. Next, SecYEG was reconstituted into detergent destabilized small unilamellar vesicles (SUVs) with a SecYEG:phospholipid molar ratio of 1:500. Using LC-MS analysis we determined that 30 μM of PG and PE, respectively. Post-reconstitution, we similarly determined that 1.4 % and 1.7 % of phospholipids in the obtained proteoliposomes were these native PE and PG species indicating that proteoliposomes containing no PE of PG can technically not be obtained. When the lysate was supplemented with SecYEG proteoliposomes, about 60 % of the MtlA synthesized was inserted (Fig. 2b, lane 3–4). Further, when empty liposomes were used instead, only a faint signal (about 12 % of MtlA inserted) could be observed (Fig. 2b, lane 1–2) which may relate to spontaneous insertion as noted by Nishiyama et al. who showed that this activity could be inhibited by diacylglycerol [40]. Our results are in agreement with the notion that correct membrane insertion of newly synthesized MtlA is dependent on the SecYEG complex (Fig. 2b).

3.2. Anionic phospholipids stimulate membrane protein insertion

To examine the influence of phospholipids on MtlA insertion, first, the concentration of acidic phospholipids was varied in the proteoliposomes. The main anionic phospholipid in *E. coli* is phosphatidylglycerol (PG). Proteoliposomes were prepared containing 30 mol% phosphatidylcholine (DOPC) to compensate for the reduction in DOPG. MtlA insertion showed a strong dependence on DOPG with an optimum around 30 mol%, which corresponds to the highest levels of PG observed in *E. coli* (Fig. 3, lane 9–16). However, also in the absence of PG, significant insertion levels (39 % of total protein synthesized) were observed showing that insertion is not strictly dependent on the exogenous DOPG (lane 9). At high PG levels, the amount of inserted MtlA was reduced. However, this appears to be a result of a decrease in the total synthesis of MtlA rather than a reduction in the fraction that is inserted, suggesting a slowdown or inhibition of translation (lane 15, 16). The efficiency of MtlA insertion appears dependent on the growth phase used to harvest the cell lysate. Lysates derived from cells grown until late log phase, and that were harvested at a higher OD_{600} (1.5–2.0) levels (Fig. 2), as compared to mid-log phase cells (OD_{600} 0.6) (Fig. 3) showed a reduced total synthesis of MtlA as well as lower fraction of MtlA inserted (40 % versus 85 %). Overall, there seems to be a correlation between the synthesis activity of the lysate and the insertion activity when employing SecYEG proteoliposomes, but this phenomenon was not studied further. Overall, these data show that membrane insertion of MtlA is strongly stimulated by the presence of PG. It should be noted that the lower level of insertion observed in the absence of exogenous DOPG could relate to the low, but significant level, of endogenously bound PG.

To establishes whether the dependence on PG is specific for this phospholipid species or due to a general dependence on anionic, i.e., negatively charged phospholipids, 30 mol% DOPG was replaced by 30 mol% phosphatidylserine (DOPS) or 15 mol% cardiolipin (CL) with di/tetraoleoyl acyl chains. To compensate for the double negative charge present on CL, only 15 mol% CL was used and combined with 15 mol% DOPC. We found no significant differences in MtlA insertion when comparing the SecYEG proteoliposomes containing PG, PS or CL (Fig. 4), indicating that the negative charge at the polar headgroup of these phospholipid species is the main determinant for the dependence.

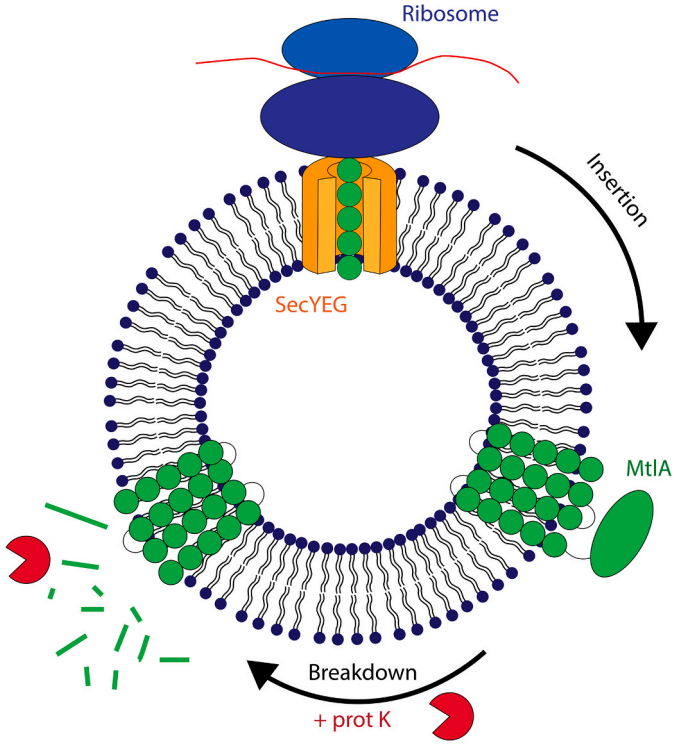


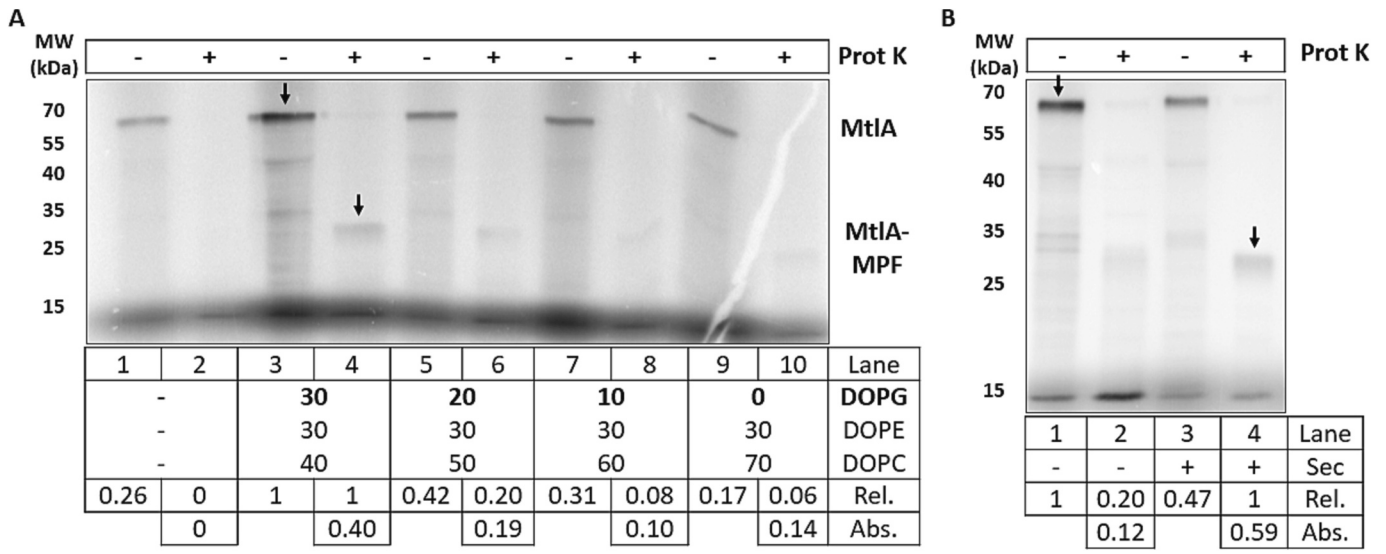
Fig. 1. Schematic illustration of MtlA synthesis and digestion by proteinase K.

3.3. MtlA insertion is enhanced by phosphatidylethanolamine

The other main phospholipid component of the *E. coli* membrane is phosphatidylethanolamine (PE), which is zwitterionic and depending on the degree of acyl chain unsaturation, also can adopt a conical non-bilayer state. SecYEG proteoliposomes were prepared with 30 mol% DOPG and 0–70 mol% DOPE, and 70–0 % DOPC. Again, there was a strong dependency with an optimal concentration of about 60 mol% DOPE (Fig. 5, lane 9–16). At 0 mol% DOPE, still substantial insertion occurred (lane 9). Again, total synthesis was also reduced at lower DOPE levels (lane 1–8). To assess if the stimulatory effect of PE is related to the non-bilayer properties of DOPE, as was observed for translocation, DOPE was replaced by the distearoyl-PE (DSPE) that harbors two saturated C18 acyl chains. Due to the high melting temperature (74 °C) of this phospholipid, the bilayer to hexagonal phase transition only occurs at a temperature far above the assay temperature [41]. The level of insertion at 50 mol% DSPE was similar to that observed with DOPE, and also the concentration dependence was similar (Fig. 6). Thus, the PE requirement cannot be attributed to the polymorphism of this phospholipid but rather depends on the zwitterionic nature of the polar head group that potentially can act on proteins through hydrogen bonding.

3.4. Membrane insertion of MtlA synthesized with the PURE system

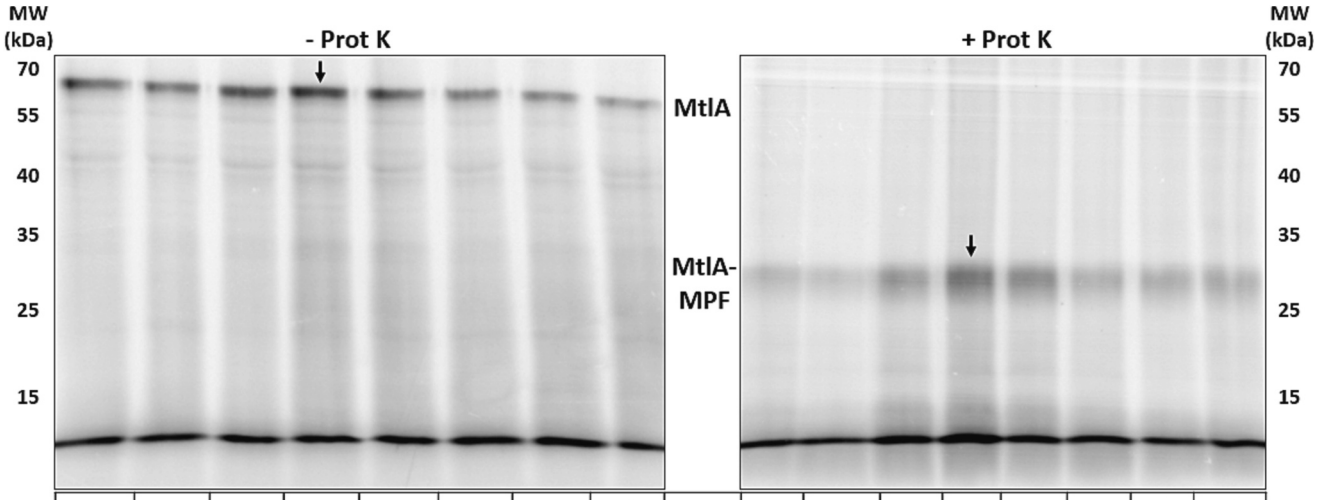
The S135 lysate of *E. coli* cells contains all cytosolic proteins, including the targeting components SRP and FtsY. In recent years, a more defined system has become available for protein synthesis which is the PURE (Protein synthesis Using Recombinant Elements) system [42]. All the components required for transcription and translation are added to the system as purified components to yield a functioning synthesis system. The PURE system lacks Ffh, the protein component that combines with 4.5S RNA to form SRP, FtsY and all other chaperones that could potentially fulfil a role in membrane protein insertion. Previous studies have shown that the PURE system can be used for membrane protein insertion in combination with urea washed inverted membrane vesicles (INVs) [43]. To examine if MtlA insertion in the PURE system shows a similar PG and PE dependence, PURE was supplemented with purified FtsY, Ffh and 4.5S RNA. It should be noted that in previous



1	2	3	4	5	6	7	8	9	10	Lane
-		**30**		**20**		**10**		**0**		**DOPG**
-		30		30		30		30		DOPE
-		40		50		60		70		DOPC
0.26	0	1	1	0.42	0.20	0.31	0.08	0.17	0.06	Rel.
	0		0.40		0.19		0.10		0.14	Abs.

1	2	3	4	Lane
-	-	+	+	Sec
1	0.20	0.47	1	Rel.
	0.12		0.59	Abs.

Fig 2. A. MtlA synthesis and digestion using 135S *E. coli* cell lysate grown to OD_{600} 2.0 in the absence and presence of proteoliposomes. The C-terminus domain is removed proteinase K addition. B. Example of MtlA synthesis and proteinase K treatment on liposomes and proteoliposomes using 30 mol% DOPG, 30 mol% DOPE and 40 mol% DOPC. Sec = presence of SecYEG. Rel. = Relative intensity to the highest signal (with or without proteinase K treatment) indicated by the arrows. Abs = Absolute insertion values calculated from the ratio between the MPF and its corresponding full length MtlA after correcting for the reduction in the number of methionine residues (correction value 1.5 assuming that the N-terminal methionine was not removed).

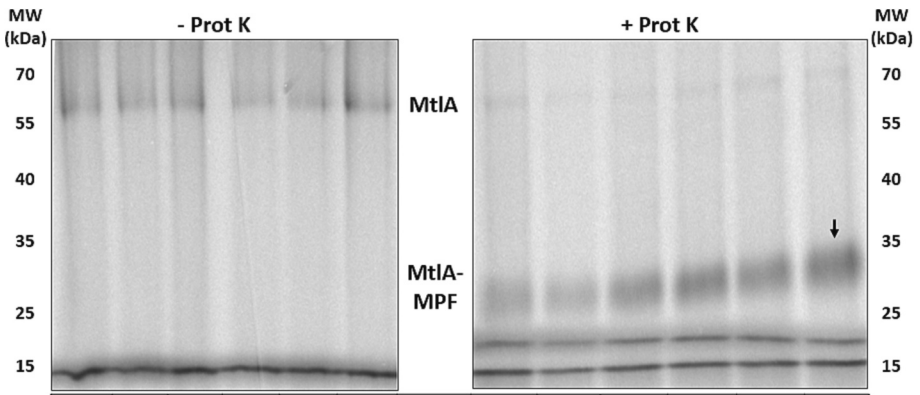


1	2	3	4	5	6	7	8	Lane	9	10	11	12	13	14	15	16
0	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**DOPG**	**0**	**10**	**20**	**30**	**40**	**50**	**60**	**70**
30	30	30	30	30	30	30	30	DOPE	30	30	30	30	30	30	30	30
70	60	50	40	30	20	10	0	DOPC	70	60	50	40	30	20	10	0
1.0	0.71	0.89	1	0.73	0.55	0.49	0.35	Rel.	0.46	0.52	0.76	1	0.86	0.51	0.53	0.50
								Abs.	0.39	0.63	0.72	0.85	1.0	0.79	0.92	1.2

Fig 3. Effect of DOPG content on total synthesis (left gel) and insertion (right gel) of MtlA in an *E. coli* cell lysate. Rel. and Abs. are defined in the legend to Fig. 2.

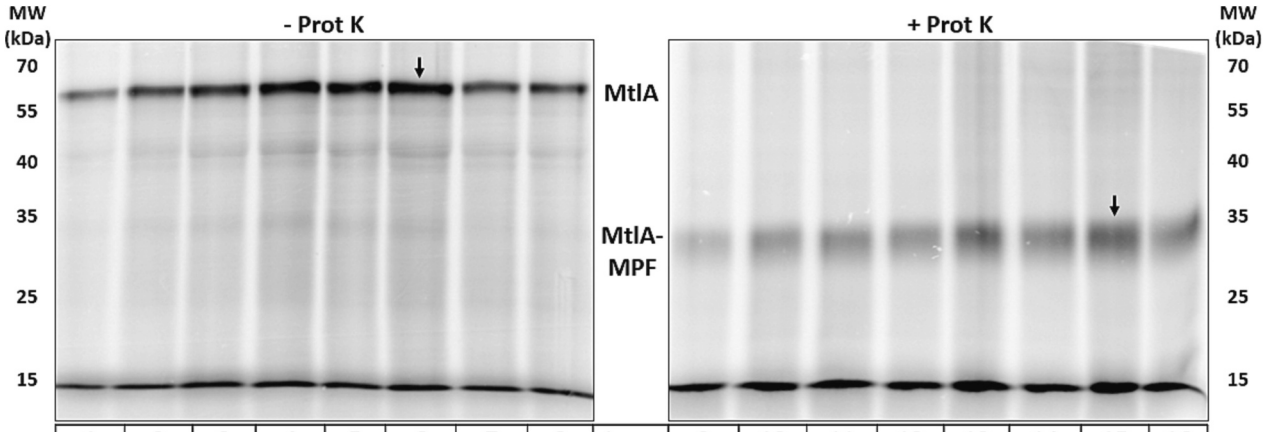
studies the addition of 4.5S RNA was not necessary as it is a contaminant of the RNA present in the PURE system [43,44]. Similar to the lysate experiments, optimum MtlA insertion occurred around 30 and 50 mol% for DOPG and DOPE, respectively (Fig. 7). Notably, however, a stronger dependence of MtlA insertion on these phospholipid species was observed as in the absence of DOPG or DOPE, insertion efficiencies of only 3 and 31 % were observed, respectively.

We also re-examined if MtlA insertion with the PURE system combined with SecYEG proteoliposomes is dependent on SRP and FtsY for insertion. For these experiments, the optimal phospholipid concentration was used employing DOPG:DOPE:DOPC in a molar ratio of 30:50:20, respectively. Only a modest increase of 20 % of inserted MtlA was observed when the reactions were supplementing with both SRP and FtsY (Fig. 8, lane 7, 10). Potentially, small amounts of SRP or FtsY are present in the commercial PURE system and associated with the ribosomes or as contaminants present in the purified SecYEG samples [43,45]. Surprisingly, when the reactions were supplementing with either only SRP or FtsY, the efficiency of insertion was strongly decreased by about 50 % suggesting the presence of an incomplete targeting pathway is inhibitory (lane 8, 9). Also, the potential



1	2	3	4	5	6	Lane	7	8	9	10	11	12
0	**10**	**20**	**30**	**30**	**15**	**DOPG DOPS CL**	**0**	**10**	**20**	**30**	**30**	**15**
30	30	30	30	30	30	PE	30	30	30	30	30	30
70	60	50	40	40	55	PC	70	60	50	40	40	55
						Rel.	0.45	0.39	0.62	0.78	0.70	1

Fig 4. Total synthesis (left gel) and insertion (right gel) of MtlA when DOPG is replaced by DOPS or cardiolipin. Rel. and Abs. defined in the legend to Fig. 2.



1	2	3	4	5	6	7	8	Lane	9	10	11	12	13	14	15	16
30	30	30	30	30	30	30	30	DOPG	30	30	30	30	30	30	30	30
0	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**DOPE**	**0**	**10**	**20**	**30**	**40**	**50**	**60**	**70**
70	60	50	40	30	20	10	0	DOPC	70	60	50	40	30	20	10	0
0.42	0.63	0.77	0.96	0.88	1	0.63	0.63	Rel.	0.46	0.69	0.75	0.69	0.83	0.80	1	0.72
								Abs.	0.59	0.59	0.52	0.38	0.51	0.43	0.85	0.61

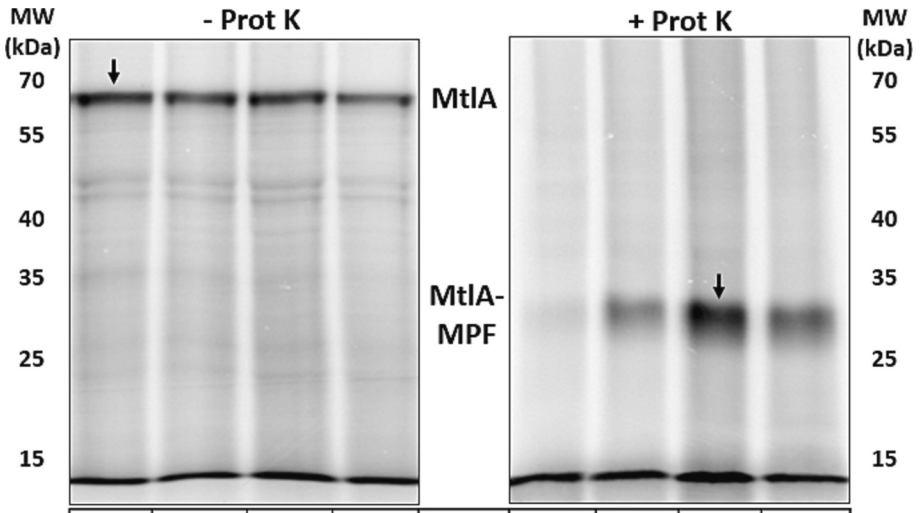
Fig 5. Effect of DOPE content on total synthesis (left gel) and insertion (right gel) of MtlA in an *E. coli* cell lysate. Rel. and Abs. defined in the legend to Fig. 2.

requirement for SecA was tested, but addition of purified SecA was without effect confirming earlier observations (lane 11) [24]. Finally, the insertase YidC was co-reconstituted with SecYEG in a 1:1:500 M ratio to the phospholipids. This also did not affect insertion indicating that YidC is not required for membrane insertion of MtlA (lane 12).

4. Discussion

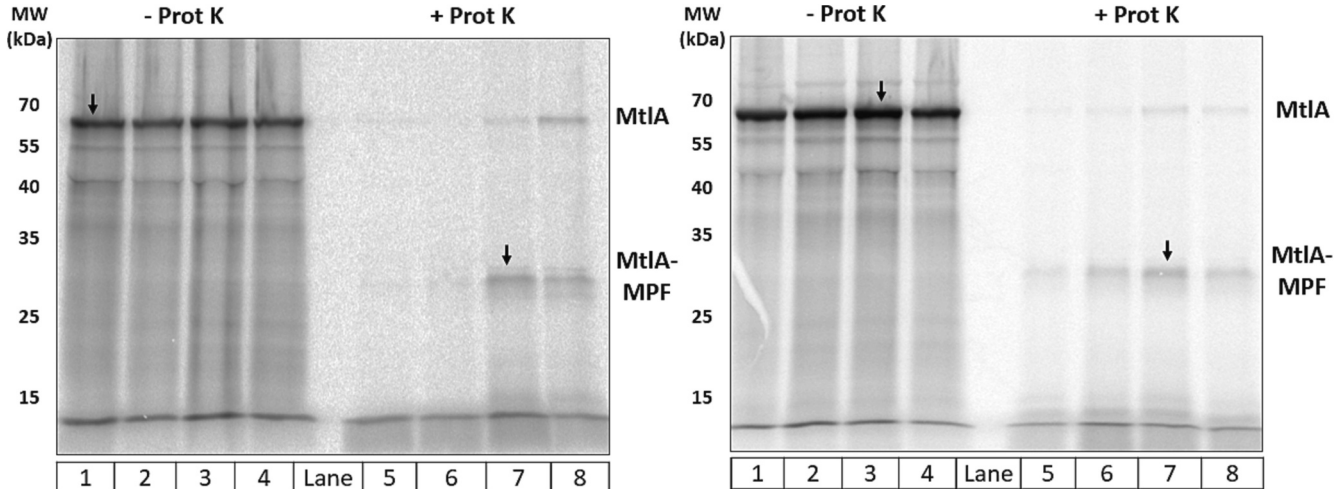
Here, we show the importance of the phospholipid composition on the optimal functioning of the SecYEG translocon for membrane protein insertion. Irrespective the lysate used for transcription and translation, i.e., an S135 cell lysate or the PURE system, optimal MtlA insertion was shown to occur around 30 mol% DOPG and 50 mol% DOPE, which is close to the native phospholipid composition of the *E. coli* membrane [11]. Since synthetic phospholipids are used, these conditions only approach the native phospholipid composition where there is a large variety of phospholipid species that differ in acyl chain length and degree of unsaturation. Since MtlA insertion is not dependent on SecA ([24], this study), the phospholipid dependence cannot be attributed to this protein, that has been shown to be strictly dependent on anionic phospholipids. Furthermore, we observed no direct dependency of any specific anionic phospholipid as long as other negatively charged phospholipids were present. It should be stressed that various studies addressed the phospholipid dependence of the spontaneous insertion of polytopic membrane proteins. However, since spontaneous insertion is a different process than SecYEG-mediated insertion, the results cannot be directly compared [46,47].

Previous in vivo studies have shown that FtsY binds to membranes [22,48,49]. Further, in its catalytic cycle, FtsY also interacts with SecYEG and hence in cells, the majority of FtsY is found membrane localized [27,50]. FtsY does not contain transmembrane helices, but it contains surface active helixes that are able to peripherally associate



1	2	3	4	Lane	5	6	7	8
30	30	30	30	DOPG	30	30	30	30
10	**30**	**50**	**70**	**DSPE**	**10**	**30**	**50**	**70**
60	40	20	0	DOPC	60	40	20	0
1	0.83	0.96	0.68	Rel.	0.08	0.47	1	0.75
				Abs.	0.08	0.54	1.00	1.06

Fig 6. MtlA insertion when DOPE is replaced by an DSPS. Proteinase K is added to all samples. Rel. and Abs. are defined in the legend to Fig. 2.



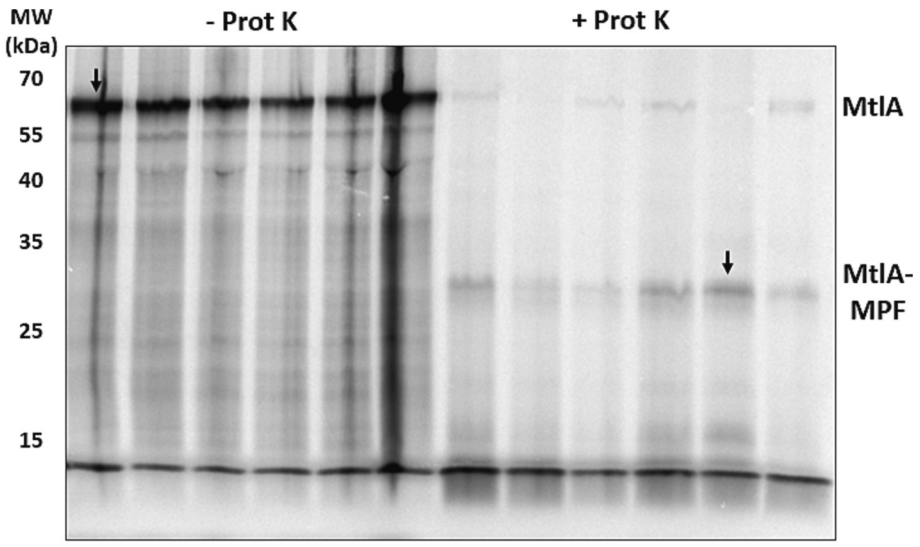
1	2	3	4	Lane	5	6	7	8
0	**10**	**30**	**50**	**DOPG**	**0**	**10**	**30**	**50**
30	30	30	30	DOPE	30	30	30	30
70	60	40	20	DOPC	70	60	40	20
1	0.69	0.96	0.74	Rel.	0.03	0.09	1	0.68
				Abs.	0.01	0.04	0.30	0.26

1	2	3	4	Lane	5	6	7	8
30	30	30	30	DOPG	30	30	30	30
0	**30**	**50**	**70**	**DOPE**	**0**	**30**	**50**	**70**
70	40	20	0	DOPC	70	40	20	0
0.72	0.98	1	0.71	Rel.	0.31	0.62	1	0.57
				Abs.	0.13	0.19	0.30	0.25

Fig 7. Effect of variations in DOPG (left gel) and DOPE (right gel) range on MtlA synthesis and insertion using PURE system DOPE. Rel. and Abs. are defined in the legend to Fig. 2.

with the membrane. The presence of anionic phospholipids results in a stronger association with the membrane [21,22]. Possibly, the enrichment of anionic phospholipids surrounding the SecYEG translocon may promote the SecYEG-FtsY interaction, which may enhance the efficiency of membrane insertion of MtlA.

Further, it is possible that the ribosome-SecYEG interaction also depends on specific phospholipids. MD simulations based on cryo-EM studies of the structure of co-translating ribosome-SecYEG complexes in lipid-filled nanodiscs show that the ribosome interacts with phospholipids in two distinct locations [19,20]. First, ribosome-phospholipid interactions were observed on the reverse side of SecY near TMH10. However, these were reportedly mostly intermittent and did not appear to have a head group preference [20]. Interestingly, during a pre-opened SecYEG state, an enrichment of PG could be observed in the vicinity of the transmembrane helix (TMH) 3 and TMH 4 of SecY and near but not directly in front of the lateral gate [19]. During translocation, this site



1	2	3	4	5	6	7	8	9	10	11	12	Lane
-	+	-	+	+	+	-	+	-	+	+	+	SRP
-	-	+	+	+	+	-	-	+	+	+	+	FtsY
-	-	-	-	+	-	-	-	-	-	+	-	SecA
-	-	-	-	-	+	-	-	-	-	-	+	YidC
1	0.85	0.53	0.55	0.74	0.97	0.83	0.45	0.33	0.94	1	0.40	Rel.
						0.23	0.14	0.17	0.35	0.28	0.09	Abs.

Fig 8. Influence of various components when inserting MtlA using PURE system. Rel. and Abs. are defined in the legend to Fig. 2.

also associates with PG (or CL) to promote SecA binding [18,51]. However, no ribosome-lipid interactions were reported. Remarkably, using POPG/POPC nanodiscs, an accumulation of PC was reported near the lateral gate [19]. However, since PC is normally not present in physiologically relevant concentrations, combined with our observations that PC cannot recover membrane protein insertion activity when PE or PG are absent, raises the question of how relevant this observation is.

Secondly, MD-simulations of the ribosome-lipid interactions during a late state of insertion, showed near but not at the lateral gate formed by the interaction of PE with the negatively charged rRNA H59 [20]. The attraction of the PE to the ribosomal RNA H59 causes an upward motion of several phospholipids within one leaflet of the membrane near the lateral gate. It was speculated that this disorder promotes the insertion by lowering the energy barrier [20,52]. This interaction was observed less strongly when during an earlier state when PC was used instead of PE [19]. It is currently difficult to determine what the influence of both variables is. However, our observation that PC is unable to compensate for the absence of PE suggests that it is indeed phospholipid headgroup dependent. We speculate that the positive charge on the zwitterionic PC is too well shielded to effectively interact with the rRNA. The dependence of insertion on PE could be a reflection of this theory, where the positive charge on the methylated N-atom of PE might interact with the rRNA. Our data suggest that the ability of DOPE to form non-bilayer structures is not critical in its function, as the completely saturated DSPE shows a similar activation profile of MtlA insertion. Obviously, other aspects of the physicochemical characteristics of phospholipids need to be considered such as membrane fluidity that is well controlled in *E. coli* membrane through the large variation in acyl chain lengths and degrees of unsaturation. This is less robust in the mono type acyl tail liposomes used in our experiments. Further, other components such as YidC or the SecDF, protein molecular crowding, or even phase separations of the phospholipid could influence the insertion activity.

Finally, a proper understanding of the minimal phospholipid requirements for membrane protein insertion is important for the utilization of the SecYEG translocon for the insertion of newly synthesized membrane proteins in a synthetic cell that is built from individual components. These data underscore that a functional membrane of a synthetic cell, at least as far as it concerns the headgroup composition, should be as native-like as possible.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamem.2023.184232.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Arnold J.M. Driessen reports financial support was provided by Dutch Research Council.

Data availability

Data will be made available on request.

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