The F286Y mutation of PrlA4 tempers the signal sequence suppressor phenotype by reducing the SecA binding affinity

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Abstract SecYEG forms the protein-conducting channel of the *Escherichia coli* translocase. It binds the peripheral ATPase SecA that drives the preprotein translocation reaction. PrlA4 is a double mutant of SecY that enables the translocation of preproteins with a defective or even missing signal sequence. The effect of the individual mutations, F286Y and I408N, was studied with SecYEG proteoliposomes. SecY(I408N) is responsible for the increased translocation of preproteins with a defective and normal signal sequence, and exhibits a stronger *prl* phenotype than PrlA4. This activity correlates with an elevated SecA-translocation ATPase and SecA binding affinity. SecY(F286Y) supports only a low SecA binding affinity, preprotein translocation and SecA translocation ATPase activity. These results suggest that the second site F286Y mutation reduces the strength of the I408N mutation of PrlA4 by lowering the SecA binding affinity.

Key words: Protein translocation; SecA; SecY; Signal sequence suppression

1. Introduction

Escherichia coli translocase consists of an oligomeric assembly of the heterotrimeric SecYEG integral membrane protein complex and the peripheral ATPase SecA (reviewed in [1]). SecA binds with high affinity to SecYEG [2,3]. By multiple cycles of ATP binding and hydrolysis, SecA drives the stepwise translocation of the preprotein across the membrane, a process that is accelerated by the proton motive force [4–7].

The N-terminal signal sequence of preproteins is important for their targeting to SecA [8,9]. Genetic studies have identified mutations in *secY* (*prlA*), *secE* (*prlG*), *secA* (*prlD*) and *secG* (*prlH*) that permit the translocation of preproteins with a defective, or even missing, signal sequence [10–14]. *Prl* mutations (protein localization) do not directly restore the recognition of the signal sequence, but instead disrupt a proofreading activity which results in broadened selectivity of the translocase [15]. *PrlA* and *prlG* mutants appear to function by similar mechanisms [13], while *PrlD* mutants seem to act differently [16].

Most of the insights in the mechanism of signal sequence suppression are derived from studies on PrlA4 [17]. Previously, we have shown that PrlA4 supports an increased SecA binding affinity both in the presence and absence of ATP. This results in a stabilization of the SecA–preprotein complex at the membrane, and consequently, a more efficient initiation of translocation [18]. Therefore, the *prlA* mutation not only reduces the need for a proper signal sequence but also improves translocation of normal preproteins. Due to the increased SecA binding affinity, the proton motive force dependency of the translocation is reduced [19]. The PrlA4 mutant has a different conformation that affects the protease-sensitivity of SecA [20]. PrlA and PrlG mutants are more unstable than wild-type SecYEG in β-octylglucopyranoside (OG) detergent solution [21].

PrlA4 is a double mutant that harbors the F286Y mutation in transmembrane segment 7 (TMS7) and I408N in TMS10. The latter is responsible for the suppressor phenotype [22], but cannot be stably maintained in *E. coli* without second site mutations such as F286Y (PrlA4) or S188L (PrlA6) [15,21]. This seems to be a common feature with strong *prlA* mutants, but the molecular mechanism by which these second site mutations work is not understood. Here, we have studied the contribution of the individual mutations to the PrlA4 phenotype using proteoliposomes reconstituted with purified SecYEG complex.

2. Materials and methods

2.1. Materials

SecA, SecB, proOmpA, and SecYEG were purified as described [23]. SecYEG was reconstituted into liposomes of *E. coli* phospholipids (Avanti polar lipids, Alabaster, AL, USA) by detergent dilution [23]. SecA and proOmpA were labeled with ^{125}I (Radiochemical Centre, Amersham, UK) using Iodo-Beads (Pierce, IL, USA). ^{35}S-labeled Δ8-proOmpA was synthesized from plasmid pET2336 using the *E. coli* T7 S30 Extract System for Circular DNA (Promega, Madison, WI, USA). Inner Membrane Vesicles (IMVs) were obtained as described [24] and treated with a polyclonal SecA antibody to remove the endogenously bound SecA [18,25]. Detergent dodecylmaltoside (DDM) was from Anatrace (Maumee, OH, USA).

2.2. Plasmids

To facilitate cloning, the *Nco*I site between the *secY* and *secE* genes was removed from pET610 [24] by PCR, yielding pET2302. SecY(I408N,F286Y), SecY(F286Y) and SecY(I408N) were introduced into pET2302 by PCR mutagenesis resulting in pET2306, pET2307 and pET2308, respectively. Δ8-proOmpA was placed behind a T7 promoter by transferring the *Hin*dIII/*Eco*RI fragment from pET25 [18] into *Hin*dIII×*Eco*RI-digested pBS2KS, yielding pET2336. All constructs were confirmed by sequencing.

2.3. Other techniques

In vitro translocation of [^{125}I]proOmpA or [^{35}S]Δ8-proOmpA into

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proteoliposomes was assayed by its accessibility to added proteinase K [26]. Translocation reactions were analyzed by SDS–PAGE and autoradiography. Binding of SecA to IMVs was assayed essentially as described [2]. The SecA ATPase activity was assayed as described [27].

3. Results

3.1. Strong signal sequence suppressor activity of SecY(I408N) is tempered by the second mutation, F286Y

To dissect the function of the two mutations in the PrlA4 signal suppressor mutant, I408N and F286Y were separately introduced into SecY and cloned into a vector that allows high-level overexpression of His_6-tagged SecYEG. Wild-type SecYEG and SecY(F286Y)EG were overproduced to similar levels, but the expression of SecY(I408N)EG was severely reduced as indicated by the levels in the membranes (Fig. 1A). Re-introduction of F286Y into the I408N mutant (=PrlA4) restored the overexpression to the level found for wild-type SecYEG. The SecYEG complexes were purified and reconstituted into proteoliposomes (Fig. 1B). Prl mutations have been reported to weaken the association between SecY, SecE and SecG [21]. Since the purification method relies on the co-purification of SecE and SecG with His_6-tagged SecY [23], the possibility existed that due to the weakened interaction, the mutant SecYEG complexes dissociate. However, SDS–PAGE, silver staining and Western blotting (Fig. 1B) showed that this was not the case. Proteoliposomes contained equal amounts of SecY, SecE and SecG. The I408N mutation caused an aberrant migration of SecY on SDS–PAGE (Fig. 1). Such behavior on SDS–PAGE is indicative of an altered conformation of SecY as the net charge is not affected by the mutation.

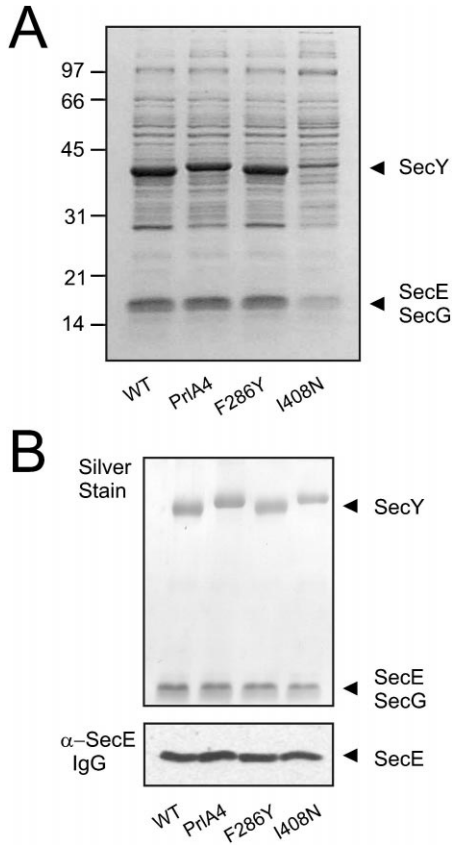

Fig. 1. Overexpression and purification of wild-type and mutant SecYEG complexes. A: Isolated inner membrane vesicles containing overexpressed wild-type SecYEG, PrlA4, SecY(F286Y)EG or SecY(I408N)EG were analyzed by SDS–PAGE and CBB staining. B: Proteoliposomes containing purified wild-type SecYEG, PrlA4, SecY(F286Y)EG or SecY(I408N)EG were analyzed by SDS–PAGE and silver staining or by immunoblotting using an antibody against SecE.

Proteoliposomes were analyzed for proOmpA-stimulated SecA (translocation) ATPase activity. Translocation ATPase was ~6-fold higher with SecY(I408N)EG as compared to the wild-type, and even exceeded that of the PrlA4 mutant (Fig. 2A). The basal SecA ATPase in the absence of proOmpA was also higher with SecY(I408N) and PrlA4 proteoliposomes. Strikingly, the F286Y mutation dramatically reduced the translocation ATPase activity to less than 15% of the wild-type. In vitro translocation assays using chemical amounts of ^{125}I-labeled proOmpA showed a similar pattern. SecY(I408N)EG supported the highest activity, while the SecY(F286Y)EG complex was five- to eight-fold less active than the wild-type (Fig. 2B). To analyze the signal sequence suppressing activity, the translocation of Δ8-proOmpA was assayed. Δ8-proOmpA carriers a deletion of the isoleucine at position 8 of the signal sequence, which results in a major translocation defect in vivo [28]. Likewise, Δ8-proOmpA is poorly translocated in vitro, but the translocation defect can be suppressed by PrlA4 mutant [18]. Since Δ8-proOmpA is poorly translocated, radiochemical amounts of ^{35}S-labeled Δ8-proOmpA were synthesized in vitro to allow for a sensitive detection of translocation in vitro. Again, SecY(I408N)EG supported the highest activity, while SecY(F286Y)EG proteoliposomes showed no measurable translocation of Δ8-proOmpA (Fig. 2B). As observed previously [29], reconstituted wild-type SecYEG translocates low amounts of Δ8-proOmpA (Fig. 2A). This activity likely originates from the high levels of SecA and SecYEG present in the assay [18]. In vivo studies have shown that signal sequence mutants, and even deletions, are somewhat leaky and can be translocated to a small extent by wild-type SecYEG [14,30]. In this respect, high amounts of SecA or SecYEG can suppress signal sequence defects in vivo [16] and in vitro [18]. Taken together, the results demonstrate that the SecY(I408N) is a stronger signal sequence suppressor than PrlA4, while the F286Y mutation dramatically reduces the activity of SecYEG. When combined with the I408N mutation, F286Y tempers the Prl activity of the SecY(I408N)EG complex.

3.2. Reconstituted PrlA4 and SecY(I408N)EG complexes are not thermolabile

Octylglucoside-solubilized SecYEG complex is thermolabile [31]. Prl mutations render the complex even more thermolabile, causing it to dissociate in detergent solution already at 37°C [21]. This thermolability has been attributed to a loosened interaction between the SecYEG subunits [21]. Since *prl* mutants are not temperature-sensitive in vivo [32], it appears that the thermolability is detergent-induced. Unlike OG, the DDM is capable of preserving the SecA–SecYEG interaction [33]. Therefore, the thermostability of the DDM-solubilized and reconstituted SecYEG complexes were compared. DDM-solubilized SecYEG was incubated at 37°C for up to 30 min, reconstituted into proteoliposomes, and the remaining SecA translocation ATPase activity was determined (Fig. 3).

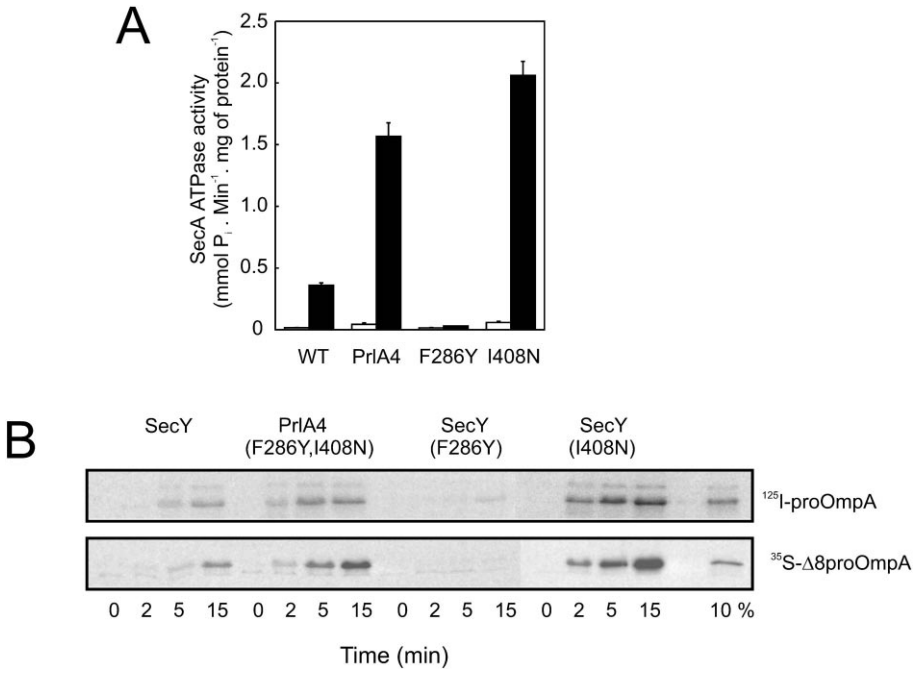

Fig. 2. Activity of purified wild-type and mutant SecYEG complexes. A: SecA ATPase in proteoliposomes in the absence (white bars) or presence (black bars) of proOmpA. B: Translocation of [^{125}I]proOmpA and [^{35}S]Δ8-proOmpA into proteoliposomes (5 and 20 μg/ml, respectively) in the presence of 10 μg/ml SecA and 2 mM ATP. Note that with proOmpA, chemical amounts of ^{125}I-labeled preprotein were used, whereas the Δ8-proOmpA was synthesized in vitro with a transcription/translation system to yield radiochemical amounts of ^{35}S-labeled preprotein. Therefore, the translocation efficiencies of these two preproteins cannot be compared directly.

Wild-type SecYEG and SecY(F286Y)EG retained more than 50% of their activity after 30 min incubation at 37°C in DDM, while PrlA4 and SecY(I408N)EG were readily inactivated under these conditions (Fig. 3A), confirming the previous observations in OG [21]. However, the wild-type and mutant SecYEG complexes reconstituted in proteoliposomes were completely stable when incubated at 37°C (data not shown) and 47°C (Fig. 3B). Similar results were obtained with IMVs (data not shown). The activity of SecY(F286Y) increased two-fold, but this is only a marginal effect as the activity is low (Fig. 1A). Therefore, the thermolability of PrlA

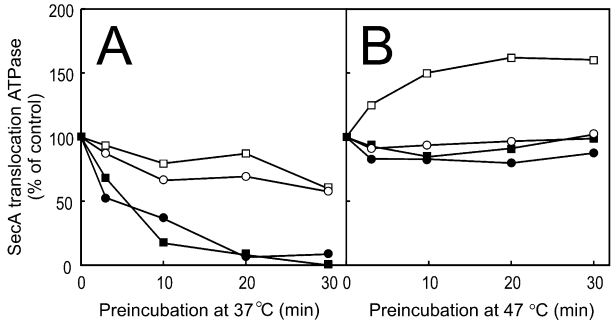

Fig. 3. Thermolability of detergent-solubilized and reconstituted wild-type and mutant SecYEG complexes. A: DDM-solubilized SecYEG (○), PrlA4 (●), SecY(F286Y)EG (□) or SecY(I408N)EG (■) were incubated at 37°C for the times indicated. The residual activity was measured after reconstitution in *E. coli* lipids as proOmpA-stimulated SecA-ATPase activity and plotted as a percentage of the activity prior to the preincubation at 37°C. B: Reconstituted SecYEG (○), PrlA4 (●), SecY(F286Y)EG (□) or SecY(I408N)EG (■) were incubated at 47°C for the times indicated. The residual SecA translocation ATPase activity was plotted as described for (A).

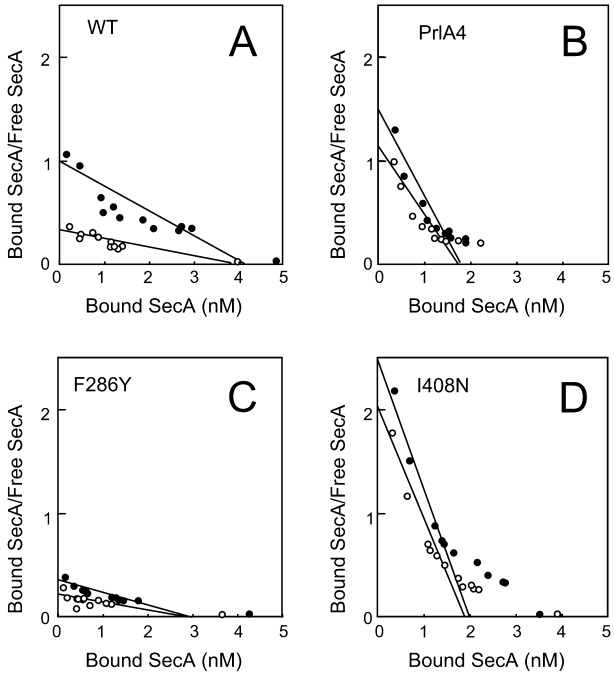

Fig. 4. Scatchard analysis of SecA binding to wild-type and mutant SecYEG complexes. [^{125}I]SecA (0.5–125 nM) binding to inner membrane vesicles (50 μg/ml) containing overexpressed wild-type SecYEG (A), PrlA4 (B), SecY(F286Y)EG (C) or SecY(I408N)EG (D) in the presence (○) or absence (●) of 2 mM ATP. Data were not corrected for background and low affinity SecA binding.

mutants as observed in detergent solution is not manifested when the enzyme is present in the lipid membrane.

3.3. I408N mutation is responsible for the increased SecA binding affinity of PrlA4

PrlA4 exhibits an increased binding affinity for SecA as compared to the wild-type [18]. To determine which of the two mutations is responsible for this phenomenon, SecA binding experiments were performed. IMVs containing overproduced wild-type and mutant SecYEG complexes were used as similar binding experiments with proteoliposomes are hampered by the high levels of SecA binding to the lipid surface. SecA binding to wild-type SecYEG in the absence and presence of ATP occurred with a K_d of 4 and 13 nM, respectively (Fig. 4A). PrlA4 binds SecA with a much higher affinity (K_d $\sim$1.3 nM) that is barely affected by ATP (K_d $\sim$1.6 nM) (Fig. 4B). Strikingly, the SecY(I408N) mutant binds SecA tighter (K_d of 0.8–1.0 nM) (Fig. 4D). In the presence of ATP, its binding affinity for SecA (K_d $\sim$1.0 nM) is even up to 13-fold higher compared to the wild-type. The convex appearance of the Scatchard plots of PrlA4 and SecY(I408N)EG (Fig. 4B,D) is due to the low affinity binding of SecA to the lipid surface. SecY(F286Y)EG binds SecA (K_d of 8 nM) with a two-fold lower affinity than wild-type SecYEG (Fig. 4C). The affinity is further reduced by ATP (K_d $\sim$20 nM) which makes it a very poor binding partner for SecA. These data indicate that I408N is responsible for the increased SecA binding affinity of PrlA4, while the second site mutation, F286Y partially reverses this effect.

4. Discussion

In this report, we have analyzed the contribution of the F286Y and I408N mutations to the phenotype of PrlA4 using IMVs and proteoliposomes reconstituted with the purified SecYEG complex. Our in vitro studies confirm previous in vivo results that show that the I408N mutation is responsible for the suppressor phenotype [22]. The suppressor phenotype, as manifested by the translocation of a signal sequence defective proOmpA derivative, is accompanied by an increased translocation of the wild-type proOmpA and correlates with a dramatically improved SecA-binding affinity. Strikingly, SecY(I408N) not only exhibits a much stronger *prl* activity than PrlA4, but it is also more effective in binding SecA both in the presence and absence of ATP. In contrast, the second mutation, F286Y, markedly reduces the SecA binding affinity and supports only a low translocation and SecA-ATPase activity. Since I408N and F286Y affect the binding of SecA in an opposite manner, we propose that the second site mutation tempers the *prl* phenotype by reducing the SecA binding affinity of SecY(I408N). Strong *prlA* mutations are often accompanied by a second site mutation [13,15]. Such mutations may function by a similar mechanism as F286Y in PrlA4.

We have previously shown that the I278C mutation in TMS7 confers a suppressor phenotype and increases the affinity for SecA [29]. The F286Y mutation of PrlA4 localizes in the same TMS, but reduces the SecA binding affinity (this paper). It is of interest to note that in a regular α-helix, I278 and F286 are predicted to be located on same site of the helix. Recently, the sites of interaction between TSMs of SecY and SecE have been mapped by cysteine scanning mutagenesis [37]. These data suggest that I278 and F286 are part of a helical face that points away from TMS3 of SecE. Many mutations have been described in SecY that cause either a strong or weak signal sequence suppression phenotype. These mutations are not only confined to TMS7 and TMS10, but can also be found in TMS2, periplasmic loop 1 and, less frequently, in other parts of the protein. Although mutations in the other regions have not been studied in detail, it is likely that they also alter the SecA binding affinity of SecY.

Prl mutants are less thermostabile in detergent solution than the wild-type SecYEG complex, and readily dissociate upon prolonged incubation at 37°C [21]. The exact mechanism of inactivation is unclear, as a functional SecYEG complex can be reconstituted from the individual SecY, SecE and SecG subunits [34,35]. The low expression of SecY(I408N)EG compared to PrlA4 (Fig. 1A) points to a reduced stability. Although SecY(I408N) is a stronger Prl suppressor than PrlA4, the proteins did not differ significantly in thermolability in detergent solution ([21], this study). Therefore, the restoration of overexpression of SecY(I408N) by F286Y cannot be explained by an effect of F286Y on the (thermo-)stability of the PrlA4 complex. Thermal inactivation of SecYEG in detergent solution is accompanied by a dissociation of the complex. This has been taken to suggest that a loosened SecY–SecE interaction is responsible for the *prl* phenotype [21]. Hypothetically, the Prl phenotype would emerge from a relaxation of a putative signal sequence binding site on SecYEG, rendering the complex less stringent in accepting preproteins with defective signal sequences. The transmembrane SecY–SecE contact surface would be part of such a signal sequence binding site. It is, however, important to note that current biochemical evidence in favor of such a specific signal sequence binding site on SecYEG is weak as the helical contacts between SecY and SecE persist when a preprotein inserts into the translocation channel in an ATP- and SecA-dependent manner [24,37]. Moreover, the model does not readily account for the translocation of preproteins without a signal sequence [14,30] and the improved translocation of homologous and heterologous preproteins [36]. Since the increased thermolability of the SecYEG complex is not manifested when the protein is functionally embedded in the membrane, it appears that the phenomenon is detergent-induced. It may well be that also in the membrane the SecY–SecE interaction is altered, but this does not lead to thermolability in a physiologically relevant temperature range. In contrast, the altered SecA binding affinity by PrlA mutants is observed both with the membrane-embedded ([18], this study) and detergent-solubilized complexes [29]. We therefore propose that the primary effect of the *prlA* mutations is the modulation of the SecA binding affinity rather than a relaxation of signal sequence recognition. The enhanced stability of the SecA–SecY complex in the presence of ATP allows for an improved initiation of translocation, which is reflected by increased rates of the translocation of normal and signal sequence defective preproteins [18]. The improved SecA binding affinity may well arise from an altered physical interaction between SecY and SecE.

Biochemical and electron microscopy studies indicate that upon the activation of SecA by the non-hydrolyzable ATP analogue AMP–PNP, a large translocation pore is formed that comprises four SecYEG complexes [23]. This process mimics the ATP-dependent initiation of translocation in the

absence of a preprotein [5]. Recruitment of SecYEG complexes by SecA likely represents a critical step in the translocation reaction. A recent equilibrium ultracentrifugation study suggests that PrlA4 forms more stable SecYEG dimers as compared to wild-type SecYEG [38]. The increased SecA binding affinity and an altered oligomeric state of the idle SecYEG complex may facilitate the SecA-mediated formation of SecYEG tetramers. This would provide the PrlA4 mutant with a kinetic advantage for translocation of preproteins as compared to the wild-type. Alternatively, the enhanced SecA binding may be a result of the increased stability of the PrlA4 mutant SecYEG dimer. Future studies should be directed to analyze the dynamics of the assembly of the translocase complex.

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