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In Vivo Cross-Linking of the SecA and SecY Subunits of the *Escherichia coli* Preprotein Translocase

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Precursor protein translocation across the *Escherichia coli* inner membrane is mediated by the *translocase*, which is composed of a heterotrimeric integral membrane protein complex with SecY, SecE, and SecG as subunits and peripherally bound SecA. Cross-linking experiments were conducted to study which proteins are associated with SecA in vivo. Formaldehyde treatment of intact cells results in the specific cross-linking of SecA to SecY. Concurrently with the increased membrane association of SecA, an elevated amount of cross-linked product was obtained in cells harboring overproduced SecYEG complex. Cross-linked SecA copurified with hexahistidine-tagged SecY and not with SecE. The data indicate that SecA and SecY coexist as a stable complex in the cytoplasmic membrane in vivo.

In *Escherichia coli* and other bacteria, secretory proteins are translocated across the cytoplasmic membrane as precursors (or preproteins) by a multimeric integral membrane protein complex termed the translocase (reviewed in references 17 and 48). Core components of the translocase are the SecA, SecY, SecE, and SecG polypeptides (6, 23). SecA is a large homodimeric protein (16), with preprotein-stimulated ATPase activity (32). It is found both free in the cytosol and peripherally associated with the inner membrane (8, 9, 12). SecY, SecE, and SecG are integral membrane proteins that can be purified in detergent solution as a heterotrimeric complex (6, 7). This complex, or a mixture of the individual purified subunits, suffices for reconstitution of the SecA- and energy- (ATP and proton motive force) dependent preprotein translocation into proteoliposomes (6, 7, 15, 23, 36).

A variety of biochemical and genetic evidence indicates that the subunits of the translocase are interacting polypeptides. SecY, SecE, and SecG can be isolated and coimmunoprecipitated as a stable complex (6, 7). SecE stabilizes SecY (35) and protects it against proteolysis by FtsH, a membrane-bound ATP-dependent protease (27). Further studies have defined the interacting regions of SecY and SecE (2, 20, 41). In wild-type cells, newly synthesized SecY immediately associates with SecE to form a complex that does not dissociate over time or during translocation, whereas SecG may be a less stable subunit of the translocase (26). In contrast, from genetic data, it can be argued that SecE and SecY are dissociating proteins (3, 4).

SecA is a dissociable subunit of the translocase and appears to cycle in an ATP-dependent fashion between cytosol and membrane (5, 18, 46), although it has been suggested that this process may not be necessary for the translocation reaction per se (11). Membrane binding of SecA occurs with high affinity at the SecYEG complex (14, 22) and with low affinity to phospholipids (24, 33). By use of ligand affinity blotting, a direct interaction between SecA and SecY has been demonstrated (43). This interaction appears to involve the amino-terminal half of SecY and the carboxy-terminal third of SecA. During translocation, both SecY and SecA can be photoaffinity cross-linked to the preprotein proOmpA, indicating that these two Sec proteins are in the nearest vicinity of a translocating polypeptide chain (25). The SecYEG-bound SecA is accessible to trypsin digestion from the periplasmic surface (11, 28, 45), suggesting that it may traverse the membrane. In the presence of preprotein and ATP, a 30-kDa carboxy-terminal domain of SecA has been shown to adopt a membrane-protected, proteinase-inaccessible conformation (18, 19, 38). This has led to the suggestion that the stepwise translocation of preproteins across the membrane (42) is coupled to ATP-dependent cycles of membrane insertion and deinsertion of the carboxy-terminal SecA domain (18). Strikingly, this process seems to be coupled to a reversible membrane topology inversion of the SecG subunit of the translocase (37).

Most of the data on the interaction between Sec proteins has been gathered from in vitro studies using isolated membrane vesicles and cytosolic components. We have now employed an in vivo cross-linking approach to identify SecA-interacting proteins in intact cells. Cross-linking results in the formation of a 150-kDa protein complex, which in addition to SecA harbors the SecY protein. These data support recent in vitro suggestions (11, 45) that SecA is an intrinsic subunit of the translocase.

MATERIALS AND METHODS

Materials. Formaldehyde (37% [wt/vol] in H_2O) was from Sigma Chemical Co. (St. Louis, Mo.), Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) agarose was obtained from QIAGEN Inc. (Chatsworth, Calif.), and Wizard miniprep columns were from Promega Co. (Madison, Wis.). A mixture of mouse monoclonal antibodies (oligoclonal) (13) was used to detect SecA on immunoblots. Antibodies against SecE and SecG were obtained by immunizing rabbits with synthetic peptides corresponding to amino acids 64 to 81 for SecE (KGKATVAFAREARTEVRK) and 89 to 98 for SecG (APAKTEQTQP), coupled to a multiantigen peptide (Research Genetics, Huntsville, Ala.). Rabbit polyclonal antiserum against SecB and a synthetic peptide corresponding to the 22 amino-terminal amino acids of SecY were generously provided by W. Wickner (Dartmouth College, Hanover, N.H.).

Bacterial strains and plasmids. *E. coli* D10 (21) and NO2947 (30) were used. For overproduction of SecYEG in strain NO2947, pET340 that harbors the *secY*, *secE*, and *secG* genes in tandem under control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *trc* promoter was used (45). pET324 was used as the control plasmid lacking the *sec* genes. Plasmids overproducing the SecYEG complex with amino-terminally His-tagged SecY (pET349) and SecE (pET320) were obtained by insertion of a linker region in the *Nco*I sites in the start codons of the *secY* and *secE* genes in pET340. The inserted linking sequence 5′-CC ATGCATCACCATCACCATCACGATGACGATGACAAAGCCATGG-3′

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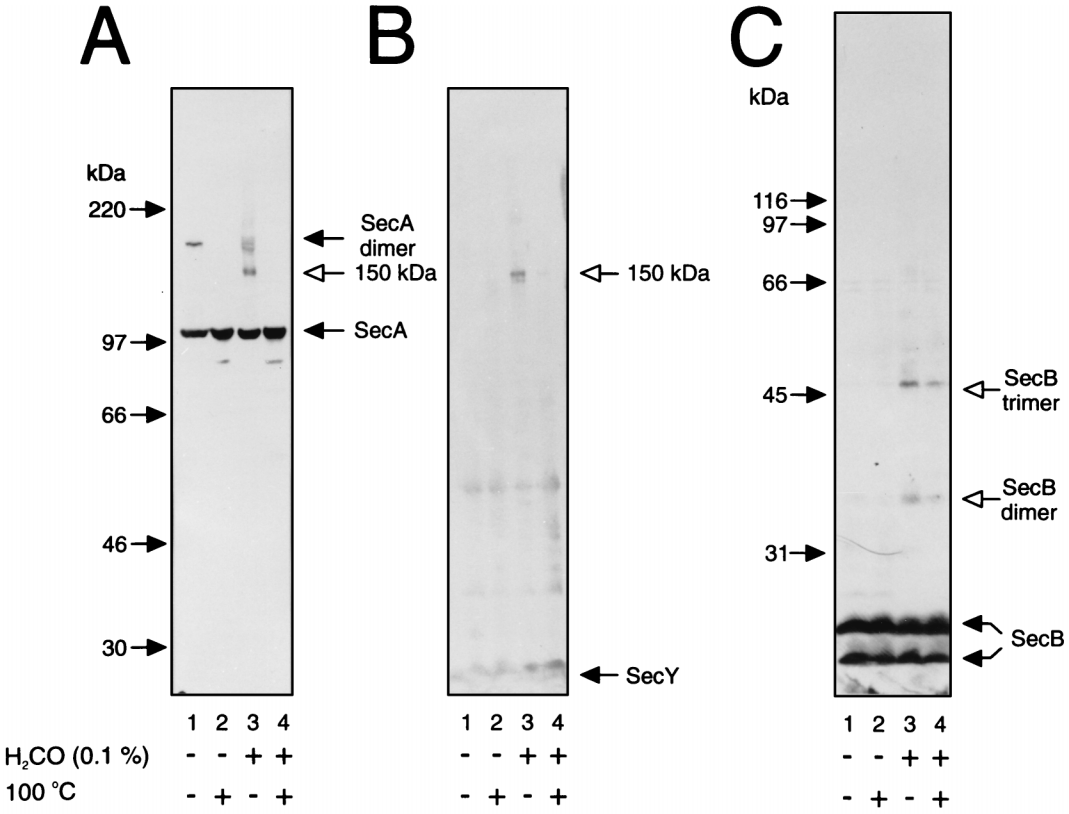

FIG. 1. In vivo formaldehyde cross-linking of SecA to SecY. Anti-SecA (A), -SecY (B), and -SecB (C) immunoblots of *E. coli* D10 cells incubated without (lanes 1 and 2) and with (lanes 3 and 4) 0.1% (wt/vol) formaldehyde (H_2CO) are shown. Prior to SDS-PAGE, parts of the samples were heated at 100°C (lanes 2 and 4) to reverse the cross-linking. Cross-linked complexes are indicated by open arrows, while the positions of SecA, SecY, and SecB proteins are indicated by closed arrows. For the detection of SecA and SecY cross-links, SDS–7.5% PAGE was used with an optimal separation of proteins with a molecular mass between 50 and 300 kDa. For the detection of SecB, SDS–12% PAGE was used.

codes for H_6D_4KA, i.e., a six-His tag followed by an enterokinase recognition site. A His tag preceded by an enterokinase site was introduced at the carboxy terminus of SecY via PCR. The oligonucleotide sequence 5′-CCAGGAATT CGTCCGGGA-3′, encoding amino acids 354 to 358 of SecY and an *Eco*RI site, was used as a forward primer. The *Sal*I-digestible reverse primer 5′-GAGAGTC GACTTAATGGGTGATGGTGATGGTTTGTCATCGTCATCTCGGC CGTAGCCTTTCAG-3′ encodes amino acids 437 to 442 of SecY and adds the amino acid sequence $(D)_4K(H)_6$ to the carboxy terminus. The PCR product was cloned into pBluescript II SK^+ (Stratagene, La Jolla, Calif.) via *Eco*RI-*Sal*I digestion and finally cloned in *Eco*RI-*Cla*I-digested pET340 via *Eco*RI-*Acc*I digestion, yielding pET512. All constructs were confirmed by sequence analysis on a Vistra DNA sequencer 725 with the automated Δ*taq* sequencing kit (both from Amersham, Buckinghamshire, United Kingdom). Isolation of plasmid DNA and other DNA techniques followed standard procedures (40).

Growth of bacteria and isolation of cytoplasmic membranes. NO2947 cells transformed with the various plasmids were grown aerobically at 37°C on LB broth containing 50 mg of ampicillin per liter to an optical density at 660 nm (OD_{660}) of 0.7. Exponentially growing cultures were then supplemented with 0.5 mM IPTG, and growth was continued to an OD_{660} of 1.0. Membrane vesicles were prepared by French pressure treatment (10). SecA translocation ATPase activity in the presence of urea-extracted membranes (12) was measured as described previously (32), with 100 μg of membrane proteins per ml, 20 μg of SecA per ml, 2 mM ATP, 50 μg of SecB per ml, and in the absence or presence of 30 μg of proOmpA per ml.

Cross-linking and protein analysis. In vivo cross-linking of intact cells was performed as described previously (39). Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5 to 15% acrylamide gels (31), followed by Western blotting (immunoblotting) onto Immobilon-P polyvinylidene fluoride membranes (Millipore, Bedford, Mass.). The Protean II system from Bio-Rad (Hercules, Calif.) was used for electrophoresis to obtain maximal separation of the cross-linked complexes. Immunoblots were developed with CSPD chemoluminescent substrate as described in the manufacturer's recommendations (Tropix, Bedford, Mass.). Protein concentrations were determined by Lowry assays (34). For quantitative analysis of immunoblots developed with the chemiluminescence kit, films of various exposures were scanned densitometrically with a DF-2400T scanner (Dextra Technology Corp., Taipei, Taiwan) and analyzed with SigmaScan/Image (Jandel Corp., San Rafael, Calif.).

Purification of His-tagged proteins. After cross-linking, cells (10 ml, OD_{660} of 1.0) were washed three times with 50 mM Tris-Cl (pH 8.0), containing 2 mM phenylmethylsulfonyl fluoride and 5 mM *p*-aminobenzamidine, resuspended in 1 ml of the same buffer, and disrupted through sonication. The remaining cells were spun down (10,000 × *g*, 2 min), and membranes were collected from the supernatant by centrifugation (120,000 × *g*, 20 min). The pellet was solubilized in 250 μl of buffer A (1.25% [wt/vol] *n*-octyl-β-D-glucopyranoside [octylglucoside], 20% [wt/vol] glycerol, 300 mM NaCl, 50 mM Tris-Cl [pH 8.0]) for 1 h on ice, after which nonsolubilized material was removed by centrifugation (120,000 × *g*, 20 min). To ensure the presence of equal quantities of protein during the purification procedure, 20 μl of the supernatant was analyzed by SDS-PAGE followed by Coomassie brilliant blue staining and immunoblotting with anti-SecA antibody. Solubilized proteins were incubated for 2 h at 4°C by constant shaking with Ni^{2+}-NTA agarose beads that were prewashed with buffer A. The solution was transferred into Wizard miniprep columns, and flowthrough fractions were collected by centrifugation (Eppendorf centrifuge, 2,000 rpm, 1 min). The column material was washed five times with 200 μl of 30 mM imidazole in buffer A for 15 min and then centrifuged. Proteins that were specifically bound were finally eluted with 100 μl of 250 mM imidazole in buffer A. Samples were analyzed by SDS-PAGE and immunoblotting.

RESULTS

In vivo cross-linking of SecA-associated proteins. Wild-type D10 cells were cross-linked with formaldehyde and analyzed by SDS-PAGE and immunoblotting with an oligoclonal antibody (OAb; mixture of monoclonal antibodies) directed against SecA (13). In the presence of 0.1% (wt/vol) of formaldehyde, a specific cross-linked protein complex with a molecular mass of 150 kDa was obtained (Fig. 1A, compare lanes 1 and 3). In addition, a cross-linked product (lane 3) with a molecular mass

of about 180 kDa was observed. The 180-kDa complex represents dimeric SecA protein as was confirmed by cross-linking of purified SecA (data not shown) and was also present in unboiled samples not incubated with formaldehyde (Fig. 1A, lane 1). Most of the cellular SecA is not cross-linked and remained visible as a monomer on SDS-PAGE with a molecular mass of 100 kDa. Formaldehyde-dependent cross-linking of proteins is reversible, and both complexes were found to disintegrate upon extensive boiling (lane 4). When higher concentrations of formaldehyde were employed, i.e., 0.5 to 1% (wt/vol), the specificity of cross-linking was lost, and a smear of protein aggregates with a molecular mass larger than 250 kDa was identified by the anti-SecA OAb (data not shown).

To assess whether the cross-linked complex contains any of the integral membrane subunits of the translocase, blots were developed with polyclonal antibodies (PAbs) that recognize SecY, SecG, or SecE. The anti-SecY PAb detected a protein complex with a molecular mass of 150 kDa in the cross-linked sample (Fig. 1B, lane 3), while with anti-SecE and -SecG PAbs, no specific protein complexes were found in this molecular mass range (data not shown).

Cross-linking of the cytosolic chaperone SecB was examined since SecA binds to SecB with high affinity (22), and the molecular mass of a SecB tetramer (64 kDa) could also account for a ~160-kDa SecA-associated complex. With 0.1% formaldehyde, a minor fraction of the SecB was found to be cross-linked (Fig. 1C). The products were tentatively identified as dimers and trimers, since similar cross-links were found with purified SecB (data not shown). No SecB was present in the 100- to 200-kDa range, and it is therefore unlikely that the 150-kDa complex contains cross-linked SecB. These data suggest that the 150-kDa complex is composed of at least monomeric SecA together with SecY protein.

Overproduction of SecYEG results in an increased cross-linking of the 150-kDa complex. To substantiate the polypeptide composition of the formaldehyde cross-linked 150-kDa complex, in vivo cross-linking experiments were performed with cells that overproduce the SecYEG complex. *E. coli* NO2947 was used, since it shows a better overproduction of SecYEG than strain D10 (data not shown). Cells were transformed with plasmid pET342 carrying the *secY*, *secE*, and *secG* genes in tandem with individual ribosome binding sites or with plasmid pET324, a control plasmid lacking the *sec* genes. Formaldehyde cross-linking of SecYEG-overproducing cells (termed SecYEG$^+$) again resulted in the formation of the 150-kDa complex, but the amount formed was substantially (i.e., eightfold) larger (based upon densitometric scanning), as observed with the parental strain containing the control plasmid (Fig. 2A). The elevated level of the 150-kDa complex formed in the SecYEG$^+$ strain correlated with the increase in the amount of SecA present in sucrose gradient-purified inner membranes (Fig. 2B).

Isolation of the SecA-containing 150-kDa complex. To enable purification and unequivocal identification of the polypeptide composition of the cross-linked 150-kDa complex, a six-histidine tag was introduced at the amino or carboxy terminus of SecY and at the amino terminus of SecE. The genes were cloned individually in tandem behind the *trc* promoter in the order *secY-secE-secG*, as has been described for the nontagged genes (45). Strain NO2947 was transformed with pET324 (control), pET340 (SecYEG$^+$), pET349 (SechYnEG$^+$, designating a His tag on the N terminus of SecY), pET512 (SechYcEG$^+$, designating a His tag on the C terminus of SecY), and pET320 (SecYhEnG$^+$, designating a His tag on the N terminus of SecE). Immunoblot analysis of isolated membranes with SecY, SecE, and SecG-specific PAbs revealed that the His-tagged proteins were overexpressed in similar amounts as the untagged proteins (Fig. 3A). The His tag at SecY or SecE does not interfere with the activity of the translocase, since urea-treated membranes derived from the various strains exhibited identical SecA translocation ATPase activities that were around 20-fold higher than the activity found for the parental strain (Fig. 3B). The background ATPase activity of the SecYEG$^+$ membranes was increased as well, most likely due to a stimulatory effect of the presence of high amounts of the SecYEG complex (Fig. 3B).

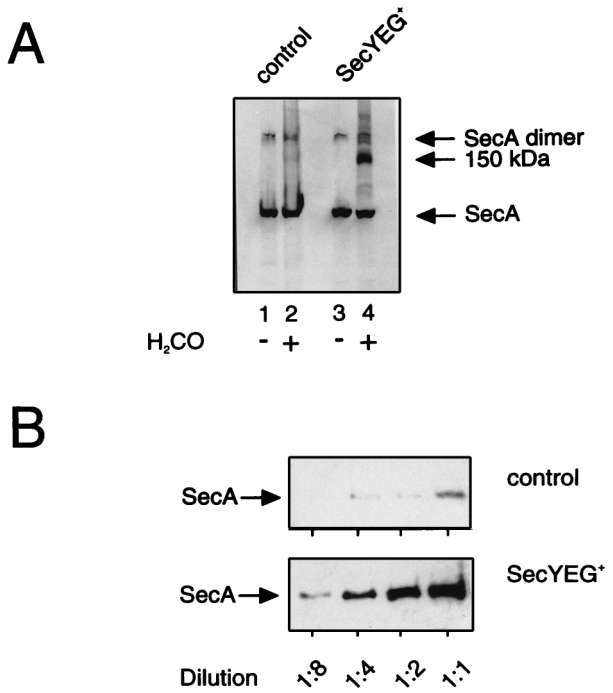

FIG. 2. Overproduction of the SecYEG complex results in increased cross-linking of SecA as a 150-kDa complex. Experiments were performed with *E. coli* NO2947 cells transformed with pET324 (control) or pET340 (SecYEG$^+$). (A) Cells were incubated without (lanes 1 and 3) or with (lanes 2 and 4) 0.1% (wt/vol) formaldehyde (H_2CO) and analyzed by SDS–7.5% PAGE and immunoblotting with anti-SecA OAb. (B) Anti-SecA immunoblots of a dilution series of sucrose gradient-purified inner membrane vesicles from control cells and SecYEG$^+$ cells are shown. A ratio of 1:1 indicates 5 μg of total membrane protein.

Cells that overproduce the SecYEG complex with His-tagged SecY or SecE proteins were incubated with 0.1% formaldehyde. After disruption by sonication, membranes were isolated, solubilized with octylglucoside, and loaded onto Ni^{2+}-NTA columns. After several washing steps, proteins were eluted with imidazole and analyzed by immunoblotting with anti-SecA OAb. Only in the case of His-tagged SecY did the eluates from the cross-linked samples show the presence of the 150-kDa complex (Fig. 4, lanes 3 and 4). No SecA or SecA-containing complex was specifically eluted from the column loaded with the sample containing the His-tagged SecE or with His-tagged SecY when the sample was not cross-linked (Fig. 4, lanes 5 and 2, respectively). The lack of a SecA-containing complex in the imidazole-eluted fractions with the His-tagged SecE is not due to the lack of binding of this protein to the column resin, since His-tagged SecY and SecE can be readily purified as individual proteins by the Ni^{2+}-NTA affinity chromatography (data not shown). As a control, elution fractions were stained with anti-SecY or anti-SecE PAb, showing that His-tagged SecY and SecE were purified specifically from the expected samples (Fig. 4, lanes 2 to 5 in the middle and lower

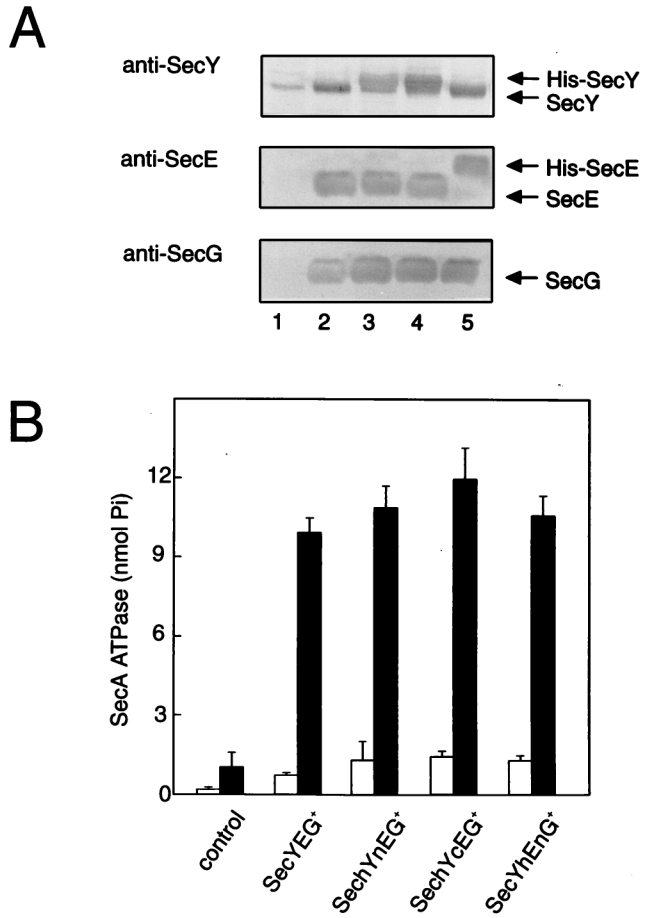

FIG. 3. Functional overexpression of the SecYEG complex with His-tagged SecY and SecE proteins. (A) Membranes derived from strain NO2947 bearing pET324 (control, lane 1), pET340 (SecYEG$^+$, lane 2), pET349 (SechYnEG$^+$, lane 3), pET512 (SechYcEG$^+$, lane 4), and pET320 (SecYhEnG$^+$, lane 5) were analyzed by SDS–15% PAGE and immunoblotting with PAbs against SecY, SecE, and SecG, as indicated. (B) Stimulation of the SecA ATPase activity by the precursor protein proOmpA with the membranes shown in panel A is illustrated. Assays were performed as described in Materials and Methods with urea-treated membranes. SecA ATPase activities in the absence (open bars) and presence (solid bars) of preprotein are indicated. The data shown are the average and standard deviation of three independent experiments.

panels). These data unequivocally identify SecA and SecY as the cross-linking partners of the 150-kDa complex.

DISCUSSION

The general protein secretory pathway in *E. coli* is an extensively studied model system for preprotein transport in bacteria. Various biochemical approaches have shown that SecY, SecE, and SecG interact, forming a heterotrimeric integral membrane protein complex. This complex functionally interacts with the SecA protein to form a catalytically active assembly termed the translocase, which satisfies the minimal requirement to reconstitute efficient preprotein translocation in vitro. In this report, we have used an in vivo cross-linking approach to analyze the SecA interaction with the SecYEG complex. The use of formaldehyde as cross-linker allowed the identification of a specific interaction between SecA and SecY in intact cells. This interaction most likely is a direct one, since it is detected in in vitro ligand affinity blotting experiments (43). It is well established that the SecY, SecE, and SecG subunits interact (6, 7, 23, 26, 35, 44) and that the entire SecYEG complex is needed for specific SecA binding (14, 29). SecA may also interact with other proteins, such as the integral membrane proteins SecD and SecF (19) and the cytosolic chaperone SecB (22). However, the cross-linking approach did not reveal a specific interaction of SecA with any other proteinaceous component of sufficient abundance. Since the detection of complexes by cross-linking is limited by the availability and the proximity of reactive groups, the absence of cross-linked complexes does not imply that there are no other in vivo protein-protein interactions than the ones shown in this paper.

The cross-linking reagent identified a 150-kDa complex that, based on the estimated molecular mass in SDS-PAGE, contains SecY together with monomeric SecA. Cytosolic SecA is normally purified as a stable dimeric protein and can be cross-linked as such by formaldehyde and succinimidyl esters (1). Since the SecA dimer does not dissociate at the translocase (16), it seems that the conformation of the translocase-bound SecA differs from that of the cytosolic SecA in a manner such that it cannot be efficiently cross-linked as a dimer. Alternatively, a single SecA-SecY cross-linking event may induce a structural rearrangement of the molecules and thereby prevent further cross-linking or cause an aberrant behavior of the complex on an SDS-PAGE gel. At high formaldehyde concentrations, SecA-containing complexes with a molecular mass of >250 kDa were detected. However, the poor specificity of

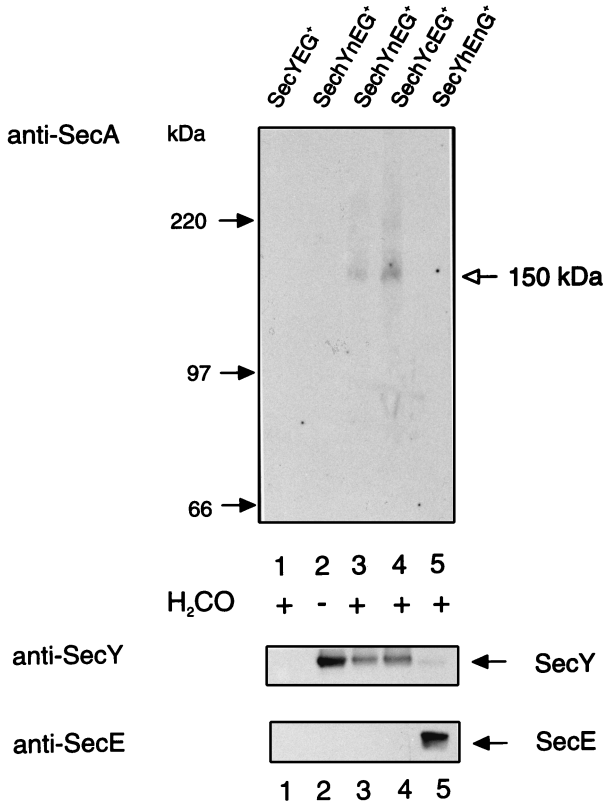

FIG. 4. Purification of the 150-kDa complex through His-tagged SecY. Immunoblots of elution fractions after Ni^{2+}-NTA purification of the indicated samples are shown. On blots developed with anti-SecA OAb, the 150-kDa complex is visible only in fractions from cross-linked samples containing His-tagged SecY (upper panel). His-tagged SecY and SecE are stained in the appropriate samples, both before and after cross-linking (middle and lower panels, respectively). For the anti-SecA blot, samples were run on an SDS–7.5% PAGE gel; for the anti-SecY and -SecE blots, they were run on an SDS–15% PAGE gel.

cross-linking under those conditions prevented the detection of discrete protein bands. The amount of 150-kDa complex formed was dependent on the strain used for cross-linking and was, for instance, consistently higher with strain D10 than with strain NO2947. The difference may be related to the growth physiology and/or due to small variations in the level of functional translocase. Nevertheless, with each strain, the same specificity of cross-linking was observed, i.e., predominant formation of the SecA-containing 150-kDa complex.

The cross-linking experiments were aimed at the detection of SecA-associated proteins under conditions that no translocation takes place, i.e., the idle state of SecA. Therefore, washed, resting cells were used. A substantial fraction of the cellular SecA, including a tightly bound subfraction, is membrane associated under these conditions (8). In SecYEG$^+$ cells, an even larger fraction of the total cellular SecA is tightly membrane associated, since it is not removed upon sucrose gradient purification of inner membranes or by urea extraction (45). The cellular redistribution is most likely due to an elevation of the number of high-affinity SecA membrane binding sites (14) and takes place at the expense of the cytosolic SecA pool, which is partially compensated for by a mild overproduction (45). The SecYEG-bound SecA molecule exposes its carboxy terminus to the periplasm and is active in in vitro preprotein translocation (45). Since the cross-linking yield correlates with the increase in the SecA membrane association upon SecYEG overproduction, it is concluded that the elevated cross-linking of SecA to SecY in SecYEG$^+$ cells is due to the enhanced level of functional translocase.

To elucidate the protein composition of the 150-kDa complex, His-tagged SecY and SecE were used. All His-tagged proteins were normally active in protein translocation, since they supported translocation reactions as efficiently as the untagged proteins did. Whereas normal preprotein translocation was increased, the His-tagged proteins did not support translocation of a precursor carrying a deficient signal sequence (data not shown). This proOmpA (termed Δ8) misses residue Ile-8 of the signal sequence and is translocated into isolated inner-membrane vesicles carrying the *prlA4* phenotype (47). Therefore, we conclude that the His-tagged SecY and SecE proteins have a normal, and not a *prlA* or *prlG*, phenotype, respectively.

By use of the same conditions as those described above for the purification of the cross-linked SecA-SecY complex, we were able to purify biochemical amounts of His-tagged SecY or SecE as individual proteins. However, the His-tagged proteins are part of an intact SecYEG complex, since they can be isolated as such in a one-step purification on an anion-exchange column (unpublished data). We can only speculate why the SecYEG complex dissociates during Ni^{2+}-NTA affinity purification. It may be due to the absence of phospholipids in buffers or to the extensive washing procedures with detergent-containing buffers, both of which are conditions that differ from other purification procedures (6, 7, 14). In a similar way as that described for the SecY and SecE proteins, a plasmid encoding amino-terminally His-tagged SecG was constructed. However, the protein could not be detected on immunoblots of membranes isolated from the overproducing strain, and the construct was therefore not used in further experiments.

In conclusion, the in vivo cross-linking approach demonstrates a specific interaction between SecA and SecY as a 150-kDa protein complex in the cytoplasmic membrane of intact cells. It appears that even in resting cells, these proteins stably interact at the membrane translocation sites. Since the SecA cross-linking method discriminates between the SecYEG-bound and nonbound fractions, it provides a convenient tool to probe the SecA membrane-association state in vivo.

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