The EMBO Journal Vol.16 No.16 pp.4871–4879, 1997

The SecDFyajC domain of preprotein translocase controls preprotein movement by regulating SecA membrane cycling

Franck Duong and William Wickner[1]

Dartmouth Medical School, Department of Biochemistry, 7200 Vail Building, Hanover, NH 03755, USA

[1]Corresponding author

Escherichia coli **preprotein translocase comprises a membrane-embedded hexameric complex of SecY, SecE, SecG, SecD, SecF and YajC (SecYEGDFyajC) and the peripheral ATPase SecA. The energy of ATP binding and hydrolysis promotes cycles of membrane insertion and deinsertion of SecA and catalyzes the movement of the preprotein across the membrane. The proton motive force (PMF), though not essential, greatly accelerates late stages of translocation. We now report that the SecDFyajC domain of translocase slows the movement of preprotein in transit against both reverse and forward translocation and exerts this control through stabilization of the inserted form of SecA. This mechanism allows the accumulation of specific translocation intermediates which can then complete translocation under the driving force of the PMF. These findings establish a functional relationship between SecA membrane insertion and preprotein translocation and show that SecDFyajC controls SecA membrane cycling to regulate the movement of the translocating preprotein.**

Keywords: membrane proteins/preprotein translocase/proton motive force/SecDFyajC/Sec proteins

Introduction

Protein export across the bacterial inner membrane is catalyzed by preprotein translocase, a multisubunit enzyme composed of an integral membrane domain, SecYEGDFyajC, and a peripheral membrane domain, SecA (for review, see Wickner and Leonard, 1996). The essential or 'core' subunits of this enzyme, identified by genetic and biochemical studies, are the SecY, SecE and SecA proteins (Schatz and Beckwith, 1990; Akimaru *et al.*, 1991; Duong and Wickner, 1997). Translocation of preproteins *in vitro* has been achieved using purified SecA and SecYEG reconstituted into proteoliposomes (Brundage *et al.*, 1990). Translocation depends upon the energy of ATP hydrolysis by SecA (Chen and Tai, 1985; Lill *et al.*, 1989) and is strongly stimulated by the proton motive force (PMF) across the membrane (Geller *et al.*, 1986; Shiozuka *et al.*, 1990).

In vitro studies have dissected the translocation reaction further into distinct subreactions, leading to a working model (Schiebel *et al.*, 1991). SecA, when in contact with a preprotein, the SecYE subunits of the translocase and acidic phospholipids (Lill *et al.*, 1990), binds ATP and supports the translocation of 20–30 residues of the preprotein across the membrane. Hydrolysis of the bound ATP then releases the preprotein from SecA, allowing the PMF to drive further translocation. Complete translocation of the preprotein can be achieved through many such cycles of ATP-driven SecA binding, limited translocation, release from the chain and PMF-driven translocation (Tani *et al.*, 1989; Schiebel *et al.*, 1991; Driessen, 1992). Analysis of SecA topology during ATP-driven translocation revealed that a C-terminal 30 kDa domain of SecA undergoes repeated cycles of membrane insertion and deinsertion (Economou and Wickner, 1994; Price *et al.*, 1996). Thus, in the absence of PMF, ATP-driven translocation may be catalyzed by repeated cycles of SecA membrane insertion and deinsertion, each insertion promoting the translocation of 20–30 residues of the preprotein across the membrane (Economou and Wickner, 1994). Cross-linking experiments showed that SecA and SecY contact the preprotein during translocation and that the 30 kDa domain of SecA is largely shielded from the lipid phase of the membrane during insertion (Joly and Wickner, 1993; Eichler *et al.*, 1997).

The SecYE domain of the translocase is sufficient to provide sites for SecA binding and insertion, to activate SecA as an ATPase and to allow some translocation (Duong and Wickner, 1997). However, the translocation that takes place at SecYE is very inefficient and results in extensive ATP hydrolysis without a proportional increase in preprotein translocation (Kawasaki *et al.*, 1993). A more efficient preprotein translocation requires the functions of SecG (Douville *et al.*, 1994; Hanada *et al.*, 1994) and the members of the *secD* operon, i.e. SecD, SecF and YajC (Gardel *et al.*, 1990; Pogliano and Beckwith, 1994a; Duong and Wickner, 1997). The topology inversion cycle of SecG may facilitate the cycle of SecA membrane insertion and deinsertion and thus increase the efficiency of preprotein translocation (Nishiyama *et al.*, 1996). Though it is not known how the SecDFyajC domain of translocase facilitates translocation, SecDFyajC-depleted membranes are unable to stabilize the inserted form of SecA (Economou *et al.*, 1995) and to use or maintain a full membrane potential (Arkowitz and Wickner, 1994). Overproduction of SecDFyajC leads to enhanced SecA membrane insertion (Kim *et al.*, 1994; Duong and Wickner, 1997).

Despite these considerable advances in our understanding of the translocation reaction, important aspects of the mechanism remain unclear. The most paradoxical aspect of the translocation mechanism concerns the contribution of SecDFyajC. Though SecDFyajC-depleted or enriched membranes have an altered SecA membrane cycle, they showed normal rates of ATP-driven preprotein translocation (Economou *et al.*, 1995; Duong and Wickner, 1997), and SecDFyajC was only found to be critical for PMF-

driven translocation (Arkowitz and Wickner, 1994). Thus, the relevance of the SecA membrane cycling was questionable since there was no apparent correlation between the effects of SecDFyajC on ATP-driven preprotein translocation and SecA membrane insertion.

We have now examined the effects of SecDFyajC and SecA membrane cycling on the movement of the preprotein chain in transit across the membrane. We find that SecDFyajC allows stabilization and accumulation of specific translocation intermediates which are driven forward rapidly by the PMF to complete translocation. We show that this SecDFyajC regulation of preprotein movement occurs via regulation of SecA membrane cycling and that SecA insertion (or deinsertion) correlates with a limited forward (or backward) movement of the preprotein. These findings link the effects of SecDFyajC on SecA membrane cycling and preprotein translocation and establish a direct relationship between SecA insertion and preprotein movement.

Results

The accumulation of translocation intermediates requires SecDFyajC

The transmembrane movement of the preprotein polypeptide chain is not a continuous process but takes place in a stepwise manner (Schiebel *et al.*, 1991; Uchida *et al.*, 1995). In the case of proOmpA, the most abundant translocation intermediates, termed I_{16} and I_{26}, have a 16 and 26 kDa translocated domain (Tani *et al.*, 1989; Schiebel *et al.*, 1991). Using membranes with depleted (DF^-), wild-type (DF^+) or enriched (DF^{+++}) levels of SecDFyajC proteins, we previously reported that SecDFyajC does not significantly affect the rate of ATP-driven proOmpA translocation (Arkowitz and Wickner, 1994; Economou *et al.*, 1995). However, we find that the translocation intermediate I_{26} is more prominent in the wild-type or SecDFyajC-enriched membranes than in the SecDFyajC-depleted ones (Figure 1A). To examine more closely the effects of SecDFyajC on I_{26} formation, translocation reactions were performed at low ATP concentrations (2 μM). At this ATP concentration, SecA function becomes limiting for translocation and, therefore, translocation intermediates are more abundant (Schiebel *et al.*, 1991). As shown Figure 1B, the formation of I_{26} requires SecDFyajC. In the absence of SecDFyajC, I_{26} is not accumulated but there is a cluster of translocation intermediates of somewhat lower molecular weight than I_{26}. Moreover, as seen most clearly at the earlier times of the reaction, SecDFyajC seems actually to reduce the rate of translocation of full-length proOmpA. These results establish a direct involvement of SecDFyajC in the ATP-driven preprotein translocation reaction.

SecDFyajC stabilizes translocation intermediates and slows full translocation

To examine the movement of polypeptide chains without release from the translocation site, a synthetic translocation-arrested intermediate was generated by cross-linking bovine pancreas trypsin inhibitor (BPTI) to proOmpA (Bassilana and Wickner, 1993). The large covalently folded structure of BPTI blocks translocation and thereby forms an arrested transmembrane intermediate at the point of

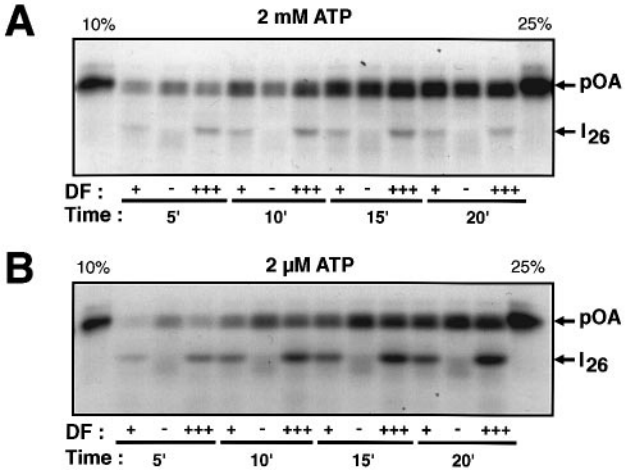

Fig. 1. SecDFyajC governs the accumulation of translocation intermediates. (**A**) At high ATP concentration. [^{125}I]proOmpA (1 μg/ml; 60 000 c.p.m.) pre-mixed with unlabeled proOmpA (10 μg/ml) was pre-incubated in 100 μl of TL buffer (50 mM KCl, 50 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.9) with DTT (1 mM), BSA (200 μg/ml), SecB (48 μg/ml), SecA (5 μg/ml) and urea-stripped IMVs (100 μg/ml) from *E.coli* BL21 (DF^+), BL325 (DF^-) or BL21 pCDF (DF^{+++}) for 2 min at 37°C. Translocation was started by the addition of 2 mM ATP and arrested after the indicated times by chilling on ice. Samples were treated with proteinase K (1 mg/ml; 15 min, 0°C), TCA precipitated and analyzed by SDS–PAGE and fluorography on a '15%' gel. [^{125}I]proOmpA (10 and 25%) added to the reaction was loaded on the gel as a standard. (**B**) At low ATP concentration. Translocation was performed as described above, except that an ATP-regenerating system (5 mM creatine phosphate, 10 μg/ml creatine kinase) was added and only [^{125}I]proOmpA (1 μg/ml; 60 000 c.p.m.) was used. Translocation was started with 2 μM ATP and arrested at the indicated time by chilling on ice.

coupling (Schiebel *et al.*, 1991). BPTI was coupled to the cysteinyl residue 302 of proOmpA using the reversible cross-linker *N*-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP). The stably folded structure of BPTI arrested the translocation of proOmpA–BPTI (and its mature form without leader sequence) at residue 302, yielding a proteinase K-resistant domain of 29 kDa termed I_{29} (Figure 2A). In the presence of 2 mM ATP, SecDFyajC levels did not affect the extent of I_{29} formation (lanes 9 and 10). Reduction of the disulfide in the cross-linker allowed release of BPTI from proOmpA and completion of translocation (lanes 11 and 12). When the translocation of proOmpA–BPTI C302 with 5 μM ATP was examined, formation of the arrested intermediate I_{29} was faster in the absence of SecDFyajC (lanes 1–8). In the presence of SecDFyajC, most of proOmpA–BPTI was arrested at the I_{26} position. Thus, by stabilizing the translocation intermediate I_{26}, SecDFyajC slows the forward movement of the chain.

To determine whether SecDFyajC also affected the stability of the earlier translocation intermediate I_{16}, translocation reactions were performed at yet lower ATP concentration (2 μM; Figure 2B). Like I_{26}, translocation intermediate I_{16} could only be clearly detected in the presence of SecDFyajC, and translocation to I_{29} was faster in the absence of SecDFyajC.

The PMF completes the translocation of the intermediates stabilized by SecDFyajC

Preprotein translocation is stimulated by the PMF, which can complete the forward movement of a translocation

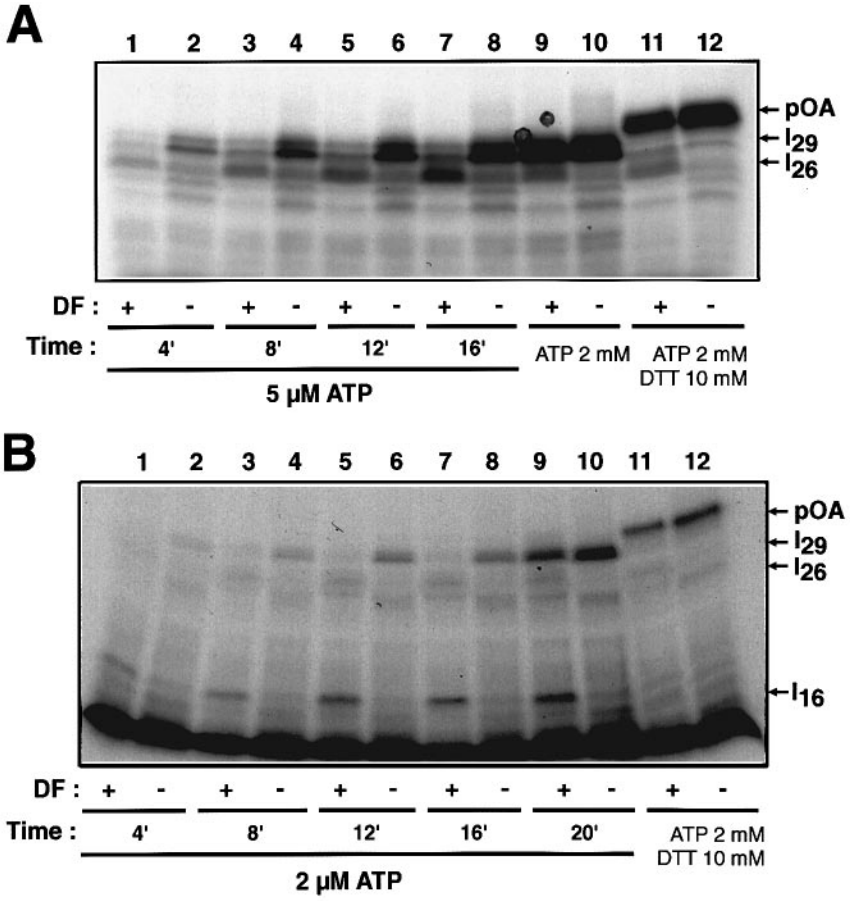

Fig. 2. SecDFyajC slows proOmpA translocation and stabilizes I_{26} and I_{16}. (**A**) Translocation of proOmpA–BPTI C302 at 5 µM ATP. Translocation reactions were as described in Figure 1A but with [^{125}I]proOmpA–BPTI C302 (1 µg/ml; 60 000 c.p.m.), 5 µM ATP and an ATP-regenerating system (5 mM creatine phosphate, 10 µg/ml creatine kinase). Translocation was arrested after the indicated times. After 16 min, 2 mM ATP (lanes 9–12) and 10 mM DTT (lanes 11 and 12) were added and the reactions incubated further for 2 min. Samples were treated with proteinase K (1 mg/ml; 15 min, 0°C) and analyzed by '15%' SDS–PAGE and fluorography. (**B**) Translocation intermediate I_{16}. Translocation reactions were performed with proOmpA–BPTI C302 and 2 µM ATP for the indicated times. After 16 min, 2 mM ATP and 10 mM DTT were added (lanes 11 and 12) and incubation was continued for 2 min. Translocation reactions were analyzed by 'high' Tris SDS–PAGE and fluorography.

intermediate in the absence of ATP hydrolysis (Schiebel *et al.*, 1991; Driessen, 1992). SecDFyajC is important for the full maintenance of the PMF and thus can stimulate the rate of proOmpA translocation in the presence of a redox substrate (NADH) at low ATP concentration (Figure 3A; Arkowitz and Wickner, 1994). In the presence of PMF, however, little translocation intermediate I_{26} was formed even in SecDFyajC-enriched membranes (Figure 3A). The I_{26} which had accumulated in the presence of ATP and SecDFyajC (Figure 1B) may have been chased rapidly to fully translocated proOmpA by the PMF. To test this hypothesis directly, proOmpA translocation was performed in two steps (Figure 3B). Translocation intermediates were formed with 2 µM ATP for the indicated time, ATP was removed by apyrase, thereby blocking new initiation of translocation or any ATP-driven chain movement, and a PMF was applied. In control samples without NADH (not shown), the level of I_{26} formed in the first incubation was dependent on SecDFyajC as seen in Figure 1B. However, in the presence of NADH, the abundant I_{26} intermediates which had formed at 2 µM ATP (Figure 1B) were swept forward to yield fully translocated proOmpA (Figure 3B). Moreover, though of slightly different sizes, the level of I_{26} which remained was independent of SecDFyajC, as also seen in Figure 3A. Thus, by increasing the formation of translocation intermediates I_{16} and I_{26}, SecDFyajC increases the translocation of proOmpA in the presence of PMF. In addition to the inability of SecDFyajC-depleted membranes to provide translocation intermediates, their inability to maintain a full PMF (Arkowitz and Wickner, 1994) may also contribute to the lower efficiency of proOmpA translocation.

Both inserted SecA and I_{26} are unstable without SecDFyajC

In the absence of SecDFyajC, either proOmpA or proOmpA–BPTI formed a cluster of translocation intermediates ranging from 24 to 26 kDa (Figures 1B and 2A, respectively). Since SecDFyajC-depleted membranes are unable to stabilize the membrane-inserted form of SecA (Economou *et al.*, 1995), the stability of both inserted SecA and I_{26} may be linked and controlled by SecDFyajC. To test this, we examined the movement of the preprotein polypeptide chain in conjunction with SecA membrane insertion, as assayed by the presence of a 30 kDa C-terminal protease-protected SecA domain (Economou and Wickner, 1994). Under the same conditions which led

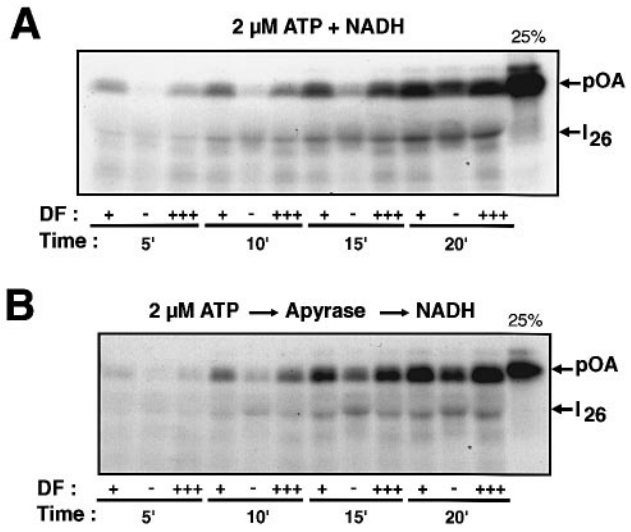

Fig. 3. The PMF chases the I_{26} accumulated in the presence of SecDFyajC to fully translocated proOmpA. (**A**) Translocation driven by both ATP and PMF. Translocation was performed as described in Figure 1B but using non-stripped IMVs from *E.coli* BL21 (DF^+), BL325 (DF^-) or BL21 pCDF (DF^{+++}). An electrochemical gradient was generated across the membrane by addition of 5 mM NADH, and the translocation was initiated with 2 μM ATP. Reactions were arrested at the indicated times and analyzed by '15%' SDS–PAGE and fluorography. [^{125}I]ProOmpA (25%) added to the reaction is shown as a standard. (**B**) Translocation can be first driven by ATP, then by PMF. Translocation was started with 2 μM ATP and, after the indicated times, arrested by apyrase addition (20 U/ml; 2 min). NADH (5 mM) was added and the reaction incubated further for 2 min. Reactions were stopped by chilling on ice and analyzed as described in Figure 1A.

to the formation of I_{24}–I_{26} in DF^- membranes (Figure 4A, lane 1; 2 μM ATP), the SecA 30 kDa domain was largely in the deinserted state and digested by protease (Figure 4B, lane 2). In the presence of SecDFyajC, however, translocation intermediates accumulated at I_{26} (Figure 4A, lane 2) and SecA was in the inserted state (Figure 4B, lane 1). Since additional ATP is required for SecA deinsertion, the removal of free ATP with apyrase still allowed bound SecA with its bound ATP to insert, but 'locked' this SecA in the inserted position (Economou and Wickner, 1994). Addition of the non-hydrolyzable ATP analog AMP-PNP also promoted SecA insertion. The level of SecA 30 kDa protected fragment obtained by these treatments was independent of SecDFyajC (Figure 4B, lanes 3–6), in agreement with the finding that SecDFyajC slows the ATP-driven deinsertion rather than being essential for SecA insertion *per se* (Economou *et al.*, 1995). Strikingly, after apyrase or AMP-PNP treatment, the translocation intermediates formed in the absence of SecDFyajC (I_{24}–I_{26}) were recovered as a unique intermediate of 26 kDa (Figure 4A, lanes 5 and 9). Thus, SecA insertion promotes the forward movement of I_{24} to I_{26}, and the instability of inserted SecA in the absence of SecDFyajC is likely to be responsible for the instability of proOmpA at I_{26}.

The imposition of a PMF at the end of the ATP-driven reaction stabilized I_{26} in the absence of SecDFyajC (Figure 4A, lane 3) and prevented the deinsertion of SecA (see Figure 5 below). In the presence of SecDFyajC, the PMF allowed a partial chase of I_{26} to fully translocated proOmpA (Figure 4A, lane 4) and, as previously observed (Figure 3), the level of I_{26} which remained after imposition of a PMF was the same in the presence or absence of SecDFyajC (Figure 4A, lanes 7 and 8). The release of the preprotein from SecA is essential to allow PMF-driven translocation, since the addition of AMP-PNP before NADH prevented the chase of I_{26} (Figure 4A, lane 12; Schiebel *et al.*, 1991). Apyrase treatment, however, locked SecA in the inserted position but without such an inhibitory effect on PMF-driven translocation (Figure 4A, lane 8). Thus, the preprotein chain is probably not tightly associated with SecA after removal of the free ATP by apyrase and can translocate in response to a PMF. These results indicate that the release of the preprotein from SecA upon the hydrolysis of its bound ATP precedes SecA deinsertion, which requires additional ATP binding and hydrolysis (Economou *et al.*, 1995). In the absence of SecDFyajC, SecA deinsertion may occur without prior release of the preprotein and thereby cause a limited reverse movement of the polypeptide chain.

SecA deinsertion and backward translocation

If the stability of I_{26} depends only on the stability of inserted SecA, it may be possible to reverse the movement of the chain by promoting SecA deinsertion. Thus, after ATP-driven translocation, SecA insertion and I_{26} were stabilized by addition of AMP-PNP (as in Figure 4A, lanes 9 and 10) and the nucleotides were then removed by centrifugation. The stability of I_{26} and inserted SecA was not affected by the centrifugation treatment (Figure 5A and B, lanes 1 and 2). Re-incubation with 2 μM ATP only led to I_{26} backward movement and SecA deinsertion in the absence of SecDFyajC (Figure 5A, lane 5, and B, lane 6). This destabilization was reversible since readdition of AMP-PNP after incubation with 2 μM ATP induced SecA insertion (not shown) and re-stabilization of I_{26} in the absence of SecDFyajC (Figure 5A, lane 9). Incubation with 2 mM ATP allowed I_{26} to translocate fully (Figure 5A, lanes 3 and 4). Even at this ATP concentration, some limited backward movement of I_{26} and SecA deinsertion were observed in the absence of SecDFyajC (Figure 5A, lane 3, and B, lane 4). Thus, in response to the ATP-driven deinsertion of SecA that occurs in the absence of SecDFyajC, the translocation intermediate I_{26} undergoes a backward movement. The presence of SecDFyajC prevents SecA deinsertion and stabilizes I_{26} against reverse translocation.

Imposition of a PMF during the re-incubation with 2 μM ATP chased I_{26} to fully translocated proOmpA in the presence of SecDFyajC (Figure 5A, lane 8) and prevented the backward movement of I_{26} which was seen previously in the absence of SecDFyajC (lane 7). Under these same conditions, the PMF also prevented the ATP-dependent SecA deinsertion that occurred in the absence of SecDFyajC (Figure 5B, lanes 6 and 8). Thus, like SecDFyajC, the PMF stabilizes the inserted form of SecA and prevents the backward movement of the chain. This result suggests that the stability of the chain is not directly dependent on SecDFyajC but rather on the inserted form of SecA.

SecDFyajC stabilizes arrested translocation intermediates at position I_{26}

To test whether the function of SecDFyajC is restricted to low ATP concentrations (2 μM) or whether it can also

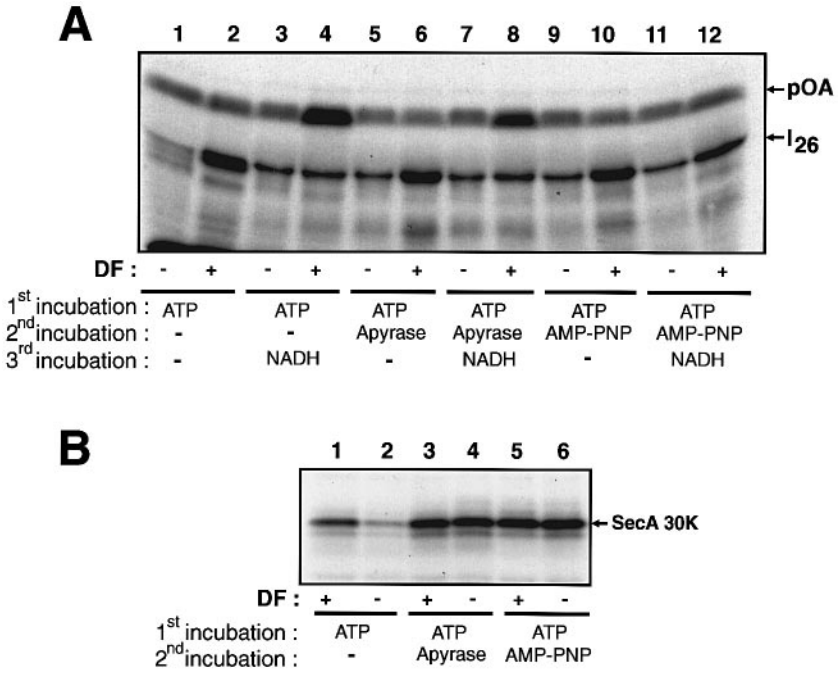

Fig. 4. (**A**) SecDFyajC stabilizes I_{26}. Translocation reactions were prepared as described in Figure 1B and incubated for 15 min with 2 μM ATP (1st incubation), using non-stripped IMVs from *E.coli* BL325 (DF^-) or BL21 (DF^+). Reactions were incubated further for 2 min (2nd incubation) without any addition (lanes 1–4), with 20 U/ml apyrase (lanes 5–8) or with 2 mM AMP-PNP (lanes 9–12). After these 2 min, 5 mM NADH was added where indicated and the incubation prolonged for 3 min (3rd incubation). Reactions were stopped by chilling on ice and analyzed as described in Figure 1A. (**B**) SecDFyajC stabilizes the inserted form of SecA. SecA membrane insertion was monitored by the appearance of a 30 kDa protease-inaccessible domain as previously described (Economou and Wickner, 1994). Reactions in 100 μl of TL buffer contained BSA, SecB, an ATP-regenerating system and IMVs from *E.coli* BL21 (DF^+) or BL325 (DF^-) at the same concentrations as in Figure 1B. Unlabeled proOmpA (1 μg/ml) and [^{125}I]SecA (80 000 c.p.m.; 5 μg/ml) were added. After pre-incubation (2 min, 37°C), the SecA membrane cycle was started with 2 μM ATP (lanes 1–6). After 15 min, 20 U/ml apyrase (lanes 3 and 4) or 2 mM AMP-PNP (lanes 5 and 6) was added and the reaction incubated further for 2 min. Reactions were stopped by chilling on ice, digested with trypsin (1 mg/ml, 15 min, 0°C), TCA precipitated and analyzed by '15%' SDS–PAGE and fluorography. The arrow indicates the trypsin-inaccessible 30 kDa domain of SecA.

stabilize preproteins which are blocked in their forward translocation at I_{26} at physiological levels of ATP (2 mM), the cysteine residue C302 was relocated genetically to residue 245 and coupled to BPTI. The translocation intermediate formed with proOmpA–BPTI C245 (and its mature form without leader sequence) arrested near position I_{26} in the presence of SecDFyajC but at earlier translocation intermediates (ranging from I_{24} to I_{26}) in its absence (Figure 6A, lanes 1 and 2). Under these same conditions, SecA was inserted in the presence of SecDFyajC and deinserted in its absence (Figure 6B, lanes 1 and 2). Addition of AMP-PNP to the reaction promoted SecA insertion as well as forward movement of the chain in the absence of SecDFyajC (Figure 6A and B, lanes 4). To determine if the inserted SecA was responsible for the stability of proOmpA–BPTI C245 at position I_{26}, membranes were treated with urea. Such treatment extracted most of the inserted form of SecA from the membrane (Figure 6B, lanes 5 and 6), and the translocation intermediate was recovered at positions I_{24}–I_{26} in either the presence or absence of SecDFyajC (Figure 6A, lanes 5 and 6), much as seen without urea extraction in the absence SecDFyajC (Figure 6A, lane 2). Thus, in the absence of inserted SecA, the stable positions of translocation intermediates are not governed by SecDFyajC. These experiments, using high ATP concentrations and intermediates where the chain is arrested near position I_{26}, confirm that it is the SecDFyajC-mediated stabilization of inserted SecA which prevents the backward movement of the chain.

While the stability of I_{26} depends on SecDFyajC, the stability of the translocation intermediate I_{29} formed using proOmpA–BPTI C302 is independent of SecDFyajC (Figures 2A, and 6A, lanes 7 and 8). However, SecA insertion was still stabilized by SecDFyajC (Figure 6B, lanes 7 and 8). Moreover, after urea treatment, most of the SecA was extracted from the inserted position but the chain remained at position I_{29} (Figure 6A and B, lanes 9 and 10). Thus, the stability of the chain at positions other than I_{26}, such as I_{29} or I_{24}, is not due to the inserted form of SecA and, consequently, is independent of SecDFyajC.

Discussion

SecD, SecF and YajC, the proteins of the *secD* operon (Gardel *et al.*, 1990), are co-expressed (Pogliano and Beckwith, 1994b) and form a complex which can be isolated along with SecYEG as a 'holoenzyme' form of the translocase (Duong and Wickner, 1997). *In vivo* studies have shown that strains with mutations or depletion of SecDF have export defects, while their overexpression stimulates translocation and can suppress leader peptide mutations (Pogliano and Beckwith, 1994a). Addition of anti-SecD antibodies to spheroplasts induced accumulation of translocation intermediates and blocked their subsequent release into the periplasm (Matsuyama *et al.*, 1993). While clearly establishing the importance of SecD and SecF for translocation, these *in vivo* studies did not define their mechanism of action. Furthermore, *in vitro* studies with

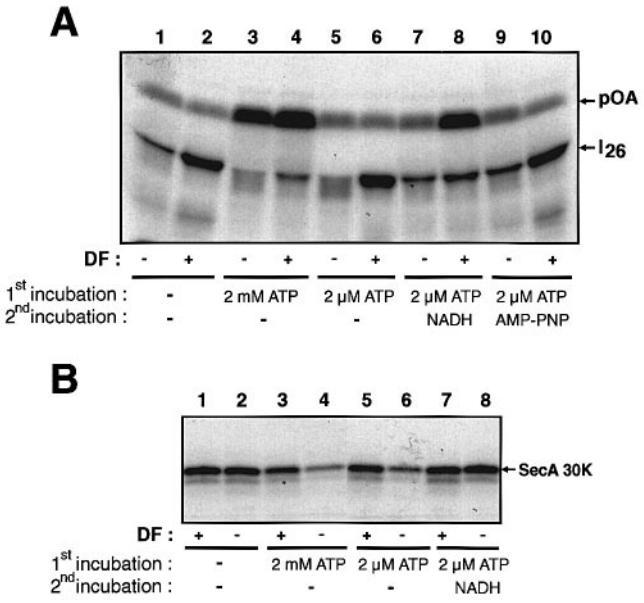

Fig. 5. SecA deinsertion and reverse translocation in the absence of SecDFyajC. (**A**) Either SecDFyajC or the PMF can prevent the reverse translocation of I_{26}. Translocation reactions were prepared as described in Figure 4A, lanes 9 and 10 (2 µM ATP for 15 min followed by the addition of 2 mM AMP-PNP). To remove nucleotides, reactions (100 µl) were layered over an equal volume of sucrose solution (0.2 M sucrose in TL buffer) and membranes sedimented by ultracentrifugation (10 min, 4°C, 73 000 r.p.m., Beckman TLA100 rotor). Sediments were resuspended in 100 µl of TL buffer and re-incubated for 2 min (1st incubation) without any additions (lanes 1 and 2), with 2 mM ATP (lanes 3 and 4) or with 2 µM ATP (lanes 5–10). After these 2 min, 5 mM NADH (lanes 7 and 8) or 2 mM AMP-PNP (lanes 9 and 10) were added and the incubation prolonged for 3 min (2nd incubation). Reactions were stopped by chilling on ice and analyzed as described in Figure 1A. (**B**) SecDFyajC or PMF prevent ATP-driven SecA deinsertion. Translocation reactions were prepared as described in Figure 4B, lanes 5 and 6 (2 µM ATP for 15 min followed by the addition of 2 mM AMP-PNP) using [^{125}I]SecA (80 000 c.p.m.; 5 µg/ml) and unlabeled proOmpA (1 µg/ml). Membranes were isolated by ultracentrifugation as described above and re-incubated for 2 min without any additions (lanes 1 and 2), with 2 mM ATP (lanes 3 and 4) or with 2 µM ATP (lanes 5–8). After 2 min incubation with 2 µM ATP, 5 mM NADH (lanes 7 and 8) was added and the incubation prolonged for 3 min. Reactions were stopped by chilling on ice, treated as described in Figure 4B and analyzed by '15%' SDS–PAGE and fluorography.

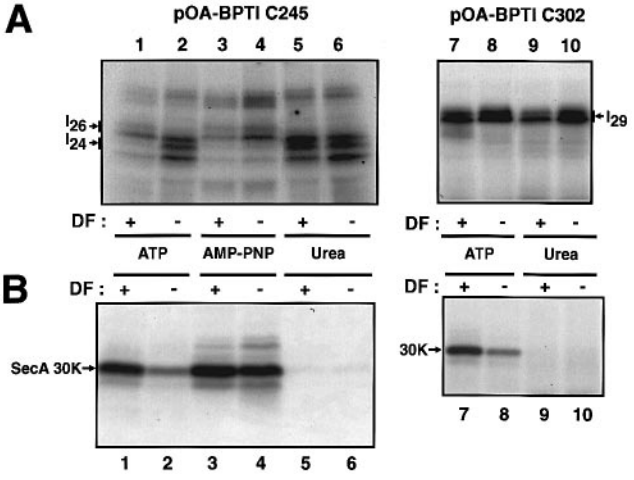

Fig. 6. SecDFyajC prevents the backward movement of proOmpA–BPTI C245 via stabilization of inserted SecA. (**A**) Translocation reactions (100 µl), as described in Figure 1A, used [^{125}I]proOmpA–BPTI C245 (lanes 1–6; 1 µg/ml; 60 000 c.p.m.) or [^{125}I]proOmpA-BPTI C302 (lanes 7–10; 1 µg/ml; 60 000 c.p.m.) and 2 mM ATP. After 15 min at 37°C, reactions were chilled on ice. An equal volume of TL buffer (lanes 1–4, 7 and 8) or 10 M urea (lanes 5, 6, 9 and 10) was added. After 5 min on ice, membranes were sedimented through a sucrose solution as described in Figure 4A and resuspended in 100 µl of TL buffer on ice. Reactions corresponding to lanes 3 and 4 were incubated further for 2 min at 37°C with 2 mM AMP-PNP. Samples were treated with proteinase K (1 mg/ml; 15 min, 0°C) and analyzed by '15%' SDS–PAGE and fluorography. (**B**) The position of the arrest of proOmpA–BPTI C245 depends on inserted SecA. Translocation reactions were as above but using [^{125}I]SecA (80 000 c.p.m.; 5 µg/ml) and unlabeled proOmpA–BPTI C245 (lanes 1–6; 1 µg/ml) or proOmpA–BPTI C302 (lanes 7–10; 1 µg/ml). Reactions were digested with trypsin (1 mg/ml; 15 min, 0°C) and analyzed by '15%' SDS–PAGE and fluorography.

inner membrane vesicles (IMVs) from strains with depleted or overproduced SecDFyajC showed very little alteration in translocation rates (Arkowitz and Wickner, 1994; Economou *et al.*, 1995; Duong and Wickner, 1997), and ATP-driven translocation of proOmpA into SecYEG-reconstituted proteoliposomes showed a rate of translocation close to that observed with intact membranes vesicles (Bassilana and Wickner, 1993). Thus, the contribution of the members of the *secD* operon to the translocation process remained largely undefined. We have now resolved this apparent discrepancy between the genetic and biochemical studies by developing more refined biochemical assays which can assess the function of SecDFyajC in translocation. Through these assays, the SecA insertion and deinsertion cycle has been tied directly to the movements of preprotein chains which are in transit through the translocase.

SecDFyajC plays a central role in the establishment, maintenance, and further movement of the translocation intermediates I_{16} and I_{26} (Figure 2). This effect, best seen at low ATP concentrations, can also be detected at physiological ATP levels (2 mM ATP, Figure 1A). However, at such ATP concentrations, the rapid kinetics of translocation obscure the regulatory function of SecDFyajC. Accordingly, arrested translocation intermediates (Figure 6) or lowered reaction temperatures (not shown) were used to reveal the effects of SecDFyajC on the stability of the intermediates at high ATP concentration. Moreover, since I_{16} and I_{26} are the major kinetic intermediates in ATP-driven translocation reactions of proOmpA (Figures 1 and 2) and almost all the chains pause at these intermediates states of translocation (Schiebel *et al.*, 1991), most proOmpA chains must be affected by SecDFyajC during their membrane transit. Without SecDFyajC, translocation intermediates are not stable but are driven forward to the fully translocated state. We emphasize that SecDFyajC does not directly determine the positions at which the intermediates are accumulated. Rather, the pattern of proOmpA translocation intermediates is determined by short hydrophobic segments of the preprotein itself (Sato *et al.*, 1997). As noted by these authors, such very short hydrophobic segments are ubiquitous in secretory proteins and thus the stepwise movement seen with proOmpA may be a general mechanism for translocation (Sato *et al.*, 1997). In this respect, the effects of SecDFyajC may not be restricted to proOmpA but will presumably apply to other preproteins which undergo a discontinuous translocation movement.

We find that SecDFyajC largely acts on the preprotein chain through regulation of the SecA membrane cycling (Figure 6). Since SecA is required for all translocase-dependent secretion, this suggests that the regulatory action of SecDFyajC will also be quite general.

SecA had been shown to insert into the membrane in response to the binding of proOmpA and ATP (Economou and Wickner, 1994). Non-hydrolyzable ATP analogs promote both SecA insertion (Economou *et al.*, 1995) and a limited, defined forward movement of translocation intermediates (Schiebel *et al.*, 1991). This had suggested that SecA membrane insertion and chain movement were coupled. However, it has also been suggested that SecA insertion plays only a structural role to form part of the protein-conducting channel (Chen *et al.*, 1996). Our current work does not address how SecA can penetrate into and across the membrane. Rather, we have examined here the relationship between the movement of translocation intermediates and a major SecA conformational change which is monitored by the appearance of a 30 kDa protease-inaccessible SecA domain, described as the membrane-inserted state of SecA (Economou and Wickner, 1994). We find that preprotein movement is coupled intimately to the insertion and deinsertion of SecA and is regulated by SecDFyajC: (i) addition of a non-hydrolyzable ATP analog promotes both a limited forward translocation of I_{24} to I_{26} and SecA insertion (Figure 4); (ii) imposition of a membrane potential or removal of ATP by apyrase stabilizes both the inserted state of SecA and I_{26} (Figure 5); and (iii) upon removal of SecA by urea (Figure 6), the intermediate I_{26} is no longer stabilized by SecDFyajC. Taken together, these results show that SecA insertion promotes a limited forward movement of the preprotein and that the translocation intermediate I_{26} is stabilized by the inserted form of SecA, itself stabilized by SecDFyajC. These data suggest that SecA membrane insertion carries 'loops' of the preprotein across the membrane in a sewing machine manner (Economou *et al.*, 1994; Wickner and Leonard, 1996) until SecDFyajC stabilizes inserted SecA and hence slows the chain movement at positions corresponding to the intermediates I_{16} or I_{26}. It is noteworthy that only I_{16} and I_{26}, whose appearance correlates with the presence of short hydrophobic stretches in proOmpA (Sato *et al.*, 1997), are affected by SecDFyajC. Indeed, the stability of I_{29}, an intermediate which is not normally detected during translocation, does not rely on inserted SecA and SecDFyajC (Figure 6). Thus, it is possible that SecA specifically recognizes these hydrophobic segments and inserts and stabilizes the preprotein chain only at these specific positions. Since SecDFyajC seems to acts through SecA, this model may explain why only I_{16} and I_{26} are affected. Thus, the insertion and deinsertion cycle of SecA may not catalyze the complete chain movement but only serves to stabilize those translocation intermediates and to hold them in place for the further action of the PMF. In the absence of PMF, further forward movement of the preprotein chain may be promoted by the other subunit of the dimeric SecA molecule (Driessen, 1993) or by parts of SecA other than the inserted 30 kDa domain. Indeed, though we monitor SecA insertion by the accessibility of the C-terminal 30 kDa domain to protease, other parts of SecA also undergo profound conformational changes and a 65 kDa N-terminal fragment of SecA also inserts into the membrane during preprotein translocation (Eichler and Wickner, 1997). When the 30 kDa domain of SecA is not inserted, intermediates such as I_{29} may be stabilized by the folding of the chain on the other side of the membrane, by association with other Sec proteins or by other domains of SecA.

SecDFyajC facilitates translocation of proOmpA in the presence of a PMF (Figure 4; Arkowitz and Wickner, 1994). The inability of SecDFyajC-depleted membranes to maintain a normal electrochemical gradient may be directly responsible for the lack of stimulation of translocation by PMF (Arkowitz and Wickner, 1994). However, in our current experiments, the PMF was able to stabilize translocation intermediates in SecDFyajC-depleted membranes (Figure 5A), indicating that the PMF is not completely abolished in these membranes. Moreover, SecDFyajC is not essential for coupling the PMF to translocation since a PMF-dependent stimulation of translocation can occur in SecYEG proteoliposomes (Brundage *et al.*, 1990; Driessen, 1992). By separately analyzing the effects of ATP and PMF (Figure 3B), we find that this PMF-dependent stimulation is at the expense of accumulated translocation intermediates. These observations led us to propose that SecDFyajC facilitates translocation by increasing, through SecA stabilization, the quantity of translocation intermediates available for the action of the PMF. It is noteworthy that the SecDFyajC-mediated facilitation of translocation by the PMF was detected at low ATP concentration *in vitro* (Arkowitz and Wickner, 1994) while the PMF and ATP are not limiting *in vivo*. The PMF dependence of preprotein translocation can be alleviated by high SecA concentration *in vitro* (Yamada *et al.*, 1989). In our studies, reducing the level of ATP may have the same effect as reducing the SecA concentration and hence render proOmpA translocation more dependent on the PMF. Even at high ATP concentration, SecDFyajC was essential for the PMF to stimulate translocation of the precursor of the maltose-binding protein (preMBP) (Arkowitz and Wickner, 1994). Clearly, the role of SecDFyajC in translocation is not restricted to proOmpA and can affect other preproteins under physiological PMF and ATP conditions. We would suggest that the pattern of pre-MBP translocation intermediates may be strongly affected by SecDFyajC even at high ATP concentrations.

The proposed mechanism for SecDFyajC action may allow a better coupling between the energies of ATP and PMF. In the absence of SecDFyajC, the unregulated preprotein movement may lead to excessive ATP consumption, to a lack of intermediates available for the action of the PMF and, finally, to the dissipation of the electrochemical gradient. Indeed, preprotein translocation is accompanied by an increase in the ionic permeability of the membrane (Schiebel and Wickner, 1992), SecDFyajC depletion decreases the magnitude of the PMF *in vitro* and of one of its components, $\Delta\psi$, *in vivo* (Arkowitz and Wickner, 1994), and IMVs with overproduced SecYE exhibit a translocation-dependent leak of protons (Kawasaki *et al.*, 1993). In the absence of SecDFyajC, dissipation of the electrochemical gradient and high ATP consumption will alter the cell physiology progressively and reduce preprotein translocation (Pogliano and Beckwith, 1994a). Interestingly, not only mutations in *secD* and *secF* but

also mutations in *secA*, and the synthesis of a hybrid preprotein which jams the translocase, induce the expression of PspA (Kleerebezem and Tommassen, 1993), a stress protein which maintains the PMF in detrimental conditions.

We recently reported that SecDFyajC can increase the activity of SecYE during ATP-driven translocation in the absence of a PMF (Duong and Wickner, 1997). Such stimulation was only seen when SecG was either absent or present at a subsaturating concentration with respect to SecYE. In such conditions, the very weak translocation activity of SecYE may depend entirely on the ability of SecDFyajC to maintain stable translocation intermediates and to prevent reverse translocation. By stabilizing the inserted form of SecA, and hence translocation intermediates at an early stage of the translocation process, SecDFyajC may help to bypass the proposed proof-reading function of SecY (Osborne and Silhavy, 1993) and thereby facilitate the translocation of precursors with defective leader peptides (Pogliano and Beckwith, 1994a). Furthermore, since each preprotein will probably have its own unique set of translocation intermediates based on the distribution of short hydrophobic segments in its sequence (Sato *et al.*, 1997), each would be affected differently by SecDFyajC, as previously observed (Arkowitz and Wickner, 1994; Pogliano and Beckwith, 1994a).

SecDFyajC appears as a regulatory domain of preprotein translocase. By controlling the SecA membrane cycling, and hence stabilizing translocation intermediates which can be driven forward further by the PMF, SecDFyajC may allow a better coordination between ATP and PMF-driven translocation and thus lower the amount of hydrolyzed ATP per molecule translocated. Further studies will be needed to establish individual functions of SecD, SecF and YajC and to determine whether SecDFyajC directly binds to inserted SecA or only influences SecA stability through alteration of the structure of SecYEG. Since it seems that only 10% as much SecDFyajC as SecYEG is present in the cell (Pogliano and Beckwith, 1994b; Duong and Wickner, 1997), the SecDFyajC complex may exchange rapidly with SecYEG complex. Alternatively, two kinds of translocase, with or without SecDFyajC, may co-exist in *Escherichia coli*. Studies of subunits exchange (Joly *et al.*, 1994) and effects of the PMF on the structure of the translocase will be needed to resolve this central question.

Materials and methods

Materials

SecA (Cunningham *et al.*, 1989), SecB (Weiss *et al.*, 1988) and the precursor form of OmpA (proOmpA) (Crooke *et al.*, 1988) were purified as described. [^{125}I]Na (~17 mCi/μg) was from Amersham. [^{125}I]SecA (~ 8×10^5 c.p.m./μg) (Economou and Wickner, 1994) was prepared as described. Proteinase K, creatine kinase, creatine phosphate, AMP-PNP and NADH were from Boehringer-Mannheim. ATP, lipid-free bovine serum albumin (BSA), TPCK-treated trypsin and potato apyrase (grade VIII) were from Sigma. Iodogen and SPDP were from Pierce. IMVs from *E.coli* strain BL21 (*hsdS, ompT*$^-$, *gal*), BL21 pCDF (plasmid pBAD33 carrying the *secD* operon) and from the SecDFyajC-depleted strain BL325 (BL21 *tgt::kan-araC*$^+$-P$_{BAD}$*::yajCsecDF*) were prepared as described (Douville *et al.*, 1995; Duong and Wickner, 1997). To render the IMVs proton-permeable, the F_1 subunit of the F_0-F_1 ATPase was removed in 6 M urea (30 min, 4°C). This treatment also removed endogenous SecA (Cunningham *et al.*, 1989).

Construction and purification of proOmpA cysteine mutants

The *omp*A gene carried on a 1.3 kb *Eco*RI–*Hin*dIII DNA fragment on pTRC-Omp9 plasmid (Crooke *et al.*, 1988) was inserted into the *Eco*RI–*Hin*dIII sites of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible plasmid pTrc99A (Pharmacia). Site-directed mutagenesis was performed using the Clontech Transformer Mutagenesis Kit. Cys290 and Cys302 were replaced by serine residues using the mutagenic primers 5′-GGCAACACCTCTGACAACGTG and 5′-CTGATCGACTCCCTGGCTCCG respectively. In the mutant deleted for both Cys290 and Cys302, Ser245 was replaced by a cysteine residue using the mutagenic primer 5′-CGCATCGGTTGTGACGCTTAC. All mutations were verified by sequencing the relevant coding regions. ProOmpA proteins with a unique cysteine at either position 302 or 245 were expressed in *E.coli* DH5α and were purified first as described (Crooke *et al.*, 1988), then on Pharmacia FPLC MonoQ resin (in 6 M urea, 1 mM 2-mercaptoethanol, 50 mM glycine, pH 8). The proteins were eluted from the column at 0.2 M NaCl.

Synthesis, purification and iodination of proOmpA–SPDP–BPTI

BPTI was coupled to the cross-linker SPDP (Carlson *et al.*, 1978). The activated BPTI molecule was then cross-linked to the unique cysteine of proOmpA C302 or C245 as described (Schiebel *et al.*, 1991). The proOmpA–BPTI was purified from the adduct by FPLC MonoS chromatography (in 6 M urea, 1 M acetic acid) where the protein was eluted at 0.3 M NaCl. Proteins were concentrated by trichloroacetic acid (TCA) precipitation and resuspended in 6 M urea, 50 mM Tris–HCl, pH 7.9. Either proOmpA (100 μg) or proOmpA–BPTI (100 μg) was added to an iodogen-coated tube (Pierce; 40 μg dissolved in chloroform and evaporated under a stream of N_2) followed by addition of [^{125}I]Na (1 μl; 500 μCi). After 10 min on ice, the iodinated proteins were desalted with a G25 spin-column (Biorad) equilibrated in 6 M urea, 50 mM Tris–HCl, pH 7.9. [^{125}I]proOmpA and [^{125}I]proOmpA–BPTI (~6×10^6 c.p.m./μg) were stored at –80°C and used within 2 months.

Miscellaneous

Protein concentration was determined using the Bradford reagent (Biorad) with BSA as standard. For protein precipitation after a translocation assay, samples were mixed with 1/3 volume of ice-cold 50% TCA, incubated for 30 min on ice and collected by centrifugation (16 000 r.p.m., 10 min, 4°C, Beckman microfuge). The sediment was suspended in 1 ml of acetone and centrifuged. The acetone was removed by aspiration followed by an incubation at 37°C for 5 min. Proteins were resuspended in SDS sample buffer and analyzed on SDS–PAGE '15%' or 'high' Tris gels (Brundage *et al.*, 1992). Autoradiography of ^{125}I-labeled polypeptides was performed at –80°C with intensifying screens. Protein molecular weight markers were from Bethesda Research Laboratories.

Acknowledgements

We thank Marilyn Rice Leonard for expert assistance, J.Eichler and Z.Xu for critical reading of the manuscript and A.Price and C.Ungermann for useful discussions. F.D. was supported by fellowship from the Institut National de la Santé et de la Recherche Médicale. This work was supported by a grant from the National Institute of General Medical Sciences.

References

Akimaru,J., Matsuyama,S.-i, Tokuda,H. and Mizushima,S. (1991) Reconstitution of a protein translocation system containing purified SecY, SecE, and SecA from *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **88**, 6545–6549.

Arkowitz,R.A. and Wickner,W. (1994) SecD and SecF are required for the proton electrochemical gradient stimulation of preprotein translocation. *EMBO J.*, **13**, 954–963.

Bassilana,M. and Wickner,W. (1993) Purified *Escherichia coli* preprotein translocase catalyzes multiples cycles of precursor protein translocation. *Biochemistry*, **32**, 2626–2630.

Brundage,L., Hendrick,J.P., Schiebel,E., Driessen,A.J.M. and Wickner,W. (1990) The purified *E.coli* integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation. *Cell*, **62**, 649–657.

Brundage,L., Fimmel,C.J., Mizushima,S. and Wickner,W. (1992) SecY, SecE and band 1 form the membrane-embedded domain of *Escherichia coli* preprotein translocase. *J. Biol. Chem.*, **267**, 4166–4170.

Carlson,J., Drevin,H. and Axen,R. (1978) Protein thiolation and reversible protein–protein conjugation. *Biochem. J.*, **173**, 723–737.

Chen,L. and Tai,P.C. (1985) ATP is essential for protein translocation into *Escherichia coli* membrane vesicles. *Proc. Natl Acad. Sci. USA*, **82**, 4384–4388.

Chen,X., Xu,H. and Tai,P.C. (1996) A significant fraction of functional SecA is permanently embedded in the membrane. *J. Biol. Chem.*, **271**, 29698–29706.

Crooke,E., Brundage,L., Rice,M. and Wickner,W. (1988) ProOmpA spontaneously folds in a membrane assembly competent state which trigger factor stabilizes. *EMBO J.*, **7**, 1831–1835.

Cunningham,K., Lill,R., Crooke,E., Rice,M., Moore,K., Wickner,W. and Oliver,D. (1989) Isolation of SecA protein, a peripheral protein of the *E.coli* plasma membrane that is essential for the functional binding and translocation of proOmpA. *EMBO J.*, **8**, 955–959.

Douville,K., Leonard,M., Brundage,L., Nishiyama,K.-i., Tokuda,H., Mizushima,S. and Wickner,W. (1994) Band 1 subunit of *Esherichia coli* preprotein translocase and integral membrane export factor P12 are the same protein. *J. Biol. Chem.*, **269**, 18705–18707.

Douville,K., Price,A., Eichler,J., Economou,A. and Wickner,W. (1995) SecYEG and SecA are the stoichiometric components of preprotein translocase. *J. Biol. Chem.*, **270**, 20106–20111.

Driessen,A.J.M. (1992) Precusor protein translocation by the *Escherichia coli* translocase is directed by the protonmotive force. *EMBO J.*, **11**, 847–853.

Driessen,A.J.M. (1993) SecA, the peripheral subunit of the *Escherichia coli* precursor protein translocase, is functional as a dimer. *Biochemistry*, **32**, 13190–13197.

Duong,F. and Wickner,W. (1997) Distinct catalytic roles of the SecYE, SecG and SecDFyajC subunits of preprotein translocase holoenzyme. *EMBO J.*, **16**, 2756–2768.

Economou,A. and Wickner,W. (1994) SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. *Cell*, **78**, 835–843.

Economou,A., Pogliano,J.A., Beckwith,J., Oliver,D.B. and Wickner,W. (1995) SecA membrane cycling at SecYEG is driven by distinct ATP binding and hydrolysis events and is regulated by SecD and SecF. *Cell*, **83**, 1171–1181.

Eichler,J. and Wickner,W. (1997) Both an N-terminal 65 kDa domain and a C-terminal 30 kDa domain of SecA cycle into the membrane at SecYEG during translocation. *Proc. Natl Acad. Sci. USA*, **94**, 5574–5581.

Eichler,J., Brunner,J. and Wickner,W. (1997) The protease-protected 30 kDa domain of SecA is largely inaccessible to the membrane lipid phase. *EMBO J.*, **16**, 2188–2196.

Gardel,C., Johnson,K., Jacq,A. and Beckwith,J. (1990) The *secD* locus of *E.coli* codes for two membrane proteins required for protein export. *EMBO J.*, **9**, 3209–3216.

Geller,B.L., Movva,N.R. and Wickner,W. (1986) Both ATP and the electrochemical potential are required for optimal assembly of pro-OmpA into *Escherichia coli* inner membrane vesicles. *Proc. Natl Acad. Sci. USA*, **83**, 4219–4222.

Hanada,M., Nishiyama,K., Mizushima,S. and Tokuda,H. (1994) Reconstitution of an efficient protein translocation machinery comprising SecA and the three membrane proteins, SecY, SecE and SecG (p12). *J. Biol. Chem.*, **269**, 23625–23631.

Joly,J.C. and Wickner,W. (1993) The SecA and SecY subunits of translocase are the nearest neighbors of a translocating preprotein, shielding it from phospholipids. *EMBO J.*, **12**, 255–263.

Joly,J.C., Leonard,M.R. and Wickner,W. (1994) Subunit dynamics in *Escherichia coli* preprotein translocase. *Proc. Natl Acad. Sci. USA*, **91**, 4703–4707.

Kawasaki,S., Mizushima,S. and Tokuda,H. (1993) Membrane vesicles containing overproduced SecY and SecE exhibit high translocation ATPase activity and countermovement of protons in a SecA- and presecretory protein-dependent manner. *J. Biol. Chem.*, **268**, 8193–8198.

Kim,Y.J., Rajapandi,T. and Oliver,D. (1994) SecA protein is exposed to the periplasmic surface of the *E.coli* inner membrane in its active state. *Cell*, **78**, 845–853.

Kleerebezem,M. and Tommassen,J. (1993) Expression of the *pspA* gene stimulates efficient protein export in *Escherichia coli*. *Mol. Microbiol.*, **7**, 947–956.

Lill,R., Cunningham,K., Brundage,L., Ito,K., Oliver,D. and Wickner,W. (1989) The SecA protein hydrolyzes ATP and is an essential component of the protein translocation ATPase of *E.coli*. *EMBO J.*, **8**, 961–966.

Lill,R., Dowhan,W. and Wickner,W. (1990) The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins. *Cell*, **60**, 259–269.

Matsuyama,S., Fujita,Y. and Mizushima,S. (1993) SecD is involved in the release of translocated secretory proteins from the cytoplasmic membrane of *Escherichia coli*. *EMBO J.*, **12**, 265–270.

Nishiyama,K.-i., Suzuki,T. and Tokuda,H. (1996) Inversion of the membrane topology of SecG coupled with SecA-dependent preprotein translocation. *Cell*, **85**, 71–81.

Osborne,R.S. and Silhavy,T.J. (1993) PrlA suppressor mutations cluster in regions corresponding to three distinct topological domains. *EMBO J.*, **12**, 3391–3398.

Pogliano,J.A. and Beckwith,J. (1994a) SecD and SecF facilitate protein export in *Escherichia coli*. *EMBO J.*, **13**, 554–561.

Pogliano,K.J. and Beckwith,J. (1994b) Genetic and molecular characterization of the *Escherichia coli secD* operon and its products. *J. Bacteriol.*, **176**, 804–814.

Price,A., Economou,A., Duong,F. and Wickner,W. (1996) Separable ATPase and membrane insertion domains of the SecA subunit of preprotein translocase. *J. Biol. Chem.*, **272**, 31580–31584.

Sato,K., Mori,H., Yoshida,M., Tagaya,M. and Mizushima,S. (1997) Short hydrophobic segments in the mature domain of proOmpA determine its stepwise movement during translocation across the cytoplasmic membrane of *Escherichia coli*. *J. Biol. Chem.*, **272**, 5880–5886.

Schatz,P.J. and Beckwith,J. (1990) Genetic analysis of protein export in *Escherichia coli*. *Annu. Rev. Genet.*, **24**, 215–248.

Schiebel,E. and Wickner,W. (1992) Preprotein translocation creates a halide anion permeability in the *Escherichia coli* plasma membrane. *J. Biol. Chem.*, **267**, 7505–7510.

Schiebel,E., Driessen,A.J.M., Hartl,F.-U. and Wickner,W. (1991) $\Delta_{\mu H^+}$ and ATP function at different steps of the catalytic cycle of preprotein translocase. *Cell*, **64**, 927–939.

Shiozuka,K., Tani,K., Mizushima,S. and Tokuda,H. (1990) The proton motive force lowers the level of ATP required for the *in vitro* translocation of a secretory protein in *Escherichia coli*. *J. Biol. Chem.*, **265**, 18843–18847.

Tani,K., Shiozuka,K., Tokuda,H. and Mizushima,S. (1989) *In vitro* analysis of the process of translocation of OmpA across the *Escherichia coli* cytoplasmic membrane. *J. Biol. Chem.*, **264**, 18582–18588.

Uchida,K., Mori,H. and Mizushima,S. (1995) Stepwise movement of preproteins in the process of translocation across the cytoplasmic membrane of *Escherichia coli*. *J. Biol. Chem.*, **270**, 30862–30868.

Weiss,J.B., Ray,P.H. and Bassford,P.J.,Jr (1988) Purified SecB protein of *Escherichia coli* retards folding and promotes membrane translocation of the maltose-binding protein *in vitro*. *Proc. Natl Acad. Sci. USA*, **85**, 8978–8982.

Wickner,W. and Rice Leonard,M.R. (1996) *Escherichia coli* preprotein translocase. *J. Biol. Chem.*, **271**, 29514–29516.

Yamada,H., Matsuyama,S.-i., Tokuda,H. and Mizushima,S. (1989) A high concentration of SecA allows proton motive force-independent translocation of a model secretory protein into *Escherichia coli* membrane vesicles. *J. Biol. Chem.*, **264**, 18577–18581.

Received on April 24, 1997; revised on May 27, 1997