SecD and SecF are required for the proton electrochemical gradient stimulation of preprotein translocation

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Mutations in secD and secF show impaired protein translocation across the inner membrane of *Escherichia coli*. We investigated the effect of SecD and SecF (SecD/F) depletion on preprotein translocation into inverted inner membrane vesicles (IMVs). Both IMVs and cells which were depleted of SecD/F were defective in their ability to maintain a proton electrochemical gradient. The translocation of pre-maltose binding protein (preMBP), which is strongly ΔψH⁺ dependent, showed a 5-fold decreased rate with IMVs lacking SecD/F. In contrast, proteolytic processing of preMBP to MBP by leader peptidase was similar in IMVs containing and lacking SecD/F, consistent with earlier findings that only ATP-dependent translocation is required for the initiation of translocation. In the absence of a ΔψH⁺, with ATP as the sole energy source, preMBP translocation into IMVs which contained or were depleted of SecD/F was identical. Translocation of the precursor of outer membrane protein A (proOmpA) in the presence of subsaturating ATP also required a generated ΔψH⁺ and, under these conditions, proOmpA translocation required SecD/F. With saturating concentrations of ATP, where ΔψH⁺ has little effect on *in vitro* proOmpA translocation, SecD/F also had little effect on translocation. These results explain why SecD/F effects are precursor protein dependent *in vitro*.

Key words: *Escherichia coli*/membrane protein/secrretion

Introduction

Genetic selections and screens have identified the sec genes which are necessary for protein export in *Escherichia coli* (Bieker *et al.*, 1990; Schatz and Beckwith, 1990). These genes encode the cytoplasmic chaperone SecB (Kumamoto and Beckwith, 1983; Collier *et al.*, 1988; Kumamoto, 1989), the peripheral membrane protein SecA (Olive and Beckwith, 1981), and integral membrane proteins SecD/F (Gardel *et al.*, 1987, 1990), SecE (Riggs *et al.*, 1988) and SecY (Emr *et al.*, 1981; Ito *et al.*, 1983). Mutations in the genes of the membrane-associated and embedded components result in pleiotropic defects in protein secretion *in vivo*. The roles of SecA, SecB, SecE and SecY in protein translocation have also been examined at a biochemical level *in vitro*. This combined genetic and biochemical approach has established the basic outlines of protein export in *E. coli* (Bieker *et al.*, 1990; Mizushima and Tokuda, 1990; Schatz and Beckwith, 1990; Wickner *et al.*, 1991).


Both ATP hydrolysis (Chen and Tai, 1985) and a proton electrochemical gradient (Date *et al.*, 1980; Daniels *et al.*, 1981; Enequist *et al.*, 1981; Zimmermann and Wickner, 1983; Geller, 1990) are necessary for protein secretion. These two energy sources are required for maximal rates of translocation *in vitro* (Chen and Tai, 1986; Geller *et al.*, 1986), yet preproteins can be translocated in the absence of a proton electrochemical gradient (Yamada *et al.*, 1989). *In vitro* studies have led to the following working model of preprotein translocation. Upon binding preprotein, the SecA subunit of translocase is activated for ATP hydrolysis (Lill *et al.*, 1989). The energy of ATP binding can drive an initial loop of preprotein across the membrane, where it gains access to leader peptidase (Schivel *et al.*, 1991). ATP binding and hydrolysis then results in net movement of the polypeptide chain of the preprotein across the inner membrane (Schivel *et al.*, 1991). During membrane transit, SecY is proximal to the translocating preprotein (Joly and Wickner, 1993) and translocation can drive the net unfolding of a preprotein structural domain (Arkowitz *et al.*, 1993). After the ATP-dependent initiation of translocation, a proton electrochemical gradient can drive large segments of protein across the membrane (Tani *et al.*, 1989, 1990; Schiebel *et al.*, 1991). These results and experiments *in vivo* using uncouplers of ΔψH⁺ (Geller, 1990) suggest that the proton electrochemical gradient acts in part during the latter stages of preprotein translocation, whereas the initiation of translocation absolutely requires ATP.

While the roles of SecB, SecA, SecY and SecE in protein translocation are well characterized, the precise functions of the integral membrane components SecD and SecF are...
less clear (Matsuyama et al., 1992). Amino acid sequence and TnphoA insertion analyses indicate that SecD and SecF are integral membrane proteins with large periplasmic domains (Gardel et al., 1990). Cold-sensitive mutants in secD or secF inhibit the export of a variety of preproteins in vivo (Gardel et al., 1987, 1990; Pogliano and Beckwith, 1994). Recently, Matsuyama et al. (1993) demonstrated that pretreatment of spheroplasts with anti-SecD IgGs inhibited secretion of proOmpA and preMBP, suggesting that SecD may be involved in the release of preproteins following translocation. We now report studies of the role of SecD/F in preprotein translocation into inverted inner membrane vesicles. Using a strain with regulated expression of SecD/F, we have depleted these proteins from the inner membrane of E. coli. SecD/F are required for the maintenance of a full ΔμH⁺, both in vivo and in vitro. They are therefore needed for the translocation of the ΔμH⁺-dependent precursor protein preMBP. Under specific conditions in which proOmpA translocation requires ΔμH⁺, SecD/F is also required, whereas when translocation is driven only by ATP, it is independent of SecD/F. These studies indicate that SecD/F are not required for ATP-dependent translocation, but are needed for ΔμH⁺-dependent translocation. Our results do not establish whether these proteins directly couple ΔμH⁺ to translocation.

Results

Inner membrane vesicles can be depleted of SecD/F

To investigate the role of SecD/F in translocation, we isolated inverted inner membrane vesicles (IMVs) from cells depleted of SecD/F (Figure 1). The strains JP91 and JP135 have a deletion for secD/F at its chromosomal location, and carry a plasmid in which secD/F expression is under the control of the inducible and repressible araB promoter (pGAPI) or its normal promoter (pCGSH1), respectively. JP91 is described elsewhere (Pogliano and Beckwith, 1994). In the presence of 0.002% arabinose, JP91 synthesizes both SecD and SecF. In the absence of arabinose and the presence of 0.2% glucose (repressor), SecD/F synthesis is undetectable (Pogliano and Beckwith, 1994). SecD/F depletion results in a slight growth defect in rich media, starting ~10 doublings after their synthesis has been shut off, and an in vivo kinetic defect in proOmpA processing. These cells remain viable at 37°C, yet exhibit cold-sensitive growth. These observations are consistent with those of Pogliano and Beckwith (1994). A culture of JP91 in LB with 0.002% arabinose was centrifuged to remove residual arabinose and then used to inoculate media containing either arabinose or glucose. After >10 doublings, cells were harvested and inverted IMVs were prepared. The IMVs were examined for SecD, SecF, SecY and total protein content (Figure 1). IMVs prepared from JP91 or JP135 cells grown in the presence of arabinose contained both SecD (lanes 1 and 3) and SecF (lanes 4 and 6). In contrast, IMVs prepared from JP91 grown in the presence of glucose had no detectable SecD (lane 2) or SecF (lane 5). Quantitation of immunoblots with [125I]protein A revealed that IMVs from arabinose-grown JP91 contained 11-fold more SecD and 26-fold more SecF than the levels in the wild-type strain KM9 (Table I). SecD/F depletion in IMVs from glucose-grown JP91 was 95% for SecD and >96% for SecF (compared to the wild-type level). The overall protein profile of the IMVs was unaffected by the depletion of SecD/F (lanes 9 and 10), suggesting that depletion of these two proteins did not perturb the protein composition of the inner membranes. Specifically, depletion of SecD/F had no effect on the SecY content of the IMVs (lanes 7 and 8) or on the content of SecE or leader peptidase (data not shown).

Fig. 1. Depletion of SecD and SecF from inverted IMVs. IMVs were prepared from JP91 and JP135 cells containing a deletion of the chromosomal SecD/F operon (Pogliano and Beckwith, in press) and either plasmid pCGSH1, in which SecD/F synthesis is directed from their wild-type promoter, or pGAPI, in which SecD/F synthesis is arabinose inducible. Cells were grown in LB containing 0.002% arabinose (+) or 0.2% glucose (−). Lanes 1, 2, 4 and 5 represent 100 μg of membrane protein. Lanes 3 and 6–10 represent 20 μg of membrane protein. Samples (lanes 1–8) were analyzed by SDS–PAGE, transferred to PVDF membranes, probed with the respective antibodies, followed by [125I]protein A, and autoradiography as described in Materials and methods. Lanes 9 and 10 were Coomassie stained.
**Inner membrane vesicles and permeabilized cells depleted of SecD/F are unable to maintain a full proton electrochemical gradient**

The IMV's $\Delta \psi$ (inside positive) and $\Delta p$H (inside acidic), components of $\Delta \mu H^+$, were first measured by the fluorescence of the potential- and pH-sensitive dyes oxonol VI (Apell and Bersch, 1987) and 9-aminoacridine (Klionsky et al., 1984), respectively. IMVs containing SecD/F displayed a change in fluorescence of oxonol VI of 176 relative units (mg protein)$^{-1}$ (SD 20; $n = 4$) upon addition of 2 mM succinate, whereas IMVs lacking SecD/F showed a change in fluorescence of only 32 units (mg protein)$^{-1}$ (SD 2; $n = 4$). In similar experiments with 9-aminoacridine, no observable $\Delta p$H was detected in IMVs lacking SecD/F. These results indicate that IMVs lacking SecD/F are strikingly defective in their ability to generate and/or maintain a $\Delta \mu H^+$. Figure 2A and Table II show quantitation of the $\Delta \psi$ defect in IMVs using $[^{14}C]KSCN$, flow dialysis, and scintillation counting. Flow dialysis measurements quantitate the steady-state distribution of a diffusible ion [SCN$^-$ for IMVs and tetraphenylphosphonium (TPP$^+$) for cells] and $\Delta \psi$ is calculated from the Nernst equations ($\Delta \psi = 590 \log \text{[ion]}_{\text{out}} / \text{[ion]}_{\text{in}}$). With IMVs containing SecD/F, a $\Delta \psi$ of 129 mV (SD 8; $n = 3$) was generated by the addition of 5 mM succinate. This transmembrane electrochemical potential was stable for $\sim 5$ min. IMVs lacking SecD/F showed no change in distribution of $[^{14}C]KSCN$ upon succinate addition. Even when five times the amount of (SecD/F)$^-$ IMVs (1 mg membrane protein; inset Figure 2A) was used, no detectable $\Delta \psi$ was observed. To determine whether the electron transport chain is affected in these IMVs, NADH oxidase activity was assayed. IMVs containing and lacking SecD/F have comparable NADH oxidase specific activities: 1.75 nmol NADH (min $\mu g$ protein)$^{-1}$ (SD 0.18; $n = 3$) and 1.47 nmol NADH (min $\mu g$ protein)$^{-1}$ (SD 0.06; $n = 3$), respectively. Taken together, these results demonstrate that (SecD/F)$^-$ IMVs are unable to maintain a proton electrochemical potential.

**Table 1.** SecD and SecF content of inner membrane vesicles

<table>
<thead>
<tr>
<th>Strain</th>
<th>Arabinose</th>
<th>SecD (SD)</th>
<th>SecF (SD)</th>
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<tr>
<td>JP91</td>
<td>+</td>
<td>10.9 (2.3)</td>
<td>26.2 (5.5)</td>
</tr>
<tr>
<td>JP91</td>
<td>−</td>
<td>0.05 (0.02)</td>
<td>&lt;0.04$^{\text{a}}$</td>
</tr>
<tr>
<td>KM9</td>
<td>−</td>
<td>1.00</td>
<td>1.00</td>
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SecD and SecF content were determined from quantitative immunoblot analysis as shown in Figure 1 and described in Materials and methods. Values are the average of five separate experiments with two different IMV preparations for SecD and three different IMV preparations for SecF. JP135 IMVs had ~2 times the amount of SecD and 3 times the amount of SecF as KM9 IMVs, consistent with pCGSH1 being a multicopy plasmid.

$^{a}$This amount of SecD was consistently observed even in cells that had grown in the presence of glucose for >30 doublings.

$^{b}$SecF was never observed on autoradiograms and 0.04 represents the level of detection.

**Fig. 2.** Inner membrane vesicles and permeabilized cells depleted of SecD/F are unable to maintain a proton electrochemical gradient. (A) IMVs depleted of SecD/F cannot maintain a $\Delta \psi$. The upper chamber of a flow dialysis cell contained 200 $\mu l$ of 1 mg/ml IMVs (SecD/F content indicated) in buffer C and was continuously mixed. The reaction was initiated by the addition of 20 $\mu M$ $[^{14}C]KSCN$ (56 mCi/mmol). Oxygen-saturated buffer C was passed through the lower chamber of the cell at 1.3 ml/min and fractions were collected. At fraction 25, succinate was added to 4.7 mM in order to generate a $\Delta \mu H^+$ (see arrow) and a stream of buffer C-saturated oxygen was passed over the reaction mixture in the upper chamber. At fraction 45, CCCP was added to 18.8 $\mu M$ to collapse the $\Delta \mu H^+$ and the oxygen stream was removed from the upper chamber. Radioactivity was quantitated by scintillation counting of 0.4 ml aliquots. The data were corrected for the slight change in volume from the succinate addition. The inset shows an identical flow dialysis determination with 5 mg/ml (SecD/F)$^-$ IMVs. (B) Cells depleted of SecD/F are unable to maintain a $\Delta \psi$. The upper chamber of a flow dialysis cell contained 200 $\mu l$ buffer C and was continuously mixed. The reaction was initiated by the addition of 100 $\mu M$ $[^{14}C]TPP^+$ (19 mCi/mmol). Oxygen-saturated buffer C was passed through the lower chamber of the cell at 0.7 ml/min and fractions were collected as in (A). At fraction 23, K ascorbate was added to 17.3 mM; at fraction 24, phenazine methosulfate was added to 173 $\mu M$; and at fraction 25, EDTA-permeabilized cells (SecD/F content indicated) were added to 4 mg/ml (see arrow). A stream of buffer C-saturated oxygen was then passed over the reaction in the upper chamber. At fraction 45, CCCP was added to 18.8 $\mu M$ to collapse the $\Delta \mu H^+$ and the oxygen stream was removed from the upper chamber. Radioactivity was quantitated and data analyzed as described in (A).

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To further investigate this effect of SecD/F depletion on the proton electrochemical gradient, we examined the electrochemical potential of permeabilized whole cells. Figure 2B shows a typical flow dialysis Δψ determination using [14C]TPP+ and 4 mg/ml cell protein. There is a 1.5-fold difference in the distribution of [14C]TPP+ between cells containing and lacking SecD/F, and as a result a Δψ difference of 11 mV (Table II). This difference in electrochemical potential is not a cold-sensitive defect, since the Δψ measurements performed at 37°C showed a similar difference (Table II). Furthermore, this difference in electrochemical potential is not observed in isogenic cells in which SecD/F are not depleted (JP135, Table II). To determine if this decreased electrochemical potential in cells depleted of SecD/F was specific for SecD/F, we examined the effect of SecE depletion using the strain CM124 with a deleted chromosomal secE gene and a plasmid with SecE synthesis directed by the araB promoter. Depletion of SecE in this strain is lethal, yet it is possible to decrease the level of SecE by allowing the cells to grow 1.8 doublings in glucose rather than arabinose. Pulse–chase analysis of proOmpA secretion after 1.8 doublings revealed a significant secretion defect (data not shown), yet flow dialysis determinations of Δψ in these cells showed similar transmembrane electrochemical potentials (Table II). Taken together, these results indicate that depletion of SecD/F results in a defect in the ability to maintain a full electrochemical potential in cells, and that this defect is specific to SecD/F and not a consequence of a general secretion block. The magnitude of the Δψ defect in cells depleted of SecD/F appears modest, yet dissipation of the ΔΔH+ by similar amounts results in substantial translocation defects in vivo (Daniels et al., 1981; Bakker and Randall, 1984).

### ΔΔH+ -dependent preMBP translocation requires SecD/F

**In vivo**, the translocation of different precursor proteins is stimulated to different extents by a proton electrochemical gradient (Daniels et al., 1981; Yamada et al., 1989). In addition, mutations in SecD and SecF show a much more
striking effect on preMBP secretion in vivo than on proOmpA or proOmpF secretion (Gardel et al., 1987; Pogliano and Beckwith, 1994). To further characterize the role of SecD/F, we examined the effect of SecD/F depletion on preMBP translocation in vitro. Purified preMBP was diluted from 2 M guanidine chloride into a reaction mixture containing SecA, SecB and IMVs lacking SecD/F (Figure 3). Translocation of preMBP was routinely assayed at a concentration of 0.1 μM [an amount in excess of the estimated number of translocation sites in IMVs (Bassiliana and Wickner, 1993)]. A proton electrochemical gradient was generated by the addition of succinate and the reactions were initiated by the addition of 2 mM ATP. After translocation, samples were precipitated with trichloroacetic acid, analyzed by SDS–PAGE, transferred to PVDF membranes and probed with anti-MBP antibodies, followed by [125I]protein A and autoradiography. Figure 3A shows that IMVs can process preMBP to MBP in an ATP-dependent fashion (compare lanes 1 versus 2 and 3 versus 4) with both IMVs which contain (lane 2) and lack SecD/F (lane 4).

Quantitation of these data show that IMVs that lack SecD/F process preMBP to a similar extent compared with IMVs containing SecD/F. These findings agree with previous studies (Geller and Green, 1989; Schiebel et al., 1991) which demonstrated that ΔpH+ is not required for initiation of translocation which results in processing by leader peptidase before most of the mature domain has crossed the membrane. Because MBP in solution is inherently proteinase K resistant (Dierstein and Wickner, 1985; Randall and Hardy, 1986), preMBP translocation into the lumen of IMVs was investigated by sedimentation of the membrane vesicles to remove untranslocated preprotein. The resuspended vesicles were then treated with proteinase K to degrade the surface-adsorbed preMBP. IMVs which contained SecD/F translocated preMBP in an ATP-dependent fashion (lanes 5 and 6), whereas IMVs lacking SecD/F appeared largely defective in their ability to translocate preMBP (lanes 7 and 8). These results indicate that SecD/F are not involved in the initial stages of translocation in which the leader domain crosses the membrane and becomes accessible to leader peptidase on the lumenal side of the vesicles, yet that SecD/F are involved in later stages of translocation, stages which are particularly dependent on ΔpH+. The kinetics of preMBP translocation was further examined (Figure 3B). The amounts of protease-inaccessible, translocated preMBP were determined at 0, 1, 2, 5, 10 and 30 min in the presence of 2 mM ATP and a generated proton electrochemical gradient. Figure 3B shows there is a striking defect in preMBP translocation in the absence of SecD/F. These experiments were quantitated (Figure 4; •, ○) by gamma counting of the [125I]protein A which bound to the anti-MBP antibody on the immunoblot. Under initial velocity conditions (first 2 min of translocation), IMVs which contained SecD/F translocated preMBP ~5-fold faster than IMVs which lacked SecD/F. A similar difference in the initial velocity of [35S]preMBP (at a concentration far less than the number of translocation sites) translocation into IMVs which contain or lack SecD/F was observed (data not shown), suggesting that the defect in preMBP translocation into vesicles depleted of SecD/F is reflected in single translocase turnover events. This difference in rate of preMBP translocation is not due to the overexpression of SecD/F in arabinose-grown JP91 since IMVs from strain JP135, in which SecD/F synthesis was under the control of its wild-type promoter, and IMVs from strain D10 translocated preMBP at a similar rate as JP91 (data not shown).

PreMBP translocation is dependent on a proton electrochemical gradient in vitro (Figure 4) as it is in vivo (Geller, 1990). Translocation reactions were carried out in the presence of the uncouplers valinomycin and nigericin (0.1 μM) prior to 2 min incubation at 37°C. Translocation reactions with (SecD/F)+ IMVs and ΔpH+ (●), (SecD/F)- IMVs and ΔpH+ (●), (SecD/F)- IMVs without ΔpH+ (□) and (SecD/F)+ IMVs without ΔpH+ (□) were initiated and analyzed as described in Figure 3B. Immunoblots were quantitated by counting the stained bands in a Beckman 8000 gamma counter. Percent translocated preMBP was determined from a standard curve of 5, 10, 20 and 40% of the added preMBP analyzed on each immunoblot. Both protease-inaccessible preMBP and MBP were counted for translocated samples, although only small quantities of the preMBP were observed. Values represent the average of three separate experiments (●, ○) and two separate experiments (■, □).

**Fig. 4.** PreMBP translocation into inner membrane vesicles in the presence of a ΔpH+ is dependent on SecD/F. PreMBP translocation into IMVs containing, or depleted of, SecD/F was carried out as described in Figure 3B. Reactions lacking a ΔpH+ were preincubated for 5 min on ice with the uncouplers valinomycin (1 μM) and nigericin (0.1 μM) to prevent preMBP degradation. After incubation at 37°C, PreMBP translocation reactions were initiated by the addition of preMBP and ATP. Reactions were incubated for 5 min at 37°C. Translocation reactions were carried out in the presence of the uncouplers valinomycin and nigericin (0.1 μM) prior to 2 min incubation at 37°C. Translocation reactions with (SecD/F)+ IMVs and ΔpH+ (●), (SecD/F)- IMVs and ΔpH+ (●), (SecD/F)- IMVs without ΔpH+ (□) and (SecD/F)+ IMVs without ΔpH+ (□) were initiated and analyzed as described in Figure 3B. Immunoblots were quantitated by counting the stained bands in a Beckman 8000 gamma counter. Percent translocated preMBP was determined from a standard curve of 5, 10, 20 and 40% of the added preMBP analyzed on each immunoblot. Both protease-inaccessible preMBP and MBP were counted for translocated samples, although only small quantities of the preMBP were observed. Values represent the average of three separate experiments (●, ○) and two separate experiments (■, □).

ΔpH+-dependent preOmpA translocation requires SecD/F

To determine whether IMVs lacking SecD/F could translocate other preproteins, [35S]proOmpA was diluted from 8 M urea into reaction mixtures with IMVs that contained or lacked SecD/F. Translocation reactions were initiated by the addition of 2 mM ATP and incubated at 37°C. Samples were treated with proteinase K at 0°C to digest untranslocated precursor protein, then analyzed by SDS–PAGE and fluorography. Under these conditions, the rate translocation of [35S]proOmpA into IMVs is independent of SecD/F (Figure 5A), with ~20% [35S]proOmpA translocated at 5 min in both IMV preparations. Reactions containing 5 mM succinate, in order
to generate a $\Delta \mu H^+$, but otherwise identical to those in Figure 5A, revealed similar rates of $[^{35}S]$proOmpA translocation into vesicles containing or lacking SecD/F (data not shown). To investigate the proton electrochemical gradient component of translocation, we carried out similar translocation reactions with subsaturating concentrations of ATP present (Figure 5B). In contrast to the above results, the limited translocation of $[^{35}S]$proOmpA into IMVs seen in the presence of only 2 $\mu$M ATP strictly required SecD/F (lanes 1–4 versus 5–8). In the absence of a generated proton electrochemical potential, no fully translocated $[^{35}S]$proOmpA was observed in both IMV preparations (data not shown). These results are consistent with the previous report that a generated $\Delta \mu H^+$ can lower the $K_{\text{m}}$ (ATP) for translocation (Shiozuka et al., 1990) and increase the coupling ratio between ATP hydrolysis and proOmpA translocated (Driessens, 1993).

Antibodies directed against SecD can block the secretion of various precursor proteins, including proOmpA, from spheroplasts, suggesting that SecD is involved in the release of preproteins from the periplasmic face of the inner membrane. The experiments shown in Figure 5 were carried out using $[^{35}S]$proOmpA at concentrations far below the number of translocation sites in IMVs (Bassilana and Wickner, 1993). In order to address whether SecD/F were involved in the release of proOmpA from the periplasmic side of the membrane, we examined proOmpA translocation using saturating concentrations of this preprotein (1 $\mu$M; Crooke et al., 1988b; Bassilana and Wickner, 1993) and 2 mM ATP. IMVs that contain (Figure 6A, lanes 4–6) or lack SecD/F (lanes 7–9) were able to translocate multiple turnovers of proOmpA, ~12 pmol proOmpA in 30 min [based on previous quantitation (Bassilana and Wickner, 1993), 10 $\mu$g of IMVs contain 1.2–1.4 pmol of translocation sites]. These results indicate that the presence of SecD and SecF is not required for clearing of the proOmpA from the translocation sites, although they could accelerate this reaction in vivo.

In vivo, mutations in SecD and SecF result in a cold-sensitive growth phenotype and the accumulation of different precursor proteins at the non-permissive temperature (Gardel et al., 1990). To determine whether the depletion of SecD/F...
from IMVs results in a cold-sensitive translocation defect in vitro, we investigated the temperature dependence of proOmpA translocation (Figure 6B). IMVs that contain or lack SecD/F were able to translocate [35S]proOmpA efficiently between 20 and 37°C (lanes 2–11), suggesting that the cold-sensitive step in vivo is not rate limiting in vitro. In the presence of 1 μM proOmpA, although the amount of translocation was significantly reduced at lower temperature, similar levels of proOmpA were translocated into vesicles that contained or lacked SecD/F (data not shown).

Proteoliposomes with purified SecY/E can translocate preMBP

The translocation of preMBP shows a striking defect in IMVs which lack SecD/F. To examine whether SecD/F are absolutely required for preMBP translocation, SecY/E was purified as described through the DEAE ion-exchange step (Brundage et al., 1990). The SecY/E was reconstituted into E. coli phospholipid proteoliposomes by detergent dilution. Figure 7B shows an immunoblot analysis of SecY/E proteoliposomes compared to IMVs. SecY/E proteoliposomes have undetectable amounts of SecF (lanes 3 and 4), <5% the SecD present in IMVs (lanes 5 and 6), and have 8.6 times the amount of SecY present compared to IMVs (lanes 1 and 2). Normalizing the SecD content of SecY/E proteoliposomes to the amount of SecY present indicates that <1% SecD remains compared to IMVs. To determine whether SecY/E proteoliposomes lacking SecD/F could translocate preMBP, [35S]preMBP was diluted from 2 M guanidine chloride into a reaction mixture containing SecY/E proteoliposomes, SecA and SecB. Translocation reactions were initiated by the addition of 4 mM ATP. Samples were chilled to 0°C, sedimented through a sucrose solution, treated with proteinase K at 0°C and analyzed by SDS–PAGE followed by fluorography. SecY/E proteoliposomes translocated preMBP (lanes 4–9) and this translocation required ATP (lane 10). At 60 min, ~10% of the [35S]preMBP had translocated into the lumen of the proteoliposomes. We conclude that ATP-driven translocation does not require SecD/F.

Discussion

The roles of SecD and SecF in protein translocation have been elusive. We have investigated the functions of these proteins using a strain in which it is possible to fully deplete the inner membrane of both SecD and SecF. Our results demonstrate a specific effect of SecD/F depletion on the translocation of a ΔψH+–dependent precursor protein (preMBP) and little or no effect on proOmpA translocation in the presence of high concentration of ATP. The processing of preMBP, which needs only ATP and reflects the translocation of the leader sequence, is largely unaffected by the depletion of SecD/F. However, the translocation of the entire mature domain of preMBP shows a striking kinetic defect in the absence of SecD/F. We have also shown that SecD/F depletion results in an undetectable ΔψH+ in IMVs and a decrease in Δψ in vivo. Although the optimal, ΔψH+-driven preMBP translocation is dependent on the presence of SecD/F, ATP-driven preMBP translocation is observed in the absence of SecD/F in both depleted IMVs and purified SecY/E proteoliposomes. These results show that SecD/F are not necessary for ATP-dependent translocation. While SecD/F are required for ΔψH+ stimulation of translocation, our results do not establish whether SecD/F act directly or indirectly in coupling ΔψH+ to translocation.

Role of SecD/F in vivo protein secretion

Both our results and other in vivo studies (Gardel et al., 1987, 1990; Pogliano and Beckwith, 1994) demonstrate that SecD/F are not strictly required for protein secretion. Mutations thus far isolated in secD/F by two different secretion screens have only identified cold-sensitive alleles (Gardel et al., 1987; Riggs et al., 1988). It appears that the cold-sensitive growth and secretion phenotypes are caused
by a different rate-limiting step in the secretion process at low temperatures and are not indicative of a thermal instability in the mutant proteins (Pogliano and Beckwith, 1993). In contrast to secY, secE and secA, secD/F are non-essential for growth at 37°C (Pogliano and Beckwith, 1994). Although secretion is impaired either with secD/F mutations or in the absence of secD/F, these proteins are not strictly required for cell viability under physiological conditions. A striking example of the difference in the cellular requirement for SecE and SecD/F is that deletion of SecE results in a growth defect starting three doublings after SecE synthesis has been turned off and, subsequently, cell inviability (unpublished data). In contrast, SecD/F deletion results in a slight growth defect starting ~10 doublings after SecD/F synthesis has been shut off and cells remain viable at 37°C. Consistent with these in vivo observations, we have shown that inner membrane vesicles and purified SecY/E/A reconstituted into proteoliposomes, both of which lack SecD/F, can translocate two different precursor proteins: an outer membrane protein and a protein residing in the periplasmic space.

From in vivo secretion studies on cells lacking secD/F, the role of these proteins in the secretion defect is unclear. The processing of proOmpA in vivo is much slower in cells lacking SecD/F, yet pulse-chase analysis indicates that all of the proOmpA initially labeled is converted to OmpA within 10 min (our work; Pogliano and Beckwith, 1994). While $\Delta_{\mu}H^+$ appears to be essential for protein secretion in vivo, different preproteins exhibit different sensitivities to dissipation of $\Delta_{\mu}H^+$ (Daniels et al., 1981). Intact cells depleted of SecD/F show a modest but reproducible decrease in the magnitude of $\Delta_{\psi}$. Dissipation of $\Delta_{\mu}H^+$ by similar amounts results in a substantial translocation defect in vivo (Daniels et al., 1981; Bakker and Randall, 1984). Taken together, these in vivo and in vitro measurements of $\Delta_{\mu}H^+$ strongly suggest that depletion of SecD/F is associated with a decrease in magnitude of $\Delta_{\mu}H^+$.

**Role of SecD/F in $\Delta_{\mu}H^+$-dependent translocation**

Recently, Matsuyama et al. (1993) have shown that antibodies directed against SecD specifically inhibit the secretion of precursor proteins from spheroplasts. These experiments suggest a role for SecD in the release of proteins from the periplasmic side of the inner membrane. Our in vitro and in vivo data indicate that such a block is not absolute and most likely results in a delay of precursor protein release. For example, in IMVs lacking SecD/F, the rate of preMBP translocation was reduced ~5-fold, yet preMBP translocation continued past 30 min. Attempts to isolate a blocked translocation intermediate under these conditions were unsuccessful.

PreMBP processing in the absence of SecD/F is largely unperturbed, suggesting that SecD/F are involved in a late step of translocation such as preMBP release. In vivo, processing of preMBP to MBP can occur prior to translocation of the entire protein across the inner membrane (Randall, 1983; Thom and Randall, 1988). Similarly, processing of proOmpA occurs in an initial ATP-dependent step and a proton electrochemical gradient is sufficient to drive the completion of OmpA translocation across the membrane in a latter step (Geller and Green, 1989; Tani et al., 1991; Schiebel et al., 1991). Moreover, it has recently been shown in vivo that dissipation of $\Delta_{\mu}H^+$ results in the accumulation of a membrane-bound processed intermediate of preMBP located on the periplasmic side of the inner membrane (Geller, 1990). These results suggest that $\Delta_{\mu}H^+$ is involved in the release of preMBP, and perhaps other preproteins, from the periplasmic side of the membrane. Genetic studies have also suggested that SecD/F are involved in a latter step of preprotein translocation (Gardel et al., 1990; Bieter-Brady and Silhavy, 1992).

The rate of proOmpA translocation into proteoliposomes co-reconstituted with purified SecY/E and the light-driven proton pump bacteriorhodopsin increases up to 7-fold upon generation of a $\Delta_{\mu}H^+$ by illumination (Brundage et al., 1990; Driessen, 1992). These results demonstrate that SecD/F are not essential factors required for coupling $\Delta_{\mu}H^+$ to stimulation of translocation. However, SecD/F could have an important role in enhancing or increasing the efficiency of translocation, or may indeed exert preprotein-specific effects.

Our results, taken with the recent work from Matsuyama et al. (1993), suggest that SecD/F are involved in a $\Delta_{\mu}H^+$-dependent release step from the periplasmic side of the inner membrane. This role for SecD/F offers an explanation as to why different precursor proteins show different severities of translocation defects with secD/F in vivo and in vitro. A striking example of this preprotein-specific SecD/F dependence is the in vitro translocation of [Y283D]preMBP [preMBP with a point mutation in the mature domain (Chun et al., 1993)], which shows no difference in its rate of translocation into IMVs which contain and lack SecD/F (unpublished data). In addition, our results explain why SecD/F were not found to be required for proOmpA translocation in reconstituted SecY/E proteoliposomes capable of multiple cycles of proOmpA translocation (Brundage et al., 1990; Bassiliana and Wickner, 1993).

The role of SecD/F at the molecular level in $\Delta_{\mu}H^+$-dependent translocation is unclear. We envision two possibilities with respect to this function of SecD/F in preprotein translocation. SecD/F could be loosely associated components of preprotein translocase which either prevent dissipation of $\Delta_{\mu}H^+$ or convert this energy source to net chain movement. Alternately, SecD/F might not be associated with translocase, but could prevent the dissipation of the proton electrochemical gradient across the inner membrane. Our results do not differentiate between these two possibilities for SecD/F function. Genetic studies which examined the titration of conditional sec alleles by suppressor-directed inactivation complexes [a toxic preprotein with a defective signal sequence targeted to the signal sequence suppressor prA (secY)] suggested that both secD and secF are components of the translocation complex (Bieker-Brady and Silhavy, 1992). Biochemically, SecY and SecE co-purify and co-immunoprecipitate (Brundage et al., 1990, 1992), strongly suggesting that these proteins form a complex; yet SecD and SecF have not yet been detected in such complexes. With the ability to generate inner membrane vesicles that contain or lack SecD/F, and the identification of precursor proteins and translocation conditions which show a SecD/F dependence, it may be possible to develop an assay for SecD/F to aid in the purification of functional SecD/F. In the absence of direct temperature-sensitive mutations in SecD/F, further genetic and biochemical studies will be necessary to determine whether SecD/F are directly involved in translocation or exert their effect via the proton electrochemical gradient.
Materials and methods

Biologicals

JP91 (ΔlacX74, rpl150, thi secDF15::kan, penBv4e-1-2, zad::Tn10, leu+, araΔ714, recA::cat/GAP[P_1BADsecE+]) and JP135 (ΔlacX74, rpl150, thi secDF15::kan, penBv4e-1-2, zad::Tn10, leu+, araΔ714, recA::cat/GSH1[secDF1+]) E. coli strains were kind gifts from Dr. Joe Poglianico and were constructed as described (Poglianico and Beckwith, 1994). E. coli extracts were prepared with (MC1000, ara-) or (MC1000, phaΔPwA, penBv4e-1, zad::Tn10etStr2, secE1911, recE::cat/Pcm22[P_1BADsecE+]).

Measurements of Δψ and ΔpH

Δψ, inside positive, and ΔpH, inside acidic, of IMVs were initially determined using oxonol VI and 9-amino-chloro-2-methoxycacidine, respectively (Arkowitz et al., 1993). Measurements were made with 25 or 50 μg IMV protein and an electrochemical potential was generated by the addition of 2 mM succinate. Δψ, inside positive, of IMVs was quantitated by measuring the distribution of [14C]KSCN in the inverted membrane vesicles using flow dialysis (Reenstra et al., 1980). The upper and lower chambers of the flow dialysis apparatus (Jens Duborg Co., MA) were separated by Spectrapor 2 dialysis membrane (Fisher Scientific Co.). The flow dialysis apparatus was kept at 25°C unless indicated. The upper chamber contained 0.2 ml of 1 mg/ml IMVs in buffer C [100 mM KCl (pH 7.0), 5 mM MgSO4, 20 μM [14C]KSCN and was stirred with a magnetic bar. Oxygen-saturated buffer C was pumped through the lower chamber at 1.3 ml/min and fractions were collected (1–5, 1.2 ml; and 6–65, 0.47 ml). Succinate (4.7 mM) was added at fraction 25 and a stream of buffer C-saturated oxygen was passed over the reaction in the upper chamber. Carboxyl cyanide m-chlorophenylhydrazonene (CCCP) (18.8 μM) was added at fraction 45 and the oxygen stream was removed from the upper chamber.

In vitro translocations

Translocation into inverted inner membrane vesicles was performed in buffer A (50 mM HEPES-KOH (pH 8.0), 50 mM KCl, 5 mM MgCl2, 2 mM DTT, 0.1 mg/ml fatty acid-free BSA) containing SecY and SecE as indicated. An ATP-regenerating system consisting of 5 mM creatine phosphate and 10 μg/ml creatine kinase was used when ATP was present. Reactions for time courses were warmed to 37°C for 2 min and then initiated by the addition of ATP. Single time point translocation reactions had ATP present at all time points and were initiated by warming to the indicated temperature. Succinate was added to 50 mM final concentration for generation of proton electrochemical gradient 1 min prior to initiation of the translocation reaction. Reactions were terminated by chilling on ice. PreOmpA translocation reactions were treated with 1 mg/ml proteinase K for 20 min at 0°C, whereas preMBP translocation reactions were sedimented through 125 μl of 1 M sucrose, 50 mM Tris-Cl (pH 7.9), 50 mM KCl in an airbag (30 min, 30 psi, 4°C). Sediment was resuspended with 50 μl of buffer B (50 mM HEPES-KOH (pH 8.0), 50 mM KCl, 5 mM MgCl2) on ice and treated with 1 mg/ml proteinase K at 0°C (20 min for the IMVs and 30 min for proteoliposomes). Digestions were stopped by the addition of an equal volume of ice-cold 50% trichloroacetic acid and incubated for at least 1 h at 0°C. Precipitated protein was collected by centrifugation (Eppendorf centrifuge, 10 min, 4°C), resuspended with 1 ml of ice-cold acetone and centrifuged. Acetone was removed by aspiration, followed by incubation at 37°C to remove residual acetone. The sediment was resuspended in 40 μl SDS sample buffer (containing 5 mM PMSF for preMBP samples) and heated for 2 min at 95°C. Samples were analyzed by SDS–PAGE followed by immunoblotting or fluorography. Gels were immunoblotted individually to PVDF with a semi-dry transfer system (ABN Polyblot, American Bioticas) for 30 min at 250 mA. PVDF membranes were probed with 1:1000 diluted anti-MBP sera and quantitated using 2–5 μCi [125I]protein A.

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References


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