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Binding, activation and dissociation of the dimeric SecA ATPase at the dimeric SecYEG translocase

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The bacterial preprotein translocase is comprised of a membrane-embedded oligomeric SecYEG structure and a cytosolic dimeric SecA ATPase. The associations within SecYEG oligomers and SecA dimers, as well as between these two domains are dynamic and reversible. Here, it is shown that a covalently linked SecYEG dimer forms a functional translocase and a high affinity binding site for monomeric and dimeric SecA in solution. The interaction between these two domains stimulates the SecA ATPase, and nucleotides modulate the affinity and ratio of SecA monomers and dimers bound to the linked SecYEG complex. During the translocation reaction, the SecA monomer remains in stable association with a SecYEG protomer and the translocating preprotein. The nucleotides and translocation-dependent changes of SecA–SecYEG associations and the SecA dimeric state may reflect important facets of the preprotein translocation reaction.

Keywords: ATPase/channel/oligomerization/preprotein translocation/SecA/SecYEG

Introduction

Preproteins containing an N-terminal classical leader peptide cross the membrane by means of a multisubunit translocase. The membrane core of the preprotein translocase is formed by the heterotrimeric SecYEG complex, universally conserved in bacteria, archaea and eukaryotes (Cao and Saier, 2003). The cytosolic part of the bacterial translocase is the dimeric ATPase SecA (Lill *et al*., 1990; Driessen, 1993). Preproteins bind to the translocase at SecA, triggering multiple rounds of ATP binding and hydrolysis (Hartl *et al*., 1990). The current model of preprotein translocation suggests that SecA interchanges between membrane-'inserted' and 'deinserted' states at the membrane-embedded SecYEG complex, thereby allowing the sequential movement of preprotein segments across the membrane (Economou and Wickner, 1994).

SecA is the motor subunit of the translocase, but the precise mechanism by which ATP-derived energy is coupled to the preprotein movement remains elusive. In addition to its high affinity for membrane-embedded SecYEG (Hartl *et al*., 1990), SecA also interacts with numerous ligands: leader and mature regions of preproteins, acidic phospholipids, SecB, nucleotides, Mg^{2+}, Zn^{2+} and its own mRNA (Economou, 2002; and references therein). Accordingly, the crystal structure of SecA reveals a complex multidomain protein with numerous binding folds (Hunt *et al*., 2002). Recent studies suggest that SecA undergoes major rearrangements upon binding to acidic phospholipids or leader peptides, leading to the dissociation or polymerization of dimeric SecA (Or *et al*., 2002; Benach *et al*., 2003). An earlier study involving heterodimers with one ATPase-inactive subunit has shown that the SecA dimer is important for translocation (Driessen, 1993), yet the recent findings suggest that SecA monomerization and polymerization may play major roles in translocation (Or *et al*., 2002; Benach *et al*., 2003).

Similarly, another current debate concerns the dynamics and stoichiometry of the SecYEG translocation channel. Electron microscopy studies have first revealed that the SecYEG protomer, or its eukaryotic counterpart, assembles into an oligomer with a pore-like structure (Hanein *et al*., 1996; Meyer *et al*., 1999; Manting *et al*., 2000). Except for one study, other evidence for oligomeric SecYEG associations comes from cross-linking, sedimentation analysis, native gel electrophoresis, electron crystallography, genetic and fluorescence resonance energy transfer (FRET) experiments (Manting *et al*., 2000; Yahr and Wickner, 2000; Collinson *et al*., 2001; Veenendaal *et al*., 2001; Bessonneau *et al*., 2002; Breyton *et al*., 2002; Matsuo *et al*., 2003; Mori *et al*., 2003). Altogether, these different studies suggest that the SecYEG oligomers are dynamic assemblies and different oligomeric states may be involved during translocation.

Knowledge of the subunit dynamics of the SecYEG oligomers, SecA dimers and SecYEG–SecA associations is thus central to understanding the mechanism of protein translocation. This is a difficult question because some of these interactions are transient and occur only at the membrane or during preprotein translocation. Novel experimental tools and methods of analysis need to be developed. The present work reports that the stabilization of the SecYEG dimer via covalent linkage allows the detection of a stable SecYEG–SecA complex in detergent solution. It is shown that nucleotides, which play a critical role in regulating SecA-mediated preprotein movement, also modulate the SecA–SecYEG associations. Furthermore, at the translocation channel, the preprotein is stably engaged with monomeric SecA and monomeric SecYEG. These results support the possibility that changes in the SecA dimeric state play a critical role during the translocation reaction. The oligomeric state of the functional SecYEG translocation channel is discussed.

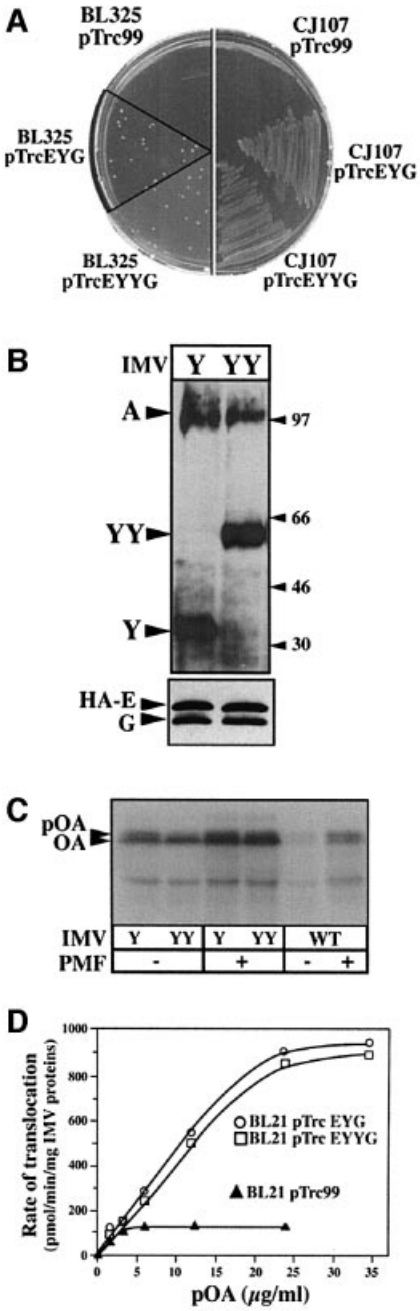

Fig. 1. The covalently linked SecYEG dimer is functional for preprotein translocation. (**A**) Overproduction of SecYYEG suppresses the SecDFyajC^{-} cold-sensitive and SecY thermo-sensitive phenotypes. Strain BL325 (BL21 *tgt::kan-araC*$^{+}$ -P_{BAD}*::yajCsecDF*) or CJ107 (Wolfe *et al*., 1985) were transformed with the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible plasmid pTrc99 or pTrc99 encoding $SecYE_{HA}G$ (pTrcEYG) or $SecYYE_{HA}G$ (pTrcEYYG). Transformants were plated on LB/ampicillin (50 μg/ml) containing 1 mM IPTG and incubated at the restrictive temperature. (**B**) Immunostaining of IMV preparations. About 1 μg of IMV proteins prepared from *E.coli* BL21 overexpressing $SecYE_{HA}G$ (IMVs-Y) or $SecYYE_{HA}G$ (IMVs-YY) were analyzed by SDS–PAGE and immunostained with the indicated antibodies. In the presence of SDS, SecY (48.5 kDa) and SecYY (97 kDa) migrate aberrantly. (**C** and **D**) Translocase activity of the IMV preparations. Translocation reactions were performed and analyzed as described in Materials and methods.

Results

A tandem of two covalently linked SecY subunits is functional for preprotein translocation

A gene encoding two SecY subunits fused in tandem (*secYY*) was constructed by linking two *secY* open reading frames (ORFs), in-frame, without an intervening stop codon. The hybrid was cloned along *secE* and *secG* in an expression-controlled plasmid vector (pTrcSecEYYG). The thermosensitive phenotype of the *secY* mutant strain (CJ107; Wolfe *et al*., 1985), as well as the cold-sensitive phenotype of the SecDFyajC-depleted strain (BL325; Duong and Wickner, 1997), were suppressed by expression of this plasmid (Figure 1A). Thus, two covalently linked SecY subunits are likely to participate in a functional translocase.

Western blot analysis of inner membrane vesicles (IMVs) prepared from the *Escherichia coli* strains carrying plasmids pTrcSecEYG (termed IMVs-Y) or pTrcSecEYYG (termed IMVs-YY) showed that SecYY/SecY, SecE and SecG were correctly synthesized and overproduced (Figure 1B). The level of endogenous SecA, which normally co-purifies with IMVs, was similar in these preparations (Figure 1B). An *in vitro* translocation assay showed that both IMVs-Y and IMVs-YY support efficient proOmpA translocation (Figure 1C), each stimulated by the proton-motive force. A quantitative translocation assay using an increasing concentration of preprotein substrate (Figure 1D) confirmed that the translocation capacity is increased ~10 times in IMVs-Y or IMVs-YY compared with wild-type IMVs (i.e. without Sec overproduction). Hence, it can be concluded that the SecYY hybrid molecule can participate in an active preprotein translocase.

The SecYY hybrid molecule forms a stable complex with SecE and SecG

Blue native gel electrophoresis (BN–PAGE; Schägger and von Jagow, 1991) of solubilized IMV proteins was performed to probe the oligomeric association of SecYY with SecE and SecG. From the IMVs-Y detergent extracts, SecYEG monomers, dimers and tetramers were immunodetected (Figure 2A, lanes 1 and 3). The SecYEG monomer:dimer ratio depends on the detergent concentration, while SecYEG tetramers and higher oligomers appear with the Sec overproduction, as previously shown (Bessonneau *et al*., 2002). From the IMVs-YY detergent extracts, oligomers of molecular weight similar to those seen with IMVs-Y were detected, with the preponderance of a $(SecYEG)^2$-like assembly (Figure 2A, lanes 2 and 4). All the assemblies cross-reacted with anti-SecY or anti-SecG antibodies (lane 7), while the SecYY-containing complex was purified by affinity chromatography using a His_6-tagged version of SecE (Figure 3A). Thus, the SecYY molecule forms stable associations with both SecE and SecG. Furthermore, the native SecYEG dimer migrated at the same position as the purified or membrane-solubilized SecYY-containing complex (Figures 2A and 3A). The relative migration of these native and SDS-dissociated assemblies strongly suggest that SecYY is associated with two SecE and two SecG (referred to as $SecYYE^2G^2$ or linked SecYEG dimer, hereafter), although a different stoichiometry cannot be excluded. The theoretical

molecular weight of these complexes is given in the legend of Figure 3.

Interestingly, when SecA was added in excess prior to IMV solubilization, two additional complexes were detected from the IMVs-YY detergent extracts (Figure 2A, labeled *). A C-terminal truncated version of SecA ($SecA_{95}$; Breukink *et al*., 1995) shifted these two complexes to lower molecular weight positions (Figure 2A, lane 9). Thus, these two complexes probably represent stable associations of SecA with the linked SecYEG dimer. Before further analysis, the oligomeric property of SecA was studied by BN–PAGE. In solution, SecA presents complex oligomeric behavior with a stoichiometry depending on protein concentration, detergent, temperature and composition of the buffer (Or *et al*., 2002; Woodbury *et al*., 2002; Benach *et al*., 2003). In this study, the comparison between the migration of molecular weight markers and SDS-dissociated SecA indicated that SecA exists as monomers and dimers, although higher oligomers were also present (Figure 2B). The $SecA_{95}$ protein, which is active for preprotein translocation but displays slightly higher endogenous ATPase activity (Breukink *et al*., 1995), also formed dimers. A monomer–dimer equilibrium was detected using increasing concentrations of SecA mixed with a constant amount of radiolabeled SecA (Figure 2C, lanes 1–4). At higher concentrations, SecA formed higher order associations (Figure 2C). These polymers may resemble those previously identified by cross-linking analysis and analytical ultracentrifugation (Hirano *et al*., 1996; Dempsey *et al*., 2002; Benach *et al*., 2003). Incubation of SecA with dodecyl-maltoside (DDM) triggered the progressive dissociation of the SecA oligomers (Figure 2C, lanes 5–7; Or *et al*., 2002). At the DDM and SecA concentrations used in this study (such as lanes 1–3), dimeric SecA was still detected.

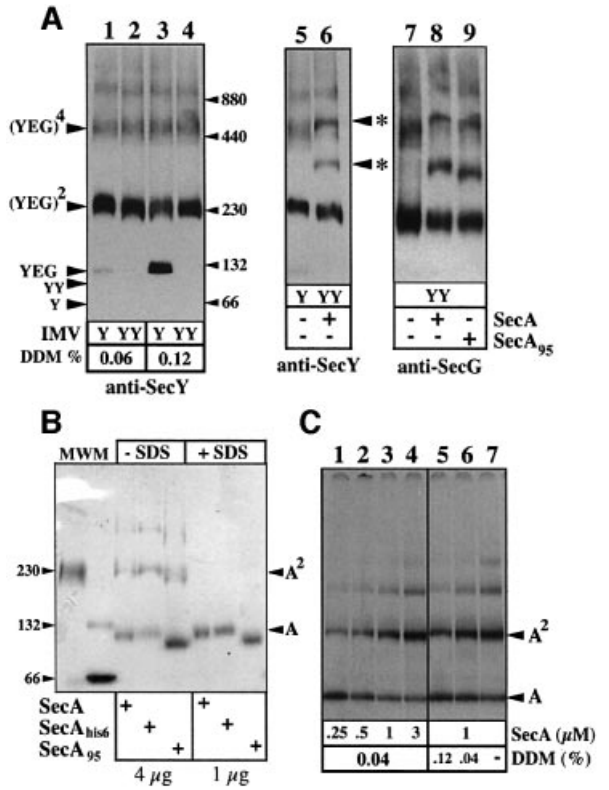

Fig. 2. Oligomeric behavior of covalently-linked SecYEG dimer and SecA. (**A**) Immunodetection of SecYEG oligomers in membranes enriched with SecYEG (IMVs-Y) or $SecYYE^2G^2$ (IMVs-YY). IMVs were solubilized in TSG buffer at the indicated detergent concentration. Protein aliquots (~0.2 μg) were analyzed by linear gradient BN–PAGE (4–12%) and immunostained. Where indicated, IMVs were incubated with SecA or $SecA_{95}$ (4 μg; 0.8 μM protomer). The asterisks indicate the probable complex of $SecYYE^2G^2$ with monomeric and dimeric SecA. (**B**) SecA, His_6-tagged SecA or His_6-tagged $SecA_{95}$ in TSG buffer (±SDS) were analyzed on a native gel followed by Coomassie blue staining. Molecular weight markers are BSA and catalase. (**C**) [^{35}S]SecA (~20 000 c.p.m., ~100 ng) was mixed at room temperature with unlabeled SecA to reach the desired concentration. Samples were diluted in TSG buffer containing DDM and analyzed by BN–PAGE (4–10%) and autoradiography. The final SecA and DDM concentrations in each sample are indicated.

The linked SecYEG dimer binds monomeric and dimeric SecA

A previous BN–PAGE analysis did not detect stable SecYEG–SecA associations or oligomerization of the SecYEG complex upon incubation of IMVs-Y with SecA (Bessonneau *et al*., 2002; and Figure 2A, lane 5). Similarly, the purified and radiolabeled native SecYEG complex did not form obvious associations with SecA (Figure 3B). Faint bands migrating above the SecYEG dimer were detected at higher SecA concentrations (Figure 3B, right lanes), suggesting that a transient or unstable interaction between native SecYEG and SecA may occur. In contrast, incubation of SecA with the purified and radiolabeled $SecYYE^2G^2$ complex readily revealed two higher molecular weight assemblies (Figure 3C; labeled $SecYYE^2G^2$–A and $SecYYE^2G^2$–A^2, respectively). Furthermore, the truncated $SecA_{95}$ shifted these two bands to lower molecular weight positions, attesting to the presence of SecA in these complexes (Figure 3D). In the absence of SecA, a low-intensity band migrating slightly below $SecYYE^2G^2$–A^2 was detected (Figures 2A, and 3A, C and D). This band migrates at the same position as the $(SecYEG)^4$ assembly (Figure 2A) and probably corresponds to an association of two $SecYYE^2G^2$ complexes [i.e. $(SecYEG)^4$]. However, the two complexes that appear upon addition of SecA migrate at positions distinct from this band and depend on both the molecular weight and concentration of SecA (Figure 3C and D). An association between $(SecYYE^2G^2)^2$ and monomeric SecA would be expected to migrate at a higher position and the shift induced by a single $SecA_{95}$ (–8 kDa) to be less obvious. In the converse experiment, radiolabeled SecA was incubated with increasing concentrations of $SecYYE^2G^2$ (Figure 3E). The same two complexes were also detected, concomitantly with the progressive disappearance of the SecA monomers and dimers. Thus, it is very likely that the $SecYYE^2G^2$ complex associates with monomeric and dimeric SecA.

This conclusion was also reached by cross-linking analysis. The radiolabeled $SecYYE^2G^2$ complex was incubated with SecA, then with an amine-reactive cross-linking reagent. Two major cross-linked products were formed in a SecA-dependent manner (labeled X-link1 and X-link2; Figure 4A, left panel). At high SecA concentrations, higher molecular weight cross-links were also detected. In the converse experiment, radiolabeled SecA

was incubated with SecYYE^{2}G^2, and cross-linked products of molecular weight similar to X-link1 and X-link2 were identified (Figure 4A, right panel). Smears and aggregates on top of the gel appeared with the increase in the SecYYE^{2}G^2 concentration. Finally, incubation of the truncated SecA$_{95}$ with the linked SecYEG dimer shifted the X-link1 and X-link2 bands to lower molecular weight positions, attesting to the presence of SecA in these cross-links (Figure 4B). These two cross-linked products were also detected with anti-SecG antibodies (Figure 4B) or anti-SecY antibodies (not shown). Thus, the X-link1 and X-link2 products probably correspond to cross-links of SecYYE^{2}G^2 with monomeric and dimeric SecA, respectively.

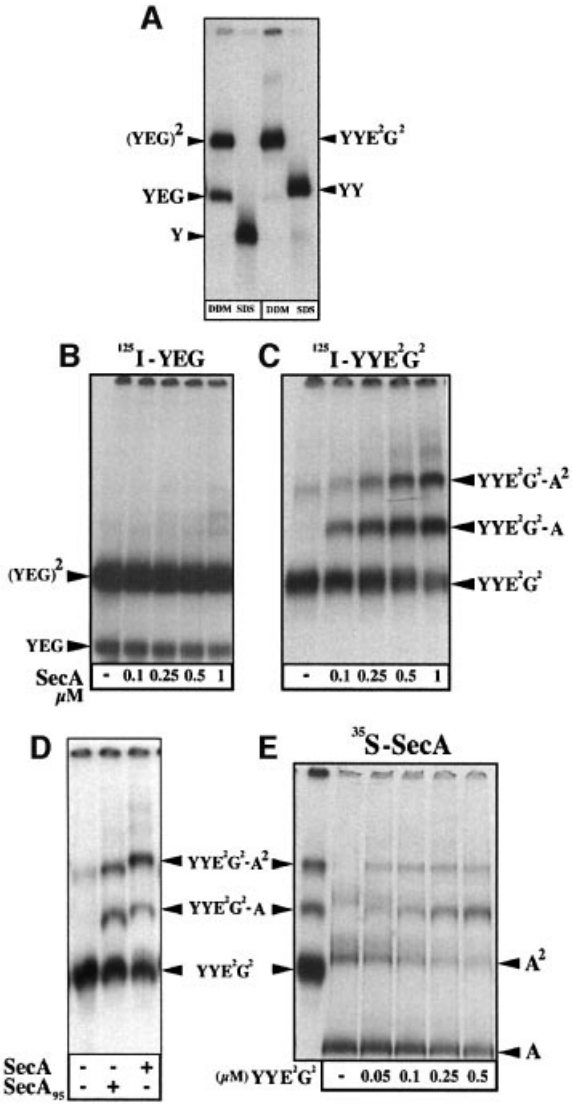

Fig. 3. The stabilized SecYEG dimer forms a stable binding site for SecA monomers and dimers. (**A**) Oligomeric state of the purified SecYEG complexes. [^{125}I]SecYEG and [^{125}I]SecYYE^{2}G^2 purified complexes (~30 000 c.p.m., ~20 ng, in TSG buffer 0.03% DDM or 0.1% SDS) were analyzed by BN–PAGE (5–13%) and autoradiography. (**B**–**D**) Complexes between radiolabeled SecYEG and unlabeled SecA. [^{125}I]SecYEG and [^{125}I]SecYYE^{2}G^2 purified complexes (~30 000 c.p.m., 0.7 μM) were incubated (5 min, 22°C) with SecA or SecA$_{95}$ at the indicated concentration in TSG buffer containing 0.04% DDM. The resulting complexes were separated by BN–PAGE (4–12%) and revealed by autoradiography. (**E**) Complexes between radiolabeled SecA and unlabeled SecYYE^{2}G^2. [^{35}S]SecA (~20 000 c.p.m., 1 μM) was incubated in TSG buffer containing 0.04% DDM with the indicated concentration of the purified SecYYE^{2}G^2 complex. The resulting complexes were analyzed by BN–PAGE (4–12%) and autoradiography. For M_r reference, a sample of the [^{125}I]SecYYE^{2}G^2 complex incubated with unlabeled SecA is loaded in the left lane of the gel. Calculated molecular weights of the Sec proteins and Sec complexes: SecY (48.5 kDa); SecYY (97 kDa); His-SecE (14 kDa); SecG (11 kDa); His-SecA (103 kDa); His-SecA$_{95}$ (95 kDa); SecYEG (73.5 kDa); SecY^{2}E^{2}G^2 (147 kDa); SecY^{4}E^{4}G^4 (294 kDa); SecY^{2}E^{2}G^2–SecA (250 kDa); SecY^{2}E^{2}G^2–SecA^2 (353 kDa).

Nucleotide-dependent SecYEG–SecA interactions

Experiments such as cross-linking, FRET, small angle X-ray scattering (Driessen, 1993; Shilton *et al*., 1998; Or *et al*., 2002) and BN–PAGE (Figure 5A) showed that ADP, ATP or ATPγS do not significantly change the SecA monomer–dimer equilibrium. However, these nucleotides were found to affect the SecA–SecYEG interactions profoundly (Figure 5B). In the presence of ADP, the SecA dimer was stably associated with the SecYYE^{2}G^2 complex (lane 2). In contrast, the SecA dimer dissociated from the SecYYE^{2}G^2 complex upon addition of ATP (lane 3 compared with lane 1). This dissociation was even stronger in the presence of ATPγS (lane 4). Rapid depletion of hydrolyzable nucleotides upon addition of apyrase restored the initial level of SecA dimers bound to SecYYE^{2}G^2 (lanes 5 and 6). Unexpectedly, the effect of ATP or ATPγS was strongly reduced when magnesium was included in the incubation buffer (lanes 7–10). Similar results were obtained by cross-linking analysis (Figure 5C). Only low amounts of X-link2, corresponding to a covalent complex of SecYYE^{2}G^2 with SecA^2, were obtained in the presence of ATP or ATPγS, compared with ADP (Figure 5C, lanes 3 and 4 compared with lane 2). This ATP- or ATPγS-dependent decrease in the amount of X-link2 was also seen in the converse experiment using radiolabeled SecA (compare lanes 13 and 14 with lane 12). The ATP-dependent decrease of X-link2 production was abolished upon addition of apyrase (lanes 5 and 6). Again, the effects of ATP or ATPγS on the X-link2 production were reduced when magnesium was present in the incubation buffer (lanes 8–10 and 16–18).

Thus, together, the data show that ATP binding to SecA tends to destabilize the association between the SecA dimer and the linked SecYEG dimer. Interestingly, both the BN–PAGE analysis and cross-linking experiments indicate that the level of SecYYE^{2}G^2–A (or X-link1) increases upon addition of ATP or ATPγS (Figure 5B and C, lanes 3 and 4). To some extent, this increase is in relation to the decrease of the level of SecYYE^{2}G^2–SecA^2 (or X-link2). This last observation may suggest that only one SecA protomer dissociates from the SecYYE^{2}G^2–A^2 complex upon binding of ATP. Alternatively, it may reflect an increased affinity of the SecA monomer for the SecYEG complex.

Activation of the SecA ATPase by the SecYEG complex in solution

The SecA ATPase activity is stimulated by acidic lipids containing membranes and by membrane-embedded SecYEG (Lill *et al*., 1990; Hendrick and Wickner, 1991). The nucleotide-dependent SecA–SecYEG associations (Figure 5) suggest that a SecYEG-dependent SecA

activation may also occur in solution. Indeed, SecA was found to hydrolyze large amounts of ATP during incubation with the SecYYE^{2}G^2 complex, and the release of inorganic phosphate augmented with the increase of SecYYE^{2}G^2 present in the reaction (Figure 6, lanes 1–5). In the absence of detergent, the SecYYE^{2}G^2 complex aggregates and the SecYYE^{2}G^2-dependent ATPase activity was lost (lanes 6 and 7). Upon addition of magnesium, the SecYYE^{2}G^2-dependent ATPase activity was strongly repressed (lane 11). The endogenous ATPase activity of SecA was also diminished by this cation (lane 10). The native SecYEG complex stimulated the SecA ATPase activity to a similar extent (Figure 6), which was also repressed by addition of magnesium (not shown). Therefore, in the absence of added magnesium and in the presence of the native or linked SecYEG complex, the SecA enzyme is stimulated and consumes ATP. Activation of the SecA ATPase by the native SecYEG complex suggests that an interaction between these two domains, at least transient, must also occur in solution. Furthermore, both the SecYEG-dependent SecA ATPase and the nucleotide-dependent SecA–SecYEG associations (Figure 5) are reduced by magnesium, suggesting that a relationship between these two events may exist.

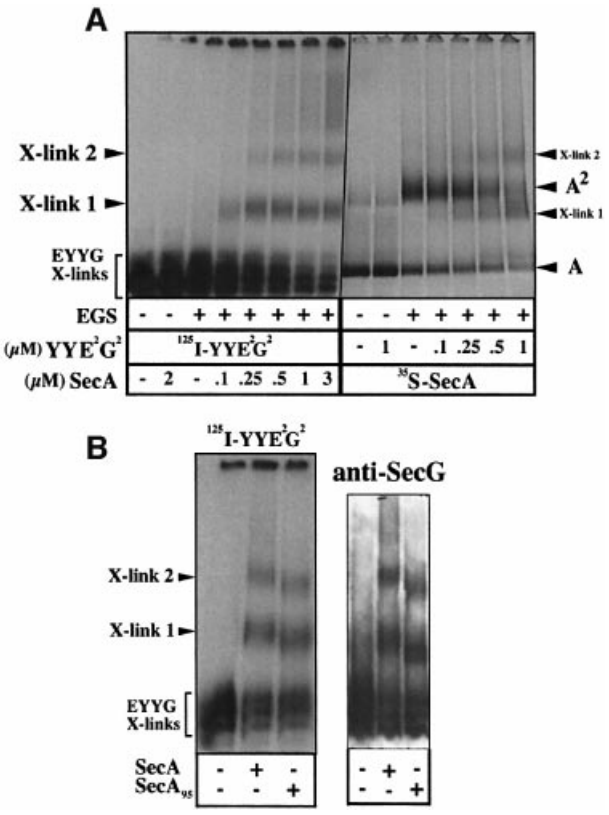

Fig. 4. Cross-linking production between SecYYE^{2}G^2 and SecA. (**A**) [^{125}I]SecYYE^{2}G^2 (0.1 μM) or [^{35}S]SecA (1 μM) were incubated in CL buffer (5 min, 22°C) with the indicated concentration of unlabeled SecYYE^{2}G^2 or unlabeled SecA, respectively. After cross-linking by EGS and dissociation of the non-covalent protein complexes by SDS, samples were analyzed by BN–PAGE (4–12%) and autoradiography. (**B**) [^{125}I]SecYYE^{2}G^2 or unlabeled SecYYE^{2}G^2 were incubated as in (A) with SecA or SecA$_{95}$, and cross-links were revealed by autoradiography or immunostaining.

Monomeric SecA and preprotein form a stable complex at the SecYEG translocation channel

We previously reported that a preprotein arrested during its translocation (intermediate I$_{26}$) forms a sufficiently stable complex with the SecYEG translocation channel to be analyzed by BN–PAGE (Bessonneau *et al*., 2002). The same technology was employed here. First, the purified SecYEG and SecYYE^{2}G^2 complexes were reconstituted into liposomes and then incubated with SecA, bovine serum albumin (BSA), ATP and [^{125}I]proOmpA conjugated to the globular BPTI molecule. Both SecYEG- and SecYYE^{2}G^2-proteoliposomes are functional for protein translocation, and the I$_{26}$ intermediate was generated in both cases, although with less efficiency in the latter case (Figure 7A, lanes 5 and 6). In the absence of SecA, ATP or at 4°C, the intermediate I$_{26}$ was not formed (Figure 7A, lanes 1–4, and 9 and 10). In the presence of dithiothreitol (DTT), which freed proOmpA from BPTI, the arrested intermediate I$_{26}$ completed its translocation across the membrane (lanes 7 and 8).

In parallel, the translocation reactions described above were treated with DDM and the solubilized materials were analyzed by BN–PAGE (Figure 7B). The molecular weight of the potential complexes is given in the legend of Figure 7. With the SecYYE^{2}G^2-proteoliposomes, proOmpA migrated as a complex of high molecular weight (lane 6), in between SecYYE^{2}G^2–A and SecYYE^{2}G^2–A^2 (250 and 350 kDa, respectively). The size of this complex (lane 6) is too large to correspond to an association of SecYYE^{2}G^2 and proOmpA (187 kDa). It is also too small to correspond to an association of proOmpA with a (SecYYE^{2}G^2)^2 complex (343 kDa). Given the affinity of the SecYYE^{2}G^2 complex for SecA, a likely composition of the complex observed in lane 6 (Figure 7B) is an association of SecYYE^{2}G^2–proOmpA with monomeric SecA (293 kDa). With the SecYEG-proteoliposomes, proOmpA was trapped with the SecYEG complex (lane 5; and Bessonneau *et al*., 2002) and migrated in between the SecYEG dimer (150 kDa) and the SecYYE^{2}G^2–SecA complex (250 kDa). This complex (lane 5) was proposed to correspond to an association of proOmpA with dimeric SecYEG (187 kDa; Bessonneau *et al*., 2002), but it may also correspond to an association of proOmpA with monomeric SecA and monomeric SecYEG (212 kDa).

To analyze further the composition of these translocation complexes, preprotein translocation was driven with truncated SecA$_{95}$ instead of wild-type SecA (Figure 7C). With the SecYYE^{2}G^2 proteoliposomes, SecA$_{95}$ shifted the SecYYE^{2}G^2–proOmpA complex to a lower molecular weight position (lanes 3 and 4). With the SecYEG-proteoliposomes, SecA$_{95}$ also decreased the molecular weight of the SecYEG–proOmpA complex (lanes 1 and 2). Thus, both the linked SecYEG dimer and the native SecYEG complex are engaged with SecA during translocation. This conclusion was confirmed by co-immunoprecipitation (Figure 7D). Anti-SecG antibodies efficiently co-immunoprecipitated a complex of SecYEG and translocation-arrested proOmpA (Bessonneau *et al*., 2002), but this study shows that SecA is also present in the immunoprecipitate (Figure 7D). In the absence of a preprotein intermediate, the complex between SecA and SecYYE^{2}G^2 may have been dissociated

during the washes of the immunoprecipitates (Figure 7D). Thus, our previous observation about the composition of the translocation complex was mistaken (Bessonneau *et al*., 2002). This study show that the translocation complex is actually formed by the ternary association of SecYEG–SecA–proOmpA. With the $SecYYE^2G^2$ proteoliposomes, the second SecYEG protomer remains associated with the translocation complex because of the covalent linkage.

Discussion

Our previous work suggested that the dimeric stoichiometry of the SecYEG channel bore the translocation intermediate (Bessonneau *et al*., 2002), but we missed the possible participation of SecA. Here, it is shown that the translocation complex bearing the preprotein is actually formed by monomeric SecYEG, as previously reported (Yahr and Wickner, 2000), in association with monomeric

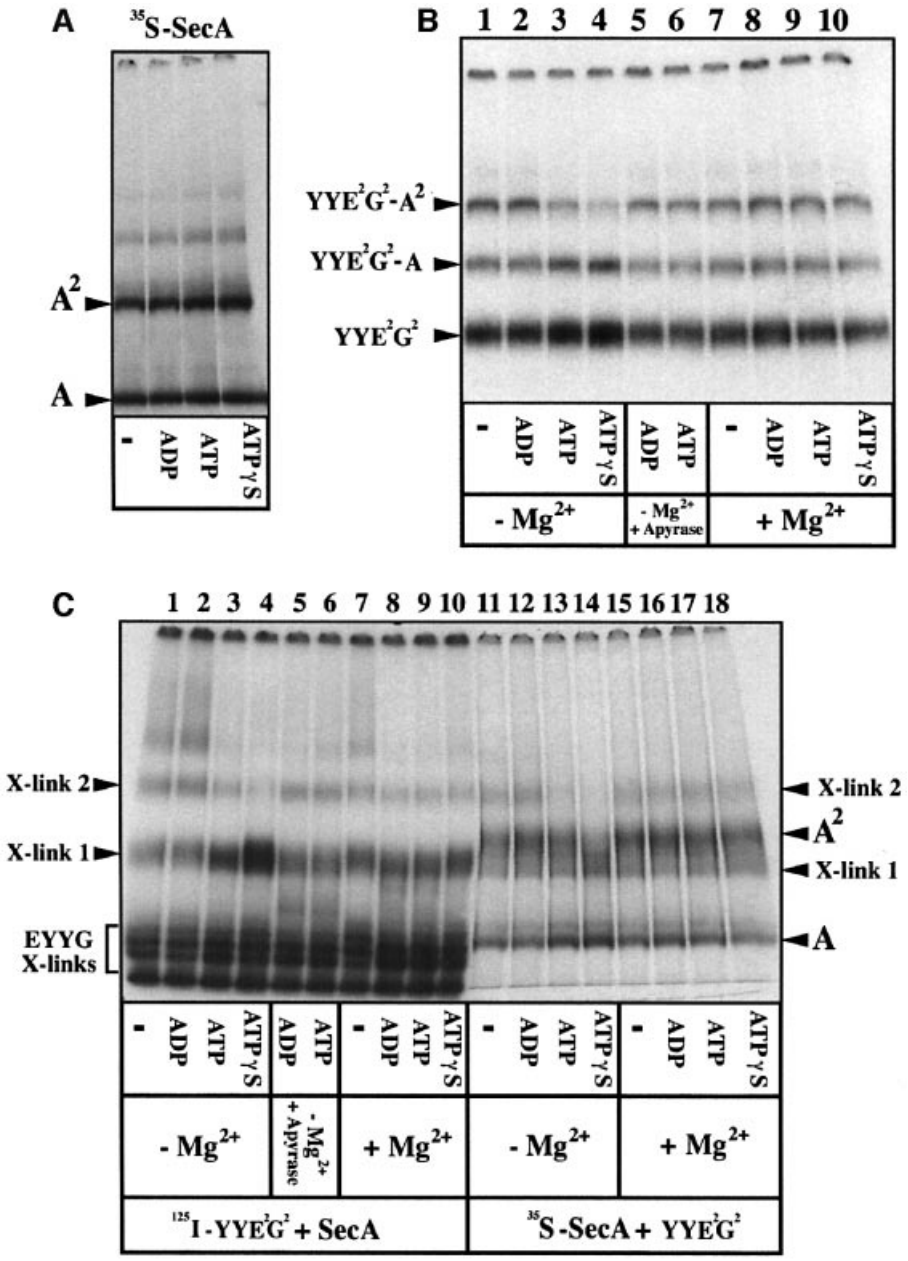

Fig. 5. Nucleotide-dependent SecA–SecYEG associations. (**A**) Nucleotides do not change the oligomeric state of SecA in solution. [^{35}S]SecA (~20 000 c.p.m., 1 μM) was incubated (10 min, 22°C) in TSG buffer containing 0.04% DDM with the indicated nucleotides (1 mM). Samples were analyzed by BN–PAGE (4–12%) and autoradiography. (**B**) Complexes between radiolabeled SecYEG and unlabeled SecA after addition of nucleotides. [^{125}I]$SecYYE^2G^2$ (0.7 μM) was mixed with SecA (1 μM) in TSG buffer (0.04% DDM; ±5 mM $MgCl_2$). After incubation (5 min, 22°C), nucleotides were added and the incubation was prolonged for 5 min. Samples (lanes 5 and 6) were treated with potato apyrase (grade VII; 20 U/ml) for an additional 2 min. All samples were returned to ice before analysis by BN–PAGE (4–12%) and autoradiography. (**C**) Cross-link production between $SecYYE^2G^2$ and SecA after addition of nucleotides. [^{125}I]$SecYYE^2G^2$ (0.1 μM) or [^{35}S]SecA (1 μM) were incubated in CL buffer (±5 mM $MgCl_2$) with unlabeleled SecA (2 μM) or unlabeled $SecYYE^2G^2$ (0.5 μM), respectively. After incubation with nucleotides (as described in B), samples were treated by EGS, then dissolved by SDS and analyzed by BN–PAGE (4–12%) and autoradiography.

SecA. Remarkably, higher concentrations of detergent further destabilized the SecYEG–SecA–preprotein complex (Bessonneau *et al*., 2002), but the intermediate of dissociation formed by monomeric SecYEG and the translocation-arrested preprotein was not detected. This suggests that the translocating polypeptidic chain may be weakly associated with the SecYEG protomer or that SecA may be structurally necessary to maintain this association during detergent solubilization. A site-specific photocross-linking study has shown that SecY and SecA simultaneously contact the translocating chain, suggesting that SecA contacts the polypeptide conduit intimately (Joly and Wickner, 1993). However, while the ternary complex SecYEG–SecA–preprotein appears as the most stable part of the translocation complex upon detergent solubilization and analysis by BN–PAGE, this result does not exclude the participation of other SecYEG protomers, either for the formation of the channel or for its activity. In fact, most current studies suggest that the SecYEG complex, in its non-translocating state, exists as oligomers. These studies include cysteine cross-linking, sedimentation analysis, native gel electrophoresis, electron microscopy, electron crystallography, genetic and FRET experiments (Meyer *et al*., 1999; Manting *et al*., 2000; Collinson *et al*., 2001; Veenendaal *et al*., 2001; Bessonneau *et al*., 2002; Breyton *et al*., 2002; van der Sluis *et al*., 2002; Matsuo *et al*., 2003; Mori *et al*., 2003). The SecYEG monomer exists in solution but readily reforms dimers upon dilution with detergent and reconstitution into lipids (Bessonneau *et al*., 2002; Breyton *et al*., 2002; Mori *et al*., 2003). Thus, a SecYEG protomer may form the central core of the translocation channel, but it is likely that the SecYEG oligomers are actively involved. The presence of SecA and an arrested preprotein in the translocation pathway may have weakened the interactions between the SecYEG protomers, thus becoming unstable in detergent solution.

The current model for post-translational translocation proposes that SecA inserts with the first loop of the

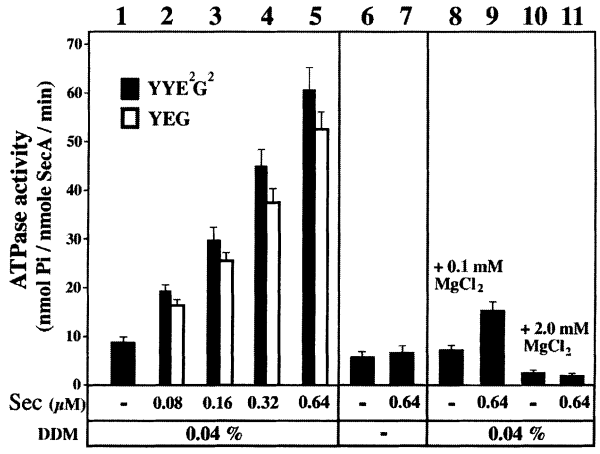

Fig. 6. Activation of the SecA ATPase by SecYEG. SecA (0.8 μM) was incubated at room temperature with the purified SecYEG or SecYYE^{2}G^2 complexes in TSG buffer (±0.04% DDM; ±$MgCl_2$) containing 2 mM of ATP. The Sec concentration refers to that of the SecYEG protomer (SecYYE^{2}G^2 = 2 × SecYEG). After 10 min incubation at 22°C, the inorganic phosphate produced by the SecA ATPase was measured by the photocolorimetric method of Lanzetta *et al*. (1979).

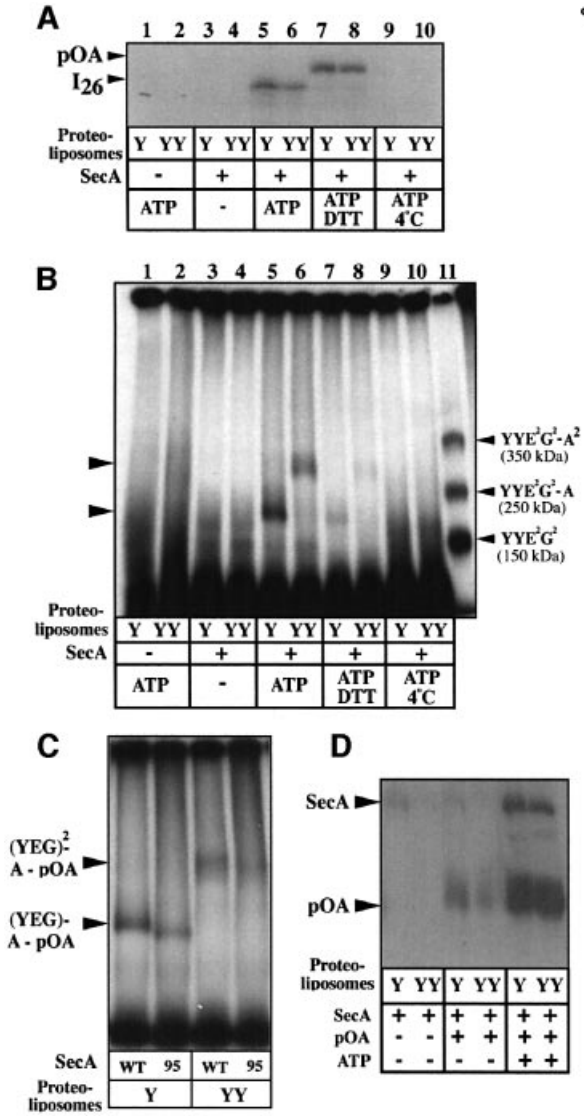

Fig. 7. The SecA monomer is engaged with preprotein and the SecYEG channel during translocation. (**A**) [^{125}I]ProOmpA–BPTI (~100 000 c.p.m.; 4 μg/ml) was incubated (10 min, 37°C) in TL buffer (50 μl final volume) with SecYEG- or SecYYE^{2}G^2-proteoliposomes (15 μg/ml), BSA (200 μg/ml), SecA (80 μg/ml; 0.8 μM) and ATP (2 mM) or DTT (2 mM) where indicated. Reactions were terminated by addition of apyrase (20 U/ml). Aliquots of the translocation reactions (1/10 vol.) were treated with proteinase K and analyzed by 12% SDS–PAGE and autoradiography. (**B**) The remainder of the translocation reaction performed in (A) was solubilized with an equal volume of TSG buffer containing 0.12% DDM. Aliquots were analyzed by BN–PAGE (4–10%) and autoradiography. For M_r reference, [^{125}I]SecYYE^{2}G^2 complexed to SecA was loaded on the same gel (lane 11). (**C**) As in (B) lanes 5 and 6, but using the truncated $SecA_{95}$ or wild-type SecA to drive translocation in SecYEG- or SecYYE^{2}G^2-proteoliposomes. (**D**) The translocation reactions described in (A) were supplemented with [^{125}I]SecA (~100 000 c.p.m.; 4 nM). At the end of the incubation (10 min, 37°C), proteoliposomes were solubilized with 300 μl of TSG buffer, 0.1% DDM (10 min, 4°C). After centrifugation (10 min, 100 000 *g*), the membrane extract was incubated with anti-SecG IgG coupled to protein A–Sepharose beads. Immunoprecipitates were washed (3 × 0.3 ml of TSG buffer) and analyzed by 10% SDS–PAGE and autoradiography. Theoretical molecular weight of the SecYEG–SecA–pOA complexes: pOA (37 kDa); SecYEG–pOA (112 kDa); (SecYEG)^2–pOA (187 kDa); SecYEG–SecA–pOA (212 kDa); (SecYEG)^2–SecA (250 kDa); (SecYEG)^2–SecA–pOA (287 kDa); (SecYEG)^4–pOA (343 kDa).

preprotein into the SecYEG channel to become both shielded from the lipid phase and partly exposed to the other side of the membrane (Economou and Wickner, 1994; Eichler and Wickner, 1997; Ramamurthy and Oliver, 1997). A single membrane-embedded SecYEG protomer may not be sufficient to allow the SecA insertion, whereas the lipid bilayer surrounding the protomer may further oppose this event. In contrast, the SecYEG protomers within the dimer are weakly associated and their interface forms a cytosolic cavity (Bessonneau *et al*., 2002; Breyton *et al*., 2002). The *prl* mutations further destabilize the SecYEG associations and increase the binding affinity of SecA and the translocation efficiency (van der Wolk *et al*., 1998; Bessonneau *et al*., 2002). The non-essential SecG subunit facilitates translocation and also stabilizes SecYEG tetramers and higher order oligomers when the SecYEG complex is overexpressed in the membrane (Bessonneau *et al*., 2002). In such a scenario, the interface of the SecYEG dimers or other oligomers may favor the initial step of the translocation reaction such as SecA binding, insertion and transfer of the first loop of the preprotein into the translocation channel. One of the SecYEG protomers would bind to preprotein and SecA, thereby forming the most stable part of the translocation complex upon detergent solubilization. Oligomeric formation of SecYEG and its eukaryotic homolog, Sec61p, is stimulated in membranes by association with SecA and ribosomes, respectively (Hanein *et al*., 1996; Manting *et al*., 2000). Different oligomeric structures may be essential for the initial steps of translocation and may specialize according to the mode of translocation, the energy source or the protein substrate.

This study also provides information about the stoichiometry and dynamics of the SecYEG–SecA association. This association was demonstrated previously by indirect assays, such as affinity binding measurements, *in vivo* cross-linking and genetic analysis (Manting *et al*., 1997; Matsumoto *et al*., 1997; Snyders et al., 1997; Lill *et al*., 1990). The current work shows that a linked SecYEG dimer readily forms a complex with monomeric and dimeric SecA in solution. Higher order SecYEG oligomers do not seem necessary for this interaction. In support of this finding, analytical ultracentrifugation and gel filtration experiments show that a SecYEG dimer stabilized with an antibody can bind dimeric SecA (I.Collinson, personal communication). Thus, the antibody or the covalent linkage may have imposed a single conformational state on the SecYEG dimer, thereby forming a high affinity SecA-binding site. In contrast, the native SecYEG complex forms dynamic assemblies in detergent solution and exists as monomers, dimers and higher order oligomers at high concentration (Manting *et al*., 2000; Collinson *et al*., 2001; Bessonneau *et al*., 2002). This oligomeric variability may prevent the stabilization and detection of the SecA-binding site. Nonetheless, the present study does not exclude other transient SecA–SecYEG associations, nor the possibility that the linkage between two SecYEG protomers induces an abnormal conformation, resulting in an artificial SecA affinity effect. However, the linked SecYEG dimer is functional for preprotein translocation, complements Sec-defective strains and stimulates the SecA ATPase activity. Furthermore, a cysteine cross-linking study has shown that both the N- and C-termini of neighboring SecY are in close proximity. The cysteine-linked dimers possess translocase activity (van der Sluis *et al*., 2002). The projection structure of dimeric SecYEG shows two monomers related to each other by an antiparallel symmetry (Breyton *et al*., 2002). Accordingly, the cytosolic loop formed by the covalent linkage between the N- and C-termini of two SecY molecules may allow sufficient conformational freedom to respect the native SecYEG dimeric structure, both in membrane and in solution. It is thus conceivable that the various analysis performed with this hybrid reflect, at least partially, the intra- and inter-molecular interactions that normally occur in the native SecYEG dimer.

These analysis show that nucleotides modulate the SecYEG–SecA associations (Figure 5). A previous work has revealed that the binding affinity of SecA for IMVs is reduced in the presence of ATP (van der Wolk *et al*., 1998). Here, it is shown that in the presence of ADP, both monomeric and dimeric SecA are stably associated with the linked SecYEG complex. Upon addition of ATP or non-hydrolyzable ATPγS, the SecA dimer tends to dissociate from the SecYEG-binding site. In contrast, in the same circumstances, the SecA monomer remains stably associated. Furthermore, an increase in the amount of the SecA monomer bound to the linked SecYEG complex is detected in the presence of ATPγS. This increase may correspond to the monomerization of the SecYEG-bound SecA dimer or to an increased affinity of the SecA monomer upon binding of the nucleotide. Clearly, nucleotides modulate the binding affinity and binding stoichiometry of SecA at the SecYEG dimer. Further studies will be required to probe the dynamics of the SecYEG–SecA interaction, but the observed nucleotide-dependent SecYEG–SecA stoichiometry may reflect important aspects of this interaction during ATP-driven preprotein translocation.

Accordingly, a link may exist between the modulation of SecYEG–SecA associations by nucleotides and the SecYEG–SecA ATPase (Figure 7) since both are diminished by magnesium in solution. However, a repression of the endogenous SecA ATPase by Mg^{2+} also takes place in solution (Kim *et al*., 2001; this study) and the lipid-stimulated SecA ATPase occurs at low Mg^{2+} concentration (Lill *et al*., 1990). The molecular mechanism of ATP binding and hydrolysis is not yet well defined, but it appears that magnesium increases the energy barrier for the SecYEG-dependent SecA activation. It is suggested from the SecA crystal structure that a low Mg^{2+} concentration favors rapid nucleotide exchange at the ATP-binding folds and contributes to the so-called SecA domain dissociation reaction (Hunt *et al*., 2002). In solution, the interaction of SecA and SecYEG may further contribute to the SecA domain dissociation, thereby leading to the activation of the SecA ATPase. The reconstitution of the SecYEG–SecA ATPase in detergent solution provides a novel *in vitro* assay to assess the mechanism of the SecA activation, without the contribution of the lipid bilayer and the SecA lipid ATPase (Lill *et al*., 1990).

A recent study shows that a monomerized SecA mutant retains some translocation activity while the SecA dimer dissociates upon contact with acidic phospholipids (Or *et al*., 2002). During translocation, only monomeric SecA

is found tightly associated with the translocation complex upon detergent solubilization (Figure 7). Altogether, the findings indicate that the SecA monomer has a key role during the translocation reaction but do not exclude the participation of the SecA dimer or other multimers. Hydrophobic peptide alters the dimeric conformation of SecA, toward dissociation but also polymerization (Or *et al*., 2002; Benach *et al*., 2003). The cross-linking experiments also suggest that polymeric SecA may be associated with the linked SecYEG dimer when SecA is present at high concentration (Figures 4 and 5). A high concentration of SecA allows proton-motive force-independent translocation (Yamada *et al*., 1989). Recently, electron microscopy studies and crystal structure analysis have revealed that the interface of the SecA dimer, and possibly higher order oligomers, forms a pore-like structure which may be important for the function of SecA (Sharma *et al*., 2003; Wang *et al*., 2003). These observations further complicate the current understanding of the SecA-dependent translocation mechanism. The results presented in this study strongly suggest that changes in the oligomeric associations within and between SecA and SecYEG protomers play a critical role during the translocation reaction. The reconstitution of the SecA–SecYEG complex in solution amenable to biochemical analysis provides us with a novel tool to analyze these molecular interactions further.

Materials and methods

Plasmids

Plasmids pTrcE$_{HA}$YG and pBadE$_{his}$YG were described previously (Duong and Wickner, 1997; Collinson *et al*., 2001). The *Bam*HI–*Sal*I DNA fragment encoding His$_6$-SecE was replaced in pTrcE$_{HA}$YG to give plasmid pTrcE$_{his}$YG. To construct pTrcE$_{HA}$YYG and pTrcE$_{his}$YYG, a 1.3 kb *Sal*I–*Sac*I DNA fragment encoding the *secY* ORF with the *Sac*I restriction site replacing the stop codon was generated by PCR and inserted in pTrc99a to give pTrcY′. Another 1.3 kb *Sac*I–*Xba*I DNA fragment encoding the *secY* ORF but with the *Sac*I restriction site replacing the initiation codon was inserted into pTrc99a to give pTrc′Y. The *secY* gene carried by pTrcY′ and pTrc′Y was sequenced. The *Sal*I–*Sac*I and *Sac*I–*Xba*I DNA fragments extracted from pTrcY′ and pTrc′Y were co-inserted into pTrcE$_{HA}$YG and pTrcE$_{his}$YG digested with *Sal*I and *Xba*I to excise the *secY* gene. To construct C-terminal His$_6$-tagged SecA, a 1.3 kb 5′*Nde*I–3′*Eco*RV DNA fragment was generated by PCR. Another 1.3 kb 5′*Eco*RV–3′*Xho*I DNA fragment encoding the second half of the *secA* ORF, but with the *Xho*I restriction site replacing the stop codon, was generated by PCR. The two PCR fragments were co-inserted into pET-21a (Novagen). The 2.5 kb *Nco*I DNA fragment encoding almost the entire *secA* gene was replaced by the same fragment extracted from pT7-SecA2 (generous gift from Dr D.Oliver) to give pET-SecA$_{his}$. A stop codon was introduced at Val831 to give pET-SecA95$_{his}$.

Protein purification

SecB, proOmpA and proOmpA–BPTI were purified as described (Hartl *et al*., 1990). The purification of the SecYE$_{his}$G and SecYYE$^2_{his}$G^2 complexes was achieved by Ni^{2+}-chelating chromatography, according to the procedure described by Collinson *et al*. (2001). The purified Sec complexes were stored at –80°C in TSG buffer (50 mM Tris–HCl pH 7.9, 150 mM NaCl, 10% glycerol, 1 mM DTT) containing 0.04% DDM. His-tagged SecA was purified by Ni^{2+}-chelating chromatography, then by gel filtration on a pre-packed Sephacryl S-200 HR column equilibrated in HSG buffer (50 mM K-HEPES pH 7, 50 mM NaCl, 10% glycerol, 1 mM DTT). His-tagged SecA is fully active and was used throughout this study.

Radiolabeling

Cell growth and ^{35}S metabolic labeling of SecA$_{his}$ were performed as described by Eichler and Wickner (1997). [^{35}S]SecA$_{his}$ was purified by Ni^{2+}-chelating chromatography and desalted on a G-25 spin column (Bio-Rad) equilibrated in TSG buffer. The specific activity of the purified [^{35}S]SecA was ~2 × 10^5 c.p.m./μg. ^{125}I-labeling of the SecYEG and SecYYE^{2}G^2 complexes, proOmpA and proOmpA–BPTI was as previously described (Duong and Wickner, 1997; Bessonneau *et al*., 2002). The specific activity of the ^{125}I-labeled SecYEG complexes was ~1.5 × 10^6 c.p.m./μg.

ProOmpA translocation assay

ProOmpA translocation assays were performed in 50 μl of TL buffer (50 mM Tris–HCl pH 7.9, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT) containing SecA$_{his}$ (40 μg/ml), SecB (40 μg/ml), BSA (200 μg/ml), IMVs (50 μg/ml), ATP (2 mM) and [^{125}I]proOmpA (~60 000 c.p.m.; 2 μg/ml). The transmembrane proton gradient was abolished by CCCP (carbonyl cyanide *m*-chlorophenylhydrazone; 20 μM) when necessary. After 5 min incubation at 37°C, translocation reactions were stopped on ice and treated with proteinase K (1 mg/ml, 15 min), trichloroacetic acid (TCA)-precipitated and analyzed by 12% SDS–PAGE and autoradiography. Quantitative proOmpA translocation was performed as described above, except that [^{125}I]proOmpA was pre-mixed with unlabeled proOmpA (2–34 μg/ml). Reactions were incubated 15 min at 37°C with 2 mM ATP. Translocated [^{125}I]proOmpA was visualized by autoradiography and analyzed by scanning densitometry using the ImageQuant software. The data were quantified by comparison with a [^{125}I]proOmpA standard curve. The [^{125}I]proOmpA–BPTI translocation assay is described in the legend of Figure 7.

Cross-linking assay

Both SecA and SecYYE^{2}G^2 were desalted a Sephadex G-25 column equilibrated in CL buffer (50 mM K-HEPES pH 7, 150 mM NaCl, 10% glycerol, 0.04% DDM). Cross-linking reactions were performed in 20 μl of CL buffer with 1 mM of EGS (ethylene glycol bis-succinimidylsuccinate; Sigma) for 15 min at 22°C. Reactions were terminated by addition of 1/10 vol. of quenching buffer (2 M glycine, 1 M Tris–HCl pH 8.0). The samples were incubated with SDS (0.1%, 10 min) under vigorous mixing followed by gel electrophoresis and autoradiography.

Other methods

Preparation of linear gradient blue native gels, electrophoretic conditions and electroblotting were as described by Schägger and von Jagow (1991). BN–gels were calibrated with high molecular weight markers (ferritin, 440 kDa/880 kDa; catalase, 232 kDa; BSA, 66 kDa/132 kDa). Immunoblots were visualized using the ECL reagents (AP Biotech). Protein concentrations were determined using the Bradford reagent (Biorad). The ATPase activity was measured by the colorimetric method of Lanzetta *et al*. (1979). Absorption was measured at 660 nm and compared with a standard curve prepared with a phosphate salt. SecA ATPase assays were performed in duplicate and the graph in Figure 6 represents an average of two independent experiments. Reconstitution of the SecYEG or SecYYE$_2$G$_2$ complex into liposomes was as previously described (Collinson *et al*., 2001).

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