PrIA4 prevents the rejection of signal sequence defective preproteins by stabilizing the SecA–SecY interaction during the initiation of translocation

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In *Escherichia coli*, precursor proteins are translocated across the cytoplasmic membrane by translocase. This multisubunit enzyme consists of a preprotein-binding and ATPase domain, SecA, and the SecYEG complex as the integral membrane domain. *PrIA4* is a mutant of SecY that enables the translocation of preproteins with a defective, or missing, signal sequence. Inner membranes of the *prIA4* strain efficiently translocate $\Delta 8\text{proOmpA}$, a proOmpA derivative with a non-functional signal sequence. Owing to the signal sequence mutation, $\Delta 8\text{proOmpA}$ binds to the translocase with a lowered affinity and the recognition is not restored by the *prIA4* SecY. At the ATP-dependent initiation of translocation, the binding affinity of SecA for SecYEG is lowered causing the premature loss of bound preproteins from the translocase. The *prIA4* membranes, however, bind SecA with a much higher affinity than the wild-type, and during initiation, the SecA and preprotein remain bound at the translocation site allowing an improved efficiency of translocation. It is concluded that the *prIA4* strain prevents the rejection of defective preproteins from the export pathway by stabilizing SecA at the SecYEG complex.

Keywords: proof-reading/SecA/SecY/signal sequence

Introduction

The selective translocation of precursor proteins (preproteins) across the cytoplasmic membrane of *Escherichia coli* is accomplished by the concerted action of the subunits of the preprotein translocase (Wickner *et al.*, 1991; Driessen, 1994; Driessen *et al.*, 1998). The core of this large enzyme consists of the membrane-embedded heterotrimeric SecYEG complex, and the peripheral homodimeric ATPase SecA (Brundage *et al.*, 1990; Hanada *et al.*, 1994). The SecD, SecE and YajC proteins form a separate heterotrimeric complex that can associate with the SecYEG complex to form a large holoenzyme (Duong and Wickner, 1997a).

Newly synthesized precursor proteins are bound by the

chaperone SecB which stabilizes the preprotein in a loosely folded conformation that is competent for translocation (Lecker *et al.*, 1990; Fekkes *et al.*, 1995). SecB and the signal sequence target the preprotein to the membrane, and both associate with SecA which is bound with high affinity to the SecY subunit (Manting *et al.*, 1997; Matsumoto *et al.*, 1997; Snyders *et al.*, 1997) of the SecYEG complex (Hartl *et al.*, 1990). As a result of the SecB–SecA interaction, the preprotein is transferred to SecA, which binds both its signal sequence and its mature domain (P.Fekkes, J.G.de Wit, J.P.W.van der Wolk, H.H.Kimsey, C.A.Kumamoto and A.J.M.Driessen, submitted). The release of SecB from the membrane requires the binding of ATP at one of the two (Mitchell and Oliver, 1993) ATP-binding sites of SecA (Fekkes *et al.*, 1997). At this stage, a loop of the signal sequence and the N-terminal region of the preprotein are presented to the periplasmic face of the membrane, allowing cleavage of the signal sequence by leader peptidase (Schiebel *et al.*, 1991). In the absence of the protonmotive force (Δp), preprotein translocation is a stepwise process (Schiebel *et al.*, 1991; Uchida *et al.*, 1995), comprising two distinct translocation events, the binding of SecA to the preprotein and the binding and hydrolysis of ATP by SecA (Van der Wolk *et al.*, 1997). The Δp can act as the sole driving force for the completion of translocation once SecA has released the preprotein upon the hydrolysis of ATP (Schiebel *et al.*, 1991; Driessen, 1992).

One of the enzymatic activities of the bacterial translocase complex is the high-fidelity discrimination between secretory and cytosolic proteins. The N-terminal signal sequence of preproteins is important for the initial targeting event, i.e. the recognition of the preprotein by the SecA subunit of the translocase (Cunningham and Wickner, 1989; Lill *et al.*, 1990; Kimura *et al.*, 1991; Joly and Wickner, 1993). Aberrant signal sequences are normally not recognized by the translocase resulting in a deficiency in translocation. By genetic selection, suppressors of such signal sequence mutations have been found, and although selected to suppress a particular signal sequence mutation, they are pleiotropic and permit the translocation of preproteins with a range of signal sequence mutations, including complete signal sequence deletions. Such suppressors have been found in *secY* (*prIA*, Emr *et al.*, 1981), *secE* (*prIG*, Stader *et al.*, 1989), *secG* (*prIH*, Bost and Belin, 1997) and *secA* (*prID*, Fikes and Bassford, 1989). However, the *prIA* mutations are the most potent and most commonly isolated suppressors (Bieker *et al.*, 1990). Therefore, it appears that all essential subunits of the translocase are involved in the preprotein recognition process. Originally it was suggested that *prl* suppressors may function by restoring the recognition of altered signal sequences (Emr *et al.*, 1981). However, this hypothesis does not explain the translocation of signal-sequenceless precursors (Derman

et al., 1993; Flower *et al.*, 1994; Prinz *et al.*, 1996). An alternative explanation proposes that the *prl* mutations disrupt a proof-reading system and thereby prevent rejection of defective preproteins from the export pathway (Osborne and Silhavy, 1993). Proof-reading of a signal sequence would imply an interaction between the signal sequence and one or more of the subunits of the translocase. This is well established for SecA (Cunningham and Wickner, 1989; Lill *et al.*, 1990; Kimura *et al.*, 1991), but there is no direct biochemical evidence for such an interaction with SecY, SecE and/or SecG.

To understand the mechanism of signal sequence suppression, we analysed the catalytic properties of the *prlA4* strain using isolated inner membrane vesicles (IMVs). These IMVs not only suppress the translocation defect of a preprotein with a defective signal sequence, but also translocate wild-type preproteins with higher efficiency. The data indicate that signal sequence suppression is not caused by an altered interaction of the translocase with the signal sequence domain of preproteins, but results from a remarkable increase in the SecA membrane-binding affinity. The reduced level of preprotein rejection in the *prlA4* strain finds its cause in the stabilization of the SecA–SecY complex during the initiation of preprotein translocation.

Results

PrlA4 suppresses signal sequence defects *in vitro* and translocates preproteins with an increased efficiency

To investigate the phenomenon of signal sequence suppression *in vitro*, we analysed the *prlA4* strain SE6004 (Emr *et al.*, 1981) in greater detail. *PrlA4* is one of the strongest *prl* suppressors *in vivo*. It contains two mutations in the *secY* gene that result in the amino acid substitutions F286Y and I408N in transmembrane segment 7 and 10, respectively (Sako and Iino, 1988). The latter mutation is responsible for the suppressor phenotype (Osborne and Silhavy, 1993), and enables the translocation of preproteins with a defective or completely missing signal sequence (Emr *et al.*, 1981; Derman *et al.*, 1993; Flower *et al.*, 1994; Prinz *et al.*, 1996). *In vitro* signal sequence suppression was analysed with purified, urea-denatured ³⁵S-labelled $\Delta 8\text{proOmpA}$. This proOmpA derivative lacks isoleucine at position 8 of the signal sequence, and is therefore only poorly translocated *in vivo* (Tanji *et al.*, 1991). Wild-type and *prlA4* IMVs were depleted for the endogenously membrane-bound SecA using a polyclonal antibody against SecA (Schiebel *et al.*, 1991). $\Delta 8\text{proOmpA}$ failed to translocate into the wild-type IMVs, but was translocated into, and processed by, the *prlA4* IMVs (Figure 1). Quantification of these results showed that the *prlA4* IMVs translocate $\Delta 8\text{proOmpA}$ with ~20% of the efficiency with which wild-type IMVs translocate proOmpA. Even after very long exposure times of the autoradiograms, it was hardly possible to detect translocation of $\Delta 8\text{proOmpA}$ by wild-type IMVs. Based on this quantification, we estimate that the translocation of $\Delta 8\text{proOmpA}$ into the *prlA4* IMVs must be at least 50-fold more efficient as compared with wild-type IMVs. Owing to the signal-sequence mutation, $\Delta 8\text{proOmpA}$ shows a slower mobility on SDS–PAGE as compared with wild-type proOmpA, yielding an improved

Fig. 1. *PrlA4* inner membrane vesicles suppress the $\Delta 8\text{proOmpA}$ translocation defect and translocate wild-type proOmpA with an improved efficiency. Translocation of [³⁵S]proOmpA or [³⁵S] $\Delta 8\text{proOmpA}$ across IMVs of wild-type (*secY*) and *prlA4* cells were performed for 15 min in the presence of 2 mM ATP, the presence or absence of 100 nM SecA and/or 20 mM CCCP to dissipate the Δp . Prior to the translocation reactions, IMVs were washed with a SecA polyclonal antibody to remove endogenous SecA. Results were quantitated by direct counting using the β -imager 2000. Translocation efficiencies are indicated as the percentage of total added [³⁵S]proOmpA or [³⁵S] $\Delta 8\text{proOmpA}$. Since the specific radioactivity of both precursors was identical, translocation efficiencies could be compared directly.

separation of the precursor and mature forms. The translocation of $\Delta 8\text{proOmpA}$ into the *prlA4* IMVs was ATP-dependent (data not shown) and SecA-dependent (compare lane 2 with lane 4), demonstrating the authenticity of the reaction. Strikingly, the translocation of proOmpA into the *prlA4* IMVs was 3- to 4-fold more efficient than translocation into wild-type IMVs (compare lane 3 with lane 4). Since these membranes harbour a functional H^+ -translocating F_1F_0 -ATPase, ATP will not only energize preprotein translocation via SecA-mediated ATP hydrolysis but also through the generation of a Δp . Therefore, translocation reactions were analysed in the presence of the uncoupler CCCP to determine the relative contribution of the Δp in the two membrane types. Translocation of proOmpA into wild-type IMVs was reduced strongly by the addition of CCCP (lane 5), whereas the uncoupler had little effect on the translocation of proOmpA or $\Delta 8\text{proOmpA}$ into *prlA4* IMVs (lane 6). Both membrane types had similar capacities to generate a Δp in the presence of ATP, as measured using the fluorescent indicators oxonol VI (bis[3-propyl-5-oxoisoxazol-4-yl]pentamine oxonol) and ACMA (9-amino-6-chloro-2-methoxyacridine) to assess the transmembrane electrical potential and pH gradient (data not shown; Van der Wolk *et al.*, 1997). It is important to note that even in the presence of a Δp , the wild-type IMVs failed to translocate $\Delta 8\text{proOmpA}$ (Figure 1, lane 3). These data confirm previous observations that the *prlA4* strain efficiently translocates preproteins in the absence of a Δp (Nouwen *et al.*, 1996), and extend this study to the translocation of a preprotein with a defective signal sequence, providing *in vitro* evidence for signal sequence suppression.

One remarkable observation is that the *prlA4* mutation not only restores the translocation of a preprotein with a defective signal sequence, but also permits a more efficient translocation of the wild-type preprotein. This would suggest that the *prlA4* strain has an improved translocation capacity. To further elaborate, the translocation ATPase

Fig. 2. *PrlA4* inner membrane vesicles exhibit a reduced SecA translocation ATPase activity. (A) ATPase activity of SecA (100 nM) in the presence of increasing concentrations of proOmpA in the presence of wild-type (●) or *prlA4* (○) IMVs. (B) SecA ATPase activity in the presence of proOmpA (WT) or Δ8proOmpA (Δ8) (20 μg/ml) with wild-type IMVs (WT, black bars) or *prlA4* (white bars) IMVs. IMVs were treated with 6 M urea to reduce the background ATPase activity, and subsequently washed with a SecA polyclonal antibody to remove the remaining endogenous SecA.

activity of SecA in the presence of wild-type and *prlA4* IMVs was compared. Membrane-bound ATPases and SecA were inactivated and removed by treatment of the IMVs with 6 M urea, followed by a repeated wash in the presence of a polyclonal antibody against SecA. After this treatment, *prlA4* IMVs remained more efficient in the translocation of proOmpA as compared with the wild-type IMVs (Figure 6). The proOmpA-stimulated SecA ATPase activity of the *prlA4* IMVs was about half that observed with the wild-type membranes (Figure 2A). This demonstrates that, compared with the wild-type, the *prlA4* mutant is more efficient in coupling the SecA ATPase activity to the translocation of a wild-type preprotein. Only with the *prlA4* IMVs was a low level of SecA ATPase activity observed when Δ8proOmpA was used as a substrate (Figure 2B).

Fig. 3. Signal sequence mutated Δ8proOmpA binds with a reduced efficiency to both wild-type and *prlA4* inner membrane vesicles. Binding of [³⁵S]proOmpA (white bars) and [³⁵S]Δ8proOmpA (black bars) to wild-type (WT) or *prlA4* IMVs was assayed as described (Fekkes *et al.*, 1997). SecA and SecB were present at concentrations of 250 and 100 nM, respectively.

***PrlA4* does not restore the SecA-dependent recognition of preproteins with a defective signal sequence**

Since the *prlA4* mutant permits the translocation of a wide range of preproteins with a defective signal sequence, it may restore the recognition of these abnormal signal sequences and thus alter the binding of Δ8proOmpA to the SecA-subunit of the translocase. The amount of membrane-bound proOmpA in the presence of SecA and SecB was comparable for the wild-type and *prlA4* IMVs (Figure 3, lanes 1 and 3), even though the *prlA4* IMVs translocate proOmpA more efficiently. In the absence of SecA, only low levels of membrane-bound proOmpA were detected (data not shown; P.Fekkes, J.G.de Wit, J.P.W.van der Wolk, H.H.Kimsey, C.A.Kumamoto and A.J.M.Driessen, submitted). With wild-type IMVs, the SecA-dependent binding of Δ8proOmpA was severely impaired (lane 2) (P.Fekkes, J.G.de Wit, J.P.W.van der Wolk, H.H.Kimsey, C.A.Kumamoto and A.J.M.Driessen, submitted) as expected for a preprotein with a defective signal sequence. However, binding was not restored with *prlA4* IMVs (lane 4), indicating that the *prlA* phenotype, at least as far as targeting is concerned, is not caused by an alteration in binding affinity of the SecYEG-bound SecA for the aberrant signal sequence.

***PrlA4* binds SecA with an increased affinity**

Both in the wild-type and *prlA4* strain, SecY is expressed from a single chromosomal copy of the *secY* gene. However, to exclude the possibility that the elevated translocation activity of the *prlA4* IMVs is caused by increased expression of SecY, the amount of SecY in both types of vesicles was compared using immunoblot analysis. As shown in Figure 4A, the polyclonal antiserum directed against the purified SecY protein detected identical levels of SecY in both membrane types. However, immunoblot analysis revealed that *prlA4* IMVs retained significantly larger amounts of SecA associated with the membrane after isolation compared with wild-type IMVs (Figure 4B, compare lane 1 with lane 4) despite the fact that the levels

Fig. 4. Wild-type and *prlA4* inner membrane vesicles harbour identical amounts of SecY but differ in the extent of SecA membrane association. **(A)** Immunoblot analysis of the relative SecY level in IMVs of the wild-type (lane 1) and *prlA4* (lane 2) strain. Immunoblots were stained with a polyclonal antibody raised against purified wild-type SecY (Van der Does *et al.*, 1998). **(B)** Immunoblot analysis of the relative amounts of SecA associated with wild-type (lanes 1–3) and *prlA4* (lanes 4–6) membrane before (lanes 1 and 4), and after (lanes 2 and 5) treatment with 6 M urea and subsequent wash with a polyclonal antibody directed against SecA (lanes 3 and 6). Immunoblots were stained with a mixture of monoclonal antibodies directed against different epitopes of SecA (Den Blaauwen *et al.*, 1997).

of SecY are identical (Figure 4A). We noted that the *prlA4* IMVs remained active for proOmpA translocation after urea-extraction (data not shown). Only after incubation with a polyclonal antibody against SecA, to completely remove and inactivate the membrane-bound SecA (Figure 4B, lane 6; Schiebel *et al.*, 1991), did translocation of proOmpA into *prlA4* IMVs become strictly dependent on the addition of SecA (Figures 1 and 6). Binding analysis using 125 I-labelled SecA and urea/antibody-treated IMVs also showed that more SecA is bound to *prlA4* than to wild-type IMVs (Figure 5A). Scatchard analysis of the binding of [125 I]SecA revealed that wild-type and *prlA4* IMVs contained an equal number of high-affinity binding sites for SecA, but differed remarkably in the SecA-binding affinity (Figure 5B and C). The K_d s for SecA binding to *prlA4* and wild-type IMVs were ~ 1.4 and 7 nM, respectively. These data demonstrate that the *prlA4* mutant of SecY binds SecA with a dramatically increased affinity.

The functional impact of the tighter SecA binding to the *prlA4* IMVs becomes evident when the interaction between SecA and SecY is analysed under conditions that allow the initiation of translocation. In the presence of ATP, a major fraction of the SecA bound to SecY at subsaturating concentration, is released from the wild-type IMVs, whereas little release seemed to occur with *prlA4* IMVs (Figure 5A). Similar observations were made in the presence of preprotein, i.e. saturating concentrations of proOmpA or $\Delta 8$ proOmpA, or when the ATP was replaced by the non-hydrolysable analogue ATP γ S (data not shown). The ATP-induced release of the SecA is due to a major reduction in the SecA membrane-binding affinity (Figure 5B and C). With the wild-type IMVs, the K_d value falls from 7 to ~ 24 nM upon the addition of ATP. Likewise, in the *prlA4* IMVs, ATP addition reduces the SecA-binding affinity from 1.4 to 3.6 nM. However,

Fig. 5. SecA binds with an elevated affinity to PrlA4. **(A)** Binding of [125 I]SecA (10 nM) to urea- and SecA-antibody-treated IMVs of wild-type (WT) and *prlA4* cells in the absence (black bars) or presence (white bars) of 2 mM ATP. **(B)** Scatchard plot analysis of specific binding of [125 I]SecA (1–500 nM) to wild-type IMVs in the absence (●) or presence (○) of 2 mM ATP. **(C)** As (B) except that *prlA4* IMVs were used. The fraction of non-specific SecA binding was 0.02. IMVs were treated with 6 M urea and washed with polyclonal antibodies raised against SecA.

Fig. 6. ProOmpA is efficiently translocated by *prlA4* inner membrane vesicles at low SecA levels. **(A)** Translocation of [35 S]proOmpA across urea- and SecA-antibody-treated IMVs of wild-type (*secY*) and *prlA4* cells in the presence of 2 mM ATP and varying concentration of SecA. The amount of wild-type and *prlA4* SecY present in the assay was identical based on quantitative immunoblotting as described in the legend to Figure 4A. Reactions were analysed using SDS-PAGE and autoradiography. **(B)** Quantification of the amount of translocated [35 S]proOmpA and [35 S]OmpA by wild-type (●) and *prlA4* (○) IMVs by direct counting using the β -imager 2000.

the binding affinity remains higher than that found with wild-type IMVs in the absence of ATP. It is important to note that the presence of nucleotide did not affect the number of high-affinity binding sites (Figure 5B and C). Therefore, the ATP-dependent release of SecB (Fekkes *et al.*, 1997) from the translocation sites is unrelated to the lowering of the SecA-binding affinity. In the presence of a saturating concentration of SecA, ATP binding indeed caused the release of SecB both from the wild-type and *prlA4* IMVs (data not shown) confirming our previous conclusion that SecB is released from the membrane at the initiation of translocation (Fekkes *et al.*, 1997).

The increased affinity of SecA binding to the SecYEG complex in the *prlA4* IMVs results in more efficient proOmpA translocation at SecA concentrations that are suboptimal for the wild-type IMVs (Figure 6). The rate of proOmpA translocation into *prlA4* IMVs increased almost linearly with the SecA concentration in the range 1–10 nM, whereas it appeared to be delayed with the wild-type IMVs (Figure 6B). Therefore, it seems that the increased binding affinity of the *prlA4* mutant stabilizes the SecA at the site of translocation during preprotein translocation.

***PrlA4* exhibits a reduced level of preprotein rejection**

To determine whether the tighter binding of SecA by the *prlA4* IMVs is accompanied by an increased retention of the preprotein at the site of translocation, the influence of ATP on the SecA-dependent binding of proOmpA to the membrane was examined (Figure 7). These experiments were performed at 0°C to prevent complete translocation

Fig. 7. *PrlA4* inner membrane vesicles exhibit a reduced level of wild-type and $\Delta 8$ proOmpA release upon the initiation of translocation. Binding of [35 S]proOmpA or [35 S] $\Delta 8$ proOmpA to urea- and SecA-antibody-treated IMVs of wild-type (WT) and *prlA4* cells in the absence (white bars) or presence (black bars) of 2 mM ATP. SecA was used at a concentration of 300 nM. Binding experiments were performed at 0°C. Data were not corrected for non-specific binding of wild-type and $\Delta 8$ proOmpA, which was ~2%.

of the preproteins. ATP caused a marked reduction of the amount of proOmpA that was bound specifically to the wild-type IMVs (compare lane 1 with lane 2). However, under these conditions, most of the proOmpA remained bound to the *prlA4* IMVs (compare lane 3 with lane 4). ATP binding to SecA also caused the release of the $\Delta 8$ proOmpA from the wild-type IMVs (compare lane 6 with lane 8), but this effect was less dramatic because of the low level of specific $\Delta 8$ proOmpA binding. From these data we infer that in the wild-type, a major fraction of preprotein is normally rejected from the translocation site at the stage of initiation. In contrast, hardly any rejection takes place in the *prlA4* IMVs. A positive correlation exists between the ATP-dependent release of the preprotein and SecA from the translocation sites, and the efficiency of preprotein translocation. Therefore, these data suggest that the increased efficiency of translocation and the suppression of signal sequence defects by the *prlA4* strain find their common cause in the increased functional binding of SecA to the translocation site. Stabilization of the SecA binding at the translocation sites prevents the premature loss of the bound preprotein from these sites, and therefore, results in a more efficient initiation of translocation upon the binding of ATP to SecA.

Discussion

In this paper we present experimental support for the proof-reading model of Osborne and Silhavy (1993), and provide a mechanistic explanation for signal sequence suppression by the *prlA4* strain. At the initial stages of translocation, the SecA subunit of the translocase interacts with the signal sequence and mature domains of the preprotein. According to the proof-reading model, the proper recognition of the signal sequence domain by SecA allows the formation of a functional complex between SecA and SecYEG. Once this complex is formed, binding

of ATP to SecA elicits a conformational change (Den Blaauwen *et al.*, 1996; Van der Does *et al.*, 1998) that drives the insertion of a SecA domain into the membrane (Economou and Wickner, 1994; Economou *et al.*, 1995) and by co-insertion, initiates the translocation of the signal sequence and part of the mature preprotein domain as a hairpin-like structure (Schiebel *et al.*, 1991). ATP hydrolysis dissociates the preprotein from its SecA-bound state, reverses the conformational change of the SecA domain, and promotes its membrane de-insertion. A defective signal sequence will be recognized by SecA, albeit with low efficiency, but the SecA will not form a functional complex with the SecYEG heterotrimer. Subsequent binding of ATP will not result in the formation of the SecA-inserted state and translocation is not initiated. Instead, hydrolysis of ATP will release the preprotein into the cytosol and translocation is aborted. *Prl* suppressor mutations would effectively bypass this proof-reading step by allowing the formation of a functional complex between SecA and the SecYEG heterotrimer even without the proper recognition of a signal sequence. Consequently, preproteins with a wide variety of mutated signal sequences, and even complete deletions, are translocated. Our current data demonstrate that in the *prlA4* strain, the interaction between SecA and the SecYEG complex is indeed altered. Our data indicate that proof-reading must precede the membrane-insertion of the SecA domain(s). This is evident from the observation that the *prlA4* IMVs are less efficient in supporting the formation of a protease-protected 30 kDa fragment of SecA (H.Tokuda, personal communications; J.van der Wolk, unpublished observations) whereas they bind SecA more tightly and translocate proOmpA more efficiently compared with wild-type IMVs. Suppression of the signal sequence defect is not a direct result of the enhanced activity of the *prlA4* IMVs as the mutation gives rise to a much greater stimulation of the $\Delta 8\text{proOmpA}$ translocation (~50-fold) than that of proOmpA (3- to 4-fold).

How can signal sequence suppression be understood mechanistically in terms of the SecA–SecY interaction? Previous studies have demonstrated that SecA binds with high affinity to the SecY subunit of the translocase (Hartl *et al.*, 1990; Manting *et al.*, 1997; Matsumoto *et al.*, 1997; Snyders *et al.*, 1997). The *prlA4* SecY protein binds SecA with a greatly enhanced affinity as compared with the wild-type. ATP binding to SecA causes a major reduction in this binding affinity, and for the wild-type this results in a strongly diminished stability of the SecA–SecY complex. Owing to the intrinsic instability of the complex, preprotein translocation is not initiated efficiently. Although a surprising finding, this even results in a significant level of rejection of wild-type preproteins, at least *in vitro*. With a defective signal sequence, the odds of a productive interaction between SecA and SecY are even lower, as the preprotein recognition during the initial stages of targeting is already unfavourable. With the *prlA4* strain, the high SecA–SecY binding affinity retains the SecA at the translocation site, preventing it from releasing (i.e. rejecting) the bound preprotein. The increased stability of the complex promotes the chance of a functional interaction, and consequently initiation of translocation is far more efficient. This model explains why wild-type preproteins (Nouwen *et al.*, 1996), as shown in this paper

for proOmpA, are translocated more efficiently in the *prlA4* strain than in the wild-type. This model seems to be applicable to other *prlA* mutant strains as similar results have been obtained with SecYEG proteoliposomes reconstituted with the *prlA5* (I278C) mutant SecY (A.Kaufmann, C.van der Does, E.Manting and A.J.M.Driessen, unpublished data). High amounts of SecA (Huie and Silhavy, 1995; J.van der Wolk, unpublished data) or SecYEG (A.Kaufmann, unpublished data) can stimulate the translocation of preproteins with a defective signal sequence by mass action. However, the efficiency of translocation under these conditions is only a fraction of that observed with the *prlA4* strain. This implies that saturation of the SecYEG complex with SecA alone does not suffice for efficient signal sequence suppression. Instead, we propose that the suppression is caused by the improved stability of complex formation. In this respect, overproduction of SecD and SecE also results in a weak *prl* suppressor phenotype (Pogliano and Beckwith, 1994), possibly by stabilizing the SecA–SecYEG interaction (Duong and Wickner, 1997b).

Our data exclude the possibility that the *prlA4* suppressor functions by restoring the recognition of altered signal sequences. Owing to the signal sequence defect, the SecA-dependent binding of $\Delta 8\text{proOmpA}$ to the wild-type IMVs is severely impaired. This phenomenon is, however, not restored in the *prlA4* IMVs. Likewise, proOmpA binds with a similar efficiency to *prlA4* and wild-type IMVs, even though it translocates much more quickly into the *prlA4* membranes. We were unable to translocate any appreciable quantities of urea-denatured OmpA into the *prlA4* IMVs. However, the removal of the signal sequence resulted in further reduction in the SecA binding activity (P.Fekkes, J.G.de Wit, J.P.W.van der Wolk, H.H.Kimsey, C.A.Kumamoto and A.J.M.Driessen, submitted) to a level that, at the most, would support a translocation activity below the detection limit. The ability of the preprotein to interact with SecA seems to be necessary for efficient suppression. Signal sequence mutations, even the deletions, are leaky for preprotein translocation. A complete deletion of the signal sequence of PhoA still allows *in vivo* secretion in a wild-type background at ~1% of the rates observed with prePhoA (Derman *et al.*, 1993). This reaction depends strictly on SecB; presumably SecB compensates for the lack of targeting information in PhoA that is devoid of the signal sequence. So far, it has not been possible to secrete cytosolic proteins in the *prlA4* strain at a detectable level (Prinz *et al.*, 1996). Such proteins may not interact or interact only poorly with SecB, and thus will not be targeted to SecA. Alternatively, their folding characteristics may be incompatible with the translocase system.

An intriguing observation is that the Δp dependency of preprotein translocation is low in the *prlA* strains (Nouwen *et al.*, 1996). These strains even translocate proOmpA containing a disulfide-bridge-stabilized tertiary loop that in the wild-type strictly requires the Δp . This has led to the suggestion that *prlA* mutations cause a general relaxation of the translocase rather than a specific change that allows bypassing of the recognition of the signal sequence. These data have been taken to indicate that the Δp stimulates translocation by altering the conformation of the translocase. How are the *prl* phenotype and the reduced Δp -

dependency related? The mechanism by which the Δp drives translocation is unknown, but various lines of evidence indicate that the Δp acts as a direct driving force only when the translocating preprotein is not bound to SecA, i.e. when SecA releases the preprotein upon hydrolysis of ATP (Schiebel *et al.*, 1991; Driessen, 1992; Van der Wolk *et al.*, 1993). The Δp has been shown to lower the apparent K_m of the translocation reaction for ATP, thereby allowing efficient translocation at low ATP concentrations (Shiozuka *et al.*, 1990). This phenomenon has been attributed to an accelerating effect of the Δp on the rate of ADP release by SecA. We would propose that this is an indirect result, for instance, through an accelerating effect of the Δp on the SecA membrane de-insertion kinetics. Indeed, imposition of the Δp reduces the steady-state SecA 30 kDa level in both wild-type and in *prlA4* IMVs (H.Tokuda, personal communication). Finally, the Δp -requirement for preprotein translocation can be suppressed by high levels of SecA (Yamada *et al.*, 1989). In all cases, it appears that the catalytic cycle of SecA is influenced by the Δp in an indirect manner. The reduced Δp -requirement of preprotein translocation in *prlA* strains is therefore most easily understood in kinetic terms. Since the *prlA4* strain binds SecA with a 5-fold enhanced affinity as compared with the wild-type, only low concentrations of SecA are required to relieve translocation of the Δp requirement. Although the Δp and *prlA* suppressor have many functional aspects in parallel, the Δp alone will not, or will only marginally, suppress signal sequence defects.

Another important question is whether all *prl* suppressors work according to the mechanism outlined for the *prlA4* strain. Our current findings also explain the suppressor phenotype of another *prlA* strain with mutations in TMS 7 of SecY (A.Kaufmann, unpublished data). *PrlG* and *prlD* mutants do not exhibit a reduced Δp -dependency of translocation (Nouwen *et al.*, 1996), indicating that SecE and SecA may perform their proof-reading function at a different step in the catalytic cycle. However, these suppressors are much weaker than the *prlA* suppressors. Many of the *prlD* suppressor mutations are clustered around nucleotide-binding site I (NBS-I) and nucleotide-binding site II (NBS-II) of SecA giving rise to either azide resistance or azide supersensitivity, respectively (Huie and Silhavy, 1995). Sodium azide is an inhibitor of Sec-dependent preprotein translocation. It blocks the SecA translocation ATPase activity (Oliver *et al.*, 1990) and traps SecA in a membrane-inserted state (Van der Wolk *et al.*, 1997). It may be, therefore, that the *prlD* mutations, like the *prlA* mutations, affect the membrane binding or cycling of SecA. Intriguingly, no suppressor mutations in SecA have been isolated that allow the translocation of signal sequenceless preproteins (Flower *et al.*, 1994). This indicates that in a normal wild-type situation, the SecA has to interact with the signal sequence in order to undergo the conformational change that is sensed by the SecYEG complex to fulfil the proof-reading function.

In conclusion, the stability of the interaction between SecA and SecY is critical for the initial steps of preprotein translocation. Mutations that increase the stability of complex formation cause a more efficient initiation of preprotein translocation at the expense of selectivity.

Materials and methods

Biochemicals

SecA (Cabelli *et al.*, 1988), SecB (Weiss *et al.*, 1988), $\Delta 8$ proOmpA and proOmpA (Crooke *et al.*, 1988) were purified as described. 35 S-labelled proOmpA and $\Delta 8$ proOmpA were synthesized from plasmid pET33 and pET25, respectively, using an *in vitro* transcription/translation reaction (De Vrije *et al.*, 1987). The radiolabelled proteins were affinity-purified as described (Crooke and Wickner, 1987). SecA was iodinated with Na 125 I as described previously (Economou and Wickner, 1994; Fekkes *et al.*, 1997). Inverted IMVs were prepared from *E.coli* strains MC4100 [F^- *araD139* (Δ lac)U169 *rpsL* *relA* *thi*] and SE6004 [F^- *araD139* (Δ lac)U169 *rpsL* *relA* *thi* *lamB60* *prlA4*] (Emr *et al.*, 1981) by the procedure of Chang *et al.* (1978), treated with 6 M urea (Cunningham *et al.*, 1989) and washed with polyclonal antibodies directed against SecA (Schiebel *et al.*, 1991) as indicated.

DNA manipulation and oligonucleotide-directed mutagenesis

$\Delta 8$ proOmpA was constructed by unique site elimination (USE) oligonucleotide-directed mutagenesis (Deng and Nickoloff, 1992) of the *ompA* gene subcloned into pBAD18 (containing the M13 intergenic region) (Guzman *et al.*, 1995). Single-strand DNA was isolated from *E.coli* strain TG1 as described by Sambrook *et al.* (1989). Mutagenesis was performed with kinased $\Delta 8$ mutagenic primer in 10-fold excess over kinased USE primer. Standard DNA sequencing was performed to identify colonies containing correctly mutagenized plasmid. The mutagenized *ompA* gene was subcloned into the *EcoRI*-*PstI* sites of the overexpression vector pTRC99A, yielding pET25.

In vitro translocation

In vitro translocation reactions (in 50 μ l) were performed at 37°C as described (Cunningham *et al.*, 1989) with 20 mg/ml of SecA, 32 mg/ml of SecB, 1 μ l of urea-denatured [35 S]proOmpA or [35 S] $\Delta 8$ proOmpA, 10 mM phosphocreatine and 50 mg/ml creatine kinase in buffer B [50 mM HEPES KOH, pH 7.5, 30 mM KCl, 0.5 mg/ml bovine serum albumin (BSA), 10 mM DTT, and 2 mM Mg(OAc) $_2$] unless stated otherwise. *E.coli* MC4100 (*secY*) or SE6004 (*prlA4*) IMVs were added to a final concentration of 300 mg/ml. Reactions were initiated by the addition of 2 mM ATP and terminated after 15 min by chilling on ice. Samples were treated with proteinase K (0.1 mg/ml) for 30 min on ice, precipitated with 7.5% (w/v) TCA, washed with ice-cold acetone and analysed by 12% SDS-PAGE. Gels were dried and exposed to Kodak Biomax MR film or quantified using the β -imager 2000 (Biospace Measures, Paris, France).

Other techniques

Protein determination was performed according to the method of Lowry *et al.* (1951) with BSA as standard. Binding of [125 I]SecA, [125 I]SecB, [35 S]proOmpA and [35 S] $\Delta 8$ proOmpA to urea- and SecA antibody-treated IMVs was performed as described (Hartl *et al.*, 1990; Fekkes *et al.*, 1997). Molar concentrations of SecA are calculated assuming SecA is a dimer. The translocation ATPase activity of SecA was assayed using the method of Lill *et al.* (1989). The membrane-bound levels of SecY and SecA were determined by immunoblot analysis using a polyclonal antibody against purified SecY (Van der Does *et al.*, 1998) and a mixture of monoclonal antibodies against SecA (Den Blaauwen *et al.*, 1997). Measurements of the transmembrane electrical potential and pH gradient were carried out with the fluorescent dyes oxonol VI (bis[3-propyl-5-oxoisoxazol-4-yl]pentamine oxonol) and ACMA (9-amino-6-chloro-2-methoxyacridine) as described previously (Van der Wolk *et al.*, 1997).

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