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Recognition of secretory proteins in *Escherichia coli* requires signals in addition to the signal sequence and slow folding

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Abstract

Background: The Sec-dependent protein export apparatus of *Escherichia coli* is very efficient at correctly identifying proteins to be exported from the cytoplasm. Even bacterial strains that carry *prl* mutations, which allow export of signal sequence-defective precursors, accurately differentiate between cytoplasmic and mutant secretory proteins. It was proposed previously that the basis for this precise discrimination is the slow folding rate of secretory proteins, resulting in binding by the secretory chaperone, SecB, and subsequent targeting to translocase. Based on this proposal, we hypothesized that a cytoplasmic protein containing a mutation that slows its rate of folding would be recognized by SecB and therefore targeted to the Sec pathway. In a Prl suppressor strain the mutant protein would be exported to the periplasm due to loss of ability to reject non-secretory proteins from the pathway.

Results: In the current work, we tested this hypothesis using a mutant form of λ repressor that folds slowly. No export of the mutant protein was observed, even in a *prl* strain. We then examined binding of the mutant λ repressor to SecB. We did not observe interaction by either of two assays, indicating that slow folding is not sufficient for SecB binding and targeting to translocase.

Conclusions: These results strongly suggest that to be targeted to the export pathway, secretory proteins contain signals in addition to the canonical signal sequence and the rate of folding.

Background

The Sec-dependent protein export pathway of *Escherichia coli* is responsible for translocation of secretory proteins across the inner membrane to final destinations in the periplasm or outer membrane. Secretory proteins, also called preproteins, are synthesized with a cleavable amino terminal signal sequence that functions both to slow folding of the preprotein and to aid in recognition of the secretory protein by export factors. Export of many, but not all, secretory proteins is dependent on interaction with SecB, a cytoplasmic chaperone that maintains the preprotein in a loosely folded conformation competent for translocation. Both SecB and the preprotein provide binding sites for SecA, a peripheral membrane ATPase. SecA targets the preprotein to the membranous translocase complex composed of SecY, SecE, SecG, SecD, SecF, and YajC. Formation of the complete translocase complex promotes an ATP binding and hydrolysis cycle by SecA that results in

segmental translocation of the secretory protein across the membrane [1–4].

The signal sequence is crucial for efficient translocation; mutations in the signal sequence significantly reduce export of the preprotein and complete deletions of the signal sequence eliminate essentially all export [5–8]. Selection for extragenic suppressors of such export defective preproteins led to the identification of the *prl* alleles of *secY* (*prlA*), *secE* (*prlG*), *secA* (*prlD*), and more recently *secG* (*prlH*) [6,9–11]. Early hypotheses predicted that the Prl suppressors function by expanded or altered interactions with signal sequences, facilitating recognition of mutant as well as wild type signal sequences [6].

More recent observations support an alternative mechanism of action for the PrlA/SecY and PrlG/SecE suppressors [8,12]. First, PrlA/G-mediated suppression does not exhibit allele specificity; all *prlA* and all *prlG* alleles that have been examined suppress all signal sequence mutations, implying that suppression is not due to a specific altered interaction that allows recognition of a mutant signal sequence [8,12]. Furthermore, all of the *prlA* and *prlG* alleles suppress complete deletions of the signal sequence, suggesting that an interaction between the signal sequence and the Prl suppressor is not necessary for suppression or even for export [5,8,13]. Based on these observations, it was proposed that the PrlA and PrlG suppressors do not function through altered interactions with signal sequences, but rather by loss of recognition [8,12]. The wild type SecE and SecY proteins are thought to function in concert to proofread the signal sequence of secretory proteins, rejecting defective precursors from the export pathway. The PrlA (SecY) and PrlG (SecE) suppressors are compromised in their ability to proofread, allowing export of proteins with mutations, or even complete deletions, of the signal sequence.

This proofreading model predicts that the critical step for translocation of a signal sequence-defective secretory protein is recognition by SecB and subsequent presentation to SecE/Y. SecB binds portions of the mature secretory protein [14–18], and SecA would bind to the SecB-precursor complex by virtue of its ability to bind SecB [19,20] as well as the preprotein [21–23]. Thus, SecA-mediated targeting of the preprotein to translocase would occur. In a wild type cell, the mutant precursor would be rejected at this point by SecY/E, but in a PrlA or PrlG suppressor strain, rejection would not occur and the mutant secretory protein would be exported.

In fact, all of the PrlA and PrlG suppressors are completely dependent on functional SecB for manifestation of suppressor activity [5,8]. Even certain proteins that are not normally SecB-dependent for translocation become SecB-dependent when they contain a signal sequence mutation and are exported via the suppressor pathway. For example, PhoA is normally exported independent of SecB. Deletion of the signal sequence from *phoA* severely compromises export; export is restored in a *prlA* suppressor strain. However, this suppression is dependent on SecB [5]. Therefore, there are characteristics of the mature portion of PhoA that promote recognition by SecB, and this recognition is essential for PrlA-mediated suppression.

Although *prlA* and *prlG* strains export secretory proteins that completely lack a signal sequence, there is no evidence of export of any cytoplasmic proteins in a *prl* suppressor strain [13]. That is, proteins that are supposed to remain in the cytoplasm are not mislocalized to the periplasm. It has been suggested that perhaps cytoplasmic proteins are not exported in *prl* suppressor strains because they fold rapidly and escape recognition by SecB, while secretory proteins, even those lacking a signal sequence, fold more slowly, allowing time for SecB recognition and binding [5,13]. SecB binds to a variety of unfolded proteins in vitro [24,25], although binding appears to be more selective in vivo [26]. This selectivity may be based on the slower folding characteristics of secretory proteins.

Therefore, we proposed previously that if a cytoplasmic protein contains a mutation that slows its rate of folding, SecB will recognize and bind to that mutant protein. Binding of SecB would result in targeting of the unfolded protein to the translocation apparatus. According to the proofreading model, in a wild type strain, SecE and SecY would reject the unfolded protein due to lack of a signal sequence. In a Prl suppressor strain, however, the proofreading function would be compromised and the mutant protein would be exported [8]. In the experiments described here, we tested this hypothesis by examining the export of such a cytoplasmic protein with a folding mutation. The results indicate that slow folding is not sufficient for SecB binding and subsequent targeting to translocase and further, that secretory proteins contain targeting information in addition to the signal sequence and the folding kinetics.

Results and Discussion

To test the hypothesis that a cytoplasmic protein containing a mutation that slowed its folding would be exported in a Prl suppressor strain, a well characterized cytoplasmic protein was obtained. The protein of choice was the amino terminal portion of the λ repressor protein, a 92 residue domain that folds into a stable, predominantly α-helical structure [27]. Folding studies were performed at a pH and ionic strength similar to physiological conditions and the folding parameters were predicted to approximate the in vivo situation. The wild type protein (N^{102LT}) is

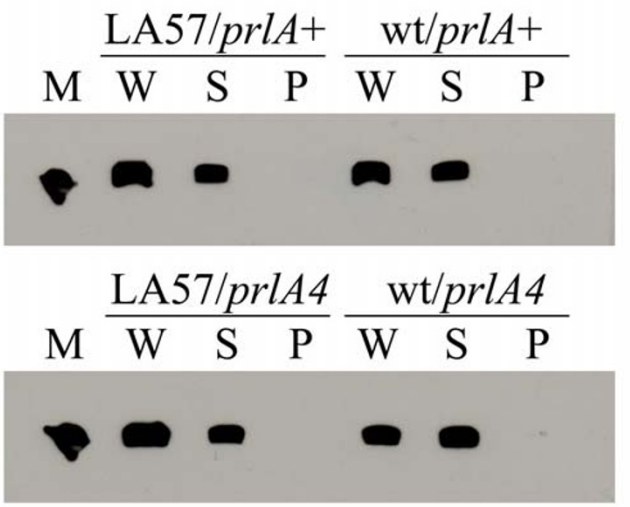

Figure 1
Immunodetection of λ repressor fragments in bacterial fractions LA57 indicates the slow folding λ repressor, LA57-N^{102LT}, while wt refers to the wild type λ repressor, N^{102LT}. The strains used were either wild type *secY*/*prlA* or *prlA4* as indicated. The individual lanes are as follows: M – purified N^{102LT} used as a molecular weight marker, W – whole cell lysate, S – spheroplast fraction (containing cytoplasmic contents and membranes), P – periplasmic fraction.

very stable at 37°C, with a T_m for unfolding of about 55°C. The mutant version that we used (LA57-N^{102LT}) contains a Leu to Ala alteration of amino acid 57 that dramatically reduces the stability of the folded structure. The LA57-N^{102LT} protein has a T_m of approximately 25°C, and is about 80% unfolded at 37°C and 60% unfolded at 30°C [27]. Further, the mutant protein has been shown to have a half-life of about 40 minutes in wild type *E. coli* strains at 37°C [27].

Localization of N^{102LT} and LA57-N^{102LT}

Plasmids pKL106 and pKL107 encoding LA57-N^{102LT}-FLAG or N^{102LT}-FLAG, respectively, were transformed into strains IM104 (wild type *secY*) or IM105 (*prlA4*). These strains contained deletion mutations in the *lon* and *degP* genes to limit proteolysis in both the cytoplasmic and periplasmic spaces. Following induction of the λ repressor fragment, whole cell lysates, spheroplasts, and periplasmic fractions were prepared from each strain. Samples were analyzed by Western blotting using antibodies directed against the FLAG epitope present at the carboxyl terminus of the λ repressor fragments.

According to our hypothesis, the largely unfolded nature of LA57-N^{102LT} should allow SecB to bind, resulting in targeting of LA57-N^{102LT} to translocase. In the *secY* wild type strain, proofreading by SecE/Y would block export of LA57-N^{102LT}, while in the *prlA4* strain, the PrlA4 suppressor would allow export of a portion of LA57-N^{102LT} to the periplasm. This was not the result we observed (Fig. 1). No LA57-N^{102LT} was detected in the periplasm, even in the *prlA4* strain, indicating that export did not occur to any significant extent. To ensure that all periplasmic contents were released during the spheroplast preparation, the blots were stripped of FLAG antibody and reprobed with antibody directed against MalE, a periplasmic binding protein. All detectable MalE was present in the periplasmic fractions (data not shown), demonstrating that complete release of the periplasmic contents had been attained. Therefore, there was no detectable export of LA57-N^{102LT} in the *prlA4* strain.

There were two reasonable explanations for this unexpected finding; 1) an unfolded state is not sufficient for SecB binding, or 2) SecB binding is not sufficient to promote export in a *prl* suppressor strain. To distinguish between these possibilities, we examined whether SecB was able to bind LA57-N^{102LT} using two different methods to identify interactions. The first approach was co-immunoprecipitation of LA57-N^{102LT}-SecB complexes, the second was an in vitro protease protection assay.

Co-immunoprecipitation of SecB complexes

Co-immunoprecipitations were performed using whole cell lysates of strains induced for expression of λ repressor. Immunoprecipitations were performed as described using antibodies directed either against SecB or against the His tag on the λ repressor. Following the immunoprecipitation reaction, samples were analyzed by Western blotting using the opposite antibody, that is, samples that had been immunoprecipitated with anti-SecB were examined with anti-His while those that had been precipitated with anti-His were probed with anti-SecB. In addition, as a positive control, the interaction of SecB with the secretory protein LamB was examined using anti-LamB antibodies. While an interaction between LamB and SecB was clearly seen (Fig. 2c), no interaction was observed between SecB and either N^{102LT} or LA57-N^{102LT} (Fig. 2a and 2b). This was true regardless of whether the precipitating antibody was directed against SecB or the His tag. The wild type N^{102LT} was not expected to interact with SecB as it would fold very rapidly and stably; it was more surprising that no interaction was detected between SecB and the destabilized LA57-N^{102LT}. This result implied that the unfolded nature of LA57-N^{102LT} was not sufficient for SecB binding.

Protease protection assays

To substantiate the results observed by co-immunoprecipitation, an in vitro protease protection assay was employed. SecB has a proteolytic-sensitive domain that is cleaved by proteinase K in a limited proteolysis reaction. When substrate is bound to SecB, the conformation is al-

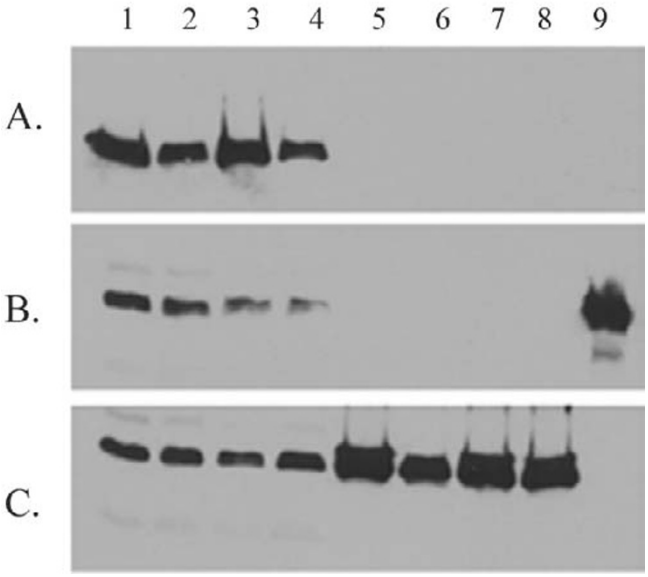

Figure 2
Immunoprecipitations of SecB complexes A. Precipitation was performed with antibody directed against SecB, followed by immunoblotting using antibody against the His tag on the λ repressor fragments. B. Precipitating antibody was directed against the His tag, immunoblotting utilized the anti-SecB antibody. C. Precipitation was carried out with antibody against LamB, anti-SecB was used for immunoblotting. In all gels, samples were as follows: lanes 1–4 – whole cell lysates prior to immunoprecipitation, lanes 5–8 – immunoprecipitated material; lanes 1 and 5 – N^{102LT}/*prlA4*, lanes 2 and 6 – LA57-N^{102LT}/*prlA4*, lanes 3 and 7 – N^{102LT}/*prlA*$^+$, lanes 4 and 8 – LA57-N^{102LT}/*prlA*$^+$, lane 9 in gel B – purified SecB.

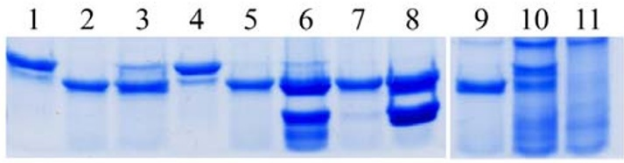

Figure 3
Protease protection assays Lane 1 – undigested SecB, lane 2 – SecB digested with proteinase K; lanes 3 through 10 – SecB was incubated with potential substrates at the indicated concentrations prior to proteinase K digestion; lane 3 – 20 nM poly-L-lysine, lane 4 – 150 nM poly-L-lysine; lane 5 – 150 nM N^{102LT} (wild type), lane 6 – 4 μM N^{102LT}; lane 7 – 150 nM LA57-N^{102LT} (slow folding mutant), lane 8 – 4 μM LA57-N^{102LT}; lane 9 – 150 nM proOmpA, lane 10 – 1.4 μM proOmpA; lane 11 – proOmpA alone digested with proteinase K. Lower molecular weight band in lanes 6 and 8 is the λ repressor fragment.

tered such that the site is no longer protease accessible, rendering SecB protease resistant [28]. Thus, this assay can be used to detect binding of SecB to a substrate. Poly-L-lysine and small peptides are routinely used as substrates for this analysis as demonstrated in Fig. 3 (lanes 3 and 4). To determine whether a larger protein was also able to protect SecB, we used purified, urea-denatured proOmpA as a substrate. The proOmpA also was able to bind to and protect SecB (Fig. 3, lanes 9 and 10) although a much higher concentration of substrate was required to achieve 50% protection. To ensure that the observed protection was not due simply to the high protein concentration in the reaction, a non-secretory protein was used as a negative control. BSA was added to the same concentration or higher as proOmpA with no resultant protection of SecB (data not shown). Therefore, this assay reliably serves as an additional method to observe SecB-substrate interactions.

The λ repressor proteins were purified on a nickel affinity chromatography column by virtue of the His tag at the carboxyl terminus of the proteins. Each was assayed for protection of SecB at concentrations up to and exceeding that used for proOmpA. Neither N^{102LT} nor LA57-N^{102LT} protected SecB from proteolysis, indicating that no binding occurred (Fig. 3, lanes 5–8). Although the mutant LA57-N^{102LT} is predicted to be in an unfolded conformation in the bacterial cell, the protease protection assay was performed at 4°C. To verify that refolding of LA57-N^{102LT} at this temperature was not interfering with interpretation of the results, we further tested the SecB binding activity by incubating LA57-N^{102LT} in 8 M urea, then diluting it into the protection reaction, similar to the conditions used for proOmpA. Again, no protection was detected (data not shown). These results demonstrated that even when fully unfolded, LA57-N^{102LT} was not a substrate for SecB binding.

Conclusions

The hypothesis driving the present studies was premised on the observation that *prl* suppressor strains are able to correctly identify secretory proteins, even in the absence of a signal sequence. It has been widely thought that this recognition was due to slow folding of secretory proteins in the cytoplasm, resulting in binding by SecB and subsequent targeting to translocase. While slow folding may be a necessary criterion for export, the data presented here indicate that an unfolded nature is not sufficient for SecB binding and targeting. This conclusion is based on failure of the LA57-N^{102LT} protein to bind SecB either in vivo or in vitro. The in vivo results are founded both on the co-immunoprecipitation experiments and on the fact that no export of the protein was detected in a *prlA4* strain, while the in vitro results are based on the protease protection assays. Therefore, we conclude that secretory proteins must contain information, in addition to the signal sequence and slow folding characteristics, that targets them to the

secretory pathway or that cytoplasmic proteins contain information that prevents export even if the folding parameters are altered. Further studies are underway to identify such targeting sequences.

Methods

Bacterial strains and plasmids

All bacterial strains used were derivatives of *E. coli* K12 strain MC4100 [29]. IM104 is MC4100 *lamB14D lon*::Tn*10 degP*::*kan* and IM105 is IM104 *prlA4*. Plasmids pKL106 and pKL107 are pET11c (Novagen) derivatives carrying tandem copies of the λ repressor fragment (derived from plasmids pAD103 and pAD105, generous gifts from Alan Davidson, University of Toronto and Robert Sauer, MIT). In pKL106, the wild type λ repressor is tagged with the myc epitope and the LA57 λ repressor has the FLAG epitope. In pKL107, the wild type λ repressor contains the FLAG eiptope and the LA57 mutant has the myc epitope. In all experiments, only antibodies to the FLAG epitope were used, therefore pKL106 resulted in detection of the mutant λ repressor and pKL107 was used to observe the wild type. Plasmids pIM101 and pIM102 were used in the co-immunoprecipitation assays and for purification of the λ repressor fragments. These plasmids are derivatives of pET11c that contain a single copy of the λ repressor, either wild type (pIM102) or LA57 (pIM101). The λ repressor inserts contain a FLAG tag at the carboxyl terminus followed by a hexahistidine motif.

Bacterial growth, induction and fractionations

Plasmid-containing bacteria were grown in LB [30] with ampicillin (100 μg ml^{-1}) at 30°C. Cells were induced for expression of the λ repressors at an OD_{600} = 0.4–0.5 by addition of 1 mM IPTG either for 30 minutes (spheroplast procedures) or for 3 hours (immunoprecipitation experiments).

Spheroplasts were prepared similarly to published protocols [31]. Briefly, 1 ml of bacterial cells was pelleted for 3 min at 16,000 rpm in an Eppendorf centrifuge. All subsequent steps were performed on ice. The cell pellet was resuspended in 0.5 ml spheroplast buffer [1 M sucrose, 25 mM TrisHCl (pH 8.0), 5 mM EDTA, and lysozyme (50 μg ml^{-1})]. After incubation on ice for 12 min, $MgCl_2$ was added to a final concentration of 3 mM and incubation continued for an additional 5 min. The spheroplasts were pelleted by centrifugation at 16,000 rpm for 10 min at 4°C. The resultant supernatant contained the periplasmic contents. The pellet, which consisted of the membranes and cytoplasmic contents, was resuspended in 0.5 ml of spheroplast buffer. A parallel sample was prepared without the final centrifugation step, resulting in a whole cell lysate. All samples were precipitated with TCA and resuspended in gel loading buffer preparatory to electrophoresis.

Immunoblotting

Fractions were electrophoresed on 17% polyacrylamide gels and transferred to nitrocellulose. Immunoblotting was performed as described [32] using monoclonal antibody M2 directed against the FLAG epitope (Eastman Kodak) at a 1:600 dilution, or monoclonal antibody against MalE (Sigma) at a 1:500 dilution. When necessary, antibodies were stripped from the nitrocellulose by incubation in stripping buffer [1 M glycine (pH 2.2), 20 mM MgAc, 50 mM KCl] followed by immunoblot blocking solution.

Immunoprecipitations

Bacterial cells were induced for 3 hours for λ repressor expression as described above and harvested by French Press lysis. Lysates were immunoprecipitated with minor alterations to previous protocols [8]. Specifically, the IP buffer contained only 50 mM NaCl and incubation with the primary antibody was performed overnight at 4°C. Antibodies used were directed against the His epitope (monoclonal, Boehringer Mannheim), SecB (polyclonal, gift from William Wickner), or LamB (polyclonal, gift of Thomas Silhavy). Immune complexes were collected with Protein A agarose (Pierce), samples were electrophoresed on 15% polyacrylamide gels, and immunoblotting performed as described above. Following immunodetection, all blots were stripped of primary antibody and reprobed with the precipitating antibody to verify that precipitation had occurred.

Protease protection assays

SecB protease protection assays were performed as described [28], followed by electrophoresis on 15% polyacrylamide gels and detection by Coomassie Blue staining. The λ repressor fragments were purified by nickel NTA affinity chromatography (Qiagen).

Authors' contributions

Localization studies were performed by IM, immunoprecipitations and protease protection assays were carried out by MAS. AMF conceived of the study and participated in its design and coordination. The manuscript was written by AMF and edited jointly by all authors. All authors read and approved the final manuscript.

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