The PrlA and PrlG phenotypes are caused by a loosened association among the translocase SecYEG subunits

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***prlA* mutations in the gene encoding the SecY subunit of the membrane domain of the *Escherichia coli* preprotein translocase confer many phenotypes: enhanced translocation rates, increased affinity for SecA, diminished requirement for functional leader sequences, reduced proton-motive force (PMF) dependence of preprotein translocation and facilitated translocation of preproteins with folded domains. We now report that both *prlA* and *prlG* mutations weaken the associations between the SecY, SecE and SecG subunits of the translocase. This loosened association increases the initiation of translocation by facilitating the insertion of SecA with its bound preprotein but reduces the stimulatory effect of the PMF during the initial step of translocation. Furthermore, the originally isolated *prlA4* mutant, which possesses a particularly labile SecYEG complex, acquired a secondary mutation that restored the stability while conserving the flexibility of the complex. Combinations of certain *prlA* and *prlG* mutations, known to cause synthetic lethality *in vivo*, dramatically loosen subunit association and lead to complete disassembly of SecYEG. These findings underscore the importance of the loosened SecYEG association for the Prl phenotypes. We propose a model in which each of the PrlA and PrlG phenotypes derive from this enhanced SecYEG conformational flexibility.**
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Introduction

Preproteins of *Escherichia coli* with classical apolar leader (signal) sequences cross the plasma membrane by means of a multisubunit preprotein translocase. The translocase is composed of two trimeric integral membrane domains, SecYEG and SecDFyajC, and a peripheral membrane domain, SecA (for reviews see Ito, 1996; Wickner and Leonard, 1996; Duong *et al.*, 1997; Danese and Silhavy, 1998; Driessen *et al.*, 1998; Economou, 1998). Genetic and biochemical studies, as well as evolutionary comparisons, demonstrate that SecY and SecE are the physical and functional core of the integral membrane domain of translocase, while SecDFyajC or SecG serve to promote SecYE function (Schatz and Beckwith, 1990; Hartmann *et al.*, 1994; Duong and Wickner, 1997a).

Biochemical dissection has led to a model of preprotein translocation (reviewed in Duong *et al.*, 1997; Danese and Silhavy, 1998). After interaction with chaperones, preproteins associate with the SecA subunit, itself bound to the SecYEG membrane domain of the translocase. The translocation of preprotein is then initiated by the binding of ATP to SecA. This results in a substantial conformational change, causing large 30 and 65 kDa domains of SecA to be inserted into the translocase (Economou and Wickner, 1994; Eichler and Wickner, 1997). This insertion of SecA carries the N-terminal loop of the preprotein across the membrane, initiating translocation (Schiebel *et al.*, 1991). Subsequent ATP hydrolysis allows SecA to release the preprotein and de-insert from the membrane, thereby preparing for additional rounds of ATP-driven preprotein movement (Economou and Wickner, 1994). During this translocation cycle, SecDFyajC stabilizes SecA in its membrane-inserted state to allow the accumulation of translocation intermediates which are then driven forward by the proton-motive force (PMF; Schiebel *et al.*, 1991; Duong and Wickner, 1997b). The PMF was also shown recently to promote the de-insertion step of the SecA cycle (Nishiyama *et al.*, 1999), thereby facilitating translocation.

Genetic studies defined most of the components of the translocase. Many of the *sec* genes were first identified by *prl* mutations (Bieker *et al.*, 1990; Schatz and Beckwith, 1990), which restore normal protein localization to preproteins with defective leader sequences. These suppressor mutations map in *secY* (*prlA*), *secE* (*prlG*) and *secA* (*prlD*) (Emr *et al.*, 1981; Fikes and Bassford, 1989; Stader *et al.*, 1989). More recently, *prlH* mutations were obtained in *secG* (Bost and Belin, 1997). Strikingly, the *prl* suppressors modify the specificity of the translocase, since these mutations allow significant export of preproteins with a wide variety of defective, or even deleted, leader sequences (Derman *et al.*, 1993; Flower *et al.*, 1994; Prinz *et al.*, 1996). This lack of allele specificity has suggested that the *prl* mutations broaden the selectivity, or disrupt the 'proof-reading' activity, of the translocase (Osborne and Silhavy, 1993).

Further biochemical analysis revealed that the *prlA* mutations in *secY* not only affect leader peptide recognition but also promote the rate of translocation of normal preproteins and obviate the stimulatory effect of the PMF on translocation (Nouwen *et al.*, 1996a; Perez-Perez *et al.*, 1996). The well-characterized *prlA4* mutation also enhances the affinity of SecA for the SecYEG domain of the translocase (van der Wolk *et al.*, 1998), enhances the dependence on SecB (Derman *et al.*, 1993; Francetic *et al.*, 1993) and relieves the *in vitro* translocation blockage caused by certain folded structures in the mature domain of a preprotein (Nouwen *et al.*, 1996a). Moreover, the *prlA4* mutation modulates the topology of an engineered integral membrane protein and corrects the membrane

insertion defects caused by positive charges in the N-terminal region of protein pIII of phage fd (Peters *et al.*, 1994; Prinz *et al.*, 1998). Thus, the mechanism of *prl* suppression is more complex than initially anticipated. The *prl* mutations might affect various subreactions of the translocase. Alternatively, these mutations may modify a single biochemical property of the enzyme which could then cause these seemingly unrelated effects. Thus, a better understanding of the *prl* mutants should provide insight into several aspects of the translocation mechanism.

We now report that a primary effect of *prlA* and *prlG* mutations is to loosen the association between SecY, SecE and SecG subunits of the SecYEG complex. The strength of the SecYEG association correlates with the efficiency of the initiation of translocation and with its PMF dependence. We propose that the diverse properties of *prl* mutants are likely to derive primarily from this enhanced translocase conformational flexibility.

Results

PrIA mutations loosen SecYEG associations

The suppressor mutations of the different *prlA* alleles are clustered in regions corresponding to three distinct topological domains of SecY; the first periplasmic loop (P1) and transmembrane helices 7 and 10 (TM7 and TM10, respectively; Emr *et al.*, 1981; Osborne and Silhavy, 1993). *prlA* mutations were also isolated in TM2 and TM5 (Francetic *et al.*, 1993; Osborne and Silhavy, 1993). These clusters of *prlA* mutations may not define an active site of SecY but may rather have a general effect on the conformation of the protein. Since SecY, SecE and SecG function as subunits of the SecYEG complex, *prlA* mutations may have altered the interactions between these subunits. Since the wild-type complex is somewhat thermolabile in detergent micellar solution (Brundage *et al.*, 1992), we have studied the thermostabilities of the SecYEG complexes with wild-type SecY or with *PrIA* subunits.

We have analyzed *prlA4*, *prlA3* and *prlA401* mutations in TM7/TM10, P1 and TM7, respectively (Emr *et al.*, 1981; Sako and Iino, 1988). For ease of analysis, each of the *PrIA*/SecY proteins was co-expressed with SecE and SecG from their genes on a plasmid-based arabinose-inducible promoter, with the SecE protein bearing a hemagglutinin (HA) epitope (Douville *et al.*, 1995). After induction with arabinose and [³⁵S]methionine radio-labeling of the cells, membranes were purified and a detergent extract was prepared on ice with β -octylglucopyranoside and *E. coli* phospholipids. As shown previously, SecY and SecE subunits do not exchange between complexes (Joly *et al.*, 1994) and the anti-HA antibodies allowed us selectively to immunoprecipitate plasmid-synthesized SecYEG complexes (Figure 1). The *prlA* mutations did not affect the synthesis levels of SecY, SecE and SecG or their ratio in each complex (Figure 1, compare odd lanes). However, when the detergent extracts were warmed to >33°C (Figure 1, even lanes), the quantity of SecYEG complexes recovered by immunoprecipitation was strongly reduced by the *prl* mutations. Since the total quantity of HA-SecE immunoprecipitated is not affected by temperature, we conclude that the *prlA* mutations confer an increased lability on the SecYEG complex.

Fig. 1. The *prlA* mutations weaken SecYEG association. [³⁵S]methionine-labeled membranes (~10⁶ c.p.m.) from *E. coli* BL21 overexpressing the wild-type or *prlA*-mutated SecYEG complex were solubilized on ice with the non-denaturing NDIP buffer containing 1.25% β -octyl glucoside, as described in Materials and methods. Extracts were incubated further at 4 or 33°C for 5 min before immunoprecipitation with anti-HA antibodies pre-bound to protein A-Sepharose beads. The immunoprecipitates were analyzed by 17% SDS-PAGE and fluorography. The following mutations were introduced into the *secY* gene on the pHA-EYG plasmid: *prlA4* (F286Y + I408N), *prlA3* (F67C), *prlA401* (S282R) and Y402 (S282L).

At temperatures >39°C, both wild-type and mutated complexes are completely dissociated (not shown).

To establish that the lowered stability of the complex is a true characteristic of the *prlA* mutations, an allelic form of *prlA401* was constructed and termed Y402. The *prlA401* mutation substitutes arginine for serine at residue 282 in TM7 of SecY (S282R; Sako and Iino, 1988) and the Y402 mutation substitutes leucine for serine at the same position (S282L; this study). In contrast to *prlA401*, the trimeric complex containing the mutation Y402 retains the wild-type thermostability (Figure 1, lanes 9 and 10) and, as expected, the Y402 mutation does not confer the *in vitro* Prl translocation phenotype (see below). Finally, an export-defective mutant of SecY (mutant *secY205*; Y429D; Taura *et al.*, 1997) exhibited the same thermostability as wild-type complex (not shown).

Both *prlA* mutations and PMF stimulate the initiation of translocation

The *prl* mutations were selected to permit the translocation of preproteins with altered leader peptides. Further *in vitro* characterization with inner membrane vesicles (IMVs) showed that *prlA* mutations also confer an improved translocation efficiency which no longer requires the stimulatory action of the PMF (Nouwen *et al.*, 1996a; van der Wolk *et al.*, 1998). We therefore tested whether the strength of SecYEG complex association correlates with its *in vitro* translocation capacity and dependence on the PMF. Two assays were employed to measure the activity of the translocase, the formation of an arrested translocation intermediate of 29 kDa from proOmpA bearing covalently coupled bovine pancreas trypsin inhibitor (BPTI; Schiebel *et al.*, 1991; Duong and Wickner, 1997b) and the ATP-driven membrane insertion of SecA, rendering its 30 kDa C-terminal domain inaccessible to proteases (Economou and Wickner, 1994). These two assays were performed in the same conditions but with trace amounts of either [¹²⁵I]proOmpA-BPTI (Figure 2A) or [¹²⁵I]SecA (Figure 2B). Since the *prlA4* mutation increases the affinity of SecA for SecYEG, experiments were performed at concentrations well above the *K_d* value of SecA for the translocase (van der Wolk *et al.*, 1998) and reactions were

Fig. 2. Initiation of translocation is facilitated by PMF and *prlA* mutations. (A) Translocation reactions were performed in 50 μ l of TL buffer containing SecA (15 μ g/ml), SecB (48 μ g/ml), BSA (200 μ g/ml), wild-type or *prlA* mutated SecYEG-enriched IMVs (100 μ g/ml) and [125 I]proOmpA–BPTI (~80 000 c.p.m.) pre-mixed with unlabeled proOmpA–BPTI (5 μ g/ml). After pre-incubation (2 min, 37°C) with either the uncoupler CCCP (20 μ M) or the PMF-generating nucleotide NADH (5 mM), translocation reactions were initiated with 200 μ M ATP and stopped after 5 min by chilling on ice. Samples were digested with proteinase K (1 mg/ml, 15 min, 0°C) and treated with 15% ice-cold TCA. Translocated material was analyzed by 15% SDS–PAGE and fluorography. (B) SecA insertion reactions were performed as described above except that [125 I]SecA (~60 000 c.p.m.) was added instead of [125 I]proOmpA–BPTI. After 5 min at 37°C in the presence of 200 μ M ATP, reactions were stopped by apyrase addition (20 U/ml, 2 min, 37°C) and chilled on ice. The inserted 30 kDa fragment of SecA was revealed by digestion with trypsin (1 mg/ml, 15 min, 0°C). (C) Insertion of [125 I]SecA–D209N (~60 000 c.p.m.) pre-mixed with unlabeled SecA–D209N (10 μ g/ml) was performed as described above except that wild-type SecA was omitted. The reaction was stopped by chilling on ice and the inserted 30 kDa fragment of SecA was revealed by digestion with proteinase K (1 mg/ml, 15 min, 0°C).

stopped by apyrase addition to lock SecA in its inserted state (Economou *et al.*, 1995). As expected, *prlA4*, *prlA3* and *prlA401* mutations in *secY* confer PMF independence to proOmpA–BPTI translocation and SecA insertion (Figure 2A and B, lanes 3–8). In contrast, both wild-type and SecY402/EG translocases remain strongly stimulated by the PMF (Figure 2, lanes 1, 2 and 9, 10). Thus, there is a complete correlation between those mutations which confer the Prl translocation phenotypes, which loosen the association of the SecYEG subunits and which render translocation independent of PMF.

The formation of translocation intermediate I_{29} and inserted SecA requires activated translocation sites. Our results also suggest that the initiation of translocation is stimulated by the *prlA* mutations and the PMF. To test this hypothesis, the SecA insertion assay was performed with SecA–D209N which carries a mutation in the nucleotide-binding site I of SecA (Mitchell and Oliver, 1993). This mutant inserts into the membrane in a preprotein-dependent manner but cannot hydrolyze ATP and de-insert (Economou *et al.*, 1995). When tested with PrlA IMVs, SecA–D209N insertion occurred as for wild-type SecA and was unaffected by the PMF (Figure 2C, lanes 3–8). In contrast, SecA–D209N insertion was still stimulated by the PMF in wild-type or SecY402/EG IMVs (Figure 2C, lanes 1, 2, and 9, 10). In all cases, ATP and preprotein were required for SecA–D209N insertion to occur (not shown). Thus both the *prlA* mutations and the PMF facilitate the initiation of translocation by increasing the co-insertion of preprotein and SecA into the membrane.

Fig. 3. Effects of mutations F286Y (*A4s*) and I408N (*A4r*) which comprise *prlA4*. (A) The mutation *A4s* restores stability to the *A4r*-mutated SecYEG complex. [35 S]methionine-labeled membranes (~10⁶ c.p.m.) from *E. coli* BL21 overexpressing the wild-type or mutated SecYEG complex were solubilized as described in Figure 1 and the extracts further incubated at 4 or 37°C for 5 min before immunoprecipitation with anti-HA antibodies. The immunoprecipitates were analyzed by 17% SDS–PAGE and fluorography. (B) The PMF dependence and the efficiency of translocation and SecA insertion correlate with the stability of the SecYEG complex. [125 I]SecA insertion and [125 I]proOmpA–BPTI translocation experiments were performed as in Figure 2.

The secondary mutation that occurred in *prlA4* has an anti-*prl* effect

Several *prlA* strains contain two distinct mutations in SecY but only one seems to be responsible for the suppression (Osborne and Silhavy, 1993). For one double mutant, the suppressor mutation causes a growth defect (Francetic *et al.*, 1993) which apparently selected for the second mutation. The best characterized export suppressor, *prlA4*, is a double mutant consisting of the I408N mutation in TM10 (termed *A4r* in this study) and the F286Y mutation in TM7 (termed *A4s* in this study). The *A4r* mutation is postulated to be responsible for the suppression (Flower *et al.*, 1995), while the *A4s* mutation is thought to compensate for the growth defect of *A4r*. To study the biochemical properties of the complex containing either the *A4r* or *A4s* mutations in *secY*, we used the plasmid-based expression of SecYEG while maintaining the wild-type chromosomal copy of *secYEG* genes to avoid deleterious effects of the *prl* mutations on cell growth.

The inherent thermolability of wild-type SecYEG complex (Figure 3A, lanes 1 and 2; 52% dissociation at 37°C in detergent micellar solution) is strongly enhanced by the *prlA4* double mutation (lanes 7 and 8; 92% dissociation). However, while the complex recovered from cells with the plasmid expressing the *A4r* mutation is still thermolabile (lanes 5 and 6; 88% dissociation), the total level of complex recovered is reduced (compare lanes 5 and 1; 52% reduction). We suggest that the reduced level of PrlA4r/SecEG proteins reflects the instability of the

complex and may explain why cells have naturally acquired the secondary mutation *A4s*. Indeed, the mutation *A4s* restores to *A4r* the normal level of PrlA4/SecEG (Figure 3A, lane 7 versus 1; 102%), and the *A4s* mutation by itself has even less dissociation at 37°C (lane 4; 33%) than the wild type (lane 2; 52%). Thus, since the original *A4r* mutation weakened the SecYEG subunit association, the secondary mutation *A4r* may have been required to partly compensate for this instability and thereby restore functionality.

Accordingly, while the *prlA4r* mutant supports only reduced levels of translocation or SecA insertion (Figure 3B, lanes 5 and 6), the mutation *A4s* combined with *A4r* restores normal levels of translocation and maintains the PMF-independent character of the *A4r* mutant (lanes 7 and 8). As for wild type, translocation and SecA insertion for the *prlA4s* mutant are stimulated by the PMF (lanes 3 and 4). Thus, a stability of SecYEG association accompanies PMF-dependent translocation while a lability of the complex confers PMF independence and the Prl translocation phenotype. We propose that the combination of the *A4s* and *A4r* mutations in the *prlA4* mutant has maintained the flexibility of SecYEG association required for the Prl suppression while conferring sufficient stability to the complex to allow function and cellular growth.

***prlG* mutations in *secE* also show PMF-independent translocation and labile SecYEG interactions**

SecY and SecE are the physical and functional core of the integral membrane domain of translocase. While *prlG* alleles of *secE* are weak suppressors, both *prlA* and *prlG* suppress the same spectrum of signal sequence mutations (Flower *et al.*, 1994). Thus, the mechanism of *prlG* and *prlA* suppression may be the same.

ProOmpA-BPTI translocation, SecA insertion and SecYEG immunoprecipitation experiments were performed with membranes from cells bearing the SecYEG expression plasmid encoding PrlG1 (mutation L108R in TM3) or PrlG3 (mutation S120F in P2), the best characterized *prlG* suppressors (Flower *et al.*, 1994, 1995). As seen for the *prlA* mutants, both the translocation of proOmpA-BPTI and the insertion of SecA are fully independent of the PMF (Figure 4A, lanes 3–6). This observation contrasts with previous *in vivo* studies which indicated that preprotein translocation in *prlG1* cells is affected by the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Nouwen *et al.*, 1996a). However, we find that these *prl* mutants, and especially *prlG1*, support only a reduced level of translocation or SecA insertion when compared with the wild type (lanes 1 and 2). As seen for the mutant *A4r* described above, the *prlG1* and *prlG3* mutations may confer instability on the SecYEG complex. Indeed, the total level of SecYEG complexes recovered by immunoprecipitation is reduced by the *prlG* mutations (Figure 4B, lanes 1–3), correlating with the observed low translocation activities (Figure 4A). This reduced amount of SecYEG complex conferred by the *prlG1* mutation, in accord with previous observations (Pohlschröder *et al.*, 1996), may explain the weak translocation and suppression activities of the *prlG1* mutant and may confer a higher sensitivity of the cells to CCCP (Flower *et al.*, 1994; Nouwen *et al.*, 1996a; Perez-Perez

Fig. 4. *prlG* mutations in SecE confer the same effects as *prlA* mutations. (A) Translocation in *prlG1* (L108R) and *prlG3* (S120F) IMVs is decreased but still PMF independent. [¹²⁵I]proOmpA-BPTI translocation and [¹²⁵I]SecA insertion experiments were performed as described Figure 2 but using IMVs prepared from BL21 carrying the *prlG*-mutated pHA-EYG plasmid. (B) The level of SecYEG complex is decreased by the *prlG* mutations but the thermolability is conserved. Immunoprecipitations of extracts from [³⁵S]methionine-labeled membranes containing the *prlG*-mutated SecYEG complex were performed as described in Figure 1.

et al., 1996). Nonetheless, the *prlG* mutations also confer a thermolability similar to the *prlA* mutants (Figure 4B, lanes 4–6). Thus, the PMF independence of translocation and the striking alterations in SecYEG stability are also found in *prlG* mutants, suggesting that *prlG* and *prlA* suppressors work by the same mechanism.

Allele-specific lethality and lability of *prlA*-*prlG* double mutants

A systematic genetic analysis revealed that certain pairs of *prlA* and *prlG* alleles cannot be combined in the same strain (Flower *et al.*, 1995). For instance, it is not possible to construct the double mutant *prlA4*-*prlG1* on the *E. coli* chromosome (Bieker and Silhavy, 1990). This synthetic lethality, which is recessive and allele specific, has been proposed to reflect inactive SecYE complexes which cause a lethal protein secretion defect (Flower *et al.*, 1995).

To understand better the molecular basis of this lethality, *prlA4* (consisting of *A4r* and *A4s* mutations) and *prlG1* mutations were combined on the same arabinose-inducible SecYEG plasmid. Cells expressing the altered proteins have only a slight growth defect during prolonged culture, confirming the recessive nature of the mutations. After [³⁵S]methionine radiolabeling, immunoprecipitations revealed that the lability of the SecYEG complex carrying the *prlG1* mutation (Figure 5A, lane 3) is greatly enhanced by the *prlA4* mutation so that only very low levels of complex are recovered (lane 4). Thus, SecYEG complex with these two mutants proteins either cannot form or readily dissociates, probably leading to rapid proteolytic

Fig. 5. SecYEG complex lability reflects allele-specific lethality of *prlA*–*prlG* double mutants. (A) The SecYEG complex is labile in the *prlA4*–*prlG1* double mutant. [35 S]methionine-labeled membranes were prepared from strains expressing SecYEG from plasmid pHA-EYG carrying mutations *prlA4*, *prlG1* or *prlA4* + *prlG1*. Solubilization of the membranes ($\sim 10^6$ c.p.m.) in NDIP buffer was at 4°C and immunoprecipitations of the SecYEG complex were performed as in Figure 1. (B) The lability of the SecYEG complex in the *prlA*–*prlG* double mutants is allele specific. Mutations *prlA726* (S68P) and *prlA8913* (S68F) were introduced on plasmid pHA-EYG or plasmid pHA-EYG carrying the mutation *prlG1* (L108R). After [35 S]methionine labeling of the membranes and solubilization at 4°C, immunoprecipitations were performed as described in Figure 1.

degradation of the Sec proteins (Akiyama *et al.*, 1996; Pohlschröder *et al.*, 1996).

To confirm the correlation between the *prl*-induced lability of the complex and the synthetic lethality, two other double mutants were constructed. The mutation *prlA726* (S68P in P1) and the allelic mutation *prlA8913* (S68F in P1) were combined with *prlG3*. Previous work has shown that the pair A726–G3 is lethal while the pair A8913–G3 is viable (Flower *et al.*, 1995). By themselves, the *prlA726* and *prlA8913* mutations allow normal synthesis of the SecYEG complex (Figure 5B, lanes 1 and 2) and confer upon the SecYEG complex a thermolability which is characteristic of the *prlA* mutations (not shown). When *prlA726* is combined with *prlG3*, very little SecYEG complex can be detected (lane 3), while the combination of *prlA8913* with *prlG3* allows the recovery of a nearly normal amount of SecYEG complex (lane 4). Thus, there is a clear correlation between the pairs of *prl* mutations which confer lethality and their effects on the lability of the complex. As proposed by Flower *et al.* (1995), the allele-specific lethality of the *prlA*–*prlG* double mutants may reflect the alteration of a contact surface between SecY and SecE. A strong alteration of such a contact surface may lead to the complete disassembly of the complex, as shown here, and to lethal translocation defects.

Discussion

prlA and *prlG* mutants do not exhibit allele specificity with respect to the signal sequence defects (Flower *et al.*,

1994). Moreover, *prlA* mutations increase normal pre-protein translocation and the affinity of SecA for SecYEG (van der Wolk *et al.*, 1998), relieve the translocation block caused by a folded structure in the mature domain of the preprotein (Nouwen *et al.*, 1996a), enhance the SecB dependence of translocation (Derman *et al.*, 1993) and even lead to modifications in the bilayer integration of certain membrane proteins (Peters *et al.*, 1994; Prinz *et al.*, 1998). Clearly, *prl* mutations do not simply alter the recognition of the mutant leader peptides but rather modify a basic property of the membrane domain of the translocase.

Our data show that *prlA* or *prlG* mutations cause a unique structural change in the translocase which loosens the association among the SecYEG subunits. Three observations support this conclusion. (i) All the *secY* and *secE* mutations which confer the Prl phenotype decrease the strength of the SecYEG subunit association. (ii) The secondary mutation acquired by the *prlA4* mutant prevents the complete disassembly of SecYEG but maintains the loosened association of the complex. (iii) The pair of *prlA* and *prlG* mutations which are lethal *in vivo* cause the complete disassembly of the SecYEG complex, while the pair which are sufficiently stable allow cellular growth. Thus, the fundamental distinction between the wild-type translocase and the *prlA* or *prlG* translocases resides in the strength of the SecYEG association and probably in the extent of the subunit conformational change which accompanies catalysis. We therefore propose that the genetic selections yielded mutants of SecY or SecE that increase the flexibility of the SecYEG subunit associations while conserving enough stability in the complex to avoid complete dissociation. In agreement with this proposal, the *prlA* and *prlG* mutations are clustered in some periplasmic and transmembrane segments which are proposed to be the contact points between SecY and SecE (Flower *et al.*, 1995), and these alleles show similar patterns of signal sequence suppression (Flower *et al.*, 1994).

What is the molecular relationship between the *prl* mutations and the PMF? The *prl* alleles are able to overcome a secretion defect caused by a defective leader peptide and can alleviate the PMF dependence of translocation (Nouwen *et al.*, 1996a). In turn, the requirement for the PMF can be altered by single point mutations in the leader peptide (Lu *et al.*, 1991; Nouwen *et al.*, 1996b). Thus, in contrast to models where the PMF only plays a role during late translocation (Schiebel *et al.*, 1991; Driessen, 1992; Duong and Wickner, 1997b), these findings suggest that the *prl* mutations and the PMF facilitate the initial steps of translocation, probably by supporting the co-insertion of SecA and the N-terminal loop of the preprotein. In agreement with this proposal, we find that the formation of the translocation-arrested intermediate I_{29} and the concomitant formation of inserted SecA occur more rapidly with the *prl* mutations or the PMF (Figure 2). It is important to note that the level of I_{29} -arrested intermediate obtained with proOmpA–BPTI reflects the actual rate of initiation of translocation and allows measurement of the yield of inserted SecA molecules at active translocation sites. With *prlA4* IMVs, proOmpA translocation occurs very rapidly and the steady-state level of inserted SecA may decrease as the proOmpA substrate is liberated into the lumen of the vesicles (Nishiyama

et al., 1999). Furthermore, by using the SecA-D209N mutant, which inserts in a preprotein-dependent manner only once at the beginning of the translocation cycle and is blocked for de-insertion (Economou *et al.*, 1995), we confirmed that the initial insertion of preprotein and SecA is facilitated by the PMF and the *prl* mutations (Figure 2). We note that AMP-PNP-driven SecA insertion, which allows SecA insertion without preprotein, is not stimulated by the PMF (data not shown; Nishiyama *et al.*, 1999), suggesting that the PMF does not exert its stimulatory effect directly on SecA or the translocase but requires the preprotein. The PMF-dependent electrophoresis of negatively charged residues in the early mature domain of the preprotein (Geller *et al.*, 1993) could facilitate the co-insertion of SecA with the N-terminal loop of the preprotein.

How can one explain the *Prl* phenotypes? Pioneering studies attributed the *Prl* suppression to a proof-reading activity by translocase (Osborne and Silhavy, 1993). It was later proposed that the increased affinity between SecA and SecYEG in the *prlA4* mutant reduces the probability of rejection of defective leader peptides (van der Wolk *et al.*, 1998). While this strengthened binding of SecA to the integral membrane domain of the translocase should facilitate the initiation of translocation of altered leader peptides, it may not necessarily represent the primary effect conferred by the *prl* mutations. Indeed, even at saturating SecA concentrations, the translocation activity for normal preproteins remains higher with *prlA4* membrane vesicles than with wild-type membrane vesicles (Figure 2; van der Wolk *et al.*, 1998; Nishiyama *et al.*, 1999). We propose that the simplest explanation is that each of the *prl* phenotypes is caused by a loosened SecYEG association, which may represent the 'relaxed' state of the translocase postulated by Nouwen *et al.* (1996a). According to this model, a relaxed SecYEG association may lower the energy barrier required to initiate translocation. If the translocase is viewed as a closed channel of SecYEG (Meyer *et al.*, 1999), a loosened interaction between its subunits may allow easier opening and thus initiation of translocation, thereby reducing the need for stimulation by the PMF. The other consequence of the loosened association will be to reduce the stringency or the specificity of the translocase so that it can accommodate preproteins with defective leader peptides. A recent model for the yeast translocase proposes the displacement of Sss1p/SecE from the Sec61p/SecY by the leader peptide during the initiation of translocation (Plath *et al.*, 1998). According to this view, the *prl* mutations should allow easier displacement of the SecYE interaction by even defective leader peptides. This flexibility could also allow a conformational expansion of the translocase channel to create a passageway for slightly folded mature domains of preprotein, alter the lateral release of hydrophobic segments of membrane proteins and strengthen interactions of SecYEG with SecA. A modulated association between the subunits of the mitochondrial outer membrane translocase has also been reported (Hönliger *et al.*, 1996). This modulation, which involves small proteins, may also modify the specificity of that translocase.

Another important question is whether all *prl* suppressors work according to the mechanism outlined for

prlA and *prlG*. Since the *prlH* mutants of SecG are phenotypically similar to *prlA* and *prlG* mutants and suppress the same spectrum of leader sequence mutations (Bost and Belin, 1997), we suspect that the *prlH* mutants function in a similar manner to weaken the SecYEG association. While it is possible that the *prlD* alleles of SecA indirectly modify the SecYEG stability, the mechanism of suppression by *prlD* may be different since the *prlD* mutants suppress a different pattern of leader sequence defects (leader sequences altered in their hydrophilic segments; Flower *et al.*, 1994; Huie and Silhavy, 1995). However, as noted by Oliver and colleagues, the *prlD* suppression relates to the destabilization of the compact quaternary structure of SecA (Ramamurthy *et al.*, 1998). Thus, the basic mechanism of *prl* suppression (destabilization of the structure carrying the *prl* mutation) may not be fundamentally different between *prlA*, *prlG*, *prlH* and *prlD*.

The dynamic interaction of the subunits of preprotein translocase probably plays a critical role in an ordered and selective mechanism of translocation. This dynamic appears particularly important for the initial steps of preprotein translocation. The conformational flexibility afforded by *prl* mutants relaxes the SecYEG subunit association to allow a more efficient initiation of translocation, but does so at the expense of both selectivity and the stimulatory effect of the PMF.

Materials and methods

Materials

ATP, lipid-free bovine serum albumin (BSA), BPTI, *n*-octyl- β -D-glucoside, potato apyrase (grade VIII) and trypsin were purchased from Sigma. *Escherichia coli* phospholipids were purchased from Avanti Polar Lipids and protein A-Sepharose CL-4B beads from Pharmacia. Iodogen and SPDP were from Pierce. AMP-PNP, CCCP, NADH and proteinase K were from Boehringer-Mannheim. SecB (Weiss *et al.*, 1988), the precursor form of outer membrane protein A (proOmpA) (Crooke *et al.*, 1988), wild-type SecA and SecA-D209N (Mitchell and Oliver, 1993) were purified as described previously (Cunningham *et al.*, 1989). SecA-D209N was expressed in strain BL21.19 [λ DE3, *supF*(Ts), *trp*(Am), *Zch*::Tn10, *secA13*(Am), *clpA319*::*kan*, *recA*::*cat*; Mitchell and Oliver, 1993] shifted to 41°C before induction. IMVs from *E. coli* strain BL21 (*hdsS*, *ompT*⁻, *gal*) transformed by the plasmid pHA-EYG and derivatives were prepared according to the procedure of Douville *et al.* (1995). [¹²⁵I]Na (≈ 17 mCi/ μ g) was from Amersham and [³⁵S]methionine protein labeling mix (≈ 1175 Ci/mmol) from Dupont-NEN.

Introduction of *prl* mutations into pHA-EYG

Plasmid pHA-EYG (pBAD 22 carrying HA-*secE*, *secY* and *secG* under control of the arabinose-inducible promoter) has been described previously (Douville *et al.*, 1995). The various *prl* mutations were introduced into plasmid pHA-EYG by site-directed mutagenesis using the Clontech Transformer Mutagenesis kit and following the manufacturer's instructions. All mutations were verified by sequencing the relevant coding regions. Plasmids were maintained in BL21 with 80 μ g/ml of ampicillin and 0.5% glucose in the culture medium to fully repress the arabinose-inducible promoter.

Radiolabeling and immunoprecipitation of SecYEG

For [³⁵S]methionine labeling, *E. coli* BL21 pHA-EYG and derivatives were grown at 37°C in 30 ml of M9 medium, ampicillin (80 μ g/ml), amino acids (40 μ g/ml, except methionine) and 0.2% (w/v) glucose. At $A_{600} = 0.4$, 15 μ Ci/ml of [³⁵S]methionine labeling mix and 0.5% (w/v) arabinose were added for 15 min. The cells were harvested by centrifugation (5 min, 3000 g, 4°C) and resuspended in 10% sucrose, 50 mM Tris-HCl, pH 7.5. [³⁵S]methionine-labeled membranes were prepared for immunoprecipitation as described by Joly *et al.* (1994), using anti-HA monoclonal antibody coupled to protein A-Sepharose beads (Douville *et al.*, 1995). Membranes were first solubilized for 5 min on

ice with 500 µl of NDIP buffer (3.4 mg/ml *E.coli* phospholipids, 35% glycerol, 1.25% β-octyl glucoside, 50 mM Tris-HCl, pH 7.9; Brundage *et al.*, 1992) and then incubated for an additional 5 min at the temperatures indicated in the text. The membrane extract was mixed with 100 µl of protein A-Sepharose-anti-HA antibodies for 60 min at 4°C. The beads were collected by brief centrifugation and washed three times at 4°C with 1 ml of washing buffer (100 mM NaCl, 1.0 mg/ml *E.coli* phospholipids, 30% glycerol, 0.25% β-octyl glucoside, 50 mM Tris-HCl, pH 7.9). Proteins were eluted from the beads by incubation with SDS sample buffer (10 min at 37°C) and electrophoresed in SDS-17% polyacrylamide gels.

Other methods

[¹²⁵I]SecA (~8×10⁵ c.p.m./µg), [¹²⁵I]SecA-D209N (~2×10⁵ c.p.m./µg) and [¹²⁵I]proOmpA-BPTI (~2×10⁶ c.p.m./µg) were prepared as described previously (Shiebel *et al.*, 1991; Economou and Wickner, 1994; Duong and Wickner, 1997b). Protein concentration was determined using the Bradford reagent (Bio-Rad) with BSA as standard. SDS-PAGE was performed using either 15 or 17% gels (Brundage *et al.*, 1992). For protein precipitation after a translocation assay, samples were mixed with one-third volume of ice-cold 50% trichloroacetic acid (TCA), incubated for 30 min on ice and collected by centrifugation (16 000 r.p.m., 10 min, 4°C). After acetone wash of the pellet, proteins were resuspended in SDS sample buffer and analyzed by 15% SDS-PAGE. Autoradiography of ¹²⁵I- or ³⁵S-labeled polypeptides was at -80°C with intensifying screens.

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