Translocase-Bound SecA Is Largely Shielded from the Phospholipid Acyl Chains†

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ABSTRACT: Protein translocation in *Escherichia coli* is mediated by the SecA ATPase bound to the SecYEG membrane protein complex. SecA translocation ATPase activity as well as protein translocation is dependent on the presence of negatively charged lipids. By using a phospholipid with an acyl chain linked photoactivatable group, the lipid accessibility of SecA bound at the translocase was explored. SecA bound to lipid vesicles containing negatively charged lipids was found to be readily accessible for labeling by the photoactivatable phospholipid. The presence of an excess amount of SecYEG complex resulted in a remarkable reduction in the amount of lipid-accessible SecA irrespective of the nucleotide-bound form of SecA. These data demonstrate that the SecYEG-bound SecA is largely shielded from the phospholipid acyl chains and suggest the presence of two distinct pools of membrane-bound SecA that differ in the degree of lipid association.

In *Escherichia coli*, proteins functioning outside the cytosol are transported across the inner membrane from the site of synthesis to their site of catalysis. Most of the proteins involved in this process, that is, components of the secretion machinery, have been identified by genetic analysis (*1*, *2*). Biochemical approaches are now successfully used to obtain more insight into the molecular mechanisms by which the secretion machinery acts.

Proteins destined for secretion contain an N-terminal signal sequence that is recognized by the secretion machinery, termed precursor protein translocase. Translocase consists of the soluble component SecB, the integral membrane protein complexes SecYEG and SecDFYajC (*3*), and SecA that is present in the cytosol and bound to the membrane (*4*). SecY, together with SecE and SecG, forms the translocation channel. SecDFYajC may only be transiently associated with the translocase. The energy required for the translocation process is derived from ATP hydrolysis by SecA and from the proton motive force (pmf)[1] (*5*). During or directly after translocation, the signal sequence is removed by leader peptidase.

Its ability to convert chemical energy into net movement of the precursor protein makes SecA the key component of the translocase. SecA is bound to the membrane with low affinity, presumably to the phospholipids, and to sites consisting of SecYEG with high affinity (*6*, *7*). SecA is a 102-kDa protein with two ATP binding sites (*8*) and is active as a dimer (*9*). Each monomer consists of two large domains that interact with each other in a nucleotide-dependent manner (*10*). SecA recognizes the signal sequence of proteins destined for secretion, and when bound at the membrane, SecA drives translocation by binding and hydrolyzing ATP, thereby cycling through a series of conformational states. In the presence of ATP and precursor protein, SecA is thought to insert into the membrane and two domains of 30 and 65 kDa become protease-inaccessible (*11*, *12*). Upon hydrolysis of ATP, SecA de-inserts from the membrane. In each cycle 20−30 amino acid residues of the precursor protein are moved across the membrane (*13*). Both the N- and C-terminal domains are accessible for modifying reagents from the periplasmic side of the membrane (*14*−*16*). The C-terminal domain is even accessible for protease digestion from the periplasmic side of the membrane (*16*).

SecB, used by a subset of precursor proteins as a targeting device (*17*), recognizes the unfolded precursor and subsequently binds to the C-terminus of SecA (*18*) at the membrane (*6*). Furthermore, SecB keeps precursor proteins

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[1] Abbreviations: AMP−PNP, adenosine-5′-[β,γ-imido]-triphosphate; ATPγS, adenosine-5′-[γ-thio]triphosphate; BSA, bovine serum albumin; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DTT, dithiothreitol; ESR, electron spin resonance; LUVs, large unilamellar vesicles; OG, *n*-octyl-β-D-glucopyranoside; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; pmf, proton motive force; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCA, trichloro acetic acid; TTD-PC, 1-*O*-hexadecanoyl-2-*O*-[9-[[[2-(tributylstannyl)-4-(trifluoro-methyl-3H-diazirin-3-yl)benzyl]oxy]carbonyl]nonanyl]-*sn*-glycero-3-phosphocholine; [^{125}I]TID-PG, 1-*O*-hexadecanoyl-2-*O*-[9-[[[2-[^{125}I]iodo-4-(trifluoro-methyl-3H-diazirin-3-yl)benzyl]oxy]carbonyl]nonanyl]-*sn*-glycero-3-phosphoglycerol; TLC, thin-layer chromatography; [^{125}I]TID-PC, 1-*O*-hexadecanoyl-2-*O*-[9-[[[2-[^{125}I]iodo-4-(trifluoro-methyl-3H-diazirin-3-yl)benzyl]oxy]carbonyl]nonanyl]-*sn*-glycero-3-phosphocholine.

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in a translocation competent state by preventing aggregation and stable folding (*19*−*21*). Insertion of SecA is accompanied by the release of SecB into the cytosol (*22*) and a complete topological inversion of SecG (*23*). Association of the SecDFYajC complex with SecYEG leads to stabilization of SecA in the inserted state (*24*).

Besides the precursor protein translocase, phospholipids also play an important role in translocation. The major phospholipids in the *E. coli* inner membrane are 75−80% phosphatidylethanolamine (PE), 20% phosphatidylglycerol (PG), and 1−5% cardiolipin (*25*). PE has a high propensity to form nonbilayer structures, and this property is important for protein translocation (*26*). Negatively charged phospholipids are needed for efficient translocation. Strains containing lower levels of PG and cardiolipin are impaired in protein translocation (*27*). Negatively charged lipids may be needed at various stages of translocation. The ability of signal sequences to insert into model membranes strongly correlates with their in vivo function (*28*, *29*). Negatively charged lipids induce α-helix formation of the signal sequence (*30*) and are thought to directly interact with positive charges of the signal sequence (*31*). Overexpression of phosphatidylglycerophosphate synthase suppresses a *secG* deletion, suggesting a role for negatively charged phospholipids in the SecG function (*32*). SecA penetrates deeply into model membranes (*33*) up to the center of the phospholipid bilayer (*34*). Insertion into lipid monolayers is strongly dependent on negatively charged phospholipids and inhibited by ATP (*35*). SecA lipid ATPase activity and SecA translocation ATPase activity are stimulated by negatively charged lipids (*36*). Reduction of the negatively charged phospholipid content in the membrane results in higher expression levels and an altered cellular distribution of SecA (*37*). These data suggest an important role for SecA lipid interactions during protein translocation.

An important question is whether SecA inserts in the membrane via a lipid environment or through a proteinaceous pore formed by the SecYEG complex. The proteolytic 30-kDa domain of SecA has recently been found not to be accessible for photolabeling from the hydrophobic region of the membrane with a lipid-soluble marker (*38*). Reconstitution of SecYEG with a photoactivatable phospholipid analogue provides a system to study SecA lipid interactions without the need of a protease resistance assay. We used this system to answer the question whether SecA at the site of translocation is in direct contact with phospholipids. SecA insertion into the phospholipid bilayer was found to be dependent on the presence of negatively charged phospholipids. However, SecA bound at the translocase was largely shielded from the hydrophobic region of the bilayer. This suggests that the negatively charged phospholipids do not play their role via direct interactions with SecA at the translocase. A possible role for the lipid-bound SecA will be discussed.

EXPERIMENTAL PROCEDURES

Materials. SecA (*39*), SecB (*21*), and proOmpA (*40*) were prepared as described. The prePhoE signal-sequence mutant G-10C was expressed from pNN102, which consists of the *Pac*I-*Bgl*II fragment from pNN7 (*41*) cloned in pNN100. The prePhoE mutant K125C containing the wild-type signal sequence was expressed from pNN105 which was constructed by changing the lysine codon 125 of PhoE on pJP29 (*42*) into a cysteine codon as described (*41*) resulting in pMS37. Subsequently, the *Pac*I-*Bgl*II fragment of pMS37 was cloned into pNN100. PrePhoE mutants were purified as described (*20*). Protein concentrations were determined with Bradford analysis using BSA as standard (*43*). SecYEG complex was purified from a SecYEG overproducing strain (*16*) as described (*44*). ATP, AMP−PNP, and DTT were from Sigma Chemical Co. (St. Louis, MO). ADP, ATPγS, ampicilin, phospholipase D (type I from cabbage), and proteinase K were from Boehringer (Mannheim, Germany). "Reactivials" were from Pierce (Rockford, IL), $Na^{125}I$ (350−600 mCi/mL in NaOH) and ^{14}C-labeled protein markers were from Amersham (Buckinghamshire, UK). DOPC and DOPG were from Avanti Polar Lipids (Alabaster, AL). *E. coli* phospholipids were isolated from strain MC4100 as described (*45*). Radiolabeling of precursor proteins was done as described (*16*). All other chemicals were analytical grade or better.

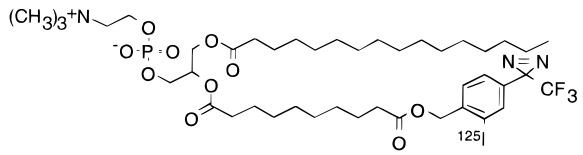

FIGURE 1: The structure of [^{125}I]TID-PC.

Preparation of Radioiodinated Photoprobes. The photoactivatable phospholipid [^{125}I]TID-PC (Figure 1) was prepared by radioiododestannylation of the tin-based precursor TTD-PC (*46*). All steps were done at room temperature under normal laboratory fluorescent light. Briefly, 50 nmol of TTD-PC was dried in a 1-mL "reactivial" under a stream of nitrogen. The dried material was redissolved in 20 μL of acetic acid to which 5 mCi of $Na^{125}I$ was added. The reaction was started by the addition of 10 μL of a 32% (w/v) peracetic acid solution in acetic acid. After 2 min, 5 μL of 100 mM NaI was added to convert all remaining TTD-PC into [^{125}I]TID-PC. After another 2-min incubation, the reaction was quenched by addition of 100 μL of 10% (w/v) $Na_2S_2O_5$. Then, 100 μL of chloroform/methanol (2:1 v/v) was added, and after vortexing, the lower organic phase was collected. This extraction step was repeated twice. The organic solvent was dried, and the radioiodinated lipid was dissolved in chloroform/methanol/water (65:25:4, v/v/v). The material was eluted on a silica gel column (5 g of silica) with chloroform/methanol/water (65:25:4, v/v/v). Fractions were collected and analyzed by TLC (same solvent) and autoradiography. [^{125}I]TID-PC-containing fractions were collected, dried, and dissolved in toluene/ethanol (1:1, v/v). Radioiodination efficiencies of 60−80% were routinely obtained.

[^{125}I]TID-PC was converted into the respective phosphatidylglycerol analogue [^{125}I]TID-PG by phospholipase D mediated headgroup exchange (*47*). Shortly, [^{125}I]TID-PC (200 μCi) was dried and dissolved in 200 μL of diethyl ether. Then 200 μL of 100 mM NaAc pH 5.6, 40 mM $CaCl_2$, and 50% glycerol containing 30 units of phospholipase D was added. The solution was stirred rigorously for 20 min at 30 °C. Lipid material was extracted as described above, separated by TLC with chloroform/methanol/water (65:25:4, v/v/v), and analyzed with autoradiography. The radioac-

tive spot containing [^{125}I]TID-PG was scraped off and eluted with chloroform/methanol (1:1, v/v). Phospholipid was analyzed on TLC as above. [^{125}I]TID-PG was found to be more than 99% pure. The total yield of the reaction was 7%.

Preparation of LUVs. Radioactive phospholipid (50 μCi) was added to 1.25 μmol of phospholipid in chloroform. Lipids were dried under nitrogen and placed under high vacuum for 1 h. Lipids were hydrated in 1 mL of 50 mM Tris/HCl, pH 7.9, and 50 mM KCl and freeze−thawed 5 times. The resulting multilamellar vesicles were extruded through a polycarbonate filter with a 200-nm pore size by applying a nitrogen pressure of 0.5 MPa to obtain large unilamellar vesicles (LUVs) (*48*).

Cross-Linking Reactions. For cross-linking reactions, samples were photolyzed by a 15-W UV light source (CAMAC universal lamp) at 366 nm for 5 min at a 1-cm distance. The half-life of the photoreactive group under these conditions was found to be less than 1 min, as determined by the decay of the diazirine characteristic absorption at 351 nm (*46*).

Determination of Penetration of SecA in Phospholipid Bilayers. SecA (100 μg), LUVs (60 nmol of Pi) composed of DOPC/DOPG 1:1 (mol/mol) containing [^{125}I]TID-PC, and various nucleotides (4 mM) were incubated in buffer (50 mM Tris/HCl, pH 7.6, 100 mM KCl, 4 mM $MgCl_2$) for 10 min at room temperature. Vesicles with bound SecA were pelleted at 70 krpm for 30 min in a Beckman TLA 100.3 rotor. Pellet and supernatant fractions were separated and photolyzed. Proteins were precipitated by TCA and dissolved in 3% SDS, 6 M urea, and 50 mM Tris/HCl, pH 7.6. Protein concentration in the samples was determined with the BCA method (10% of the sample). Radioactive labeling was analyzed after separation on 7.5% SDS−PAGE by γ-counting of the excised coomassie stained bands (80% of the sample). From these data the specific radioactivity (cpm/μg of protein) was calculated.

Reconstitution of SecYEG into Proteoliposomes. The SecYEG (200 μL) complex in mixed micellar solution (0.25−0.5 mg of protein/mL) with 1.25% (w/v) *n*-octyl-β-D-glucopyranoside (OG), 10 mM Tris/HCl, pH 8.0, 40% glycerol, 100 mM KCl, and 0.5 mg/mL *E. coli* phospholipids was mixed with 2 μL of a dispersion of sonicated *E. coli* phospholipids (50 mg/mL in 1 mM DTT) and [^{125}I]TID-PC (100 μCi, 1−3 nmol). Proteoliposomes were formed by dilution with 9 mL of 50 mM Tris/HCl, pH 7.9, 50 mM KCl, and 1 mM DTT and incubated overnight on ice. Proteoliposomes were collected by ultracentrifugation for 1 h at 45 krpm (Beckman TY65 rotor), resuspended in 200 μL of 50 mM Tris, pH 7.9, 50 mM KCl, and 1 mM DTT, and assayed for translocation ATPase activity (*36*). Liposomes were prepared in the same way by diluting 200 μg of *E. coli* lipids from a 1.25% OG solution.

Translocation Assays. Reaction mixtures (50 μL) contained SecA (20 μg/mL), SecB (34 μg/mL), ATP (2 mM), and proteoliposomes (5 μL) in translocation buffer (50 mM HEPES/KOH, pH 7.9, 30 mM KCl, 2 mM DTT, 20 mM MgAc). Precursor proteins were diluted 50-fold from a solution containing 6 M urea and 50 mM Tris/HCl, pH 7.9, and incubated in the reaction mixture at 37 °C for 20 min. Forty-five microliters of the reaction mixture was added to 1 μL of proteinase K (5 mg/mL) and incubated for 10 min on ice. Reactions were stopped by the addition of an equal volume of 20% (w/v) TCA. Samples were centrifuged for 10 min at 13 krpm in an Eppendorf centrifuge, washed with acetone, and analyzed on 15% SDS−PAGE (*49*).

Other Methods. BCA protein determination was done using a micro BCA reagent kit according to manufacturer instructions (Pierce, Rockford, IL). Phospholipid concentrations were determined as described (*50*). γ-Counting was done with a Packard crystal multidetector RIA system.

RESULTS

Negatively Charged Phospholipids Are Essential for Insertion of SecA into the Hydrophobic Region of the Bilayer. SecA insertion into phospholipid bi- and monolayers has been shown by various methods (*33*−*35*). To demonstrate that [^{125}I]TID-PC is a suitable probe for measuring the bilayer insertion of SecA, SecA was photolabeled in the presence of LUVs containing the radiolabeled phospholipid analogue (Figure 1). Upon incubation of SecA with LUVs composed of *E. coli* phospholipids and subsequent photolysis of the probe, SecA becomes radioactively labeled. Parts A and B of Figure 2 show an autoradiogram of a typical photo-cross-link experiment and the results of a quantitative analysis, respectively. SecA in the nonphotolyzed samples shows only a low level of labeling. In the presence of LUVs of DOPC very little labeling was obtained, consistent with previous observations showing that this lipid does not support SecA membrane insertion (*35*). This also demonstrates that the probe is restricted to the bilayer. The labeling efficiency increased with the amount of negatively charged phospholipid in the membrane and was maximal with pure DOPG LUVs. When instead of [^{125}I]TID-PC, [^{125}I]TID-PG was used as probe with DOPC/DOPG (1:1) LUVs, a similar labeling efficiency was obtained (data not shown). This demonstrates that the photoreactivatable PC and PG lipid analogues report the SecA membrane insertion similarly. When an increasing amount of SecA was added to DOPC/DOPG (1:1) LUVs, the amount of labeling increased, saturating at about 250 lipid molecules/added SecA monomer (Figure 2C). This value is in agreement with the stoichiometry of the SecA lipid interaction that was obtained previously (*51*). Labeling efficiencies at room temperature or at 37 °C were comparable (data not shown). On the basis of these results it is concluded that [^{125}I]TID-PC is a suitable probe for determining the insertion of SecA into phospholipid bilayers.

The interaction of SecA with phospholipids was reported to depend on the presence of nucleotides (*34*, *35*). To investigate whether the extent of membrane penetration is nucleotide-dependent, SecA was incubated with [^{125}I]TID-PC-containing LUVs composed of DOPC/DOPG (1:1) in the presence and absence of nucleotides. The vesicles were collected by centrifugation, and samples were photolyzed. In the absence of LUVs no SecA was pelleted. The specific radioactivity of the bound and labeled SecA was determined (Figure 3). Under all conditions SecA was labeled with equal efficiency. Also, variation of the phospholipid composition (pure DOPG, *E. coli* phospholipids) or the use of [^{125}I]TID-PG instead of [^{125}I]TID-PC also showed an equal labeling efficiency in the presence of different nucleotides (data not shown). It was reported previously that addition of SecA

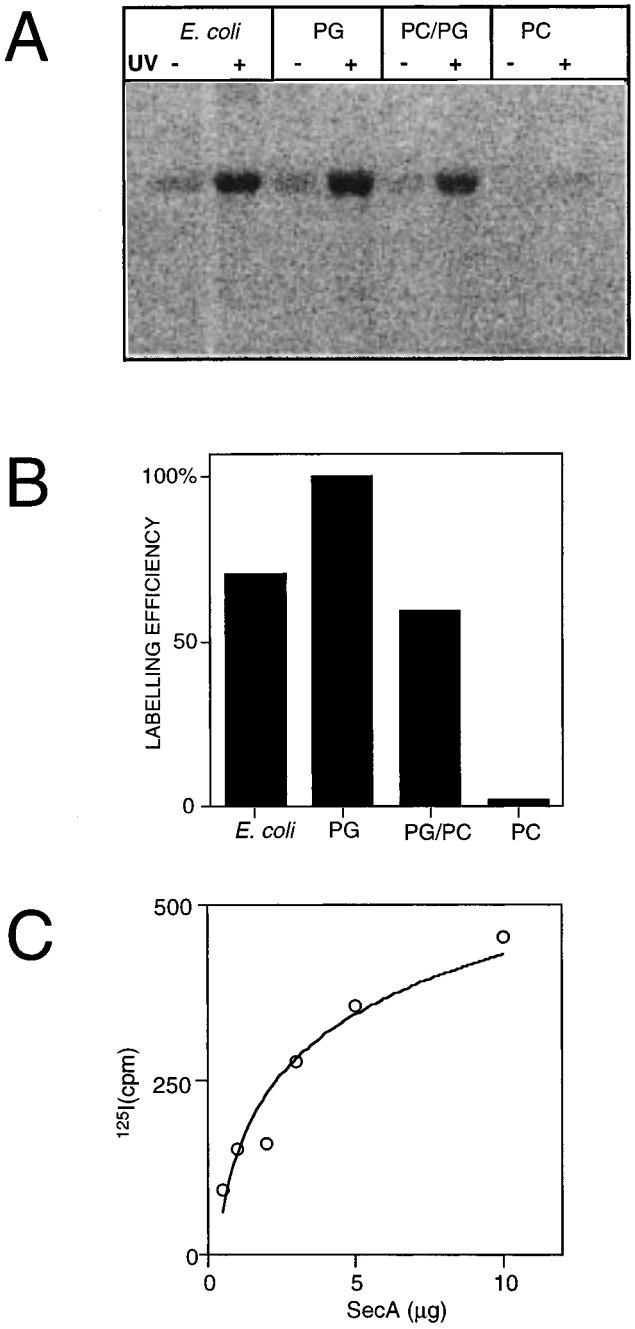

FIGURE 2: SecA inserts into the hydrophobic region of LUVs containing negatively charged phospholipids. (A) LUVs containing [^{125}I]TID-PC were prepared from *E. coli* phospholipids, DOPG, DOPG/DOPC (1:1), and DOPC. SecA (10 μg), ATP (2 mM), and LUVs (12.5 nmol of phospholipid) were incubated in translocation buffer at 37 °C. After 10 min, cross-link reactions were performed as described in Materials and Methods. (B) The amount of radioactivity is depicted as a percentage of the highest value obtained. (C) Increasing amounts of SecA were labeled in the presence of DOPC/DOPG (1:1) LUVs (20 μg).

to DOPG LUVs results in vesicle aggregation which is inhibited by ATP (*51*). This aggregation is mediated by the simultaneous interaction of a C-terminal electrostatic lipid-binding site and a hydrophobic lipid-binding site close to the N-terminus with two different vesicles. Under conditions where the nucleotide-dependent aggregation of nonradioactive vesicles was observed (not shown), we did not observe a nucleotide dependency of labeling in the presence of [^{125}I]-TID-PC-containing vesicles. This demonstrates that in our

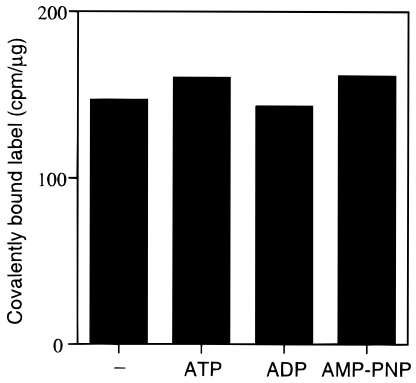

FIGURE 3: SecA labeling is not modulated by different nucleotides. SecA membrane penetration was determined as described in Materials and Methods for the nucleotides shown.

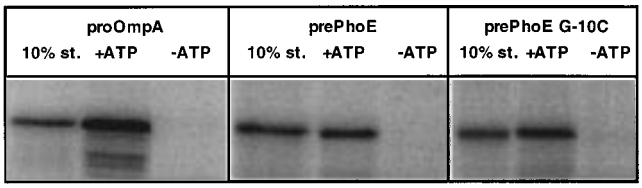

FIGURE 4: Vesicles containing [^{125}I]TID-PC support translocation of proOmpA and prePhoE. Translocation reactions were performed, using [^{125}I]-labeled proOmpA, prePhoE, and prePhoE G-10C as described in Materials and Methods. For each precursor was used a 10% standard (10% st) and a reaction with (+ATP) and without (−ATP) ATP is shown.

system the interaction of SecA with lipids is modulated by nucleotides as reported previously but the degree of exposure to the phospholipid acyl chains is not. Efficient labeling of SecA is also observed at high ionic strength (500 mM KCl) irrespective of the nucleotide present, indicating that the electrostatic binding site is not involved in photolabeling.

Proteoliposomes with Reconstituted SecYEG Containing [^{125}I]TID-PC Support Protein Translocation. Reconstitution of SecYEG into proteoliposomes provides a simple system to incorporate [^{125}I]TID-PC into membranes that support protein translocation. Proteoliposomes were prepared by rapid dilution of the octylglucoside-solubilized purified SecYEG in the presence of *E. coli* phospholipids and unlabeled TID-PC (2% of total phospholipid content). The latter is 2−10 times more than the amount of radioiodinated [^{125}I]TID-PC used in photolabeling experiments. These proteoliposomes showed a translocation ATPase activity of 120 pmol of Pi/(min μL), which is comparable to the activity found in the absence of TID-PC. Proteoliposomes were active in the translocation of different precursor proteins (Figure 4). ProOmpA and a prePhoE translocate in an ATP- and SecA-dependent manner with efficiencies of 48% and 6.5%, respectively. Interestingly, the translocation of pre-PhoE with a glycine to cysteine mutation in the hydrophobic core of the signal sequence was three times more efficient than with the wild-type signal sequence (i.e., 22% instead of 6.5%). This mutation was reported to result in a lower pmf dependency for translocation (*41*), and therefore, translocation in these pmf-deficient proteoliposomes is increased. It is, therefore, concluded that the TID-PC-containing SecYEG proteoliposomes are a genuine tool to analyze the SecA phospholipid interaction under translocation conditions.

A Fraction of SecA Is in Contact with Phospholipids during Protein Translocation. To investigate whether SecA

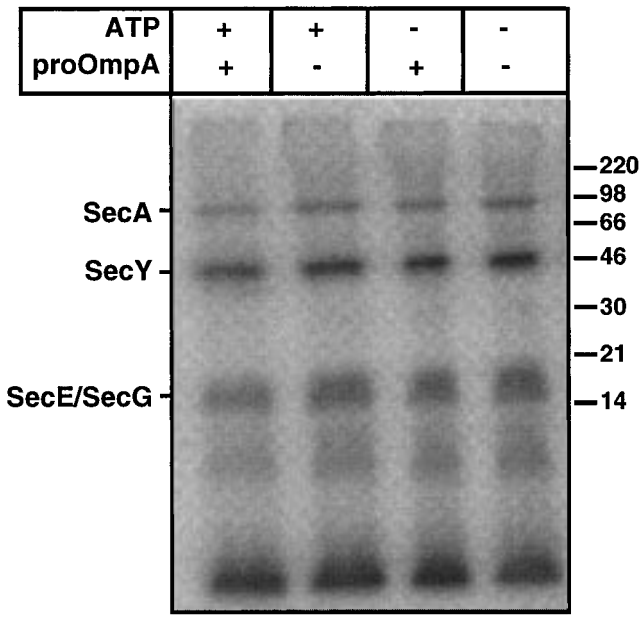

FIGURE 5: SecA is labeled under translocation conditions. Translocation reactions were performed as described. After 10 min at 37 °C, samples were photolyzed, and $CHCl_3$/MeOH was precipitated and analyzed by 15% SDS−PAGE and phosphorimaging. In lane 2 proOmpA was omitted from the reaction, in lane 3 ATP was omitted, and in lane 4 both were omitted. The positions of molecular weight markers are indicated.

is in contact with phospholipids, SecA (same amount [20 μg/mL] as used in translocation reactions) was incubated with SecYEG proteoliposomes containing [^{125}I]TID-PC in the presence of ATP and proOmpA. After 10 min of incubation at 37 °C, the samples were photolyzed. This resulted in labeling of SecA (Figure 5). Furthermore, a band corresponding to SecY and a band corresponding to SecE and SecG, which do not run separately under these conditions, were also labeled. Bands running below SecEG are unrelated to protein since these also appear when liposomes prepared by octylglucoside dilution are photolyzed. In some experiments proOmpA was also visible as a labeled band (data not shown). Labeling of SecB was never observed, demonstrating that also in proteoliposomes the [^{125}I]TID-PC probe is restricted to the hydrophobic region of the membrane. Labeling of SecA was not dependent on the presence of proOmpA or ATP (Figure 5), as evident from the quantitation of the SecA labeling on autoradiograms of several independent experiments. Also, addition of the nonhydrolyzable nucleotides AMP−PNP and ATPγS did not change the labeling efficiency of SecA (data not shown). Therefore, it is concluded that under these conditions a fraction of the SecA is in contact with the phospholipids.

SecA at the Translocase Is Largely Shielded from the Phospholipids. SecA labeled in the presence of proteoliposomes can be derived from lipid-bound SecA or from SecA bound to the SecYEG complex. To determine the relative lipid accessibility of SecA in each of these topologies, SecA labeling in the presence of liposomes was quantitatively compared to the labeling in the presence of SecYEG proteoliposomes. This experiment was performed in the presence of proOmpA and ATP, conditions that lead to stabilization of the membrane-inserted state of SecA (*11*). Liposomes and the SecYEG proteoliposomes contained identical amounts of lipids and label. When SecA was

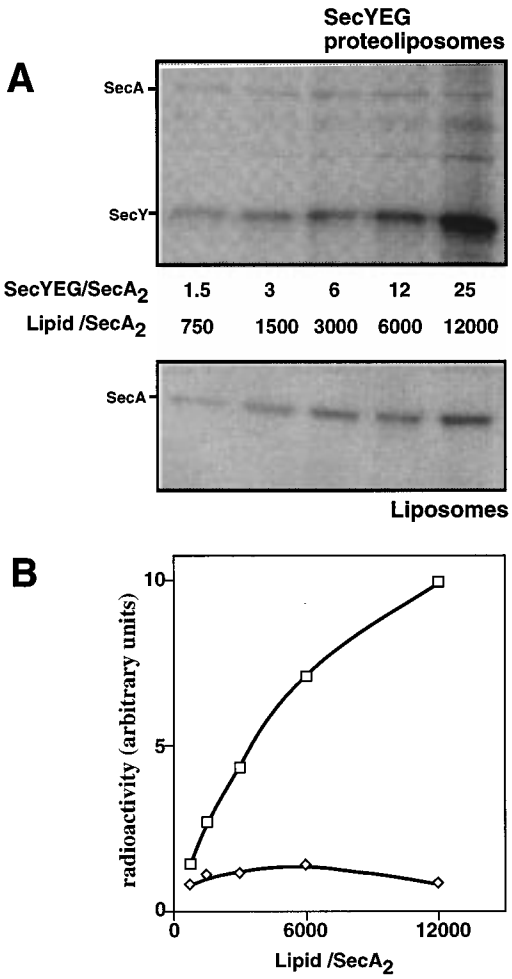

FIGURE 6: SecA is shielded from the phospholipids in SecYEG-containing vesicles. (A) To reaction mixtures containing SecA, proOmpA, ATP, and translocation buffer, increasing amounts of liposomes or proteoliposomes were added. After 20 min of incubation at 37 °C, samples were photolyzed and analyzed as described. (B) Quantitative analysis of the SecA labeled in the presence of liposomes (squares) or proteoliposomes (diamonds). The molar ratio of SecYEG over the SecA dimer is indicated (only applicable to the SecYEG-containing vesicles). This figure takes into account that only half of the SecYEG complexes are oriented correctly. The molar ratio of phospholipid over the SecA dimer is indicated and applies to both types of vesicles.

incubated with increasing amounts of liposomes, the SecA labeling also increased (Figure 6, parts A and B). This reflects an increase in binding of SecA to the liposomes (compare Figure 2C). In striking contrast, titration with increasing amounts of SecYEG proteoliposomes did not result in a significant increase of the amount of SecA labeled. At the highest point SecA labeling in the proteoliposomes is only 8% of the labeling in the presence of liposomes. The increase in SecY labeling (Figure 6A) is a result of the increased amounts of SecY. SecA binds with high affinity to SecYEG binding sites at the membrane (*6*). In proteoliposomes the binding of SecA to SecYEG can be monitored by protection of a His-tag against cleavage by enterokinase (*44*). Protection is maximal when the SecA monomer is present in slight excess relative to SecYEG (*44*). This indicates that SecA binds SecYEG with high affinity in the reconstituted system. Furthermore, this binding is functional as it correlates to the translocation ATPase and translocation activity of the proteoliposomes. Given the stoichiometries

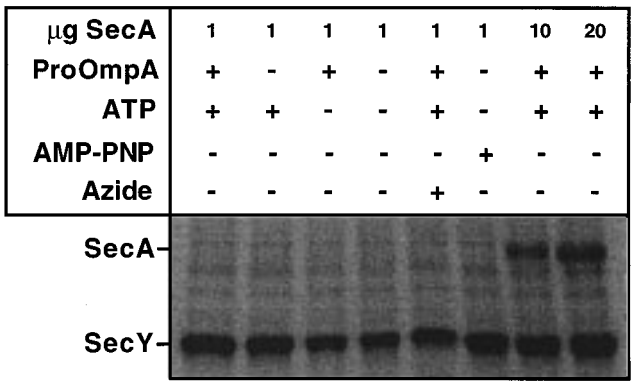

FIGURE 7: SecA shielding from the phospholipid acyl chains does not depend on translocation ligands. Cross-link reactions with SecYEG proteoliposomes (10 nmol of lipid, 55 pmol of SecYEG in the correct orientation) were performed as described in the legends to Figure 5. ATP and proOmpA were included where indicated. Reactions were performed in the presence of 10 mM sodium azide or 2 mM AMP−PNP as indicated. Control reactions were supplemented with 10 and 20 μg of SecA, respectively.

of this experiment (Figure 6A) ranging from 1.5 to 25 SecYEG heterotrimers/SecA dimer, most of the SecA will be bound to the SecYEG complex which is present in excess. This low amount of labeling compared to the labeling observed in the presence of liposomes combined with the knowledge that the amount of SecA bound to the SecYEG proteoliposomes is maximal lead to the conclusion that SecA bound at the translocase has at least a 10-fold lower access to the acyl chains than lipid-bound SecA.

The experiment described above was performed under conditions that lead to formation of the membrane-inserted state (*11*, *44*). Binding to the translocase does not require nucleotides or precursor proteins (*44*). To investigate the lipid accessibility of SecA bound at the translocase under various conditions, SecA was incubated with an excess of SecYEG proteoliposomes (Figure 7), and either the precursor protein proOmpA or ATP or both were omitted. Under none of these conditions was SecA accessible from the lipid bilayer. Also in the presence of AMP−PNP or azide, two agents that block SecA in the membrane inserted state (*11*, *52*), SecA was largely shielded from the label in the phospholipid bilayer. To exclude that SecY inhibits SecA labeling in these vesicles per se, the amount of SecA was increased to allow binding to the phospholipids. Under these conditions labeling of SecA does occur (lanes 7 and 8). These results let us conclude that SecA bound to the SecYEG complex is largely shielded from the hydrophobic region of the membrane independent of its SecYEG-bound conformation.

DISCUSSION

In this study the insertion of SecA in the lipid bilayer in the absence and presence of the SecYEG complex was studied with a cross-linking approach using the photoactivatable phospholipid analogues [^{125}I]TID-PC and [^{125}I]TID-PG. Efficient labeling was observed when SecA was incubated with vesicles prepared from *E. coli* phospholipids. Labeling was dependent on the presence of negatively charged lipids. With pure DOPC vesicles no labeling was observed. Previous experiments revealed that SecA is able to insert into a DOPG monolayer, but not into a DOPC monolayer at a surface pressure comparable to that in a phospholipid bilayer (*35*, *53*). These findings indicate that labeling directly correlates with membrane insertion. The absence of labeling with DOPC vesicles further demonstrates that the probe is restricted to the membrane. Importantly, labeling occurred irrespective of whether the probe was attached to a PC or PG molecule, implying that there is no large scale enrichment of acidic phospholipids in the periphery of the lipid-bound SecA.

SecA interacts in a nucleotide-dependent manner with model membranes containing negatively charged phospholipids (*34*, *35*, *51*). However, no nucleotide dependency of labeling with [^{125}I]TID-PC was observed under similar conditions. The nucleotide-dependent aggregation of vesicles is mediated by two distinct lipid-binding sites on SecA. One is located at the positively charged extreme C-terminus and is involved primarily in electrostatic interactions with the lipids. The other is located in the N-terminal region and is involved in hydrophobic interactions with the vesicles (*51*). In the presence of ATP one of these interactions is lost, resulting in the inhibition of vesicle aggregation. The photolabeling experiments now show that in the presence of ATP the hydrophobic interaction still exists. It can therefore be concluded that the interaction with the C-terminal binding site is lost. In monolayer experiments both binding and insertion are reduced albeit to a different extent by nucleotides (*35*). Because the relation between the molecular area and surface pressure is known for DOPG monolayers (*54*), it is possible to estimate the molecular area of SecA for different lipid-bound conformations. The molecular area in the presence of ATP is approximately 1.5 times more as compared to the situation without nucleotides (or with nonhydrolyzable nucleotides) from ~5 to ~8 nm^2, respectively. ESR studies have provided insight into the depth of insertion (*34*). By the use of spin-labeled lipids it was demonstrated that the SecA penetrates into a DOPG bilayer up to the 14th carbon atom of the acyl chain. This penetration is strongly reduced in the presence of ATP. Together these results show that there is a marked conformational change of SecA within the bilayer upon interaction with different nucleotides. However, these changes apparently do not result in large differences in the area exposed to the hydrophobic region.

SecA has the ability to insert into the phospholipid bilayer. Therefore it is conceivable that SecA bound at the translocase is inserted in the phospholipid bilayer. Alternatively, insertion could occur in a proteinacious environment. To distinguish between these two possibilities, photolabeling of SecA was assayed in the presence of SecYEG containing proteoliposomes under conditions where the largest fraction of SecA was bound to the translocase. This was directly compared to liposomes not containing the translocase. Under the applied conditions most of the SecA was expected to be bound to the SecYEG complex on the basis of the affinity (k_d = 7 nM, (*22*)) and the efficient protection of the His-tag from enterokinase cleavage (*44*). Binding to the translocase was functional since both translocation ATPase activity and translocation of different precursor proteins were observed. Under these conditions the labeling efficiency of SecA from the phospholipid acyl chain region is only 8% compared to the vesicles not containing SecYEG. This low level of labeling could be derived from lipid-bound SecA or might

indicate that the SecYEG-bound SecA is to a small extent accessible from the phospholipid bilayer. The latter would imply that binding of SecA to the SecYEG complex reduces the contact of SecA with the acyl chains by at least 92%. The reduction in lipid accessibility appears not to be dependent on nucleotides and precursor protein and is neither inhibited nor promoted by azide or AMP−PNP, compounds that stabilize SecA in a membrane-inserted state (*3*, *11*, *52*). This demonstrates that SecA bound at the translocase is largely shielded from the phospholipid acyl chains.

SecA membrane insertion during precursor protein translocation is usually assayed as the formation of two large protease-resistant fragments of 65 and 30 kDa that are derived from the N-domain and C-domain, respectively (*12*). In *E. coli* inner membrane vesicles, these fragments could be labeled only to a minor extent by an externally added hydrophobic membrane marker (*38*). It thus appears that these SecA fragments are largely shielded from the phospholipid acyl chains under the conditions that stabilize SecA in the membrane-inserted state. However, since these labeling studies were performed only after proteolysis of the SecA, it is not clear whether the lack of labeling concerned an entire molecule or if SecA fragments that were accessible to the label were degraded by the protease. Moreover, it is not known whether the 65- and 30-kDa fragments originate from the same molecule or from different subunits of the SecA dimer. The reconstitution of SecYEG together with a photoactivatable lipid analogue, used in the present study, provided a system in which the SecA lipid interaction could be studied without the need of a protease resistance assay, that is, while retaining the active state. Therefore, also conditions that do not lead to protease-resistant fragments could be analyzed, showing that the intact and active state of SecA bound to SecYEG is only poorly accessible from the lipid bilayer. Our data therefore not only correlate but also further extend the studies obtained with *E. coli* membranes. Furthermore, the low level of labeling demonstrates that shielding must occur for both subunits of the SecA dimer. In the experiments with *E. coli* membranes, labeled proteolytic fragments were obtained that were not derived from the SecYEG-bound SecA fraction but that presumably reflected the lipid-bound SecA (*38*).

Negatively charged phospholipids are involved in protein translocation (*27*). Since the SecA translocation ATPase activity is dependent on acidic phospholipids (*36*), it is likely that SecA during translocation interacts with negatively charged lipids. Surprisingly, translocase-bound SecA is largely shielded from the phospholipid acyl chains. Acidic lipids could be bound very specifically and function as cofactors of SecA activity. However, this is not very likely since high levels of acidic lipids are required for obtaining wild-type levels of translocation, while a large variety of anionic headgroup structures are able to restore translocation activity of PG-depleted vesicles (*55*). How can the effect of the depletion of acidic lipids from the membrane on translocation be explained? One possibility is that the anionic lipids enhance translocation by directly interacting with the SecYEG complex in the absence of extensive SecA lipid interactions. The requirement of nonbilayer preferring phospholipids at the periplasmic site of the membrane suggests that this type of phospholipid indeed acts directly on SecYEG (*26*). Acidic lipids may also be involved in interacting with the signal sequence (*31*). Since one can only study protein translocation activity in the presence of the SecYEGA holoenzyme and a functional precursor protein, it remains to be determined at what level these lipids exactly act. SecA is localized in the cytosol, bound to the membrane phospholipids and to the high-affinity binding sites consisting of SecYEG (*6*). The presence of lipid-bound SecA offers an attractive alternative explanation for the acidic lipid requirement. Translocase-bound SecA can be replaced by soluble SecA during translocation (*11*), but no significant amount of SecA is released from the membrane during translocation (*56*). The latter thus restricts cycling to the translocase and lipid-bound SecA pools. Decreasing the amount of acidic phospholipids in the inner membrane results in a decrease in the amount of membrane-bound SecA, and this is compensated by an increase of the cytosolic SecA levels (*36*). Cells apparently respond to a lower lipid-bound fraction by increasing the cytosolic amount of SecA to drive the equilibrium to the membrane. The lipid-bound fraction of SecA may therefore represent a critical intermediate in translocation and, for instance, function as a translocation cue that rapidly replaces the "unloaded" form of SecA bound at SecYEG. Since diffusion of the lipid-bound SecA is restricted to only two dimensions, cycling may occur with faster rates than with the free cytosolic fraction.

The core of the eukaryotic translocon is formed by a heterotrimeric Sec61p complex that is homologous to the SecYEG complex (*57*). The Sec61p complex forms oligomeric channels that assemble upon interaction with ribosomes (*58*). The large degree of shielding from the phospholipid region suggests that SecA inserts in the membrane via a proteinaceous channel, possibly an oligomer of SecYEG trimers. Such a proteinaceous channel could possibly assemble upon interaction with lipid-inserted SecA and protect the 30- and 65-kDa fragments of SecA against proteolysis or prevent labeling from the lipid bilayer. However, recent studies have shown that the SecYEG is highly susceptible to proteolysis and that it is completely degraded (fragments smaller than 6 kDa) under the conditions that stabilize the SecA fragments in a protease-resistant and lipid-inaccessible state (*44*). This may suggest that the SecYEG-bound SecA is more peripherally associated with the membrane, while the lipid-bound SecA is able to insert into the membrane.

The finding that translocase-bound SecA is largely shielded from the phospholipid acyl chains suggests that acidic phospholipids are involved in translocation by acting on the lipid-bound fraction of SecA. More detailed experiments will be needed to address the role of SecA lipid insertion during protein translocation.

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