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Distinct catalytic roles of the SecYE, SecG and SecDFyajC subunits of preprotein translocase holoenzyme

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Escherichia coli **preprotein translocase contains a membrane-embedded trimeric complex of SecY, SecE and SecG (SecYEG) and the peripheral SecA protein. SecYE is the conserved functional 'core' of the SecYEG complex. Although sufficient to provide sites for high-affinity binding and membrane insertion of SecA, and for its activation as a preprotein-dependent ATPase, SecYE has only very low capacity to support translocation. The proteins encoded by the *secD* operon—SecD, SecF and YajC—also form an integral membrane heterotrimeric complex (SecDFyajC). Physical and functional studies show that these two trimeric complexes are associated to form SecYEGDFyajC, the hexameric integral membrane domain of the preprotein translocase 'holoenzyme'. Either SecG or SecDFyajC can support the translocation activity of SecYE by facilitating the ATP-driven cycle of SecA membrane insertion and de-insertion at different stages of the translocation reaction. Our findings show that each of the prokaryote-specific subunits (SecA, SecG and SecDFyajC) function together to promote preprotein movement at the SecYE core of the translocase.**

Keywords: membrane proteins/preprotein translocase/Sec proteins

Introduction

Genetic and biochemical studies have led to the identification of seven *sec* genes, *secA, B, D, E, F, G* and *Y,* which are involved in the export of proteins across the *Escherichia coli* inner membrane (Schatz and Beckwith, 1990; Wickner *et al.*, 1991; Ito, 1996). Three of the *sec*-encoded proteins, SecA, SecY and SecE, are essential for cell viability and for preprotein translocation, both *in vivo* (Oliver and Beckwith, 1982; Murphy *et al.*, 1995) and in proteoliposomes reconstituted from purified components (Brundage *et al.*, 1990; Akimaru *et al.*, 1991). Strikingly, membrane-embedded proteins homologous to SecY and SecE have been identified in many bacterial species, in chloroplasts and in yeast and mammalian endoplasmic reticulum (ER) (Hartmann *et al.*, 1994; Rensing and Maier, 1994). SecY and SecE, as well as their mammalian and yeast counterparts, Sec61α/Sec61γ and Sec61p/Sss1p respectively, have been isolated as a complex (Brundage *et al.*, 1992; Hartmann *et al.*, 1993; Panzner *et al.*, 1995). In light of such conservation, it is possible that the SecYE complex forms the 'core' of the protein-conducting pathway. Indeed, the multi-spanning SecY subunit is in proximity to the polypeptide chain as it moves across the membrane (Joly and Wickner, 1993).

A third membrane component of the translocase, SecG, was identified as a subunit of a trimeric complex with SecYE (Brundage *et al.*, 1990; Nishiyama *et al.*, 1994). Although dispensable for cell viability at 37°C, SecG dramatically increases the efficiency of preprotein translocation, both *in vivo* and *in vitro* (Hanada *et al.*, 1994; Nishiyama *et al.*, 1994). Though they share no sequence homology, SecG is of similar size and hydrophobicity to Sec61β and Sbh1p, the third membrane component of the mammalian and yeast protein translocation machineries, respectively (Hartmann *et al.*, 1994). The SecYEG heterotrimeric complex forms a membrane-embedded portion of the *E.coli* preprotein translocase (Brundage *et al.*, 1990).

The other subunit of preprotein translocase, SecA, is an ATPase that exists in cytosolic, peripherally membrane-bound and membrane-inserted forms. SecA binds to the leader and mature domains of the preprotein, to acidic phospholipids and to SecYEG (Hartl *et al.*, 1990; Lill *et al.*, 1990). These associations activate the ATPase activity of SecA, which converts the energy of ATP binding and hydrolysis to the translocation of the polypeptide chain across the membrane (Lill *et al.*, 1989; Schiebel *et al.*, 1991). During translocation, SecA undergoes repeated cycles of ATP-driven membrane insertion and de-insertion (Economou and Wickner, 1994). SecA also possesses a binding site for the molecular chaperone SecB, which maintains the preprotein in a translocation-competent conformation (Randall and Hardy, 1995). Recently, SecG was shown to undergo a cycle of topological inversions which may be coupled to and stimulate the SecA membrane insertion cycle (Nishiyama *et al.*, 1996).

The functions of the membrane components encoded by the *secD* operon, comprised of *secD*, *secF* and the *yajC* gene (which encodes an unknown protein; Gardel *et al.*, 1990), are less clear. Protein export in strains lacking SecD and/or SecF is reduced but not abolished, while overexpression of SecD and SecF improves the export of proteins with defective leader peptides (Pogliano and Beckwith, 1994a). Addition of anti-SecD antibodies to spheroplasts leads to the partial accumulation of an advanced preprotein translocation intermediate (Matsuyama *et al.*, 1993), whereas cells depleted for SecDF are non-viable at low temperatures (Pogliano and Beckwith, 1994a).

Despite extensive biochemical characterization of the *in vitro* translocation reaction, direct involvement of the SecDF proteins has yet to be shown. Inner membrane vesicles (IMVs) depleted of SecDF cannot maintain a stable proton-motive force (PMF) (Arkowitz and Wickner, 1994) and thus do not support PMF-stimulated translocation at subsaturating ATP concentrations. However, it is

unclear whether SecDF act directly in coupling the PMF to the translocation reaction. Though SecDF are not essential for *in vitro* ATP-driven translocation (Matsuyama *et al.*, 1992; Arkowitz and Wickner, 1994), recent studies suggest that they regulate SecA membrane cycling. Overexpression of a 10 kb chromosomal region containing the *secDF* locus leads to enhanced SecA membrane association and insertion (Kim *et al.*, 1994), while SecDF depletion results in a lack of stably membrane-inserted SecA (Economou *et al.*, 1995). However, the effect of SecDF depletion on the rate of preprotein translocation was only modest. Studies of the stabilization of SecY by SecDF and suppressor-directed inactivation experiments suggested that SecDF are components of the translocation complex (Bieker-Brady and Silhavy, 1992; Sagara *et al.*, 1994), though purification and co-immunoprecipitation studies revealed the presence of only SecYEG subunits (Brundage *et al.*, 1990; Akimaru *et al.*, 1991).

We now report the identification of a holoenzyme form of preprotein translocase with an integral membrane domain composed of SecY, SecE, SecG, SecD, SecF and YajC subunits. Functional studies of membranes in which these subunits are systematically depleted or overproduced reveal their distinct functions. The SecYE complex is sufficient to activate SecA as a preprotein-dependent ATPase and to provide sites for SecA binding and insertion. However, efficient preprotein translocation and SecA membrane cycling also require the functions of either SecG or the SecDFyajC complex. Analysis of the SecA insertion/de-insertion cycle shows that SecDFyajC facilitates and stabilizes SecA insertion while SecG stimulates SecA insertion after initiation of the translocation. These studies reveal physical and functional integration of the proteins of the *secD* operon into the preprotein translocase holoenzyme.

Results

Identification of a SecDF YajC complex

SecD and SecF are in low abundance in *E.coli* (<30 copies per cell; Matsuyama *et al.*, 1992; Pogliano and Beckwith, 1994a). Hence, as a first step toward the identification of a possible multi-subunit Sec complex, the genes for the integral membrane proteins SecY, SecE and SecG, as well as the *secD* operon-encoded proteins YajC, SecD and SecF, were cloned into an expression-controlled system. SecE [tagged with an influenza hemagglutinin (HA) epitope], SecY and SecG were synthesized from plasmid pHA-EYG (referred to as pEYG hereafter), a pBR322-derived plasmid carrying the *ara* promoter (pBAD22; Douville *et al.*, 1995). The *secD* operon was expressed from pCDF, a pACYC-derived plasmid also controlled by the *ara* promoter (pBAD33; Guzman *et al.* 1995). These plasmids, pEYG and pCDF, were transformed into the *E.coli* OmpT-deficient strain BL21. After induction with arabinose, the cells were radiolabeled with [^{35}S]methionine and the membranes were purified. Despite their overproduction, the Sec proteins remained minor components of the total membrane fraction under these induction conditions (Figure 1A). Since the membrane preparation did not separate the inner and outer membrane, the most abundant protein was the outer membrane protein A, OmpA, which appears in both heat-modified and unmodified forms (Schnaitman, 1974), as shown by immunoprecipitation with anti-OmpA (α-OmpA) antibodies (Figure 1A). The specificity of the affinity-purified antibodies used in this study was confirmed by immunoprecipitation using SDS-solubilized membranes (Figure 1A).

To explore the associations among the Sec proteins, membranes from cells carrying plasmids pEYG or pEYG/pCDF were solubilized with *n*-octyl-β-D-glucoside (β-octyl glucoside) and their proteins immunoprecipitated (Figure 1B) with anti-HA (α-HA; lanes 1 and 2) or anti-SecG (α-SecG; lane 3 and 4) antibodies. A complex of three major polypeptides corresponding to SecY, HA-SecE and SecG was recovered, as previously reported (Brundage *et al.*, 1992). A band corresponding to non-tagged SecE encoded by the chromosome, running immediately below the position of SecG, was also captured in immunoprecipitation reactions using α-SecG antibodies (or α-SecY, data not shown) but not with α-HA antibodies, suggesting that only one copy of SecE is present in a given SecYEG complex (Joly *et al.*, 1994). Neither SecD nor SecF were found in these immunoprecipitates. However, when the immunoprecipitation reactions were performed using anti-SecF antibodies (α-SecF; lanes 5 and 6), two major polypeptides corresponding to SecF (30 kDa) and SecD (62 kDa) were clearly immunoprecipitated from the cells carrying pCDF (lane 6), whereas none of the SecYEG proteins were captured. Therefore, after solubilization in β-octyl glucoside, SecD and SecF remain associated as a complex, distinct from SecYEG.

Closer examination of the lower portion of the autoradiogram (Figure 1B) reveals the presence of a low molecular weight protein which co-immunoprecipitated with α-SecG, α-HA or α-SecF antibodies, but not with the SecG pre-immune sera (lane 7). Since this band is most prominent in immunoprecipitation reactions using membranes prepared from cells carrying pCDF, it may correspond to YajC, an 8 kDa, single membrane-spanning protein encoded within the *secD* operon (Gardel *et al.*, 1990; Pogliano and Beckwith, 1994b). Indeed, when the co-immunoprecipitation studies were performed with the plasmid pDF (plasmid pCDF deleted for *yajC*; lanes 9 and 11), this low molecular weight protein was no longer detected. It is noteworthy that YajC was found associated with both SecYEG (lane 8) and SecDF (lane 10) under these solubilization conditions.

Identification of a larger complex of SecYEGDF and YajC

Conditions previously used to solubilize *E.coli* membranes for immunoprecipitation studies may not have preserved the full integrity of the membrane-embedded domain of translocase. Digitonin, a detergent commonly employed for membrane solubilization (Helenius and Simons, 1975), has been used successfully in studies of the Sec system of the ER as well as the import machinery of mitochondria (Berthold *et al.*, 1995; Panzner *et al.*, 1995). Immunoprecipitation reactions were therefore performed using radiolabeled *E.coli* total membranes and a non-denaturing buffer containing digitonin instead of β-octyl glucoside. Whereas solubilization in β-octyl glucoside allowed immunoprecipitation of Sec HA-EYG using either α-HA or α-SecG antibodies, similar immunoprecipitation in the

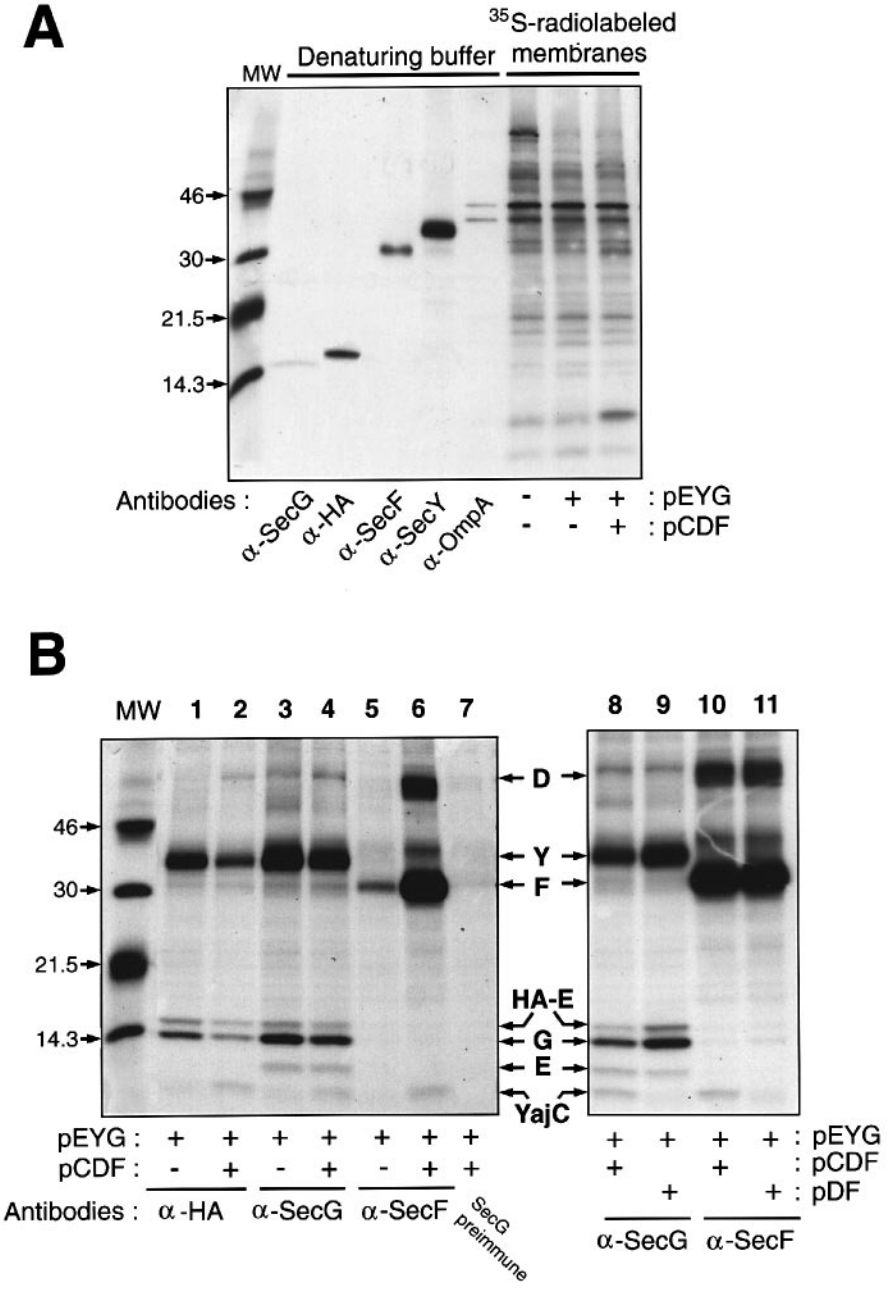

Fig. 1. SecYEGyajC and SecDFyajC form separate complexes in β-octyl glucoside. (**A**) Detection of the individual Sec proteins. [^{35}S]methionine-labeled membranes (5×10^5 c.p.m.) from *E.coli* BL21 transformed with pHA-EYG (referred to as pEYG) and pCDF were solubilized in denaturing buffer (RIPA buffer) and incubated with the indicated affinity-purified antibodies pre-bound to protein A–Sepharose beads, as described in Materials and methods. Immunoprecipitates were analyzed by SDS–PAGE and fluorography. To monitor membrane composition, radiolabeled membranes (6000 c.p.m.) prepared from *E.coli* BL21 or BL21 transformed with the indicated plasmids were dissolved in SDS sample buffer and analyzed on the same gel. Lane MW contains the ^{14}C-labeled molecular weight markers (kDa). (**B**) Immunoprecipitation of the SecYEGyajC and SecDFyajC complexes. [^{35}S]Methionine-labeled membranes (2×10^6 c.p.m.) from *E.coli* BL21 overexpressing Sec HA-EYG (plasmid pEYG), Sec HA-EYG and SecDFyajC (pEYG and pCDF), or Sec HA-EYGDF without YajC (pEYG/pDF), were solubilized with the non-denaturing NDIP buffer containing 1.25% β-octyl glucoside, as described in Materials and methods. Extracts were incubated with the indicated affinity-purified antibodies pre-bound to protein A–Sepharose beads. The beads were suspended three times with the same buffer and the immunoprecipitates were analyzed by SDS–PAGE and fluorography. The various Sec proteins are labeled.

presence of digitonin revealed a much larger complex consisting of Sec HA-EYG, SecD, SecF and YajC (Figure 2, lanes 2 and 4). The various proteins were identified by using membranes prepared from cells carrying pEYG alone (lane 3), pEYG and pCDF (lanes 2 and 4), or pEYG and pDF (lane 5). In addition to the Sec HA-EYGDFyajC complex, a high molecular weight protein of unknown identity as well as a protein of ~25 kDa were detected. The specificity of the immunoprecipitations was confirmed using α-OmpA antibodies, α-SecG pre-immune sera or protein A–Sepharose beads only (lanes 1, 6 and 9, respectively). When the experiment was performed using α-SecF antibodies (lanes 7 and 8), very little Sec HA-EYG co-immunoprecipitated with the SecDFyajC complex. This may reflect some instability of the larger complex when immunoprecipitated with α-SecF anti-

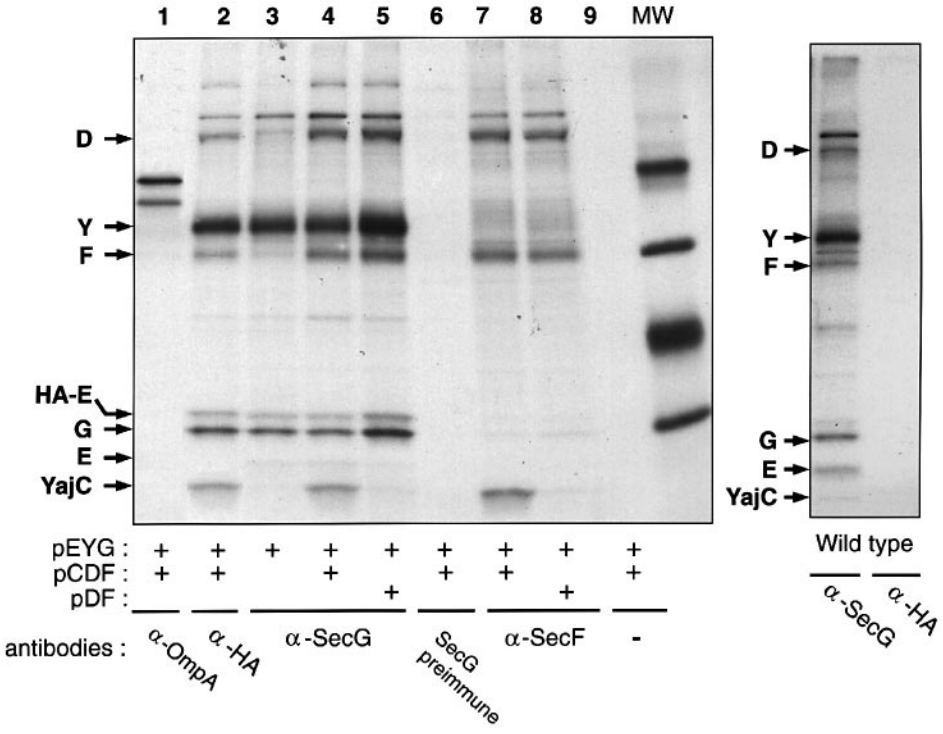

Fig. 2. SecYEGDF and YajC form a stable complex in digitonin. Immunoprecipitation of the Sec HA-EYGDFyajC complex from *E.coli* BL21 overproducing the indicated Sec proteins (left panel) or *E.coli* wild-type BL21 (right panel). Approximately 5×10^6 c.p.m. of [^{35}S]methionine-labeled membranes from *E.coli* BL21 transformed (where indicated) with the plasmids pEYG, pEYG/pDF or pEYG/pCDF were solubilized with the non-denaturing NDIP buffer containing 1% digitonin, as described in Materials and methods. For the detection of the SecYEGDFyajC complex in wild-type *E.coli*, 10^7 c.p.m. of radiolabeled membranes were used. Extracts were incubated with the indicated antibodies or with underivatized protein A–Sepharose beads (–). Immunoprecipitates were treated as described in Figure 1.

bodies. Alternatively, some epitopes recognized by α-SecF antibodies may be masked by the association of SecDFyajC with the SecYEG complex, thus allowing immunoprecipitation of only uncomplexed SecDFyajC molecules.

To determine whether the SecYEGDFyajC complex was an artifact of the overproduction of these proteins, experiments were also conducted using wild-type *E.coli* BL21. As above, the anti-SecG antibodies co-immunoprecipited the SecYEGDFyajC proteins from the wild-type BL21 membranes (Figure 2, right panel). The Sec proteins were, however, immunoprecipitated in different proportions. SecY, SecD and SecF each possess ~17 methionyl residues which can be labeled, yet the intensity of the immunoprecipitated SecD and SecF was much weaker than that of SecY. While SecE and SecG (containing four and five methionyl residues, respectively) were present in approximately equal amounts, YajC (six methionyl residues) was less abundant in the immunoprecipitate. These differences are in agreement with previous estimates of the number of Sec protein molecules per cell (Pogliano and Beckwith, 1994b), and may indicate that in wild-type *E.coli* some SecYEG complex exists alone, without YajC and SecDF proteins.

SecG is not essential for the integrity of the complex

SecG is not essential for either cell viability or translocation at 37°C. Deletion of *secG* does, however, dramatically reduce preprotein translocation *in vivo* at 20°C or *in vitro* at 37°C (Nishiyama *et al.*, 1994). The possibility was examined that SecG affects translocation through a modification of translocase stability, composition or stoichiometry. Co-immunoprecipitation studies were performed with *E.coli* BL21 and BL425, a derivative of BL21 in which chromosomal *secG* has been inactivated (Δ*secG::kan*). Each strain was transformed with pHA-EY (a derivative of pHA-EYG deleted for *secG*) and pCDF. When the membrane proteins were solubilized with β-octyl glucoside (Figure 3, left panel) and subjected to immunoprecipitation with either α-HA (lanes 1 and 2) or α-SecF antibodies (lanes 3 and 4), the separate Sec HA-EYyajC and SecDFyajC complexes were recovered whether SecG was present or not. When the membranes were solubilized with digitonin (Figure 3, right panel), the Sec HA-EYD-FyajC complex was obtained using α-HA antibodies (lanes 5 and 6), regardless of the presence or absence of SecG. Thus, SecG is not essential for SecYEDFyajC complex formation. However, we noted that α-SecF antibodies (lanes 7 and 8) consistently immunoprecipitated more Sec HA-EY proteins when SecG was present at a low amount or absent (compare Figure 3, lanes 7 or 8 with Figure 2, lane 7). Thus, the absence of SecG may increase the stability of the SecYEDFyajC complex. Alternatively, epitopes recognized by α-SecF antibodies may be partially unmasked by the absence of SecG.

Functional studies: SecYE binds and activates SecA

In vitro systems have helped to dissect the translocation process into distinct subreactions (Wickner *et al.*, 1991). In the present study, the contributions of the subunits of the membrane-embedded translocase domain were

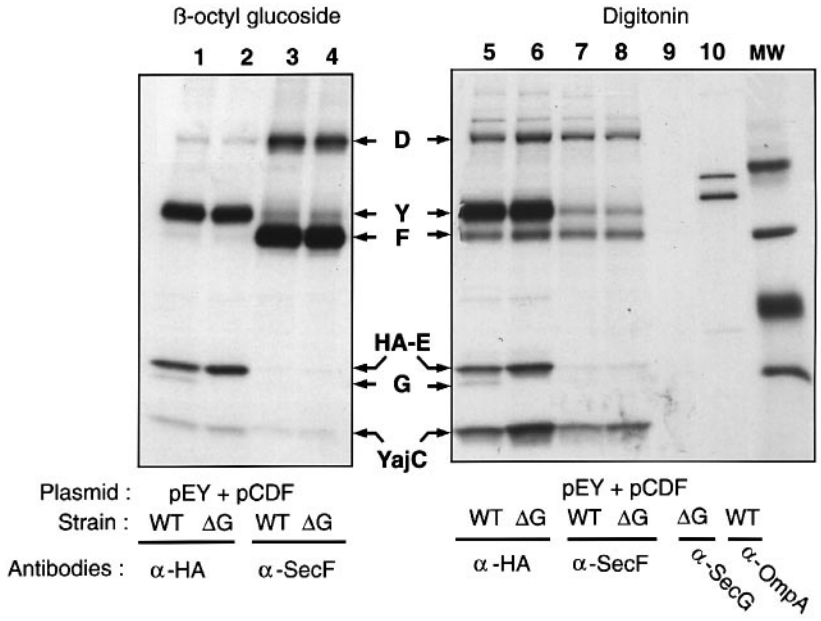

Fig. 3. Detection of the Sec HA-EYDFyajC complex in the absence of SecG. Radiolabeled membranes (5×10^6 c.p.m.) from BL21 ('WT') and BL425 (BL21 Δ*G::kan*; 'ΔG'), each transformed with the plasmids pHA-EY and pCDF, were solubilized with NDIP buffer containing either 1.25% β-octyl-glucoside (left panel) or 1% digitonin (right panel) and incubated with the indicated antibodies. Immunoprecipitates were treated as described in Figure 1.

examined. IMVs containing different relative amounts of SecYE, SecG and SecDFyajC were prepared from three *E.coli* background strains: BL21 (hereafter referred to as 'WT'), BL21 Δ*secG::kan* ('ΔG') and BL325 ('DF$^-$'). BL325 is a derivative of BL21 in which the chromosomal *secD* operon is regulated by the *ara* promoter, allowing depletion of SecDF and YajC (BL21 *tgt::kan-araC*-P_{BAD}-*yajCsecDF*; Pogliano and Beckwith, 1994a). Some of these strains were transformed further with arabinose-inducible plasmids expressing either SecG ('pG'), Sec HA-EY ('pEY'), Sec HA-EYG ('pEYG') or SecDFyajC ('pCDF'). The content of Sec proteins of each membrane preparation was examined by immunostaining (Figure 4A; plasmid-borne gene expression is denoted by +++, wild-type gene expression by +, and deletion or depletion by –). Induction of the various plasmids led to 15- to 20-fold overexpression of the corresponding Sec proteins. In agreement with previous observations, SecDF or SecG depletion/overproduction does not detectably affect expression levels or stability of HA-SecE and SecY (Arkowitz and Wickner, 1994), of leader peptidase (not shown; Economou *et al.*, 1995) or of SecE synthesized from the chromosomal gene (Figure 4A).

The various IMVs were first characterized for their abilities to bind SecA. SecA binding to membranes has been described as consisting of a low-affinity, non-saturable binding to lipid and a high-affinity, saturable binding at SecYEG (Hartl *et al.*, 1990; Douville *et al.*, 1995). These earlier studies did not, however, measure the individual contributions of the different Sec proteins to high-affinity SecA binding. Furthermore, measurement of SecA binding using proteoliposomes with purified SecYEG protein are difficult because of the high ratio of phospholipids to SecYEG necessary for efficient reconstitution (Hanada *et al.*, 1994). In the present study, SecA binding parameters were examined using membranes genetically enriched in different Sec proteins (Figure 4B). Scatchard analysis (Scatchard, 1949) revealed that the number of SecA high-affinity binding sites reflected the level of SecYE molecules present in a given strain (i.e. ≈1300 pmol of SecA specifically bound/mg of IMV protein from 'pEY' versus ≈200 pmol from 'WT'). Neither SecG nor SecDFyajC, separately or in combination, significantly affected the number of SecA-binding sites. While IMVs prepared from 'pEYG' or 'WT' displayed equal SecA-binding affinities (≈140 nM), a reproducible 30% reduction in SecA affinity was observed for IMVs prepared from 'pEY' (≈180 nM), indicating some contribution of SecG to the binding affinity of SecA at SecYE. Overproduction of SecDFyajC did not modify this binding parameter.

The various IMVs were also characterized for their abilities to activate SecA as a preprotein-dependent ATPase (Lill *et al.*, 1989). In the presence of the preprotein proOmpA and ATP, the enhancement of SecA translocation ATPase activity reflected the increase of high-affinity SecA binding to SecYE (Figure 4C, 'pEY'). SecG only caused a modest increase in the translocation ATPase activity (compare 'ΔG pEY' versus 'WT pEYG'), whereas no effects of SecDFyajC were observed. These data show that the overproduced SecYE proteins are sufficient to bind SecA and support its translocation ATPase activity. As compared with the wild-type strain, neither SecDFyajC overexpression nor SecG deletion detectably modified this activity (Figure 4C). We conclude that SecA high-affinity binding and SecA translocation ATPase activity are determined mainly by the SecYE content of the membrane.

SecG or SecDF stimulates the translocation activity of SecYE

Though hydrolysis of ATP accompanies preprotein translocation, a high level of ATP hydrolysis does not necessarily reflect an efficient translocation process (Kawasaki *et al.*, 1993). To examine directly the contribution of the

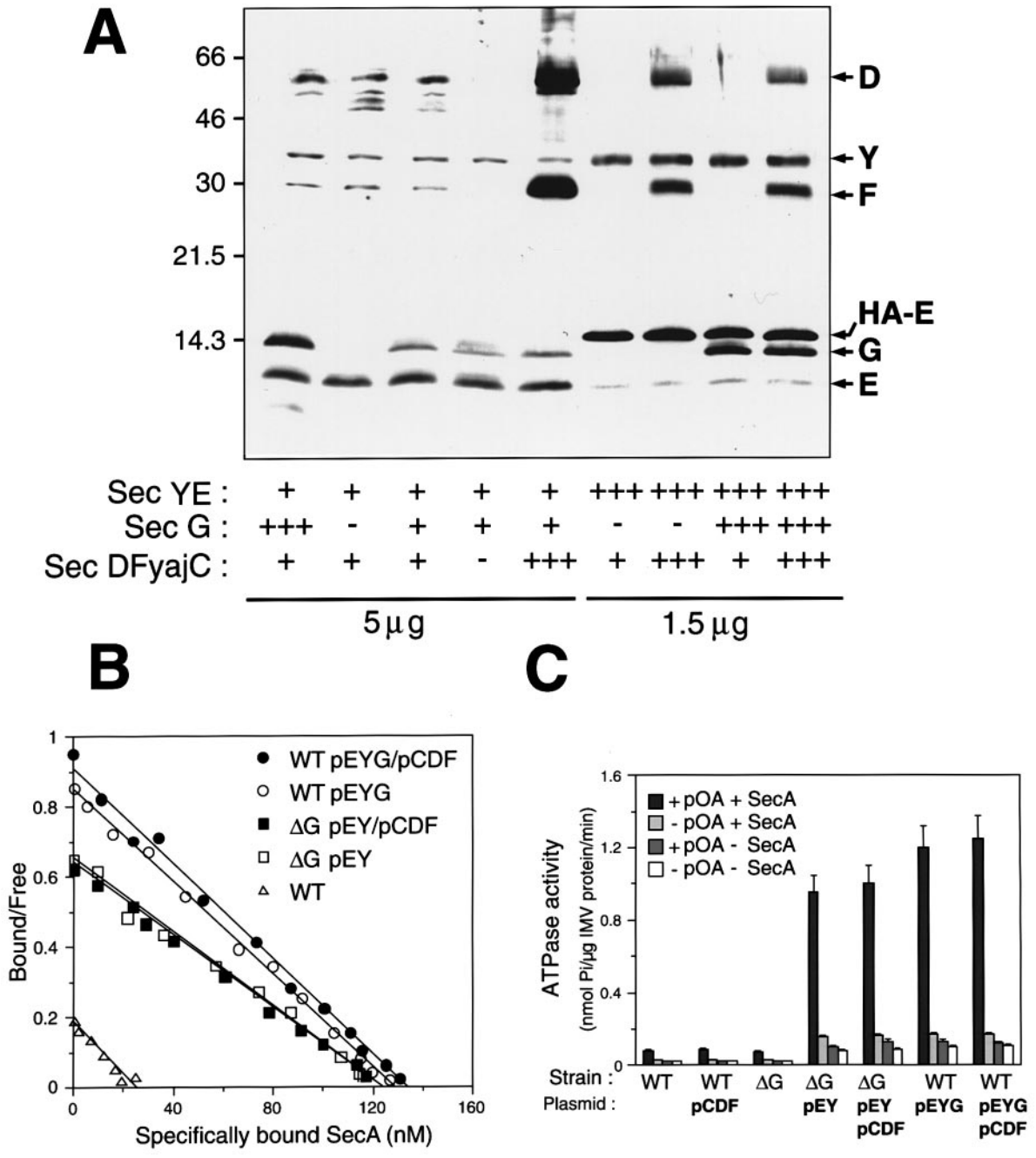

Fig. 4. SecYE determines SecA high-affinity binding and translocation ATPase activity. (**A**) Immunostaining of IMV preparations from cells with various levels of SecHA-EY, SecG and SecDFyajC. IMVs proteins were analyzed by SDS–PAGE, transferred to nitrocellulose and immunostained with a mixture of antibodies to HA, SecE, SecG, SecY, SecF and SecD. The positions of molecular weight markers (in kDa) and the quantity of IMV protein loaded onto the gel are indicated. (+++) indicates overexpression, (+) wild-type level, (–) deletion for SecG or depletion for SecDFyajC. (**B**) Scatchard analysis of SecA binding to urea-treated IMVs was performed as described in Materials and methods. Data from binding assays were analyzed by the LIGAND modeling program (Munson and Rodbard, 1980), as described by Hartl *et al.* (1990). Binding reactions contained 0.1 mg/ml IMVs proteins. The LIGAND program calculated ≈130 nM of high-affinity bound SecA for SecEY-enriched IMVs. Thus, 130 nmol/l = 130 pmol/ml×1 ml/0.1 mg IMV = 1300 pmol SecA specifically bound per mg of IMV proteins. (**C**) Translocation ATPase activity was measured as described by Lill *et al.* (1990) as modified by Douville *et al.* (1995). Urea-treated IMVs (100 µg/ml) were incubated at 37°C in 50 µl of TL buffer with BSA (200 µg/ml), SecA (40 µg/ml) and proOmpA (60 µg/ml) for either 2.5 min (IMVs enriched in SecYE) or 15 min (IMVs with wild-type level of SecYE). In control incubations, SecA buffer (TL buffer containing 10% glycerol) or urea buffer (6 M urea, 1 mM DTT, 50 mM Tris–HCl, pH 7.9) were used in place of SecA and proOmpA, respectively. The release of Pi was measured in 5 µl aliquots, as described by Lill *et al.* (1990).

various Sec proteins in preprotein translocation, reactions were performed using ^{35}S-labeled proOmpA, SecB, SecA, ATP and the IMVs described above (Figure 5A). To exclude the contribution of the PMF to preprotein translocation, IMVs were made proton permeable by removal of the F_1 subunit of the F_1F_0-ATPase (Cunningham *et al.*, 1989). In agreement with previous studies, SecDFyajC depletion (lane 2) or overproduction (lane 3) has little effect on *in vitro* ATP-driven translocation, as compared with 'WT' (lane 1) (Arkowitz and Wickner, 1994;

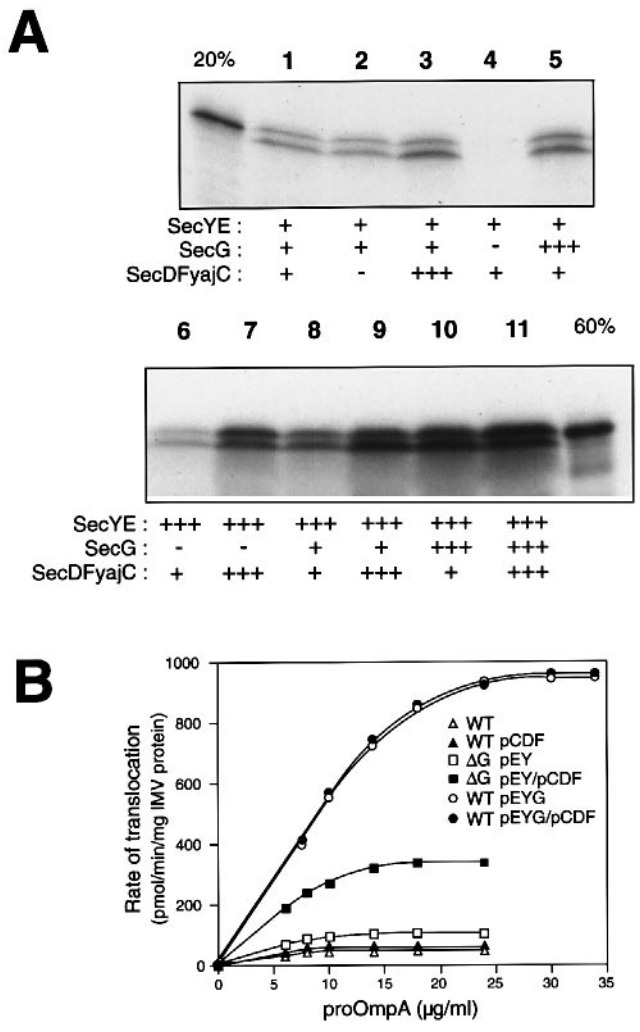

Fig. 5. Either SecG or SecDF can stimulate the translocation activity of SecYE. (**A**) Translocation reactions were performed in 100 μl of TL buffer containing SecA (40 μg/ml), SecB (48 μg/ml), BSA (200 μg/ml), an ATP-regenerating system (5 mM creatine phosphate, 10 μg/ml creatine kinase), [^{35}S]proOmpA (120 000 c.p.m.) pre-mixed with unlabeled proOmpA (5 μg/ml), and urea-stripped IMVs (100 μg/ml). The levels of the Sec proteins in the various IMVs are indicated as in Figure 4A. After pre-warming (2 min, 37°C), translocation reactions were initiated with 2 mM ATP and stopped after 10 min by chilling on ice. Samples were digested with proteinase K (1 mg/ml, 15 min, 0°C) and treated with 15% ice-cold trichloroacetic acid (TCA). Translocated [^{35}S]proOmpA was analyzed by SDS–PAGE and fluorography. Standards of 20 and 60% of [^{35}S]proOmpA added to the reaction are indicated. (**B**) The effect of SecDFyajC on translocation was analyzed in three different backgrounds: wild-type (WT), Δ*secG* pEY or WT pEYG. Initial rates of proOmpA translocation in these IMVs were determined as described above, except that [^{35}S]proOmpA (120 000 c.p.m.) was pre-mixed with unlabeled proOmpA (6–34 μg/ml). Reactions were incubated at 37°C for 5 min with 2 mM ATP and treated as described above. Translocated [^{35}S]proOmpA was visualized by fluorography and analyzed by scanning densitometry. The data were quantified by comparison with a [^{35}S]proOmpA standard curve.

Economou *et al.*, 1995). While SecG deletion strongly depresses translocation (lane 4) (Nishiyama *et al.*, 1994), the overexpression of SecG alone does not stimulate translocation (lane 5) unless SecYE is simultaneously overexpressed (lane 10). Moreover, SecYE overproduction is able to compensate for SecG deletion and result in a translocation level comparable with that of 'WT' (lane 6). These findings indicate that SecYE possesses a weak translocation activity which is strongly stimulated by SecG. Since overexpression of SecDFyajC has little effect on translocation in either 'WT' background (lane 1 versus 3) or when co-expressed with SecYEG (lane 10 versus 11), we examined whether SecG masks an effect of SecDFyajC on SecYE-based translocation. Indeed, when translocation reactions were performed with membranes containing either reduced levels or no SecG protein, SecDFyajC was found to stimulate the translocation activity of SecYE (compare lane 6 versus 7 and lane 8 versus 9). This stimulation was also seen when plasmid pDF, carrying SecDF without YajC, was used (not shown).

To examine more closely the influence of SecG and SecDF on SecYE activity, the initial rates of translocation were assayed as a function of preprotein concentration (Figure 5B). SecDFyajC and SecG increased the rate of translocation of SecYE by factors of 4 and 12, respectively. Comparisons of the 'ΔG' strain versus 'ΔG pCDF' also gave a 4-fold increase in the rate of translocation (not shown). However, the SecG and SecDF stimulatory effects were not additive, since the SecDF-mediated stimulation of translocation was no longer observed when SecG was present in stoichiometric amounts with SecYE (Figure 5B). It remains to be determined whether SecG and SecDFyajC possess interchangeable stimulatory functions or stimulate different steps during the translocation cycle.

*Overexpression of SecYE suppresses both Δ*secG* and SecDFyajC⁻ growth defects*

Certain mutations that reduce the rate of protein export render cell growth sensitive to low temperatures (Pogliano and Beckwith, 1993). Thus, we tested whether the growth defect of Δ*secG* and *yajCsecDF*$^{-}$ strains at low temperatures (Nishiyama *et al.*, 1994; Pogliano and Beckwith, 1994a) can be suppressed by overexpression of other Sec proteins. While BL325 (*tgt::kan-araC*-P_{BAD}-*yajCsecDF*) was unable to grow on rich media even at 37°C in the absence of arabinose (Pogliano and Beckwith, 1994a), the cold-sensitive phenotype of Δ*secG* null mutants was reported to be strain dependent (Bost and Belin, 1995). The strain used in this study, BL425 (BL21 Δ*secG::kan*), did not manifest any growth defect at 20°C unless the *unc* genes coding for the F_1F_0-ATPase were also deleted. The combined actions of the *secG* and *unc* deletions on the severity of the growth defect may be related to the observation that SecG is also critical for *in vitro* translocation in the absence of PMF (Hanada *et al.*, 1996). BL325 (SecDFyajC^{-}) and BL525 (BL21 Δ*secG::kan*, *unc::*Tn*10*) were transformed with plasmids overexpressing SecYE, SecG or SecDFyajC and their growths were examined at low temperature. In both cases, the cold-sensitive phenotype of the strains was suppressed when SecYE was overexpressed (Figure 6A and B). Interestingly, SecG overexpression did not complement the growth defect of BL325 (SecDFyajC^{-}), nor did SecDFyajC overexpression complement the growth of BL525 (Δ*secG::kan*, *unc::*Tn*10*) (Figure 6A and B). Thus, when increased in number, the weak translocation activity of SecYE is able to compensate for the absence of SecG in *in vitro* translocation reactions (Figure 5) and can also complement the *in vivo* growth defects of SecG-deleted or SecDFyajC-depleted mutants. However, the stimulatory activities of SecDFyajC or SecG do not compensate for the deficiency

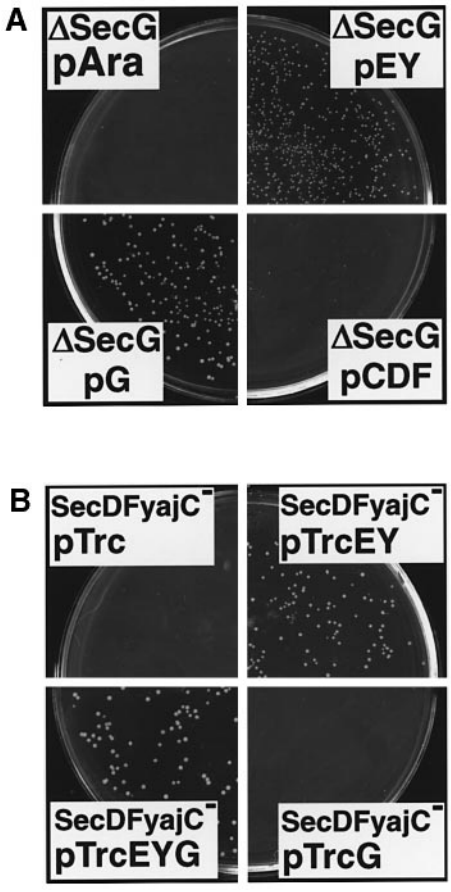

Fig. 6. Complementation of the Δ*secG* and SecDFyajC^{-} cold-sensitive phenotypes. (**A**) *E.coli* BL525 (BL21 Δ*secG::kan*, *unc::*Tn*10*) was transformed with either pG/pBAD33, pEY/pBAD33, pCDF/pBAD22 or pBAD22/pBAD33 (indicated as pAra in the figure) and recovered at 37°C with antibiotics. After growth in liquid media at 37°C, a dilution was plated onto LB/ampicillin/chloramphenicol (50 μg/ml) containing arabinose (0.5%) and incubated at 20°C for 3 days. No complementation was observed when arabinose was omitted (not shown). Only one quarter of each plate is shown. (**B**) *E.coli* BL325 (BL21 *tgt::kan-araC*$^{+}$ -P_{BAD}*::yajCsecDF*) was transformed with the IPTG-inducible plasmid pTrc99A, alone or carrying Sec HA-EYG (pTrcEYG), Sec HA-EY (pTrcEY) or SecG (pTrcG). Transformants were recovered at 37°C on LB plates containing 0.5% arabinose and antibiotics. After growth in liquid media at 37°C with 0.2% arabinose, a dilution was plated on LB/ampicillin (50 μg/ml) containing 1 mM IPTG and incubated for 2 days at 30°C. In each case, growth occurred when the LB plates were supplemented with 0.5% arabinose (not shown).

of each other *in vivo* and may, therefore, affect distinct processes.

Distinct effects of SecG and SecDFyajC on the SecA membrane insertion cycle

In the presence of preprotein and ATP, SecA undergoes cycles of membrane insertion and de-insertion (Economou and Wickner, 1994). Membrane-inserted SecA is partly inaccessible to added protease, yielding a C-terminal 30 kDa-protected fragment upon digestion (Price *et al.*, 1996). Addition of a non-hydrolyzable ATP analog, adenyl-imidodiphosphate (AMP-PNP), instead of ATP at the beginning of the translocation reaction promotes SecA insertion and leader peptide cleavage, while addition of AMP-PNP during translocation blocks both the de-insertion of SecA and preprotein translocation (Schiebel *et al.*, 1991; Economou *et al.*, 1995). These data suggest that nucleotide binding promotes SecA insertion, while de-insertion requires ATP hydrolysis (Economou *et al.*, 1995). ATP-driven SecA insertion/de-insertion reactions were studied with the various IMV preparations (Figure 7A, top panels). SecDFyajC-depleted membranes showed a clear reduction in the steady-state level of inserted SecA, assayed by a SecA 30 kDa fragment obtained upon proteolysis, as previously reported (Economou *et al.*, 1995) (compare lane 1 versus 2, Figure 7A, top panel). In contrast, the steady-state level of inserted SecA increased upon overexpression of SecDFyajC (lane 3), presumably reflecting that these proteins are present in substoichiometric amounts in wild-type cells (Pogliano and Beckwith, 1994b). SecG appears also to be an important factor for SecA insertion since 'ΔG' IMVs showed a reduction in the amount of SecA 30 kDa fragment obtained upon proteolysis (lane 4). The same effects of SecDFyajC overproduction (lanes 7 versus 8, and 9 versus 10) or SecG deletion (lanes 7 versus 9, and 8 versus 10) on SecA insertion were also seen when IMVs enriched in SecEY were tested. In these reactions, a higher SecA concentration with lower [^{125}I]SecA specific activity was used in order to saturate the additional SecYE insertion sites (see below).

Since ATP binding and hydrolysis catalyze both SecA insertion and de-insertion, the level of SecA 30 kDa fragment formed upon proteolysis reflects the steady-state between the inserted and de-inserted forms of SecA. To analyze the contributions of the various Sec proteins to the first part of the SecA cycle, i.e. SecA insertion, reactions were performed using AMP-PNP, thereby preventing SecA de-insertion (Figure 7A, middle panels). When SecG is present at a level below that of SecYE, SecA insertion is slightly impaired (compare lanes 1 versus 4, and 7 versus 9). In agreement with previous findings (Economou *et al.*, 1995), SecDFyajC depletion has no effects on SecA insertion *per se* (lane 2), indicating that SecDFyajC is not essential for the SecA insertion reaction. When overexpressed, however, SecDFyajC systematically increased SecA insertion (compares lanes 1 and 3, 4 and 5, 7 and 8, and 9 and 10). These results indicate that SecDFyajC not only inhibits the ATP-driven de-insertion, as reported (Economou *et al.*, 1995), but also stimulates the SecA insertion step.

Quantitative analysis of the amount of SecA 30 kDa fragment formed with the various IMVs, using AMP-PNP and increasing concentrations of SecA, showed that the concentration of SecA needed to half saturate the insertion sites was not affected by SecDFyajC or SecG but was only dependent upon SecYE levels (Figure 7B, left panel). This observation indicates that SecYE, which is sufficient for high-affinity SecA binding (Figure 4B), also constitutes the insertion site for SecA. Accordingly, the quantity of SecA necessary to saturate the insertion sites (≈1500 pmol SecA/mg IMVs) was close to the value of high-affinity SecA-binding sites predicted by Scatchard analysis (Figure 4B).

Both SecDFyajC and SecG increased the efficiency of AMP-PNP-driven SecA insertion at SecYE, by factors of 3 and 2, respectively (Figure 7B, left panel). We noted, however, that only 10% of the SecA bound at SecYE inserted into the membrane, even using membranes

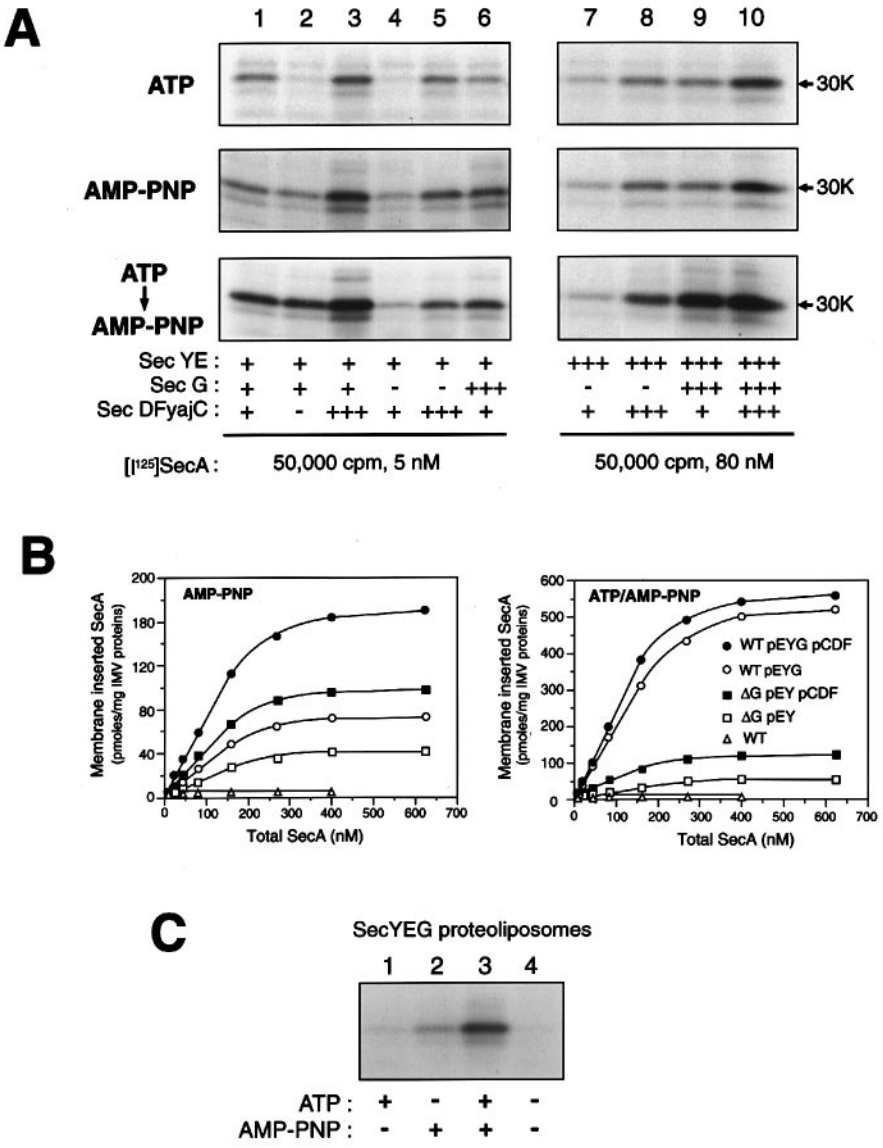

Fig. 7. SecA insertion occurs at SecYE and is stimulated by SecG and SecDFyajC. (**A**) Upper panel: assay of the SecA membrane insertion/de-insertion cycle (Economou and Wickner, 1994) was performed in 100 μl of TL buffer containing SecB (48 μg/ml), BSA (200 μg/ml), proOmpA (20 μg/ml), urea-stripped IMVs (100 μg/ml) and [^{125}I]SecA as indicated (50 000 c.p.m., 80 nM for IMVs enriched in SecYE; 50 000 c.p.m., 5 nM for IMVs with a wild-type level of SecYE). After pre-warming (2 min, 37°C), the SecA membrane cycle was initiated with 1 mM ATP. After 10 min, the samples were chilled on ice, digested with trypsin (1 mg/ml; 15 min, 0°C), TCA-precipitated and analyzed by SDS–PAGE. Protease-protected material was visualized by fluorography. The arrowhead indicates the trypsin-inaccessible 30 kDa domain of SecA. Middle panel: AMP-PNP-driven insertion was performed as above, except that 4 mM AMP-PNP was added instead of ATP and the reaction was incubated at 37°C for 3 min. Lower panel: after initiation of the SecA cycle (1 mM ATP, 10 min, 37°C), 4 mM AMP-PNP was added to block SecA in the inserted state and the reaction was incubated for an additional 3 min. (**B**) SecA insertion reactions were initiated using 4 mM AMP-PNP (3 min; 37°C) (left panel) or 1 mM ATP (10 min; 37°C), followed by the addition of 4 mM AMP-PNP (3 min; 37°C) (right panel). Each tube received a constant amount of [^{125}I]SecA (50 000 c.p.m., 80 nM) pre-mixed with non-radioactive SecA (0–60 μg/ml). After autoradiography, [^{125}I]SecA 30 kDa protected material was quantitated by scanning densitometry. When using SecYE-enriched IMVs (0.1 mg/ml), the increase of SecA insertion was linear until ≈150 nM SecA was added. Reactions contained 0.1 mg/ml IMVs proteins; thus 150 nmol/l = 150 pmol/ml×1 ml/0.1 mg = 1500 pmol SecA added per mg of IMV proteins. Correcting for the non-specific binding of SecA (≈10%), this value coincides with the number of high-affinity SecA-binding sites predicted by Scatchard analysis (Figure 4B). (**C**) SecA insertion into SecYEG proteoliposomes. [^{125}I]SecA (50 000 c.p.m.; 5 nM) was added to SecYEG proteoliposomes (10 μl; 50 μg of reconstituted proteins/ml) in TL buffer containing SecB (48 μg/ml), BSA (200 μg/ml) and proOmpA (20 μg/ml). SecA insertion reactions were performed with 1 mM ATP (10 min, 37°C, lane 1), 4 mM AMP-PNP (5 min, 37°C, lane 2) or 1 mM ATP (10 min, 37°C) followed by the addition of 4 mM AMP-PNP (3 min, 37°C, lane 3). As control, no nucleotide was added (lane 4). Samples were transferred to ice and digested with trypsin (1 mg/ml, 15 min, 0°C). The [^{125}I]SecA 30 kDa protease-protected domain was analyzed as above.

enriched in both SecG and SecDFyajC (Figure 7B, left panel). This may indicate that other factors or conditions are required to activate SecYE sites to allow a higher proportion of the bound SecA to insert. Indeed, using membranes enriched in SecYEG, the initiation of SecA insertion/de-insertion cycles with ATP and subsequent blockage with AMP-PNP (Figure 7A, lower panel) led to a drastic increase in the amount of inserted SecA (compare lanes 9 of middle and lower panels). Moreover, this increase in the level of SecA 30 kDa fragment formed was dependent on the presence of SecG rather than SecDFyajC, since a far smaller increase in the level of

inserted SecA was observed using IMVs deleted for SecG (compare lanes 7 and 9 or 8 and 10, middle and lower panels). Quantitative analysis of the SecA 30 kDa fragment formed showed that SecDFyajC and SecG stimulated the efficiency of SecA insertion at SecYE by a factor of 3 and 12, respectively (Figure 7B, right panel). Thus, in the presence of SecG and when the SecA membrane cycle is initiated with ATP and subsequently blocked with AMP-PNP, 40–50% of the SecA bound at SecYE is able to insert. Since the requirements for SecA membrane insertion and preprotein translocation are the same, SecG appears to stimulate SecA insertion at SecEY mainly after initiation of the translocation has begun. Similar results were seen with proteoliposomes containing purified SecYEG (Figure 7C). ATP-driven SecA insertion did not allow the detection of stably inserted SecA (lane 1), presumably reflecting the absence of SecDFyajC, while AMP-PNP allowed only a small fraction of the SecA to insert (lane 2) (Economou *et al.*, 1995). In contrast, if the SecA cycle was first initiated with ATP and the de-insertion blocked by AMP-PNP (lane 3), a significant increase in insertion efficiency was observed.

Discussion

Genetic experiments have pointed to the importance of the SecE and SecY interaction for preprotein translocation (Bieker-Brady and Silhavy, 1992; Pohlschröder *et al.*, 1996), to the specific effect of SecE on the stability of SecY (Matsuyama *et al.*, 1990) and to the stability of the SecYE complex during successive cycles of cell growth (Joly *et al.*, 1994). Our data clearly demonstrate that the SecYE pair form the physical and functional core of the membrane-embedded translocase. Each of the subreactions of the translocation process, SecA high-affinity binding, SecA translocation ATPase activity and SecA membrane insertion, can be performed by SecYE. Furthermore, overexpressed SecYE can suppress the growth defect of a SecG or SecDFyajC mutant. However, overproduction of SecYE did not result in a proportional increase in preprotein translocation (Figure 5 and Kawasaki *et al.*, 1993). Thus, though SecYE is essential, other factors are required for an efficient coupling between the ATP hydrolysis, SecA membrane insertion and preprotein translocation.

SecG, which is one of these stimulatory factors, has been isolated in complex with SecYE (Brundage *et al.*, 1990). The weak translocation activity of proteoliposomes reconstituted with pure SecY and SecE can be enhanced greatly by the inclusion of SecG (Hanada *et al.*, 1994), and the SecYEG complex is fully active for translocation (Bassilana and Wickner, 1993). Conversely, IMVs deleted for SecG showed a reduced translocation activity (Figure 5 and Nishiyama *et al.*, 1994). The SecG-mediated stimulation of translocation occurs via SecYE, since overproduction of SecG without simultaneous overproduction of SecYE has no effect on preprotein translocation. Overproduction of SecYE restored translocation to IMVs which lacked SecG, indicating that the increased number of inefficient 'core' translocase molecules can compensate for the absence of the SecG stimulatory factor. This notion was supported further by the suppression of the cold-sensitive growth of a *secG* null mutant by overproduction of SecYE. This last finding suggests that SecG does not specifically catalyze a cold-sensitive step. The growth defect at 20°C may rather be the result of simultaneous reduction of translocation by lower temperature (Pogliano and Beckwith, 1993) and *secG* deletion. Indeed, the Δ*secG* cold-sensitive phenotype appears to be strain-dependent (Bost and Belin, 1995).

In vivo studies had suggested that the SecDF proteins have a stimulatory function (Gardel *et al.*, 1990; Pogliano and Beckwith, 1994a). However, lack of evidence for an *in vitro* stimulatory function led to the hypothesis that SecDF may only act late in translocation, such as by releasing the translocated polypeptide from the translocase or recycling translocase subunits after each round of translocation. We have now established that SecD and SecF directly support *in vitro* ATP-driven translocation. As is the case with SecG, SecDF acts on the core translocase by stimulating the SecYE-based translocation activity. Accordingly, overexpression of SecYE is able to compensate for the growth defect of a SecDFyajC-depleted strain. *In vitro*, the SecDF stimulatory activity was only seen when SecG was either absent or present at a sub-saturating concentration with respect to SecYE. This explains why previous studies of depletion or overexpression of SecDF, done in the presence of SecG, showed only a modest effect of SecDF on preprotein translocation (Arkowitz and Wickner, 1994; Economou *et al.*, 1995; Figure 5). Several hypotheses may account for these findings: (i) SecG may stimulate a step of the *in vitro* translocation process which is normally dependent on SecDF; (ii) SecDF may manifest a stimulatory activity only when translocation is impaired or reduced; (iii) The stimulatory effects of SecDF and SecG on SecYE may be mutually exclusive. However, SecG and SecDF appear to act differently since they do not complement each other in terms of *in vivo* growth defects.

It has been difficult to define the function of SecDF since it is present at only 10–30 copies per cell, 10 times less abundant than SecYEG (Matsuyama *et al.*, 1992; Pogliano and Beckwith, 1994b), and there has been no biochemical evidence for interaction of SecDF with translocase, or even between SecD and SecF. We have now established by co-immunoprecipitation experiments that SecD and SecF are physically linked to translocase. Unexpectedly, we find that YajC is also part of the translocase complex, associated with both SecYEG and SecDF complexes when membranes were solubilized in β-octyl glucoside. An interaction of YajC with SecY was proposed previously (Taura *et al.*, 1993, 1994), based on the observation that overexpression of YajC suppresses the lethality induced by *secY*$^{-d}$*1*, a *secY* 'dominant-negative' allele, and partially stabilizes overproduced SecY. Thus, YajC may bridge the SecYEG and SecDF complexes either structurally or functionally. There is, however, no evidence that YajC is required for translocation *per se* (Pogliano and Beckwith, 1994b). Just as the contribution of SecDF was only observed clearly in the absence of SecG, specific conditions may also be required to reveal a YajC function.

It is noteworthy that the yeast ER translocase is also composed of seven distinct subunits—Sec61, Sbh1, Sss1, Sec62, Sec63, Sec71 and Sec72—which co-purify in digitonin (Panzner *et al.*, 1995). In β-octyl glucoside,

the yeast translocase is also isolable as two separable complexes (Feldheim and Schekman, 1994). Though there is considerable post-translational translocation into the yeast ER, there is no known SecA homolog, and ATP energy is coupled to preprotein movement via ER lumenal Hsp70 (BiP) (Sanders *et al.*, 1992). This raises intriguing questions as to the function of the yeast translocase subunits in addition to Sec61p and Sss1p, the homologs of the bacterial SecYE 'core'. In *E.coli*, it is not known how SecG and SecDF perform their stimulatory functions. SecA, SecD, SecF, YajC and SecG appear to be unique to bacterial translocation and have not been described in eukaryotic cells. During active translocation, SecG undergoes a significant topological alteration which may facilitate the SecA membrane insertion/de-insertion cycle (Nishiyama *et al.*, 1996), while IMVs depleted for SecDFyajC display a reduced steady-state level of inserted SecA (Economou *et al.*, 1995). Perhaps SecDFyajC and SecG have evolved to facilitate SecA membrane cycling at SecYE. Indeed, SecG or SecDFyajC were not found to affect the number of SecA insertion sites but rather modulate the SecA insertion cycle (Figure 7). The modulation by SecDFyajC and by SecG differ, however, in both extent and timing during the translocation process. SecDFyajC exerts a double effect: to prevent the de-insertion of SecA, which requires hydrolysis of ATP (Economou *et al.*, 1995), and to increase SecA insertion, which involves only binding of the nucleotide. This dual activity is compatible with a regulatory function for SecDFyajC. It may increase the translocation of normal preproteins, as seen in the absence of SecG, and facilitate translocation of preproteins with defective leader peptides, as previously reported (Pogliano and Beckwith, 1994a). The effects of SecG on SecA cycling appear equally complex. Studies of SecG showed that its topological inversion does not occur if translocation is initiated with AMP-PNP (Nishiyama *et al.*, 1996). We also found that SecG had a modest effect on SecA insertion when the reaction was driven by AMP-PNP. In marked contrast, when translocation and SecA insertion were started with ATP, and SecA de-insertion subsequently blocked with AMP-PNP, the level of inserted SecA was significantly increased by the presence of SecG. Since SecG topological inversion was only seen under the same conditions (Nishiyama *et al.*, 1996), we propose that SecG stimulates translocation after the initiation of translocation has taken place. Accordingly, the events that probably occur early in the catalytic cycle of SecA, binding at SecYE and activation as an ATPase, were not stimulated significantly by SecG (Figure 4). Moreover, classical genetic screens designed to identify genes involved in the first step of the translocation process, such as the recognition of the leader peptide, did not select *secG* (Bieker *et al.*, 1990).

Our current study shows that SecA, bound with high affinity to SecYE, constitutes the core of preprotein translocase. SecG and SecDFyajC, associated with this core in a stable fashion to form a holoenzyme, probably enhance translocation through their support of the cycle of SecA insertion and de-insertion. The genetic and biochemical tools are now available to address further fundamental questions, such as the dynamics of translocase subunit associations, how SecDFyajC stabilize SecA insertion and thus the movement of the preprotein, and which subunits directly contact the preprotein and SecA.

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 and [^{35}S]-methionine protein labeling mix (≈1175 Ci/mmol) from Dupont-NEN. [^{35}S]proOmpA (Crooke *et al.*, 1988) and [^{125}I]SecA (Economou and Wickner, 1994) were prepared as described. Proteinase K, creatine kinase, creatine phosphate and AMP-PNP were from Boehringer-Mannheim. ATP, lipid-free bovine serum albumin (BSA), TPCK-treated trypsin, *n*-octyl-β-D-glucoside and digitonin were from Sigma. *Escherichia coli* phospholipids were purchased from Avanti Polar Lipids and protein A–Sepharose CL-4B beads from Pharmacia. IMVs from *E.coli* strain BL21 (*hsdS, ompT*$^{-}$, *gal*) and its derivatives were prepared as described by Douville *et al.* (1995). To inactivate endogenous SecA, IMVs were treated with 6 M urea (30 min, 4°C) (Cunningham *et al.*, 1989).

Strains and plasmids

Escherichia coli KN425 (W3110 M25 Δ*secG::kan*) and JP325 (MC4100 *ara*Δ*714 tgt::kan-araC*$^{+}$ -P$_{BAD}$*::yajCsecDF*) were generous gifts from the laboratories of Drs H.Tokuda and J.Beckwith, repectively (Nishiyama *et al.*, 1994; Pogliano and Beckwith, 1994a). These mutations were introduced into *E.coli* BL21 by P1 transduction (Miller, 1972), giving BL425 and BL325, respectively. The *unc* deletion of KM9 (*unc*$^{-}$*::*Tn*10, relA1, spoT1, metB1*; Cunningham *et al.*, 1989) was transduced to BL21 and BL425.

Plasmids pHA-EY, pHA-EYG and pSecG, expressing SecE (tagged with an influenza HA epitope), SecY and SecG under the control of the *araBAD* promoter of pBAD22 (Guzman *et al.*, 1995), were described previously (Joly *et al.*, 1994; Douville *et al.*, 1995). To allow the overproduction of SecDFyajC along with that of SecYEG, the *secD* operon (*yajC-secD-secF*) was cloned into pBAD33, a pBAD18-derived vector with the origin of replication of pACYC184 (Guzman *et al.*, 1995). pGAP1, carrying the *secD* operon on pBAD18 (Pogliano and Beckwith, 1994a), was digested at the polylinker-encoded *Sac*I and *Hin*dIII sites. The resulting 3.9 kb DNA fragment containing the *secD* operon was gel purified and inserted into the *Sac*I–*Hin*dIII sites of pBAD33, giving plasmid pCDF33. To delete *yajC*, pGAP1 was first digested with *Apa*LI, the sites filled-in with the Klenow fragment and then digested with *Hin*dIII. The resulting 3.8 kb *Apa*LI–*Hin*dIII DNA fragment, containing *secDF* but only the 3′ part of the *yajC* gene, was inserted into the *Sma*I–*Hin*dIII sites of pBAD33 to give plasmid pDF33.

To clone *sec HA-EYG* on the IPTG-inducible plasmid pTrc99A (Pharmacia), the 2.3 kb *Nco*I–*Sac*I fragment from pHA-EYG was cloned into the corresponding sites of pTrc99A to give pTrcHA-EYG. To remove *secG*, carried on a 0.6 kb *Hin*dIII fragment, pTrcHA-EYG was digested with *Hin*dIII and the resulting 5.8 kb fragment was gel purified and religated to give pTrcHA-EY. To remove *sec HA-EY*, carried on a 1.5 kb *Eco*RI fragment, pTrcHA-EYG was digested with *Eco*RI, and the 5.0 kb fragment was gel purified and religated to give pTrcG. These plasmids complemented the growth defect of BL525 (not shown).

Growth conditions

For IMV preparation, *E.coli* BL21 transformed with the various plasmids were grown at 37°C in 3 l of Luria-Bertani (LB) broth with 50 μg/ml of ampicillin and chloramphenicol where needed (Miller, 1972). At A_{600} = 0.4, the *ara* promoter (pBAD) was induced with 0.5% (w/v) arabinose. Cells were collected at A_{600} = 1.0 and frozen as described by Douville *et al.* (1995). To deplete the YajC SecDF content of BL21, BL325 (BL21 *tgt::kan-araC*$^{+}$-P$_{BAD}$*::yajCsecDF*) was grown as described by Economou *et al.* (1995) except that LB medium was used instead of M63. Kanamycin was used at 25 μg/ml.

For [^{35}S]methionine labeling, *E.coli* BL21 and derivatives were grown at 37°C in 70 ml of M9 medium with thiamine (1 μg/ml), the appropriate antibiotic, amino acids (40 μg/ml, except methionine) and 0.2% (w/v) glucose. At A_{600} = 0.5, 15 μCi/ml of EXPRE^{35}S^{35}S protein labeling mix and 0.5% (w/v) arabinose were added for 30 min. The cells were harvested by centrifugation (5 min, 3000 *g*, 4°C), resuspended in 10% sucrose, 50 mM Tris–HCl, pH 7.5 and frozen in liquid nitrogen.

Co-immunoprecipitation studies

Crude [^{35}S]methionine-labeled membranes were prepared for immunoprecipitation (IP) as described by Joly *et al.* (1994). Peptide antibodies directed against SecY, E, G, D, F or proOmpA and an anti-HA monoclonal antibody have been described (Lill *et al.*, 1989; Arkowitz and Wickner, 1994; Joly *et al.*, 1994; Douville *et al.*, 1995). Antibodies were affinity purified using Sulfolink® gel (Pierce) according to the specifications of the manufacturer. Coupling of the affinity-purified antibodies to protein A–Sepharose beads was as described by Joly *et al.* (1994). Typically, 2.5 µl of affinity-purified antibodies (≈100 µg/ml), coupled to 10 µl of protein A–Sepharose beads in 100 µl of the appropriate IP buffer (see below) containing 2% (w/v) BSA, were used per immunoprecipitation.

For denaturing IP, membranes were solubilized in 500 µl of RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 7.9; Brundage *et al.*, 1990) for 15 min on ice before the addition of antibodies. For non-denaturing IP, membranes were first solubilized for 15 min on ice with 500 µl of extraction buffer (150 mM NaCl, 3.4 mg/ml *E.coli* phospholipids, 35% glycerol, 50 mM Tris–HCl, pH 7.9, 1.25% β-octyl glucoside or 1% digitonin) (Brundage *et al.*, 1992). After centrifugation (15 min, 16 000 *g*, 4°C), the supernatant was incubated with the antibodies (60 min, 4°C). The beads were collected by brief centrifugation and suspended three times with 1 ml of RIPA buffer or NDIP buffer (150 mM NaCl, 1.5 mg/ml *E.coli* phospholipids, 35% glycerol, 50 mM Tris–HCl, pH 7.9, 1.0% β-octyl glucoside or 1.0% digitonin). Proteins were eluted by incubation with 40 µl of SDS sample buffer for 10 min at 37°C, the beads removed by brief centrifugation and the supernatant electrophoresed in 'high Tris' SDS–polyacrylamide gels (Joly *et al.*, 1994). Digitonin was solubilized as follows: 10 g of digitonin were dissolved in 20 ml of ethanol, incubated in a boiling water bath until dissolved, and cooled (2 h, 23°C). After incubation (16 h, –20°C), the solution was centrifuged (10 min, 7000 *g*). The pellet was dried in a vacuum-desiccator, then dissolved in 10 ml of water and stored at –80°C.

SecA-binding assays

Binding of [^{125}I]SecA to urea-treated IMVs (100 µg/ml) prepared from *E.coli* BL21 and its derivatives was performed in 50 µl reactions as previously described (Economou and Wickner, 1994; Douville *et al.*, 1995). Non-radioactive SecA (0–2370 nM for IMVs with high SecYE content and 0–237 nM for IMVs from BL21) was mixed with [^{125}I]SecA (50 000 c.p.m., 51 ng) in TL buffer (50 mM KCl, 50 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM Tris–HCl, pH 7.9) prior to the addition of IMVs (0.1 mg/ml) and BSA (200 µg/ml). After 15 min on ice, samples were layered over 50 µl of buffer S (TL buffer containing 0.2 M sucrose) and membranes sedimented by ultracentrifugation (30 min, 4°C, 65 000 r.p.m., Beckman TLA100 rotor). Radioactivity was measured in the membrane pellet and in the supernatant. Binding parameters were determined using the computer modeling program LIGAND (Munson and Rodbard, 1980)

Other methods

SecYEG purification from *E.coli* carrying pHA-EYG and reconstitution of pure proteins into proteoliposomes was as described (Brundage *et al.*, 1990; Douville *et al.*, 1995). Proteoliposomes bearing pure SecYEG were assayed for translocation ATPase and translocation of [^{35}S]proOmpA (Douville *et al.*, 1995) prior to use. SecA insertion reactions were performed as described by Economou and Wickner (1994).

Protein concentration was determined using Bradford reagent (Biorad) with BSA as standard. SDS–PAGE was performed using either '15%' gels or 'high Tris' gels, and electrophoretic transfer of proteins to nitrocellulose was as previously described (Brundage *et al.*, 1992). Goat anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase (Sigma) and a chemiluminescence kit (ECL, Amersham) were used for immunodetection. Autoradiography of ^{125}I- or ^{35}S-labeled polypeptides was performed at –80°C with intensifying screens. Densitometry was performed using a Silverscan III scanner (La Cie Ltd., Beaverton, OR) and IPLabH software.

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