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

Franck Duong¹,² and William Wickner²,³

¹Laboratoire Transports et Signaliisation Cellulaires, CNRS UMR 8619, Bâtiment 430, Université de Paris XI, Orsay, 91405, France and ²Department of Biochemistry, Dartmouth Medical School, 7200 Vail Building, Hanover, NH 03755-3844, USA
³Corresponding author
e-mail: William.Wickner@Dartmouth.edu

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. Keywords: preprotein translocase/prl mutants/proton-motive force/SecA/SecYEG/subunit association

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

**PrlA 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 mutants 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 thermostable in detergent micellar solution (Brundage et al., 1992), we have studied the thermostabilities of the SecYEG complexes with wild-type SecY or with PrlA 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 PrlA/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 radiolabeling of the cells, membranes were purified and a detergent extract was prepared on ice with β-octylglucoside 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 prlA 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.

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 prlA 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 linked radiolabeled BPTI (Nouwen et al., 1996; van der Wolk et al., 1998) 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 Kd value of SecA for the translocase (van der Wolk et al., 1998) and reactions were...
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 I29 and inserted SecA requires activated translocation sites. Our hypothesis, the SecA insertion assay was performed (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.

**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 4r in this study) and the F286Y mutation in TM7 (termed 4s in this study). The 4r mutation is postulated to be responsible for the suppression (Flower et al., 1995), while the 4s mutation is thought to compensate for the growth defect of 4r. To study the biochemical properties of the complex containing either the 4r or 4s 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 4r mutation is still thermostable (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

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**Fig. 2. Initiation of translocation is facilitated by PMF and prlA mutations.** (A) Translocation reactions were performed in 50 μl of TL buffer containing SecA (15 μg/ml), SecB (48 μg/ml), BSA (200 μg/ml), wild-type or prlA mutated SecYEG-enriched IMVs (100 μg/ml) and [35S]methionine-labeled membranes pre-mixed with unlabeled proOmpA–BPTI (5 μg/ml). After pre-incubation (2 min, 37°C) with either the uncoupler CCCP (20 μM) or the PMF-generating nucleotide NADH (5 mM), translocation reactions were initiated with 200 μM 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 17% SDS–PAGE and fluorography. (B) SecA insertion reactions were performed as described above except that [35S]methionine-labeled membranes (~60 000 c.p.m.) pre-mixed with unlabeled SecA-D209N (10 μg/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).

**Fig. 3. Effects of mutations F286Y (4s) and I408N (4r) which comprise prlA4.** (A) The mutation 4s restores stability to the A4s-mutated SecYEG complex. [35S]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. [35S]methionine insertion and [35S]proOmpA–BPTI translocation experiments were performed as in Figure 2.
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 transloca. 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 (Pohlschroeder 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 et al., 1996). Nonetheless, the prlG mutations also confer a thermal stability 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. [35S]methionine-labeled membranes were prepared from strains expressing SecEYG from plasmid pHAEYG carrying mutations prlA4, prlG1 or prlA4 + prlG1. Solubilization of the membranes (~10⁶ 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 pHAEYG or plasmid pHAEYG carrying the mutation prlG1 (L108R). After [35S]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; Pohlschroeder 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 preprotein 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 I3a 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 I3a-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 the actual rate of initiation of translocation and allows measurement of the yield of inserted SecA molecules at the steady-state level of inserted SecA may decrease as the proOmpA substrate is liberated into the lumen of the vesicles (Nishiyama et al., 1994). Moreover, prlA mutations increase normal preprotein 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.

Discussion

prlA and prlG mutants do not exhibit allele specificity with respect to the signal sequence defects (Flower et al.,
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 (Hönlinger 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 the compact quaternary structure of SecA (Ramanurthy 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 appearance 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. Iodojen 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., 1998), 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 L [DE3, supF(Ts), trp(Am)], Zch: Tn10, secA13 (Am), cip4319: kan, recA: cat; Mitchell and Oliver, 1993] shifted to 41°C before induction. IMVs from E.coli strain BL21 (hsdS,omp T, gal) transformed by the plasmid pHA-EYG and derivatives were prepared according the procedure of Douville et al. (1995). [125I]Na (1175 Ci/mmol) was from Amersham and [35S]methionine protein labeling mix (1175 Ci/mmole) 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 μg/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 μg/ml), amino acids (40 μg/ml, except methionine) and 0.2% (w/v) glucose. At A600 = 0.4, 15 μg/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, 0.5% [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 form the beads by incubation with SDS sample buffer (10 min at 37°C) and electrophoresed in SDS–17% polyacrylamide gels.

**Other methods**

[121]SecA (8×10^6 c.p.m./μg), [122]SecA-D209N (2×10^6 c.p.m./μg) and [123]PrpOmpA–BPT (2×10^6 c.p.m./μg) were prepared as described previously (Shiebel et al., 1993; Economidou 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., described previously). 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 c.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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**References**

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