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SecA Is Not Required for Signal Recognition Particle-mediated Targeting and Initial Membrane Insertion of a Nascent Inner Membrane Protein*

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In *Escherichia coli*, signal recognition particle (SRP)-dependent targeting of inner membrane proteins has been described. *In vitro* cross-linking studies have demonstrated that short nascent chains exposing a highly hydrophobic targeting signal interact with the SRP. This SRP, assisted by its receptor, FtsY, mediates the transfer to a common translocation site in the inner membrane that contains SecA, SecG, and SecY. Here we describe a further *in vitro* reconstitution of SRP-mediated membrane insertion in which purified ribosome-nascent chain-SRP complexes are targeted to the purified SecYEG complex contained in proteoliposomes in a process that requires the SRP-receptor FtsY and GTP. We found that in this system SecA and ATP are dispensable for both the transfer of the nascent inner membrane protein FtsQ to SecY and its stable membrane insertion. Release of the SRP from nascent FtsQ also occurred in the absence of SecYEG complex indicating a functional interaction of FtsY with lipids. These data suggest that SRP/FtsY and SecB/SecA constitute distinct targeting routes.

Going across or integrating into the inner membrane presents two different challenges to proteins synthesized in the cytosol of a prokaryotic cell, and this is reflected by the existence of two main targeting routes. The SecB pathway is specialized for the targeting of periplasmic and outer membrane proteins. SecB is a cytosolic chaperone that binds to the mature region of a subset of preproteins (1). The SecB-preprotein complex is targeted to SecA, which is bound with high affinity to the membrane-embedded SecYEG complex (for review, see Ref. 2).

Both *in vivo* and *in vitro* experiments indicate that the signal recognition particle (SRP)[1] pathway is primarily used for the targeting of integral inner membrane proteins (3–7). This pathway resembles SRP-mediated targeting of proteins to the membrane of the endoplasmic reticulum (ER) in eukaryotes (for review, see Ref. 8). The eukaryotic SRP interacts with nascent membrane and secreted proteins and targets them to the SRP receptor SRα at the ER membrane. The eukaryotic SRP is a complex consisting of six proteins arranged on an RNA scaffold, the 7 S RNA. *Escherichia coli* contains a smaller SRP composed of the P48 protein and the 4.5 S RNA, which are homologous to the eukaryotic SRP54 and the 7 S RNA, respectively (for review, see Refs. 9 and 10). In addition, an SRα homologue has been identified in *E. coli,* designated FtsY (7, 11). Both P48 and FtsY (and their eukaryotic counterparts) are GTPases, and GTP binding and hydrolysis regulate the targeting cycle in a mechanism that has not yet been fully defined (12–14). *In vivo* overproduction of polytopic inner membrane proteins titrated out the limited amount of endogenous *E. coli* SRP (5), whereas depletion of essential SRP components affected membrane targeting of both polytopic and bitopic inner membrane proteins (3, 4).

We have developed an *in vitro* cross-linking approach to dissect subsequent stages in SRP-mediated protein targeting. Short nascent inner membrane proteins synthesized in a membrane-free *E. coli* cell extract were shown to interact with the SRP via their amino-terminal hydrophobic targeting signal (6). The SRP receptor FtsY, GTP, and inverted *E. coli* inner membrane vesicles were shown to be required for release of the SRP from the nascent chain (7). Upon release of the SRP at the membrane, the nascent chain inserts into the membrane in the vicinity of the translocase components SecA, SecY, and SecG (7). Hence, it seems that the SecB- and SRP-targeting pathways converge at a common membrane translocation complex. The exact composition of the translocase that is reached by the different routes may differ and has not yet been determined. Of particular interest is the cross-linking of SRP-targeted precursors to SecA, which functions as the molecular motor for translocation in the SecB pathway (2).

In an attempt to further define the minimal requirements for SRP-mediated targeting and membrane insertion, we have used proteoliposomes instead of inner membrane vesicles in our targeting assay. Short nascent chains of the inner membrane protein FtsQ were efficiently targeted to the *E. coli* SecYEG complex reconstituted in *E. coli* phospholipids. Hence, SecA seems dispensable for SRP-mediated protein targeting to the *E. coli* translocase. Before stable membrane insertion, the SRP was efficiently released from the nascent chains by FtsY in a GTP-dependent reaction. Protein-free liposomes also induced significant FtsY-mediated release of the SRP from the nascent chains indicating that the interaction of FtsY with lipids is

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[1] The abbreviations used are: SRP, signal recognition particle; ER, endoplasmic reticulum; DSS, disuccinimidyl suberate; RNC, ribosome nascent chain complex; SRNC, SRP ribosome nascent chain complex; GMP-PNP, guanylyl-β,γ-imidodiphosphate; INV, inverted membrane vesicle.

sufficient to induce a conformational change in the SRP that lowers its affinity for the nascent chains.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were from Roche Molecular Biochemicals GmbH (Mannheim, Germany). Megashortscript T7 transcription kit was from Ambion Inc. (Austin, TX). [^{35}S]Methionine and protein A-Sepharose were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Disuccinimidyl suberate (DSS) was from Pierce. *E. coli* total phospholipid extract was from Avanti Polar Lipids Inc. (Pelham, AL). Aurintricarboxylic acid was from ICN Biomedicals Inc. (Aurora, OH). Nucleotides and all other chemicals were supplied by Sigma.

Strains and Plasmid Constructs—Strain MC4100 was used to obtain translation lysate and inverted membrane vesicles (INVs) (15). Strain Top10F′ was used as host for plasmid pC4Meth108FtsQ (6), and the OmpT and OmpP protease-deficient strain SF100 was used as expression host for plasmid pET349 to allow the overexpression of SecYEG (16).

In Vitro Transcription and Translation, Formation of SRNC Complexes—pC4Meth108FtsQ plasmid was linearized with *Hin*dIII and transcribed using T7 polymerase. The resulting truncated mRNAs were translated for 20 min at 25 °C in an S-135 *E. coli in vitro* translation system to produce ribosome nascent chain complexes (RNCs) (6). After 3 min of incubation, aurintricarboxylic acid (1 mM) was added to inhibit further initiation of translation resulting in homogeneous nascent chain length. Translation was stopped by adding 3 mM chloramphenicol. To allow SRP-RNC (SRNC) complex formation, 260 nM reconstituted SRP was added to the translation reaction (7). After 5 min of incubation at 25 °C, samples were chilled on ice, and the resulting SRNC complexes were purified from the translation mixture by centrifugation through a high salt sucrose cushion (17) and resuspended in RN buffer (100 mM KOAc, 5 mM $Mg(OAc)_2$, 50 mM Hepes-KOH, pH 7.9).

Purification of the Translocase and Its Reconstitution into Proteoliposomes—The *E. coli* SecYEG complex that carries a hexahistidine tag at the amino terminus of SecY was purified and reconstituted in acetone/ether-washed *E. coli* phospholipids as described (16). Standard proteoliposomes were obtained by the reconstitution of 200 μl of SecYEG (50 μg/ml) in 40 μl of lipids (20 mg/ml). Proteoliposomes used in the experiment described in Fig. 3 were prepared using different amounts of 0.2 mg/ml SecYEG (0–50 μl) diluted in a total volume of 200 μl of purification buffer (1.25% (w/v octylglucoside, 0.3 mg/ml phospholipids, 40% (w/v) glycerol, and 50 mM KCl in Tris-HCl, pH 7.8). These amounts of SecYEG were reconstituted with 40 μl of phospholipids, yielding (v/v) ratios of 0–1.25. Before use, proteoliposomes were sonicated 3 times for 10 s; *E. coli* SecA was purified as described (18).

Targeting and Cross-linking—Targeting reactions were done as described previously by incubating purified SRNCs together with 1 mM FtsY, 50 μM GTP, 50 μM ATP, and either INVs (1.25 mg/ml protein) or proteoliposomes for 5 min at 25 °C (7). Modifications to this standard reaction protocol are described in the figure legends. Cross-linking was induced with 1 mM DSS for 10 min at 25 °C and quenched at 4 °C by adding one-tenth volume of quenching buffer (1 M glycine, 100 mM $NaHCO_3$, pH 8.5). To separate integral membrane from soluble and peripheral cross-linked complexes, samples were treated with 0.18 M Na_2CO_3, pH 11.3, for 15 min on ice. The membrane fractions containing integral membrane proteins were pelleted by ultracentrifugation (10 min, 110,000 × *g*) and resuspended in RN buffer. Supernatant fractions were trichloroacetic acid-precipitated, washed with cold acetone, and resuspended in RN buffer. Samples were immunoprecipitated as described (19) or mixed directly with 2× SDS-gel loading buffer before gel electrophoresis.

Immunodepletion of SecA from Translation Lysate—Anti-SecA serum was added to protein A-Sepharose slurry and incubated for 1 h at room temperature. After incubation the Sepharose was washed twice with incubation buffer (30 mM Tris-HCL pH 7.5, 150 mM NaCl, 30 mM Tris-HCL pH 7.5) and once with lysate buffer (10 mM triethylammonium, 10 mM $Mg(OAc)_2$, 22 mM $N(CH_3)_4Ac$). To prepare a control lysate, protein A-Sepharose was prepared in the same way except that no anti-SecA serum was added. Both Sepharose preparations were collected by centrifugation and resuspended in MC4100 translation lysate. After 2 h of incubation at 4 °C, the Sepharose beads were spun down and the supernatants recovered. Supernatant corresponding to the incubation with anti-SecA-loaded Sepharose was referred to as $SecA^-$ lysate, and supernatant corresponding to the incubation with Sepharose only was referred to as $SecA^+$ lysate. Both lysates were analyzed by immunoblotting using anti-SecA serum to verify the extent of depletion.

Sample Analysis and Quantification—Samples were analyzed on 12, 15, or 4–15% gradient SDS-polyacrylamide gels. Radiolabeled proteins were visualized by phosphor imaging using a Molecular Dynamics PhosphorImager 473 and quantified using the Imagequant software from Molecular Dynamics.

RESULTS

Nascent FtsQ Is Efficiently Targeted to the Translocase Contained in Proteoliposomes—FtsQ is a type II *E. coli* inner membrane protein, which was used as a model protein for SRP-mediated targeting throughout this study. An *in vitro* SRP-dependent targeting assay was developed in which short FtsQ RNCs were efficiently targeted to the translocase contained in purified INVs. Interaction of the labeled nascent chain with SecY and SecA was demonstrated by formation of covalent bonds using the bifunctional cross-linker reagent DSS (7). In this study we further investigated this SRP-dependent targeting process. To define the essential membrane components required for targeting and membrane insertion, proteoliposomes composed of *E. coli* phospholipids and purified SecYEG complex were used instead of INVs.

Short (108-mer) FtsQ nascent chains were produced *in vitro* using a membrane-free *E. coli* translation lysate. Under these conditions the endogenous SRP contained in the lysate is titrated out by the preponderance of exposed hydrophobic targeting signals. SRP loading efficiency was increased by adding reconstituted *E. coli* SRP after the translation reaction (7). The resulting SRP-RNCs complexes (SRNCs) were purified through a high salt sucrose cushion and incubated in the presence of FtsY, the *E. coli* homologue of the eukaryotic SRP receptor SRα (9) and GTP, which were shown to be essential for their proper targeting to the membranes and for efficient release of the SRP from the nascent chain (7). As accepting membranes, we compared INVs and proteoliposomes. Each reaction contained equal amounts of SecY as judged by quantitative immunoblotting (data not shown). After the targeting reaction cross-linking of 108FtsQ was induced using the membrane-permeable bifunctional cross-linker DSS and the membranes were extracted with Na_2CO_3 to separate membrane integral from soluble and membrane peripheral cross-linked complexes.

Either with INVs or with proteoliposomes, a 108FtsQ cross-linking adduct appeared at about 50 kDa in the Na_2CO_3 pellet (Fig. 1, *lanes 3* and *5*, respectively). This complex represents a membrane integral complex of the radiolabeled 108FtsQ and SecY, as demonstrated by immunoprecipitation (Fig. 1, *lanes 7* and *11*). The SecY adduct, which originates from the proteoliposomes, migrates slightly slower than the SecY from the INVs due to the histidine extension that is attached to the purified SecY used to reconstitute the SecYEG complex in the proteoliposomes (16). In addition, 108FtsQ was also found to be cross-linked to the other membrane integral components of the translocase, SecE and SecG (Fig. 1, *lanes 8* and *9* for INVs, *lanes 12* and *13* for proteoliposomes). High molecular weight (~69 kDa) cross-linked complexes in the Na_2CO_3 pellet of both INVs and proteoliposomes were also immunoprecipitated with antibodies directed against SecE (Fig. 1, *lanes 8* and *12*) and SecG (Fig. 1, *lanes 9* and *13*). These complexes probably represent trimeric structures consisting of 108FtsQ, SecY, and either SecE or SecG (7).

As previously reported (7), cross-linking to SecA (~120 kDa product) is detected in the Na_2CO_3 pellet samples when INVs are used in the targeting reaction (Fig. 1, *lanes 3* and *7*). A cross-linking adduct of ~220 kDa is also immunoprecipitated from the Na_2CO_3 pellet with anti-SecA serum and might correspond to cross-linked SecA dimer (Fig. 1, *lanes 3* and *7*). Upon immunoprecipitation a weak SecA adduct is also detected in the Na_2CO_3 pellet from proteoliposomes (Fig. 1, *lane 10*). Because the proteoliposomes do not contain any SecA initially, we

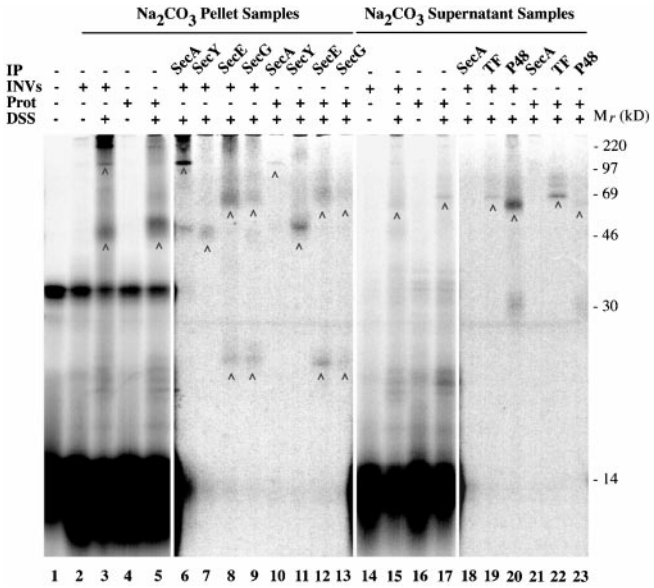

FIG. 1. **108FtsQ RNCs are efficiently targeted to proteoliposomes.** 108FtsQ RNCs were incubated with 260 nM reconstituted SRP. The SRNC complexes thus formed were purified by sedimentation through a high salt sucrose cushion and used for targeting as described under "Experimental Procedures." Either INVs or proteoliposomes (*Prot*) containing SecYEG complex were added to the targeting reaction that contained 1 μM FtsY. After cross-linking with DSS, soluble and peripheral membrane cross-linked complexes were extracted with Na_2CO_3. Both Na_2CO_3 pellet samples and trichloroacetic acid-precipitated Na_2CO_3 supernatant samples were examined by immunoprecipitation (*IP*) using the indicated antisera. Relevant cross-linking products are indicated with an *arrow*.

assume that some SecA contained in the translation lysate is co-purified with the SRNCs and is incorporated into the proteoliposomes during the targeting reaction. This point will be addressed in the last paragraph under "Results."

In the Na_2CO_3 supernatant from the INVs adducts to the SRP component P48 and trigger factor were detected (Fig. 1, *lanes 15*, *19*, and *20*) as observed before (7). Trigger factor may act as a general folding catalyst via its prolyl isomerase activity and was shown to interact with all nascent chains regardless of their hydrophobicity (6). In contrast, hardly any P48 adduct could be detected in the Na_2CO_3 supernatant when proteoliposomes were used as target membranes, whereas cross-linking to trigger factor was relatively efficient (Fig. 1, *lanes 17*, *22*, and *23*). This suggests an efficient release of the SRP from the FtsQ RNCs followed by an efficient targeting to the translocase as indicated by the strong cross-linking signal to SecY in the Na_2CO_3 pellet samples.

From these data we conclude that proteoliposomes composed of *E. coli* phospholipids and purified SecYEG complex support the transfer of 108FtsQ in a reconstituted SRP-mediated targeting assay from the SRP to the translocase. Compared with targeting reactions that contain INVs, the release of the SRP and the association with the translocase is even more efficient when proteoliposomes are used.

Targeting to the Translocase Contained in Proteoliposomes Only Requires FtsY and GTP Binding—SRP-mediated targeting of nascent FtsQ to SecY in the INVs requires FtsY and GTP or its nonhydrolyzable analogue GMP-PNP (7). We investigated whether release of the SRP from nascent FtsQ and association with SecY in the proteoliposomes occurs via the same mechanism by studying its dependence on FtsY and nucleotides in the targeting assay described above. As shown in Fig. 2, the release of the SRP and the association with SecY did not occur in the absence of FtsY (Fig. 2, *lanes 9*, *10*, and *11*). Proteoliposomes in combination with FtsY were essential for the release of the SRP (Fig. 2, *lanes 2* and *4*). GTP or GMP-PNP (*lanes 5* and *7*) but not AMP-PNP or GDP (*lanes 6* and *8*) supported the transfer reaction. Consistently, mutant FtsY, FtsY A449 (13) that is unable to bind GTP, was not functional in the targeting assay (data not shown).

Based on these results, we conclude that targeting of nascent FtsQ to proteoliposomes mechanistically resembles the targeting to INVs and thus provides a valid and attractive model for detailed studies on the mechanism of nascent chain transfer and membrane insertion.

Liposomes and FtsY Are Sufficient to Induce the Dissociation of SRP-RNCs Complexes—In vivo, FtsY is equally distributed between the cytosol and inner membrane (11). We have obtained evidence that FtsY has affinity for lipids (20).[2] To study the influence of the protein:lipid ratio on the efficiency of nascent chain transfer, proteoliposomes of different composition were prepared and tested in our targeting assay in the presence of FtsY and GMP-PNP. The efficiency of 108FtsQ targeting was monitored by quantifying the P48 cross-linking adducts in the Na_2CO_3 supernatant and the SecY cross-linking adducts in the Na_2CO_3 pellets (Fig. 3). As expected, the strongest cross-linking to P48 was observed when membranes were omitted in the targeting reaction and this signal was taken as 100%. Different proteoliposomes were made by mixing a fixed amount of *E. coli* phospholipids with increasing amounts of purified SecYEG complex. The targeting efficiency correlated with the SecYEG concentration in the lipid bilayer (Fig. 3). Proteoliposomes containing a SecYEG:lipid ratio of 1.25 resulted in the most efficient targeting as indicated by the weakest cross-linking to P48 and the strongest cross-linking to SecY.

Surprisingly, when liposomes that do not contain any SecYEG complex were used in the targeting reaction a significant SRP release was observed (~53%, Fig. 3). This release was completely dependent on the addition of both FtsY and GTP or GMP-PNP (not shown). Apparently, the interaction of FtsY with the lipids is sufficient to induce significant GTP-dependent release of the SRP from the nascent chain. However, this SRP release is not followed by effective membrane insertion of the 108FtsQ as judged by Na_2CO_3 extraction (data not shown). Apparently, the SecYEG complex is necessary and sufficient to obtain a stable integration into the lipid bilayer.

SecA Is Not Required for the SRP-dependent Targeting and Membrane Insertion of 108FtsQ RNCs—SecA is a peripheral translocase component that plays a key role in the post-translational translocation process by functioning as a receptor for precursor proteins and as an ATP-driven molecular motor (2). In a previous study (7), we demonstrated that 108FtsQ RNCs targeted to INVs were in close contact not only to the "core" translocase component SecY but also to SecA (see also Fig. 1, *lanes 3* and *6*). It remained unclear whether SecA plays an active role in the observed SRP-mediated targeting and membrane insertion of 108FtsQ or whether the SecA cross-linking adducts were obtained just because SecA is located in the vicinity of the nascent chain as a structural subunit of the *E. coli* translocase (2). If this last hypothesis is correct, SecA should be dispensable in the SRP-dependent targeting reaction. This could be tested by using SecYEG proteoliposomes as SecA-free target membranes. However, as shown in Fig. 1 (*lane 10*) weak cross-linking of SecA to 108FtsQ was observed in the Na_2CO_3 pellet of proteoliposomes. Apparently, SecA is co-purified with the SRNC preparation and can be incorporated into proteoliposomes during the targeting reaction. The affinity of SecA for SecY and for acidic phospholipids might underlie this

[2] E. de. Leeuw, K. te Kaat, C. Moser, G. Menestrina, R. Demel, B. de Kruijff, J. Luirink, and I. Sinning, manuscript submitted.

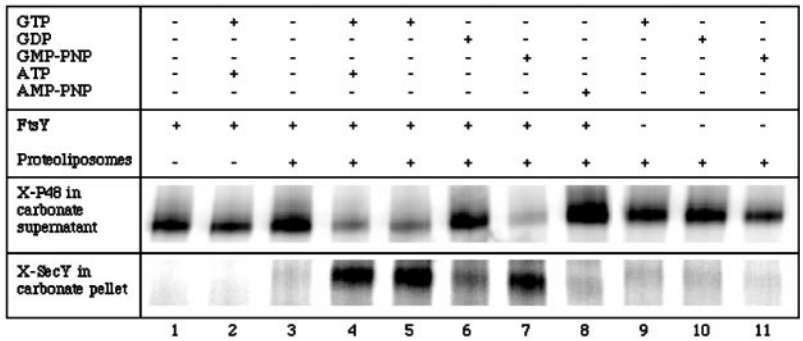

FIG. 2. **GTP binding is required for the release of SRP from 108FtsQ SRNCs and for the concomitant interaction of 108FtsQ with SecY contained in the SecYEG proteoliposomes.** Different nucleotides (50 μM each) were used in the targeting assay (see the legend to Fig. 1) that either contained or did not contain proteoliposomes. Cross-linking adducts to P48 (X-P48) in the Na_2CO_3 supernatant fractions and to SecY (X-SecY) in the Na_2CO_3 pellet fractions are shown.

Membranes used in the targeting assay	-	INVs	Proteoliposomes Ratio SecYEG:Lipid (v:v) 1.250	0.625	0.312	0.155	0.077	0.000
X-P48 in carbonate supernatant								
% Cross-linking	100	13.7	17.0	17.0	18.1	25.4	30.0	47.1
X-SecY in carbonate pellet								
% Cross-linking	0.0	56.4	100	77.6	40.7	9.9	5.4	0.0

FIG. 3. **Effect of the SecYEG:lipid ratio on SRP-mediated targeting to proteoliposomes.** 108FtsQ SRNCs were incubated in presence of FtsY (1 μM) and GMP-PNP (50 μM). INVs or proteoliposomes of different composition were used in the targeting reaction (see the legend to Fig. 1). The ratios between phospholipids (fixed volume) and purified SecYEG used to reconstitute proteoliposomes are given. In the Na_2CO_3 supernatant fractions, the strongest cross-linking to P48 (X-P48) was taken as 100%. In the Na_2CO_3 pellet fractions, the strongest cross-linking to SecY (X-SecY) was taken as 100%.

transfer of SecA from the SRNCs to the proteoliposomes (2). We estimated by Western blotting that about 0.3% of the SecA present in the translation lysate co-purifies with the SRNCs upon sedimentation of the ribosomes through a high salt sucrose cushion (data not shown). Two observations suggest that it is most likely that SecA is co-purified via its general affinity for ribosomes (21), rather than via a direct interaction with the FtsQ nascent chains. First, SecA was never found cross-linked to nascent FtsQ or any other nascent polypeptide in the absence of membranes even when added in excess (6). Second, when translation reactions were either not supplemented with any mRNA or supplemented with truncated mRNA encoding nascent chains of a cytosolic protein, the same amount of SecA was co-purified with the ribosomal fraction (data not shown). To remove this residual SecA, we immunodepleted the translation lysate using anti-SecA serum (see "Experimental Procedures" for details). After this procedure no SecA could be detected by immunoblotting in the translation lysate ($SecA^-$ lysate) compared with a mock treated lysate ($SecA^+$ lysate) (data not shown). Both lysates remained active for translation.

108FtsQ RNCs were produced using either $SecA^-$ or $SecA^+$ lysate and loaded with reconstituted SRP. After purification the two kinds of SRNCs were used in different targeting reactions to which purified SecA was added (0, 0.1, and 0.5 μM, final concentrations). Interaction with proteoliposomes was assessed by DSS-induced cross-linking to SecY (Fig. 4, *A–C*) and by quantification of the resistance of the RNCs to Na_2CO_3 extraction (Fig. 4*D*). When a $SecA^-$ lysate was used for translation and when no additional SecA was added to the targeting reaction, cross-linking to SecY was observed (Fig. 4*A*, *lane 4*) and the 108FtsQ was inserted into the membrane in a Na_2CO_3 resistant conformation (Fig. 4*D*). Thus, the FtsQ RNCs are able to reach the translocase and become inserted into the membrane in the absence of SecA. Upon addition of SecA to the targeting reaction cross-linking to SecY in the Na_2CO_3 pellet was slightly reduced irrespective of the translation lysate used, whereas SecA cross-linking adducts (**) appeared (Fig. 4*A*, *lanes 1–6* and Fig. 4*B*). Part of the SecA adducts were extracted by Na_2CO_3 (Fig. 4*A*, *lanes 7–12*). For both translation lysates, addition of purified SecA to the targeting reaction slightly reduced the Na_2CO_3 resistance of the targeted RNCs (Fig. 4*D*) consistent with the decrease in cross-linking to SecY.

To confirm that the different cross-linking adducts observed were indeed related to either SecA or to SecY, immunoprecipitations were carried out after cross-linking. The $SecA^+$ lysate was used to generate the RNCs, and 0.5 μM SecA was added to the targeting reaction. In the Na_2CO_3 pellet sample the 50-kDa product (*) was indeed immunoprecipitated with anti-SecY serum (Fig. 4*C*, *lane 3*). Both the major 120-kDa cross-linking adducts (**) and minor 220-kDa products present in the Na_2CO_3 pellet and supernatant were immunoprecipitated with the anti-SecA serum (Fig. 4*C*, *lane 2*). We assume that the ~220-kDa product corresponds to cross-linked complexes between the RNCs and the dimeric form of SecA. The SecA related adducts of ~150 and ~45 kDa ( ) in the Na_2CO_3 pellet are most likely proteolytic fragments of the SecA dimer and monomer cross-linking products.

From these data, we conclude that SecA is not required for efficient targeting and initial membrane insertion of FtsQ nascent chains *in vitro*. Furthermore, at high SecA concentrations there is a weak competition effect between SecA and SecY for the binding of RNCs delivered to the membrane via the SRP pathway. This competition seems to reduce the 108FtsQ insertion into the membrane.

DISCUSSION

We have further developed our homologous *in vitro* targeting assay that supports SRP-mediated targeting and membrane insertion of nascent *E. coli* inner membrane proteins (7). The assay now contains purified 108FtsQ RNCs saturated with SRP, FtsY, GTP, and proteoliposomes that consist of purified *E. coli* phospholipids and the core translocon complex SecYEG. In this system, the SRP was released from the nascent chain in a GTP-dependent reaction, associated with SecY and acquired a Na_2CO_3-resistant conformation in the proteoliposomes as efficiently as in crude INVs. Thus, we have defined the minimal requirements for an efficient *in vitro* SRP-dependent targeting to the core components of the *E. coli* translocon. Preliminary experiments on the topology of targeted 108FtsQ in both INVs and proteoliposomes using cysteine replacement coupled to chemical labeling, suggest that it has the same topology as the native protein (data not shown). No additional proteinaceous membrane factors are required in these early steps of the targeting process. Notably, the SecDF/YajC subcomplex is dispensable, and may therefore be specifically devoted for the

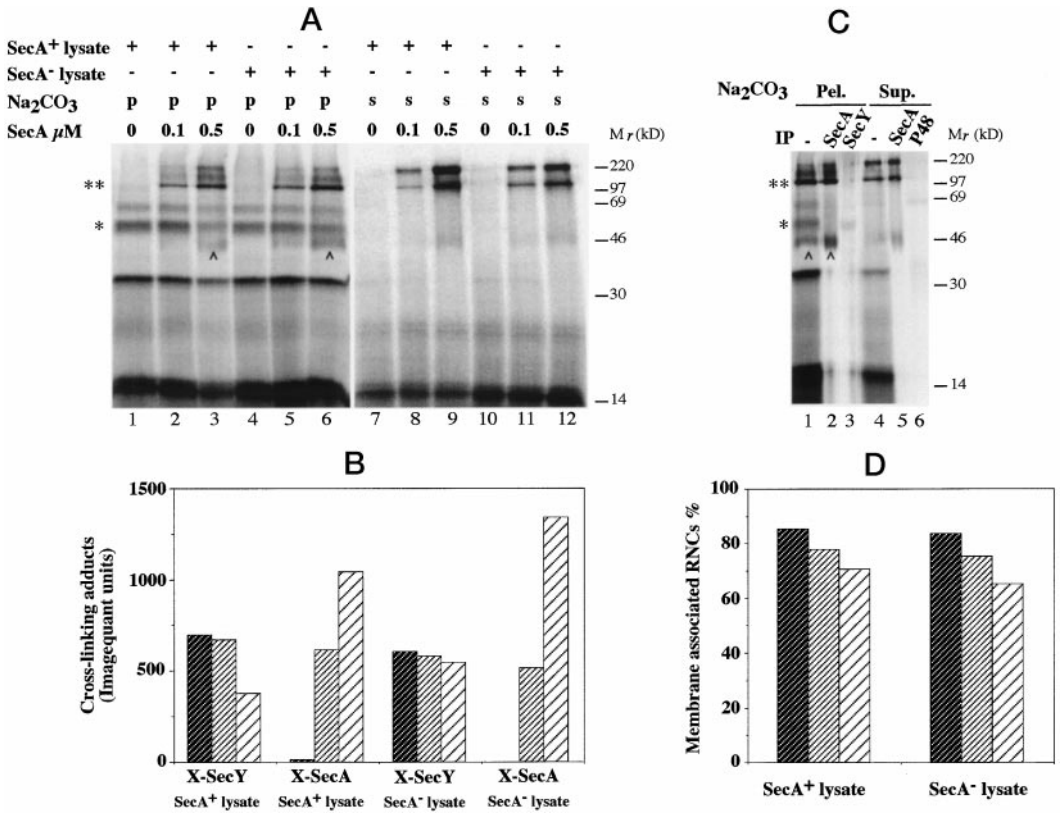

FIG. 4. **SecA is not required for the SRP-dependent targeting of nascent 108FtsQ to SecY and membrane insertion.** A $SecA^-$ lysate was prepared by immunodepletion and used to produce 108FtsQ RNCs in a comparison with mock-treated control ($SecA^{+}$) lysate. SRNCs were prepared as described in the legend to Fig. 1. The targeting reactions containing SRNCs, 1 μM FtsY, 50 μM ATP, 50 μM GTP, and proteoliposomes were supplemented or not with additional SecA (0, 0.1, or 0.5 μM, final concentrations). *A*, after cross-linking with DSS and carbonate extraction, Na_2CO_3 pellet samples (*p*) and trichloroacetic acid-precipitated Na_2CO_3 supernatant samples (*s*) were examined by SDS-polyacrylamide gel electrophoresis. Positions of the relevant cross-linked complexes, X-SecY (*) and X-SecA (**), are indicated. *B*, cross-linking adducts observed in Na_2CO_3 pellet samples in *panel A* were quantified by phosphor imaging. For each product the total Imagequant units measured are given (0 μM SecA, ▩; 0.1 μM SecA, ▨; 0.5 μM SecA, ▤). *C*, the same targeting reaction was done as in *panel A* using $SecA^+$ lysate for translation and 0.5 μM extra SecA in the targeting reaction. After cross-linking with DSS and Na_2CO_3 extraction, the cross-linked complexes present in the Na_2CO_3 pellet fraction (*Pel.*) and in the trichloroacetic acid-precipitated Na_2CO_3 supernatant fraction (*Sup.*) were immunoprecipitated (*IP*) with the indicated antisera. *D*, the same experiment was done as described in the legend to *panel A* except that no DSS treatment was carried out before the Na_2CO_3 extraction. The targeting reactions were supplemented or not with purified SecA (0 μM SecA, ▩; 0.1 μM SecA, ▨; 0.5 μM SecA, ▤). The percentage of RNCs remaining associated with the membrane pellets after Na_2CO_3 extraction was determined by SDS-polyacrylamide gel electrophoresis and phosphor imaging.

translocation or the membrane insertion of preproteins delivered via the SecB-dependent pathway (2).

Apparently, for the initial insertion of the inner membrane protein FtsQ, SecA, and ATP are not required. However, as judged by protein cross-linking, SecA was in close proximity to nascent FtsQ targeted to INVs (7) or when proteoliposomes were supplemented with SecA (this study). Probably, SecA is juxtaposed to the nascent chain due to its affinity for SecY (22, 23) rather than playing a crucial role in the initial insertion process or in the transfer of the nascent chain to SecY. As suggested by this study, when present in excess, SecA actually interfered with the transfer to SecY. Interestingly, a point mutation in SecY has been reported that specifically impaired the integration of integral membrane proteins but not the secretion of SecB-targeted precursors. In this conditional strain, expression of SecA was up-regulated (24).

SecA may be involved at a later stage in the insertion of inner membrane proteins especially when large periplasmic domains are translocated. The role of SecA in the complete assembly of inner membrane proteins is difficult to study *in vivo* and may differ for different substrate proteins (25–27). It should be noted that full-length FtsQ did not integrate into SecYEG proteoliposomes either in the presence or absence of SecA (data not shown) suggesting that additional components are required for complete *in vitro* reconstitution of inner membrane protein biogenesis.

Interestingly, liposomes that consist solely of *E. coli* phospholipids are still able to support the FtsY-mediated release of the SRP from the nascent chain, albeit less efficiently than SecYEG containing proteoliposomes. We have previously demonstrated that FtsY is able to associate with RNC-SRP complexes in the absence of GTP and membranes (7). INVs and GTP-binding but not hydrolysis were required to dissociate the SRP from the nascent chain (7). Apparently, FtsY pilots the targeting complex to the lipids without the requirement of any specific membrane receptor. This might cause a conformational change that is transferred to P48 in the SRP thus increasing the affinity of both P48 and FtsY for GTP. By analogy with the eukaryotic SRP cycle, binding of GTP to P48 may reduce its affinity for the nascent chain (28, 29). The nascent chain that is released at the membrane does not insert stably into the lipid bilayer in an alkali resistant conformation but requires the SecYEG complex to acquire a stable membrane association.

FtsY is located in part in the cytosol and in part in the cytoplasmic membrane (11). It is a highly charged protein that does not contain any predicted membrane spanning segments and its mechanism of association with the membrane is obscure (20). The data presented in this study indicate that in contrast to its mammalian counterpart SRα, FtsY can function in the release of the SRP in the absence of a dedicated membrane anchoring protein. This might explain the ability of FtsY to

cooperate with *E. coli* SRP in the targeting of proteins to microsomal membranes in a heterologous *in vitro* targeting assay (30). Consistent with this suggestion, we have recently observed that FtsY interacts with lipids using a phospholipid monolayer technique.[2] Through its affinity for lipids, FtsY may increase the effective concentration of SRNCs at the membrane (30). Still, proteoliposomes function more efficiently in the release of the SRP, which may be related to additional targeting functions of the ribosome and SRP. It is well established that eukaryotic ribosomes have affinity for Sec61p, the protein conducting channel in the ER membrane (31). Alternatively FtsY, in addition to its association with lipids, may interact directly with the SecYEG complex. However, an association of this kind could not be detected by cross-linking, immunoprecipitation or co-purification approaches.[3]

The reconstituted targeting system described in this study will be invaluable for a detailed analysis of the consecutive steps during SRP-mediated protein targeting and membrane insertion, allowing the intermolecular interactions, dynamics, and energy requirements of this process to be studied. The assay will also provide a means to identify soluble and membrane components specifically devoted to the proper assembly and folding of inner membrane proteins. Ultimately, this approach should lead to the full reconstitution of inner membrane protein biosynthesis.

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[3] E. de Leeuw and J. Luirink, unpublished results.

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