Non-bilayer lipids stimulate the activity of the reconstituted bacterial protein translocase

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Summary

To determine the phospholipid requirement of the preprotein translocase *in vitro*, the *Escherichia coli* SecYEG complex was purified in a delipidated form using the detergent dodecylmaltoside. SecYEG was reconstituted into liposomes composed of defined synthetic phospholipids, and proteoliposomes were analysed for their preprotein translocation and SecA translocation ATPase activity. The activity strictly required the presence of anionic phospholipids, whereas the non-bilayer lipid phosphatidylethanolamine was found to be stimulatory. The latter effect could also be induced by dioleoylglycerol, a lipid that adopts a non-bilayer conformation. Phosphatidylethanolamine derivatives that prefer the bilayer state were unable to stimulate translocation. In the absence of SecG, activity was reduced, but the phospholipid requirement was unaltered. Remarkably, non-bilayer lipids were essential for the activity of the *Bacillus subtilis* SecYEG complex. Optimal activity required a mixture of anionic and non-bilayer lipids at concentrations that resemble the natural lipid composition.

Introduction

Complementary genetic and biochemical approaches have shown that the translocation of proteins across the inner membrane of *Escherichia coli* is mediated by the translocase (574-576). The essential subunits of the translocase are the dissociable peripheral ATPase SecA, and the integral membrane proteins SecY and SecE (226,227). The SecYE complex forms a preprotein-conducting channel that may associate with SecG or the heterotrimeric SecDFYajC complex (274). SecA binds with high affinity to the SecYEG complex, and functions as a molecular motor that utilises the binding and hydrolysis of ATP to drive the stepwise translocation of a preprotein across the membrane (347). In addition, the proton motive force accelerates the translocation reaction (340).

Phospholipids have been shown to play an important role in protein translocation. The *E. coli* inner membrane mostly consists of the zwitterionic phospholipid phosphatidylethanol-amine (PE, 70-75%), and two anionic phospholipids, phosphatidylglycerol (PG, 20-25%) and cardiolipin (CL, 5-10%). The membrane lipid composition is normally tightly regulated, but by the use of strains engineered in the expression of enzymes involved in phospholipid biosynthesis, the *in vivo* manipulation of the bulk phospholipid composition has been achieved (577). The major anionic phospholipids PG and CL can be

depleted in a strain in which the expression of the *pgsA* gene encoding the phosphatidylglycerophosphate synthase is controlled. At low PG and CL content (2-3%), protein translocation is severely compromised, whereas lack of only CL did not affect translocation (308). Only the negative charge of the polar lipid headgroup is important as many other anionic phospholipids are able to restore the protein translocation activity of PG and CL-depleted inner membrane vesicles (IMVs) or in reconstituted proteoliposomes (227,311,312). The efficiency of protein translocation is directly proportional to the amount of anionic phospholipids (309). Anionic phospholipids influence various steps in the preprotein translocation cascade: i) they promote the interaction of SecA with the membrane surface (313-315) and SecYEG (311) and are needed for the SecA translocation ATPase activity, i.e., the preprotein-stimulated ATPase activity of the SecYEG-bound SecA (312,315). At low levels of anionic phospholipids, excess SecA can compensate for the reduced translocation activity (310) suggesting a role of these lipids in the targeting of preproteins and SecA to the membrane. In addition, the endogenous SecA ATPase activity at low Mg^{2+} concentration is stimulated by the presence of anionic phospholipids, an activity termed SecA lipid ATPase (315). ii) Membrane insertion of the positively charged signal sequence of a preprotein is dependent on anionic phospholipids (12,15,317,318,578). iii) Anionic phospholipids stabilise the SecYEG complex during octylglucoside solubilisation (225,312), and iv) influence the acquisition of the correct topology of membrane proteins (322).

The cold-sensitive growth defect of a *secG* null strain (263,264,264,265,265,366) can be suppressed by various gene products involved in phospholipid biosynthesis among which PgsA. Likewise, the cold-sensitive growth defect of the *secAcsR11* mutant strain is suppressed by overproduction of the PgsA protein (366). These effects have been attributed to an increase in the anionic phospholipid content which restores the secretion defect of these strains by facilitating the SecA catalytic cycle at low temperature (366). The exact mechanism of this activation, however, remains obscure as studies with photo-reactive phospholipid analogues suggest that the SecYEG-bound, membrane-inserted form of SecA is not in contact with phospholipids (323,324).

The other main phospholipid of the *E. coli* inner membrane is the type II lipid PE. PE adopts a non-bilayer hexagonal II phase conformation (579). Deletion of the *pssA* gene (580) which encodes the phosphatidylserine (PS) synthase, renders cells devoid of the amino-based phospholipids PS and PE. This results in severe pleiotropic effects on membrane protein function, among others the inactivation of protein translocation (327,581,582). For growth, this strain is dependent on the presence of a high concentration of divalent cations (Ca^{2+}, Mg^{2+}, or Sr^{2+}) (327,580). These cations stimulate the bilayer to non-bilayer transition of CL, which comprises 44% of the total phospholipid in this strain (328). It therefore appears that the polymorphic behaviour of these lipids is essential for growth, a role normally fulfilled by PE. The requirement for non-bilayer lipids for protein translocation appears less strict than for anionic lipids (327) but the mechanism by which these lipids act on protein translocation is unknown. Non-bilayer lipids only marginally affect the SecA lipid ATPase activity (329), and appear not essential for the functional reconstitution of protein translocation using octylglucoside- (OG-) purified SecYEG complex (311).

The reconstitution of preprotein translocation with only a limited set of purified

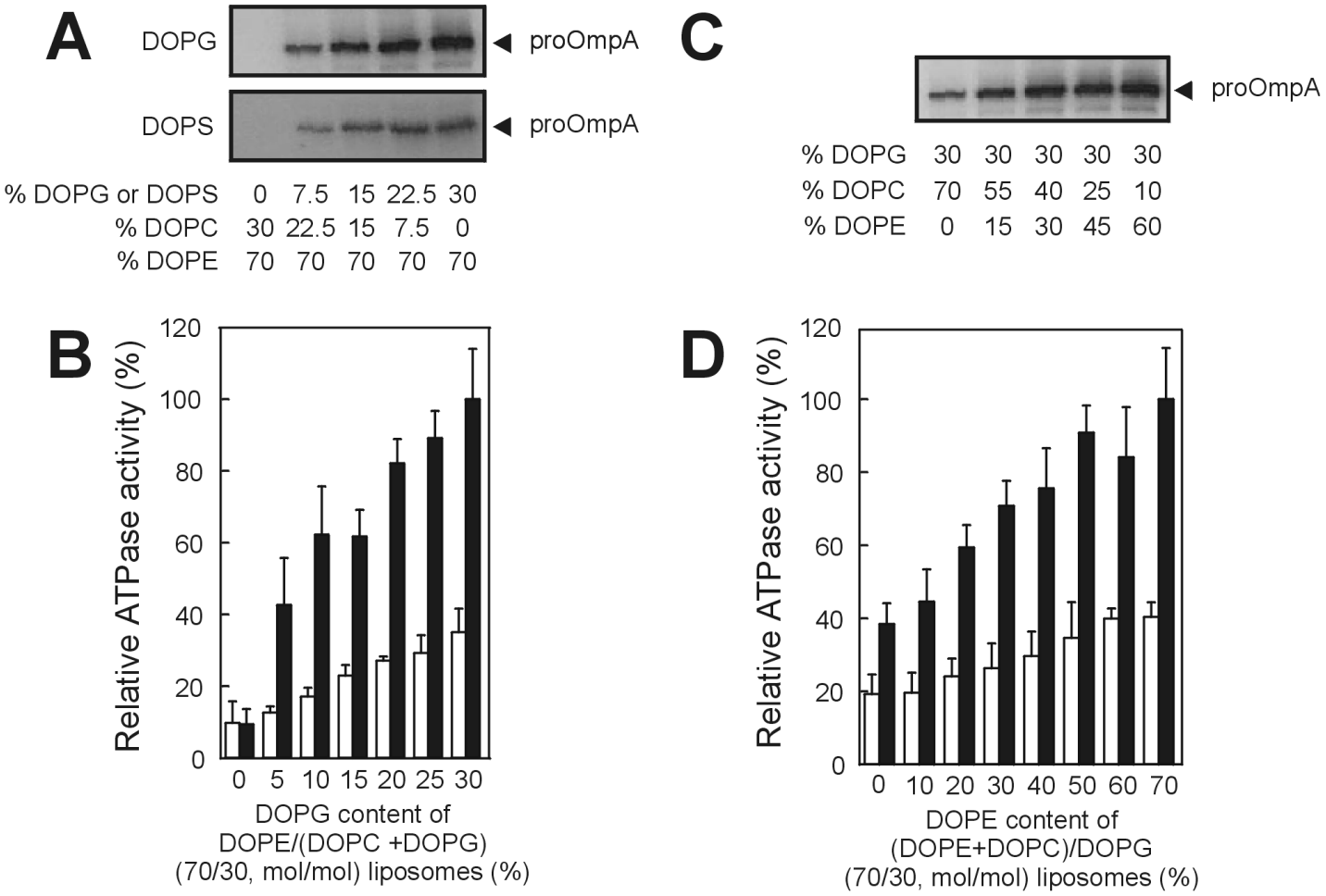

Figure. 1. Anionic lipid and phosphatidylethanolamine requirement for the functional reconstitution of the *E. coli* SecYEG complex. Dodecylmaltoside purified SecYEG was reconstituted in liposomes composed of the indicated synthetic phospholipids and analysed for the ATP-dependent translocation of ^{125}I-proOmpA (A/C) and the SecA ATPase activity in the presence (*filled bars*) and absence of proOmpA (*open bars*) (B/D).

Sec-proteins provides a unique opportunity to systematically assess the lipid requirement for preprotein translocation. We now report on a method that allows purification of the SecYEG complex in a delipidated state. The activity of the delipidated SecYEG can be restored after reconstitution into liposomes with a defined phospholipid composition. The data with the purified *E. coli* SecYEG complex not only confirms the hypothesis that anionic lipids are essential for activity, but also shows that non-bilayer lipids stimulate the activity of the reconstituted translocase. Strikingly, with the *B. subtilis* SecYEG both lipid classes are essential for activity. Overall, optimal activity is observed when anionic and non-bilayer lipids are present at a concentration that matches that of the natural membrane.

Results

Purification and delipidation of SecYEG complex

For the purification of the octylglucoside (OG) solubilised SecYEG complex, the presence of at least 0.2 mg/ml of *E. coli* phospholipids is essential to retain activity (225,257,312). To assess the phospholipid dependency of the translocase in a systematic manner, it is necessary to deplete the purified SecYEG complex of endogenous lipids. When OG was replaced for dodecylmaltoside (DDM), the addition of phospholipids was no longer required to purify the SecYEG complex in a functional state. DDM-solubilised and purified SecYEG complex was as active as complex purified from OG-solubilised membranes in the

Table 1. Purification of the *E. coli* SecA translocation ATPase was measured in the presence of proOmpA using SecYEG proteoliposomes reconstituted into *E. coli* phospholipids. N.D. : not detectable

Detergent	Lipid added (mg/ml)	Protein content (mg/ml)	Phospholipid content (μmol phosphate/ml)	Specific SecA translocation ATPase activity (nmol/mg of protein . min)
Octylglucoside	0.2	0.2	0.3	625 ± 50
	0	0.2	N.D.	N.D.
Dodecylmaltoside	0	0.4	N.D.	700 ± 50

presence of added phospholipids (Table 1). The final amount of endogenous phospholipid present in the DDM-solubilised SecYEG complex was below the detection limit, which corresponds to less than 3 mole of phospholipid per mole of SecYEG complex, assuming an equimolar stoichiometry of the subunits. In contrast, the OG-purified enzyme retained about 200 mole of phospholipid per mole of SecYEG. These data demonstrate that the SecYEG complex can be purified in a delipidated and functional state with the detergent DDM.

Reconstitution of SecYEG and lipid dependency

Delipidated SecYEG complex was used to examine the phospholipid requirement of protein translocation. The phospholipid composition of the *E. coli* inner membrane corresponds to about 70 mol% of the non-bilayer lipid PE and 30 mol% of the acidic PG and CL. To mimic the native polar headgroup composition, the SecYEG complex was reconstituted into liposomes composed of 70 mol% of DOPE and 30 mol% of DOPG. The amount of DOPC was gradually replaced by DOPG to analyse the requirement for anionic phospholipids. Total amounts of SecY, SecE and SecG reconstituted in the liposomes were determined by Western blotting, and were equal for each of the proteoliposomes (data not shown). Proteoliposomes were supplemented with SecA and analysed for the SecA ATPase activity in the absence and presence of the precursor proOmpA (Figure 1B), and for the translocation of chemical amounts of ^{125}I labeled proOmpA (Figure 1A). The activity was determined after 10 min of incubation because within this time interval, the amount of translocated proOmpA increased linearly in time (data not shown). SecYEG was completely inactive when reconstituted into DOPC:DOPE (30:70, molar ratio) (Figure 1AB), but became activated when the DOPC was replaced for DOPG or DOPS (Figure 1A). A similar observation was made for the endogenous and proOmpA-stimulated SecA ATPase activity (Figure 1B). The requirement for the other main lipid constituent of the *E. coli* inner membrane, PE, was determined by reconstitution of the SecYEG complex into liposomes composed of DOPG:DOPC (30:70, molar ratio) whereby the DOPC was gradually replaced for DOPE. Unlike DOPG, DOPE appears not to be essential for protein translocation activity. A low, but significant translocation (Figure 1C) and translocation ATPase (Figure 1D) activity was observed with the SecYEG complex reconstituted in the absence of DOPE. However, DOPE markedly stimulated the activity of SecYEG up to three-fold. These results show that anionic phospholipids are essential for the reconstitution of a functional translocase using purified and delipidated SecYEG, while the non-bilayer lipid PE is only stimulatory.

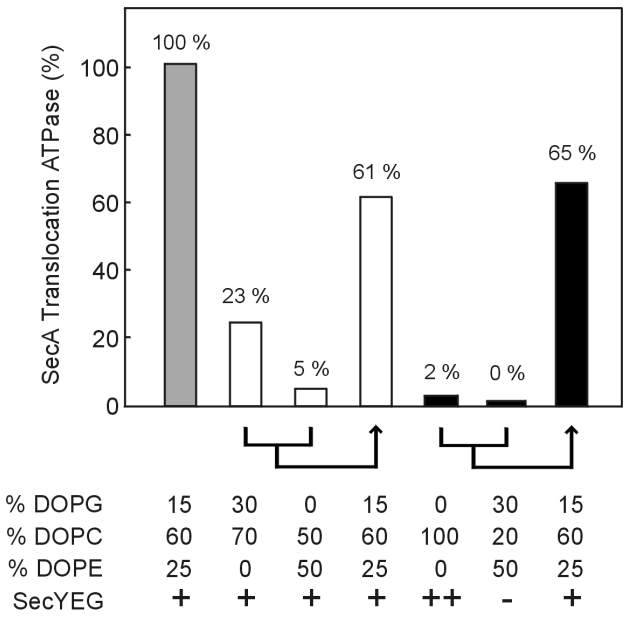

Figure. 2. Reversible activation of reconstituted SecYEG by DOPE. SecYEG was reconstituted in proteoliposomes of the indicated lipid compositions, and as indicated by the arrows, equal volumes of proteoliposomes were fused by repeated freeze-thawing steps. The amount of reconstituted SecYEG was 0, 10 and 20 μg, indicated by -, + and ++ respectively. The proOmpA-stimulated SecA ATPase activity of the original and fused proteoliposomes was measured as described in the Experimental Procedures. The activity of the SecYEG reconstituted into liposomes composed of DOPG:DOPC:DOPE (15:60:25, molar ratio) which had undergone similar freeze thawing cycles was set to 100%.

Phospholipids reversibly influence translocase activity

To exclude the possibility that the observed phospholipid requirement is due to a difference in reconstitution efficiency, the SecYEG complex was first reconstituted into proteoliposomes composed of a lipid mixture that does not support activity. Subsequently other lipids were introduced into these inactive proteoliposomes by freeze-thaw-induced fusion with liposomes of different phospholipid composition. SecYEG proteoliposomes composed of DOPC were essentially inactive (2% activity) for the proOmpA-stimulated SecA ATPase activity (Figure 2). Activity could, however, be restored by fusion of these proteoliposomes with DOPG:DOPC:DOPE (30:20:50, molar ratio) liposomes yielding a final lipid composition of DOPG:DOPC:DOPE (15:60:25, molar ratio). The activity of these fused proteoliposomes was about 65% of a control in which the SecYEG complex was reconstituted directly into this lipid mixture (Figure 2). In another experiment SecYEG was reconstituted with DOPG:DOPC (30:70, molar ratio) yielding proteoliposomes that support a low SecA translocation ATPase activity. Re-introduction of DOPE into these proteoliposomes by fusion with DOPC:DOPE (50:50, molar ratio) liposomes, again yielding a final lipid composition of DOPG:DOPC:DOPE (15:60:25, molar ratio), resulted in an increase of the activity to about 60% of the control. These results demonstrate that the activity of the reconstituted SecYEG complex can be reversibly modulated by the bulk phospholipid composition.

Non-bilayer lipids stimulate protein translocation

To further examine the effect of the non-bilayer lipid PE on protein translocation, the headgroup and acyl chain properties were varied. In a lipid mixture of DOPC:DOPG (70:30, molar ratio), the DOPC was gradually replaced for either DOPE, methyl-DOPE, or dimethyl-DOPE. Although introduction of DOPE significantly stimulated the proOmpA translocation activity (Figure 3A) and SecA translocation ATPase activity (Figure 3B) of SecYEG proteoliposomes, this effect was largely abolished when the DOPE was mono- or dimethylated. When the DOPE was exchanged for dimyristoyl-PE (DMPE), a PE derivative with a shorter hydrophobic acyl chain, hardly any stimulation of the activity was observed (Figure 3). Since the methylated forms of PE and DMPE are all bilayer lipids (31, 48) it appears that the stimulatory effect of DOPE is indeed due to its ability to form non-bilayer structures. To further confirm this hypothesis, the effect of dioleoylglycerol (DOG), a lipid

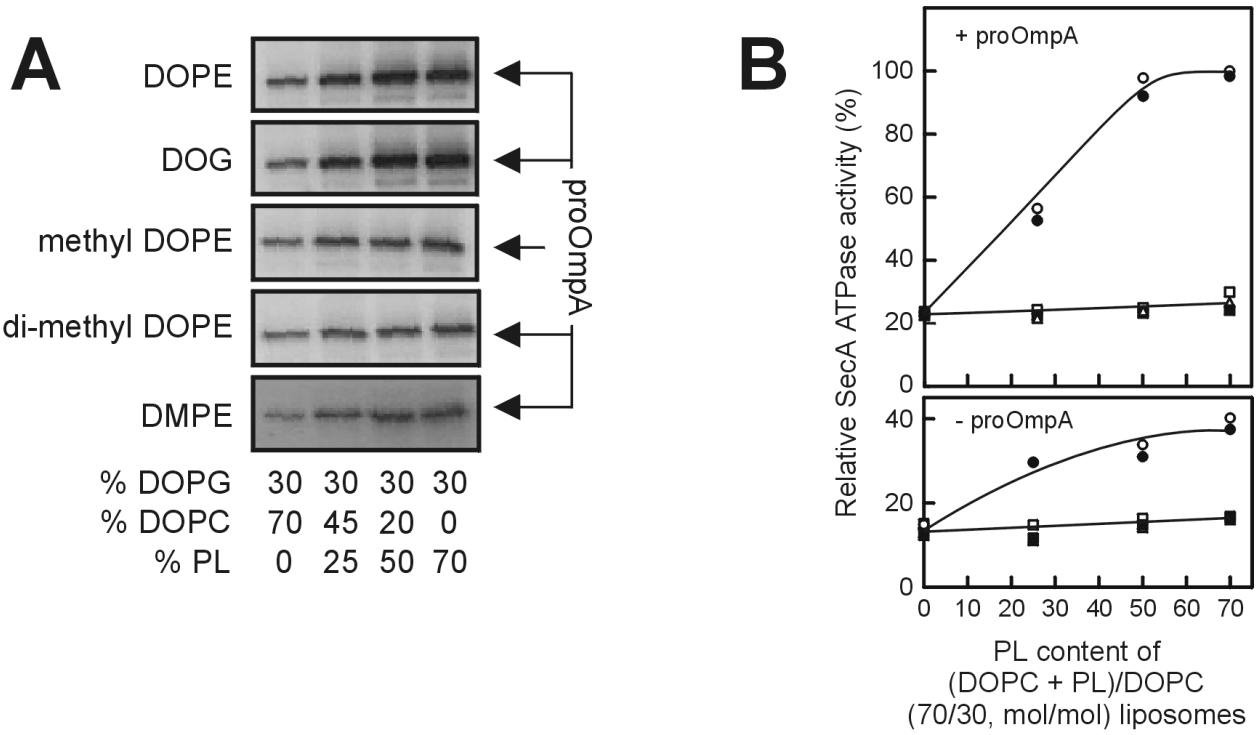

Figure 3. Non-bilayer lipids stimulate preprotein translocation. Purified SecYEG complex was reconstituted in DOPG:DOPC (30:70, molar ratio) proteoliposomes in which the DOPC was gradually replaced for DOPE (open circles), methyl-DOPE (open squares), dimethyl DOPE (closed squares), DMPE (open triangles) or DOG (closed circles). Proteoliposomes were supplemented with SecA and assayed for the ATP-dependent translocation of ^{125}I-proOmpA (A) and for the SecA-ATPase activity in the absence and presence of proOmpA (B).

with strong non-bilayer forming properties, was examined. DOG markedly stimulated the activity of the SecYEG complex to the same extent as DOPE (Figure 3). Taken together these data demonstrate that PE affects protein translocation by its ability to adopt a non-bilayer conformation.

The lipid requirement of the SecYE complex is not affected by SecG

To determine if the presence of SecG influences the lipid requirement of translocation, the SecYE complex was purified from an overexpressing strain and reconstituted into various lipid mixtures. Although some endogenous SecG co-purified with SecYE, western blotting demonstrated that the amount of SecG in the SecYE proteoliposomes was at least 25 fold lower than in the SecYEG proteoliposomes (data not shown). The absence of SecG resulted in a dramatic reduction of the activity of the SecYE complex. However, the requirement for anionic phospholipids (DOPG) and non-bilayer lipids (DOPE) (Figure 4) as observed with the SecYE complex was indistinguishable from that found for SecYEG. This indicates that there is no direct mechanistic relation between the SecG function and the activating effect of lipids.

Lipid requirement of the Bacillus subtilis SecYEG complex

To determine if the lipid requirement found for the *E. coli* SecYEG complex extends to other bacterial species, the lipid specificity of the *B. subtilis* SecYEG complex was determined. Unlike *E. coli*, anionic phospholipids are the major constituents of the *B. subtilis* cytoplasmic membrane, i.e., 70% PG, 4% CL, and 12% PE (330). To obtain functional *B. subtilis* translocase, the SecY, SecE and SecG proteins were overproduced in *E. coli* (583). IMVs

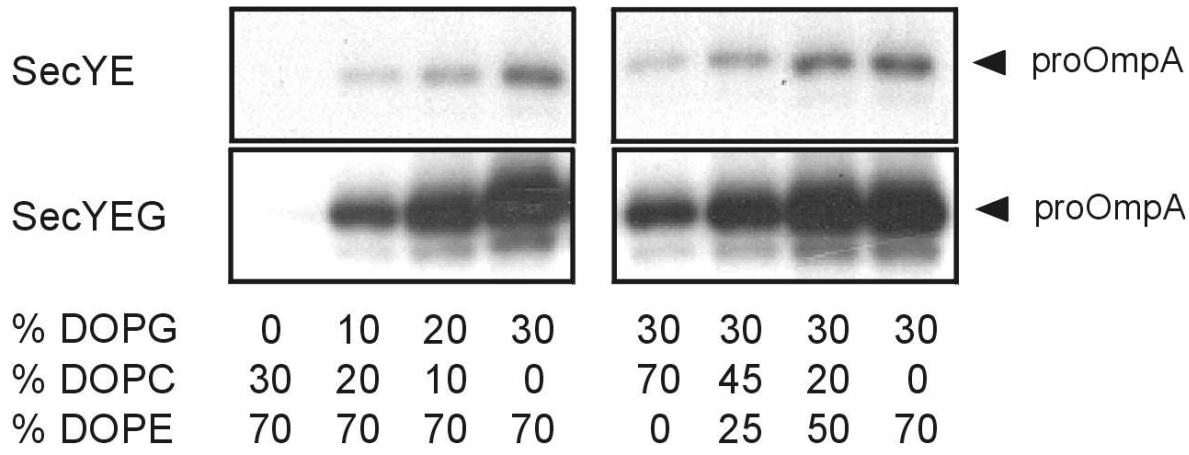

Figure 4. Lipid dependence of SecYEG and SecYE-mediated proOmpA translocation. Purified SecYE and SecYEG was reconstituted into liposomes with the indicated lipid compositions and analyzed for the translocation of ^{125}I-labelled proOmpA.

derived from cells overexpressing either the *B. subtilis* or *E. coli* SecYEG were solubilised in OG without the addition of exogenous lipids, and directly reconstituted into a 20-fold excess of synthetic lipids by rapid dilution. A mixture of DOPG:DOPE (70:30, molar ratio) was used to mimic the lipid composition of *B. subtilis*. Either the DOPG or DOPE was replaced for DOPC to analyse the requirement for anionic and non-bilayer lipids, respectively. The translocation activity of the *B. subtilis* SecYEG complex was assayed in the presence of purified *B. subtilis* SecA and ^{125}I-labeled prePhoB, a *Bacillus*-specific precursor (583,584). ^{125}I-proOmpA was used with the *E. coli* SecYEG proteoliposomes. Translocation of proOmpA by the *E. coli* SecYEG proteoliposomes again showed the strict requirement for PG and stimulation by PE (Figure 5). The presence of 25 mol% of PG and 30 mol% of PE in the *E. coli* SecYEG proteoliposomes was already sufficient to saturate the activity (See also Figure 1C and 1D). Therefore, it seems that the *E. coli* SecYEG is most active in a synthetic lipid mixture resembling the polar headgroup composition of the *E. coli* inner membrane. Remarkably, translocation of prePhoB by *B. subtilis* SecYEG proteoliposomes appeared much more critical with respect to the lipid composition. Maximal translocation activity required a very high DOPG concentration (Figure 5), and was optimal in a lipid mixture corresponding most closely to the polar headgroup composition of the *B. subtilis* membrane, i.e., DOPG:DOPE (70:30, molar ratio) (Figure 5). The data suggests that the requirement for anionic and non-bilayer lipids for preprotein translocation is a general feature of bacterial translocase complexes, and further show that in *B. subtilis*, non-bilayer lipids are essential for activity.

Discussion

The *E. coli* preprotein translocase depends for its activity on specific classes of phospholipids. Previous *in vivo* and *in vitro* studies have shown that anionic phospholipids are essential for activity while non-bilayer lipids are stimulatory (257). A systematic study of the phospholipid requirement of the purified translocase has not been reported yet, despite the fact that the lipid composition of the reconstituted proteoliposomes can be manipulated in a convenient and systematic manner. We now show that functional reconstitution of the purified, delipidated *E. coli* SecYEG requires anionic phospholipids, while non-bilayer lipids stimulate translocation. These results confirm

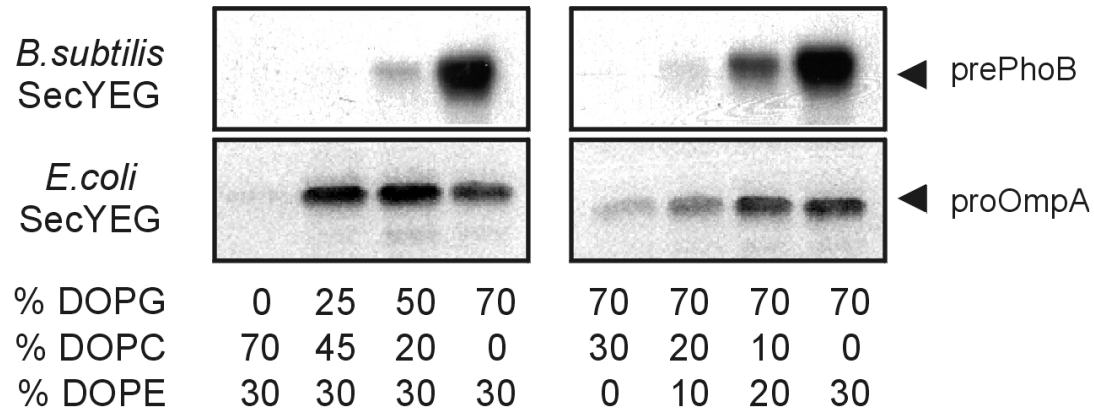

Figure 5. Comparison of the lipid dependence of *B. subtilis* and *E. coli* SecYEG-mediated preprotein translocation. IMVs containing highly overproduced *B. subtilis* or *E. coli* SecYEG complex were solubilized in octylglucoside and reconstituted in proteoliposomes with the indicated lipid compositions. Proteoliposomes were supplemented with purified *B. subtilis* or *E. coli* SecA protein, and analyzed for the ATP-dependent translocation of ^{125}I-labelled prePhoB or proOmpA as indicated.

the studies performed in the crude membrane system, but in addition, extend these observations to the *B. subtilis* SecYEG. Remarkably, the activity of the *B. subtilis* SecYEG strictly depends on the presence of non-bilayer lipids. For optimal activity, anionic and non-bilayer lipids need to present at amounts corresponding to the native membrane phospholipid composition.

In order to analyse the phospholipid requirement of the purified translocase, it is desirable to first delipidate the enzyme and subsequently restore its activity by reconstitution in liposomes with a defined phospholipid composition. The SecYEG complex has been purified from IMVs after solubilisation with OG. However, to obtain a functional SecYEG complex it is necessary to include phospholipids in the buffers used during the purification (225,226,312) (this study). Delipidation of OG-purified SecYEG leads to the irreversible inactivation of the enzyme (Table 1). We now show that SecYEG can be purified in a delipidated and functional form when instead of OG, DDM is used as a detergent. OG is a detergent with a rather short acyl chain of only eight carbon moieties. The acyl chain of DDM is longer, and thus may more closely resemble the interaction of the phospholipid acyl chain with the SecYEG complex present in detergent micelles. In this respect, it was previously noted that OG-solubilised SecYEG is thermolabile (257,362,379). DDM-purified SecYEG complex is less susceptible to such thermal inactivation (A.Veenendaal, manuscript in preparation). The DDM purified SecYEG complex, like the OG-purified, lipid-supplemented SecYEG complex (379) supports the high affinity binding of SecA, the nucleotide-induced conformational states of SecA, and the endogenous SecA ATPase activity (unpublished results). However, it has not been possible to detect SecA translocation ATPase activity with the detergent solubilised translocase even though the enzyme is stable at the temperatures that support this activity in reconstituted liposomes. Electron-microscopical studies on the *B. subtilis* SecYE complex indicate the presence of oligomeric forms that may represent the preprotein conducting channel (380). This oligomeric form may be a stable state of the SecYE complex, but alternatively, the SecYE oligomer may be a dynamic entity, disassemble, and assemble in response to the demand for translocation. When reconstituted into a lipid membrane, the kinetics of such an assembly event will largely be

dictated by the lateral protein diffusion rate. In detergent micelles, however, assembly will be limited by the rate of collision within the three-dimensional space and /or the ability of the micelles to fuse. These events may be effective only when the enzyme is present at a very high concentration. Strikingly, preprotein translocation in detergent solution has been reported for the Sec61p isolated from the endoplasmic membrane of yeast (536), but is observed only at a very high Sec61p concentration.

Although the purification method in the absence of phospholipids was developed to be able to examine effects of small quantities of phospholipids on the activity of the translocase, the data shows that high concentrations of anionic and non-bilayer lipids are needed to saturate the translocation activity. It thus appears that phospholipids act on protein translocation in a more global sense. The *E. coli* SecYEG complex is maximally active in a mixture of 30% DOPG and 70% DOPE. As far as the ratio between anionic and non-bilayer lipids concerns, this mixture 'more or less' corresponds to the lipid composition of the *E. coli* inner membrane. The activity in the optimal synthetic mixture is about 75% of that found with natural *E. coli* phospholipids, showing that substantial activity can be recovered with the defined system. Reconstituted *B. subtilis* SecYEG complex is maximally active in 70% DOPG and 30% DOPE, i.e., at a ratio that closely matches that of anionic to non-bilayer lipids in the native *B. subtilis* inner membrane. It is remarkable that both systems differ in their quantitative lipid requirement and are most active at their physiological lipid conditions. In this respect, the lipid requirement of the *E. coli* SecYEG complex did not change significantly when instead of proOmpA, prePhoB translocation was assayed (unpublished results).

Anionic phospholipids have been shown to fulfill at least a dual role, i.e., they promote SecA membrane binding and insertion and stimulate the interaction of the signal sequence of preproteins and the membrane (326). These lipids may indirectly stimulate targeting of SecA to the SecYEG complex, for instance, by promoting the low affinity membrane binding of SecA. This may result in a membrane bound pool of SecA protein that could have a kinetic advantage relative to the cytosolic pool to associate with SecYEG complexes that have completed a translocation reaction. Experimental evidence for such a mechanism is, however, difficult to obtain. Another possible role for anionic phospholipids may be found in the putative assembly of the SecYEG complex into larger functional oligomers. The collective cold-sensitive secretion defect of the *secG* null and *secAcsR11* strain (366) and many other Sec-mutants may be found in a compromised channel assembly activity.

The reconstitution studies with the purified SecYEG complex provide compelling evidence that non-bilayer lipids stimulate translocation. The activating effect of DOPE can be mimicked with dioleoylglycerol, a lipid that like DOPE adopts a non-bilayer conformation, while bilayer forming PE-derivatives fail to stimulate translocation. These data provide strong evidence that the lipid shape is the major factor for activation rather than the amino group of PE. In contrast to the *E. coli* SecYEG, the activity of the *B. subtilis* SecYEG, strictly required the presence of non-bilayer lipids. In contrast, the amino group of PE is needed for the functional reconstitution of the lactose permease of *E. coli* (585,586) and leucine permease of *Lactococcus lactis* (587). PE stimulates the folding of the lactose permease into its active conformation (588,589). Likewise, non-bilayer lipids may be needed for folding and/or assembly of the SecYEG complex.

Our data suggest that the absolute

amounts of bilayer (or anionic) and non-bilayer lipids are essential for optimal activity. Since these lipids are needed at high concentration, it seems that the protein translocation activity is determined by the collective, physical properties of the membrane. The requirement for non-bilayer lipids could relate to their effect on the lateral membrane pressure and/or optimal matching of the protein-lipid interface, and thus affect the conformation of the active translocase.

Materials and methods

Materials

E. coli SecA (549), *B. subtilis* SecA (183), SecB (77), proOmpA (562), and prePhoB (584) were purified as described. ProOmpA and prePhoB were labeled by iodination with $K^{125}I$ (584) and stored frozen in 6 M urea, 50 mM TrisCl, pH 7.5. 1,2 dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), N-methyl 1,2 dioleoyl-*sn*-glycero-3-phosphoethanolamine (methyl-DOPE), N,N,-dimethyl 1,2 dioleoyl-*sn*-glycero-3-phosphoethanolamine (dimethyl-DOPE), 1,2 dioleoyl-*sn*-glycero-3-phospho-ethanolamine (DOPE), 1,2 dimyristo-oleoyl-*sn*-glycero-3-phosphoethanolamine (DMPE), 1,2 dioleoyl-*sn*-glycero-3-[phospho-L-serine] (DOPS), 1,2 dioleoyl-*sn*-glycero-3-phospho-glycerol (DOPG) and dioleoylglycerol (DOG) were from Avanti Polar lipids, Inc., Birmingham, AL. Polyclonal antibodies (pAb) raised against purified his-tagged SecY and SecE, and against a synthetic peptide corresponding to a SecG domain were obtained as described previously (379).

Plasmids

pET610 (248) was partially digested with *Nco*I/*Bam*HI and the large *secYE* fragment was cloned into pET302 (379) to yield pET320 allowing the overproduction of the *E. coli* SecYE.

Purification of delipidated SecYEG and SecYE complexes

IMVs overexpressing his-tagged SecYE(G) were isolated from E. coli SF100 cells transformed with pET610 (*E. coli* SecYEG) (379), or pET320 (*E. coli* SecYE) (this study) as described (379). IMVs (60 mg of protein) were solubilised on ice for 30 min at 1 mg/ml in 10 mM TrisCl, pH 8.0, and 20% glycerol (buffer A) supplemented with 2% (w/v) dodecylmaltoside (DDM). Non-solubilised proteins and aggregates were removed by centrifugation (30 min at 40,000 x g at 4 °C) and the supernatant was loaded onto a DEAE column (volume 60 ml) (Whatman, DE-52) equilibrated with buffer A supplemented with 0.03% (w/v) DDM. The column was washed with 2 volumes of equilibration buffer, and proteins were eluted with a linear gradient of 0-300 mM KCl in 3 volumes of the same buffer. Alternatively, the SecYEG complex was extracted from the IMVs and purified with 1.25% (w/v) OG in the presence or absence of 0.2 mg/ml of E. coli phospholipid. Fractions were analysed by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue (CBB). Purified protein was either stored on ice or frozen in liquid nitrogen and stored at – 80 °C.

Reconstitution of SecYEG and SecYE complexes

Synthetic phospholipids were mixed in desired ratios in chloroform solution, dried under vacuum, washed with ethanol and dried again. Next, the lipid film was slowly (1-2 hrs) hydrated at a final concentration of 10 mg/ml by incubation at room temperature under a nitrogen atmosphere in a buffer containing 10 mM TrisCl, pH 8.0, and 1 mM dithiothreitol. The suspension was finally dispersed using a bath sonicator. For reconstitution, 100 µl of

purified *E. coli* SecYEG or SecYE protein (about 0.1 mg/ml) was mixed with 20 µl of lipids (10 mg/ml), incubated for 5 min on ice, and diluted rapidly with 8 ml of 50 mM TrisCl, pH 8.0, 50 mM KCl. After 5 min, proteoliposomes were collected by centrifugation (30 min, Ti-70, 200,000 x g, 4°C) and resuspended in 100 µl of 50 mM TrisCl, pH 8.0, and 50 mM KCl. Proteoliposomes were analysed for the amount of incorporated protein using the DC Protein assay (Bio-Rad, Hercules, CA), by 15% SDS-PAGE stained with CBB, and by immunoblotting on PVDF membranes using antibodies directed again SecY, SecE and SecG. Reconstituted SecYE(G) proteoliposomes were frozen and stored in liquid nitrogen. Before use, samples were thawn at 37°C and sonicated 3 times for 10 sec in a bath sonicator. Control experiments showed that the SecA translocation ATPase activity after rapid dilution was linear with the amount of SecYEG added at lipid-to-protein ratios (W/W) above 10.

To reconstitute the *B. subtilis* SecYEG into liposomes of different lipid compositions, IMVs were isolated from *E. coli* SF100 cells transformed with pET822 that directs the functional overexpression of the *B. subtilis* SecYEG complex (583). IMVs (100 µl; 0.1 mg of protein/ml) were solubilised in 1.25% (w/v) OG in buffer A as described (379). After 30 min incubation on ice, the non-solubilised material was removed by centrifugation (TLA100.4, 30 min, 180,000 x g, 4°C). The supernatant fraction (100 µl) was subsequently mixed with 20 µl of lipids (10 mg/ml), rapidly diluted, and isolated as described above. In control experiments, IMVs were used derived from *E. coli* SF100 cells transformed with pET610 overproducing *E. coli* SecYEG (248).

Fusion of SecYEG proteoliposomes

SecYEG (proteo)liposomes (100 µl; containing 0, 10 or 20 µg of SecYEG protein and 0.2 mg of phospholipids) were mixed with an equal volume and amount of (proteo)liposomes of different lipid composition. Samples were quickly frozen in liquid nitrogen and slowly thawn on ice. This procedure was repeated three times. Before use in activity assays, samples were sonicated 3 times for 10 sec in a bath sonicator.

Translocation assays

Translocation assays were performed in a final volume of 50 µl consisting of 50 mM HEPES-KOH, pH 7.6, 30 mM KCl, 5 mM $Mg(Ac)_2$, 2 mM ATP, 10 mM creatine phosphate, 0.5 µg of creatine kinase, 25 µg of bovine serum albumin, 1.6 µg of SecB, 1 µg of purified *E. coli or B. subtilis* SecA and proteoliposomes containing 6.5 µg of SecYEG or SecYE complex. Samples were preincubated for 10 min at 37 °C, and the translocation reaction was started by the addition of 1 µl of ^{125}I-labeled proOmpA or prePhoB (0.2 mg/ml in 6 M urea, 50 mM TrisCl, pH 7.5). After 10 min, reactions were terminated by chilling on ice. Samples were treated with proteinase K (0.1 mg/ml) for 15 min, precipitated with ice-cold 10% (w/v) trichloric acid, acetone-washed and analysed by SDS-PAGE on 10% (prePhoB) and 12% (proOmpA) polyacrylamide gels.

Other analytical techniques

Translocation ATPase activity of urea-treated IMVs or SecYEG proteoliposomes was measured with proOmpA as substrate as described (179). Protein concentration was determined with the DC Protein assay (Bio-rad, Hercules, CA). Phospholipid phosphorus was assayed after heat destruction of chloroform/methanol extracted phospholipids using the method of *Rouser et al.* (590).

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