Discrimination between SRP- and SecA/SecB-dependent substrates involves selective recognition of nascent chains by SRP and trigger factor

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Besides SecA and SecB, *Escherichia coli* cells possess a signal recognition particle (SRP) to target exported proteins to the SecY translocon. Using chemical and site-specific cross-linking *in vitro*, we show that SRP recognizes the first signal anchor sequence of a polytopic membrane protein (MtlA) resulting in cotranslational targeting of MtlA to SecY and phospholipids of the plasma membrane. In contrast, a possible interaction of SRP with the secretory protein pOmpA is prevented by the association of trigger factor with nascent pOmpA. Trigger factor also prevents SecA from binding to the first 125 amino acids of pOmpA when they are still associated with the ribosome. Under no experimental conditions was SecA found to interact with MtlA. Likewise, virtually no binding of trigger factor to ribosome-bound MtlA occurs even in the complete absence of SRP. Collectively, our results indicate that at the stage of nascent polypeptides, polytopic membrane proteins are selected by SRP for co-translational membrane targeting, whereas secretory proteins are directed into the SecA/SecB-mediated post-translational targeting pathway by means of their preferential recognition by trigger factor.

Keywords: polytopic membrane proteins/SecA/SecD/signal recognition particle (SRP)/trigger factor

Introduction

The bacterial signal recognition particle (SRP) and SRP receptor (SR) were originally identified via the structural homology between their components Ffh (P48), 4.5S RNA and FtsY, and their eukaryotic counterparts, the 54 kDa subunit of SRP, the 7S RNA and the α -subunit of the SRP receptor (Poritz *et al.*, 1988; Struck *et al.*, 1988; Bernstein *et al.*, 1989; Römisch *et al.*, 1989). An involvement of the bacterial SRP in protein export was initially inferred from studies with conditional mutants of the SRP/SR components that exhibited a retardation in the processing of several precursor proteins (Poritz *et al.*, 1990; Phillips and Silhavy, 1992; Lührink *et al.*, 1994).

Components of the bacterial SRP also proved to be active when tested in chimeric eukaryotic–prokaryotic SRP particles or in heterologous assay systems of non-bacterial origin (Ribes *et al.*, 1990; Bernstein *et al.*, 1993; Hauser *et al.*, 1995). A participation of the bacterial SRP/SR in the biogenesis of plasma membrane proteins was first demonstrated *in vivo* for lactose permease (MacFarlane and Müller, 1995) and later confirmed for several other membrane proteins (de Gier *et al.*, 1996; Seluanov and Bibi, 1997; Ulbrandt *et al.*, 1997). Consistent with plasma membrane proteins being substrates for the bacterial SRP, recent cross-linking studies revealed a preferential recognition by SRP of the more hydrophobic signal sequences, such as the signal anchor sequences of membrane proteins (Valent *et al.*, 1997; de Gier *et al.*, 1998).

A homologous *in vitro* system allowing assay of the authentic functions of the bacterial SRP was only recently described. It thereby became possible to reproduce *in vitro* the integration of polytopic membrane proteins into plasma membrane vesicles in an Ffh (P48)-, 4.5S RNA- and FtsY-dependent manner (Koch *et al.*, 1999). Integration did not require SecA and SecB in contrast to the transmembrane translocation of a secretory protein (pOmpA), which was exclusively dependent on the Sec proteins and could not be driven by SRP/SR (Koch *et al.*, 1999). These results therefore suggested that in *Escherichia coli*, SRP/SR and SecA/SecB constitute non-overlapping targeting devices for polytopic membrane and secretory proteins, respectively. Contradictory to those findings, however, other membrane proteins were found cross-linked to SecA when targeted to the plasma membrane (Valent *et al.*, 1998) or to require SecA for integration *in vivo* (Qi and Bernstein, 1999). In an attempt to clarify this issue, we have analysed early events in the biogenesis of an SRP-dependent polytopic membrane protein (MtlA) lacking major translocated loops by various cross-linking approaches, and compared it with an SRP-independent secretory protein. As such we used OmpA, which in *E. coli* is secreted into the periplasm by means of a cleavable signal sequence before being inserted into the outer membrane. The results clearly confirm the independent targeting functions of SRP and SecA/SecB for membrane and secretory proteins, respectively, and reveal that the decision for either pathway is taken early during protein synthesis at the ribosome by the discriminatory properties of Ffh (P48) and trigger factor, a ribosome-associated chaperone (Scholz *et al.*, 1997) and peptidyl–prolyl isomerase (Stoller *et al.*, 1995).

Results

Co-translational recognition of a polytopic membrane protein by Ffh (P48)

Using a recently described oligodeoxynucleotide-dependent method to generate ribosome-associated nascent chains

Fig. 1. Co-translational binding of the *E. coli* SRP to a polytopic membrane protein. The *mtlA* gene encoding mannitol permease of *E. coli* was transcribed and translated by a reconstituted *in vitro* system (see Materials and methods) in the presence of an anti-sense oligodeoxynucleotide giving rise to a 189 amino acid N-terminal fragment of MtlA (MtlA189). 35 S-labelled translation products were precipitated with trichloroacetic acid (TCA), separated by SDS-PAGE, and visualized by phosphorimaging. The reason why MtlA189 was resolved into two species is unclear. Co-synthesis of some full-length MtlA was usually obtained under the applied conditions. Molecular masses are indicated to the left of (A). Ffh (P48) at 20 nM, 4.5S RNA at 20 nM, and inside out membrane vesicles (INV) of *E. coli* were present during synthesis. Where indicated, cross-linking was followed by centrifugation through a sucrose cushion and immunoprecipitation (IP) using anti-Ffh (P48) antibodies (α Ffh). The cross-link between MtlA189 and Ffh (P48) is indicated ($\times$ Ffh).

(RANCs) of bacterial proteins (Behrmann *et al.*, 1998), we have synthesized *in vitro* the 189 amino acid N-terminal fragment of *E. coli* mannitol permease (Figure 1A; MtlA189). This fragment encompasses four transmembrane segments including an ~60 residue-long cytoplasmic loop between transmembrane segments 2 and 3 (Sugiyama *et al.*, 1991). When purified Ffh (P48) was present during synthesis of MtlA189 and translation products were post-translationally treated with the chemical cross-linker *N*-hydroxysulfosuccinimidyl 4-azidobenzoate (sulfo-HSAB) a distinct adduct of ~67 kDa was obtained (Figure 1A, lane 5, $\times$ Ffh). Due to its size and its cross-reactivity with anti-Ffh (P48) antibodies (Figure 1A, lane 7), this material must represent a cross-link product between the 19 kDa nascent chain of mannitol permease and Ffh (P48); its

sedimentation through a sucrose cushion (lane 6) indicates that it is associated with the ribosome.

As the reconstituted transcription/translation system used here is largely free of endogenous Ffh (P48) (Koch *et al.*, 1999), the cross-link was not detected without addition of Ffh (P48) (Figure 1A, compare lanes 2 and 5; Figure 1B, lanes 2 and 4). In contrast, addition of 4.5S RNA was not required for cross-linking to occur (Figure 1B, lanes 4 and 6), consistent with the fact that sufficient amounts of 4.5S RNA are contained in at least one of the subfractions of the reconstituted system (Koch *et al.*, 1999). No cross-link to Ffh (P48) was observed when the MtlA189 nascent chain was released from the ribosome by puromycin (Figure 1B, lanes 6 and 10). Similarly, binding to Ffh (P48) was abolished by the addition of inside out inner membrane vesicles (INV) of *E. coli* (Figure 1B, lanes 6 and 12) and by the non-hydrolyzable GTP-analog GMP-PNP (not shown) as previously described (Valent *et al.*, 1998). These results are compatible with a co-translational binding of the *E. coli* SRP to nascent chains of a polytopic membrane protein that requires the presence of ribosomes and is not sustained after targeting to the plasma membrane.

In order to demonstrate binding of Ffh (P48) to the first signal anchor sequence of MtlA as an expected recognition site for SRP, L-4'-[3-(trifluoromethyl)-3H-diazirin-3-yl] phenylalanine (Tmd-Phe), a photoactivatable derivative of phenylalanine (Graf *et al.*, 1997), was incorporated at a single position within the first transmembrane helix of MtlA. To this end, a TAG stop codon mutant of *mtlA* at position 37 was constructed by site-directed mutagenesis and a translation product was obtained by *in vitro* suppression of the nonsense codon using a suppressor tRNA that had been loaded chemically with Tmd-Phe. UV irradiation of Tmd-Phe generates a highly reactive carbene that attacks any adjacent component. Figure 2A illustrates that with DNA of the MtlA-37 TAG mutant as template, synthesis of nascent MtlA189 was achieved in the presence of the specific suppressor tRNA (MtlA189, lanes 11–14). Under similar conditions using a different oligodeoxynucleotide, a shorter nascent chain of 130 amino acids of MtlA (MtlA130) was also obtained (Figure 2A, cf. lanes 7–10). Synthesis of both nascent MtlA chains by suppression of the stop codon mutation was less efficient than with wild-type DNA (Figure 2A, see lanes 1–4 for comparison) and was strongly dependent on the nature of the stop codon-bearing tRNA (not shown; see Cloud *et al.*, 1996).

If Ffh (P48) was present during synthesis of both nascent chains of MtlA, and if samples were UV irradiated, two individual cross-links appeared (Figure 2A, $\times$ Ffh, framed area and Figure 2B, lanes 1–8), which were identical in size to those obtained by the chemical cross-linker sulfo-HSAB (Figure 2B, lanes 9–12). Similar results were obtained with stop codons engineered at positions 27, 29, 32 and 42 within the first transmembrane segment of MtlA (not shown). This indicates that within nascent chains of the polytopic membrane protein MtlA, the first signal anchor sequence serves as recognition site for Ffh (P48).

Trigger factor discriminates between nascent secretory and polytopic membrane proteins

Since Ffh (P48) had also been found cross-linked *in vitro* to secretory proteins (Valent *et al.*, 1995, 1997), we examined

Fig. 2. Ffh (P48) recognizes the first transmembrane segment of a polytopic membrane protein. (A) MtlA189 and a shorter nascent chain of 130 amino acids (MtlA130) were synthesized *in vitro* as outlined in the legend to Figure 1 by use of the indicated anti-sense oligodeoxynucleotides (Oligo). Template DNA was either wild-type *mtlA* (MtlA-WT) or a mutant *mtlA* gene carrying a stop codon at amino acid position 37 (MtlA-37 TAG). In the latter case, expression of MtlA130 and MtlA189, together with some full-length MtlA, was dependent on the addition of a suppressor tRNA loaded with Tmd-Phe (Tmd-Phe). Ffh (P48) was present during synthesis as indicated. UV, irradiation. (B) As (A) except that samples shown in lanes 9–12 were prepared as in Figure 1.

Fig. 3. Trigger factor specifically recognizes nascent chains of secretory proteins. Nascent chains of pOmpA, 125 and 191 amino acids in length, and of MtlA, 189 residues long, were synthesized in the reconstituted transcription/translation system by use of the appropriate anti-sense oligodeoxynucleotides as described before, and collected by centrifugation through a sucrose cushion (Isolated RANCs). After resuspension, they were treated with the cross-linker sulfo-HSAB as indicated. (*), cross-links of pOmpA125 and pOmpA191 with trigger factor; (x), that of MtlA189 with Ffh (P48). Lanes 2 and 3, the splitting of the lower trigger factor adduct is most likely to stem from that of the pOmpA125 band.

nascent chains of the precursor of OmpA for early molecular interactions. As shown in Figure 3, two RANCs of pOmpA, 125 and 191 amino acids in length, were synthesized *in vitro*,

isolated by sedimentation and treated with the chemical cross-linker sulfo-HSAB. Two cross-links each were thus obtained (Figure 3, lanes 2 and 5, asterisks), which gave signals of similar intensity to that between MtlA189 and Ffh (P48) (lane 8, x). Because the cross-links of the isolated pOmpA RANCs had formed without addition of any further protein they must involve a component tightly associated with the RANCs. By immunoprecipitation, this cross-linking partner was identified as *E. coli* trigger factor (Figure 3, lanes 3 and 6). Trigger factor is a 48 kDa protein (Guthrie and Wickner, 1990), which on SDS-PAGE migrates with an apparent molecular mass of 58 kDa (Hesterkamp *et al.*, 1996). The smaller of each pair of cross-links therefore corresponds arithmetically to a complex of trigger factor and the 125 and 191 residue-long fragments of pOmpA, respectively. Since the larger trigger factor adducts differ in size they cannot be derived from full-length pOmpA. They are most likely to be complexes between trigger factor and pOmpA nascent chains harbouring aberrant electrophoretic mobilities (cf. Behrmann *et al.*, 1998; corresponding doublets were obtained with other cross-linking partners of pOmpA, cf. Figure 6).

When RANCs of MtlA189 were prepared under identical conditions to those of pOmpA, i.e. in the presence of the same amount of ribosome-associated trigger factor, yet with exogenously added Ffh (P48), only SRP and no trigger factor was found cross-linked (Figure 3A, lanes 9 and 10). Thus, if trigger factor and Ffh (P48) were both present during synthesis the membrane protein was recognized only by Ffh (P48). This, however, was not due to competition between TF and Ffh (P48) for the same binding sites, because in the absence of Ffh (P48), MtlA189 was not found cross-linked to any major protein (cf. Figure 1). Only upon immunoprecipitation with anti-trigger factor antibodies did an extremely faint cross-link

Fig. 4. Recognition of nascent pOmpA by trigger factor is followed by the individual binding of SecA and SecB upon release from the ribosome. (A) *In vitro* synthesis of pOmpA125 in the presence of exogenously added Ffh + 4.5S RNA (SRP), trigger factor (TF), SecA and SecB. RANCs were collected by centrifugation and treated with the cross-linker DSS either before or after dissociation by puromycin. The antisera used are indicated. Cross-linking products with SecA ($\blacktriangleright$) and SecB ($\rightarrow$) are marked. (B) As (A) except that RANCs of pOmpA125 were first isolated and then incubated with Ffh (P48), 4.5S RNA, trigger factor, SecA and SecB. (C) Isolated RANCs of pOmpA125 were incubated with the indicated concentrations of SecA (dimer) and SecB (tetramer) prior to treatment with puromycin and DSS.

between MtlA189 and trigger factor become detectable, which disappeared upon addition of SRP (not shown). These results suggest that trigger factor has a far lower affinity for nascent chains of a polytopic membrane as opposed to a secretory protein, and that *in vivo* any interaction between MtlA189 and trigger factor is likely to be overridden by Ffh (P48).

At the ribosome, trigger factor is the primary reaction partner of nascent secretory proteins

Both SecA and SecB have been reported to bind to nascent secretory proteins (Kumamoto and Francetić, 1993; Chun and Randall, 1994; Randall *et al.*, 1997; Behrmann *et al.*, 1998). To examine this by cross-linking, SecA and SecB were added to a synthesis reaction of pOmpA125. Subsequently, RANCs were isolated and cross-linked with disuccinimidyl suberate (DSS) (Figure 4A). Despite the addition of both Sec proteins, the only cross-links of pOmpA125 detected and verified by immunoprecipitation were those with trigger factor (Figure 4A, lanes 2–6, asterisks). However, if RANCs were dissociated by puromycin prior to cross-linking, the trigger factor cross-links were completely lost (Figure 4A, lane 8) in favour of two faint adducts that by size and immunoreactivity

were identified as complexes between pOmpA125 and SecA and SecB (lanes 9 and 10, arrows). To bind to pOmpA125 in these conditions both Sec proteins must have been co-isolated with RANCs, which has in fact previously been found to occur (Behrmann *et al.*, 1998). Stronger cross-linking signals of pOmpA125 with SecA and SecB were obtained when the Sec proteins were added only after isolation of RANCs (Figure 4B, lanes 8–10). Since our antibodies raised against SecB did not consistently recognize the pOmpA125–SecB cross-links, isolated pOmpA125 RANCs were incubated with varying amounts of SecA and SecB before treatment with puromycin and DSS (Figure 4C). The cross-links in question were in fact dependent on the presence and the amount of the respective Sec proteins, thus confirming their identity (Figure 4C, arrows). Furthermore, SecA was found to bind in the absence of SecB and vice versa indicating an individual recognition of this fragment of pOmpA by SecA and SecB. The conclusion from these results therefore is that a secretory protein when still attached to the ribosome is bound with high affinity by trigger factor. This association is released immediately upon leaving the ribosome whereafter the protein is recognized by SecA and SecB.

Fig. 5. No interaction between SecA/SecB and a polytopic membrane protein after release from the ribosome. *In vitro* synthesis and isolation of RANCs of MtlA189. For further details see legend to Figure 4B. The cross-linking product between MtlA189 and Ffh (P48) is indicated (x Ffh).

Even after release from the ribosome, polytopic membrane proteins do not bind SecA/SecB

We have previously demonstrated that MtlA requires exclusively the bacterial SRP/SR for integration into plasma membrane vesicles, whereas translocation of pOmpA was only dependent on SecA and SecB (Koch *et al.*, 1999). In full agreement, when nascent MtlA189, which was recognized only by Ffh (P48) (Figure 5, lanes 2–6), was released from the ribosomes by puromycin, association with Ffh (P48) was lost but no binding of SecA and SecB could be detected by cross-linking (lanes 8–12).

Trigger factor prevents binding of Ffh (P48) and SecA to a nascent secretory protein

To corroborate the dominant role of trigger factor in the recognition of nascent secretory proteins, pOmpA125 was synthesized by a reconstituted transcription/translation system whose constituents had been prepared from a trigger factor null mutant. In the experiment depicted in Figure 6A, small residual amounts of trigger factor giving rise to cross-links with pOmpA125 (lane 4, asterisks) stemmed from the T7 RNA polymerase preparation used. In contrast to the levels of trigger factor in previous experiments, however, both Ffh (P48) and SecA were now also found associated with pOmpA125 when they had been present during synthesis (Figure 6A, lanes 3 and 6). In contrast, SecB did not bind to the first 125 amino acids of pOmpA when still associated with the ribosome (Figure 6A, lane 5) but only after dissociation of RANCs by puromycin (cf. Figure 4). This is consistent with the previously described critical length of a nascent chain required for binding of SecB (Randall *et al.*, 1997).

When SecA was added post-translationally to isolated RANCs, similar strong cross-links between pOmpA125 and SecA were obtained if trigger factor was missing

(Figure 6B, lanes 2 and 3). This result indicates that SecA can in fact recognize a substrate already at the stage of a nascent chain bound to the ribosome. In this experiment, synthesis had proceeded virtually in the complete absence of trigger factor (Figure 6B, lane 4). If trigger factor was now exogenously added during synthesis, it totally displaced SecA from the RANCs of pOmpA125 (Figure 6B, lanes 6–8).

Likewise, the fact that Ffh (P48) was found associated with RANCs of pOmpA125 in conditions in which trigger factor was either limiting (Figure 6A) or completely absent (Figure 6C, lanes 2 and 3) clearly demonstrates that nascent secretory proteins also have the inherent capability to bind to Ffh (P48). As with SecA, this interaction was completely suppressed by trigger factor supplemented in addition to SRP (Figure 6C, lanes 5–7). Therefore, a secretory protein when bound to the ribosome can in principle associate with Ffh (P48) and SecA and, after having reached a critical length, presumably also with SecB. At native ribosomes, however, these interactions are obviously overwhelmed by the high affinity of trigger factor for nascent secretory proteins.

Interaction of nascent MtlA with SecY and phospholipids reflects co-translational targeting and insertion of polytopic membrane proteins in *E. coli*

Next, the cross-linking strategy was used to obtain information on the molecular environment of membrane and secretory proteins during their initial stages of integration and translocation, respectively. As illustrated above, the Ffh (P48)-specific adduct disappeared when RANCs of MtlA189 were synthesized in the presence of inside out membrane vesicles (see band x in Figure 7A, lanes 2 and 5). Instead, anti-SecY antibodies now recognized an ~50 kDa cross-linking product in INV-containing samples (Figure 7A, compare lanes 3 and 6, arrowhead), which, however, was not maintained after addition of puromycin (lane 9). Due to its cross-reactivity with anti-SecY antibodies, and its size, this adduct represents a complex between MtlA189 and SecY, the latter migrating aberrantly on SDS-PAGE as a 35 kDa protein (Ito, 1984). Thus, nascent MtlA189, as long as it is still in contact with the ribosome, is found in close vicinity to SecY, indicating attachment of MtlA189 RANCs to the plasma membrane. When in these experimental conditions cross-linking was performed by use of Tmd-Phe positioned at three different locations within the first transmembrane segment of MtlA (residues 27, 32, 37), a 52 kDa INV protein was obtained as binding partner of MtlA (not shown). This protein did not react with antibodies directed against SecD, SecY, FtsY and FtsH, and its identity so far remains unknown.

Confirmatory of a co-translational mode of integration, the signal anchor sequence of a short ribosome-bound chain of MtlA (MtlA130) was also found in immediate proximity to membrane lipids (Figure 7B), similar to what has been reported for microsomal membrane proteins (Martoglio *et al.*, 1995). Upon UV irradiation, the three different stop codon mutants of MtlA130 gave rise to an INV-dependent cross-linking product that was slightly larger than the polypeptide itself (Figure 7B, arrow). These products disappeared upon treatment with phospholipase A2 (PLA₂) indicating that the cross-linked material

Fig. 6. Trigger factor prevents Ffh (P48) and SecA from binding to nascent secretory proteins. (A) *In vitro* synthesis of pOmpA125 by a reconstituted transcription/translation system whose components were prepared from a trigger factor knock-out strain of *E. coli*. Residual amounts of trigger factor are derived from the T7 RNA polymerase preparation as verified by immunoblotting (not shown). SRP and Sec proteins were present during synthesis. Marked are cross-links between pOmpA125 and SecA (→), trigger factor (*) and Ffh (x). (B) As (A) except that a trigger factor-free preparation of T7 RNA polymerase was used. RANCs of pOmpA125 were first isolated and then incubated with SecA. Where indicated, trigger factor had been added during synthesis. (C) As (A) except that a trigger factor-free preparation of T7 RNA polymerase was used. All indicated proteins were present during the period of synthesis. The two labelled bands of molecular masses higher than the pOmpA125–Ffh adduct (x), which are most clearly seen in lanes 1 and 2, are not cross-linking products because they appeared even in the absence of DSS.

contained phospholipids. The interactions of ribosome-associated MtlA chains with phospholipids and membrane proteins both demonstrate that in *E. coli* membrane insertion of polytopic membrane proteins occurs co-translationally.

A secretory protein when spanning the translocon is in immediate proximity to SecA, SecY and SecD, and a 60 kDa membrane protein

In contrast to membrane proteins, RANCs of secretory proteins, such as pOmpA, do not bind to INV (Behrmann *et al.*, 1998) and could therefore not be used to obtain translocation intermediates. These were instead generated in the form of pOmpA chains COOH-terminally coupled to tRNA by dissociating nascent pOmpA125–ribosome complexes with 8 M urea, which leaves the covalent linkage between the peptide and the tRNA intact (Matlack *et al.*, 1997). When incubated with INV, pOmpA–peptidyl-tRNA leads to a jamming of the SecY translocon as shown by a loss in both the translocation and integration capacity of these membrane vesicles (Koch *et al.*, 1999). Although the pOmpA–tRNA complexes were in general not detectable after SDS–PAGE, presumably because of the instability of the peptidyl–tRNA bond, most of pOmpA125 prepared by treating RANCs with 8 M urea was precipitated with cetyltrimethylammonium bromide (CTAB), indicative of a covalent linkage to tRNA [Figure 8, see (p)OmpA125 in lanes 2 and 6].

When the pOmpA125–tRNA complexes were incubated with INV in the presence of SecA and SecB, treatment with the membrane-permeable cross-linker DSS resulted in several cross-linking products (Figure 8, lane 7) of which SecA (lane 9; the antiserum used was raised against SecD and SecA) and SecY (lane 10) were clearly identified

by immunoprecipitation. An adduct of ~29 kDa (Figure 8, ★, lane 7) was recognized by antibodies directed against the periplasmic chaperone Skp (lane 8). This cross-link was also obtained when pOmpA125 had been allowed to translocate completely into INV following release from the ribosome by puromycin (Figure 8, lane 3). To probe the molecular environment exclusively of those pOmpA125 chains arrested within the translocon, immunoprecipitation was performed only on DSS-treated samples that had been precipitated with CTAB thus enriching the tRNA-coupled pOmpA125 chains. These true translocation intermediates were found cross-linked to SecA (Figure 8, lane 13) and SecY (lane 14) and also faintly to SecD (lane 13, ⇨). In addition, an unidentified membrane protein of ~60 kDa (Figure 8, ? in lanes 7 and 11) was also found in the immediate vicinity of pOmpA125, whereas no cross-links with SecE were detected (not shown). In these conditions, no more significant binding of pOmpA to Skp was observed (Figure 8, lane 12) indicating that a 125 amino acid N-terminal fragment of OmpA spanning the membrane is not yet recognized by the chaperone Skp involved in the folding of outer membrane proteins in the periplasmic space (Schäfer *et al.*, 1999).

Discussion

Polytopic membrane proteins of *E. coli* are specifically and exclusively recognized by SRP

A biochemical analysis employing purified SRP/SR components and membrane vesicles has recently demonstrated that in bacteria polytopic membrane proteins exclusively require SRP/SR for membrane integration (Koch *et al.*, 1999). This has now been confirmed by an *in vitro* cross-linking approach revealing Ffh (P48) as the only relevant

Fig. 7. A polytopic membrane protein of *E. coli* inserts cotranslationally into the plasma membrane. (A) *In vitro* synthesis of MtlA189 in the absence or presence of membrane vesicles (INV). Cross-links between MtlA189 and Ffh (x) and SecY (arrowhead) are indicated. To obtain the SecY cross-reacting signal, reaction size was scaled up 15-fold. (B) *In vitro* synthesis of MtlA130 derived from wild-type *mtlA* or stop codon mutants whose positions are indicated by numbers (e.g. 27-Tmd-Phe). Where applicable, INVs were present during synthesis. PLA₂, following irradiation samples were treated with 1% Triton X-100 and 2 U of phospholipase A₂ for 5 min at 42°C. The arrows point to the cross-linking product between MtlA and membrane phospholipids. For better resolution, these samples were separated on 13–20% polyacrylamide gradient gels.

protein interacting with nascent chains of the polytopic membrane protein MtlA. Consistent with previous findings (Valent *et al.*, 1997; de Gier *et al.*, 1998), site-specific cross-linking reveals the hydrophobic signal anchor sequence of MtlA as a recognition site for Ffh (P48).

Besides Ffh (P48), no other targeting factor of nascent MtlA was detected, suggesting that the only way a polytopic membrane protein can reach the membrane is via recognition by SRP. The precursor of the M13 major coat protein, termed procoat, is as yet the only membrane protein known to insert spontaneously into the plasma membrane of *E. coli*. Insertion of procoat involves a low hydrophobic, cleavable signal sequence and proceeds independently of SecA and SecY (Wolfe *et al.*, 1985), SecE and SRP (de Gier *et al.*, 1998). Procoat, however, is rendered SRP-dependent by increasing the hydrophobicity of its signal sequence (de Gier *et al.*, 1998). In contrast to wild type, the SRP-dependent version of procoat does not integrate any more spontaneously but requires an active translocon (de Gier *et al.*, 1998). Spontaneous integration therefore appears to be an inherent property of procoat and does not represent an alternative to the SRP-mediated pathway for other membrane proteins.

The fact that no cross-links between SecA/SecB and MtlA could be detected following release from the ribo-

Fig. 8. In the translocon, a secretory protein is in contact with SecA, Sec and SecD, and an unidentified membrane protein. RANCs of pOmpA125 were synthesized and isolated as described. Lanes 1–4, after resuspension RANCs were incubated with SecA, SecB and INV, and finally with puromycin. Following cross-linking with DSS, samples were either precipitated with TCA or with CTAB. Lanes 5–14, pOmpA125-tRNA complexes were incubated with SecA, SecB and INV. After treatment with DSS, samples were further treated as specified at the top of the figure. Marked are cross-links between pOmpA125 and SecA (●), SecD (◄), SecY (►) and the periplasmic Skp (★). An unidentified cross-linking partner (?) is also shown.

some, is in full agreement with the SecA/B-independent targeting and integration of MtlA observed *in vivo* and *in vitro* (Werner *et al.*, 1992; Koch *et al.*, 1999). Likewise, polytopic lactose permease has been shown to integrate independently of SecA into the plasma membrane of *E. coli* (Yamato, 1992; MacFarlane and Müller, 1995). In contrast, MalF (Traxler and Murphy, 1996), leader peptidase (Wolfe *et al.*, 1985), FtsQ (Valent *et al.*, 1998) and AcrB-PhoA fusion proteins (Qi and Bernstein, 1999) are all membrane proteins that were reported to require SecA for integration. The common denominator of these proteins is that in addition to an integral membrane domain of varying sizes, all four examples contain extended periplasmic loops or domains that have to be translocated across the plasma membrane. That these hydrophilic moieties determine dependence on SecA was convincingly shown for leader peptidase, whose requirement for SecA is lost by inverting the membrane topography and thereby avoiding translocation of the long periplasmic tail (von Heijne, 1989). However, the inverted enzyme remains dependent on an active SRP for membrane integration (de Gier *et al.*, 1996). The combined results therefore suggest that a plasma membrane protein of *E. coli*, provided it contains a sufficiently hydrophobic signal sequence, utilizes SRP for targeting and integration. SecA in addition would only be required if the membrane protein harbours extended hydrophilic parts that have to be translocated across the membrane.

Trigger factor prevents recognition of secretory proteins by SRP

Original studies with conditional *ffh* (Phillips and Silhavy, 1992) and *ftsY* (Luirink *et al.*, 1994) mutants had reported

reduced export kinetics for secreted proteins under conditions limiting the intracellular levels of Ffh (P48) and FtsY. This phenomenon is most likely to be caused by a depletion of the SRP/SR-dependent SecY protein (Seluanov and Bibi, 1997; Koch *et al.*, 1999) rather than by a direct negative influence of the lack of SRP/SR on the export of secreted proteins. In fact, biochemical investigations so far have ruled out any participation of SRP/SR in the translocation of pOmpA across the plasma membrane (Behrmann *et al.*, 1998; Koch *et al.*, 1999). In full agreement, Ffh (P48) was not found cross-linked to nascent pOmpA chains when attached to *E. coli* ribosomes. Only upon removal of trigger factor *in vitro* did the potential of Ffh (P48) to recognize nascent secretory proteins become visible. Likewise, interaction of the secreted protein PhoE with Ffh (P48) in the presence of trigger factor was observed only at high concentrations of SRP (Valent *et al.*, 1997). These results suggest that the documented SRP-independent export of secreted proteins in *E. coli* is ultimately due to trigger factor preventing SRP from binding to secretory proteins at the ribosome.

The failure of Ffh (P48) to bind to nascent secretory proteins in the presence of trigger factor, however, is not the result of competition for the same binding site. Analysis of signal sequence mutants of alkaline phosphatase (PhoA) revealed a direct correlation between the hydrophobicity of a signal sequence and the degree of cross-linking to Ffh (P48) but not to trigger factor (Valent *et al.*, 1997). These findings suggested that the signal sequence of a secreted protein is the likely recognition site for Ffh (P48) but not for trigger factor. In addition, trigger factor does not associate with the polytopic membrane protein MtlA despite the presence of a hydrophobic signal anchor sequence. On *E. coli* ribosomes, trigger factor has now been found in close proximity to nascent chains of secreted proteins (pPhoA; Valent *et al.*, 1997) (pOmpA; this study), membrane proteins with substantial translocated domains (FtsQ, Lep; Valent *et al.*, 1997, 1998) and cytosolic proteins (chloramphenicol acetyltransferase; Valent *et al.*, 1997). The actual recognition site(s) for trigger factor on the various substrates, however, are unknown. Our results indicate that they must harbour specific information that is not contained in the polytopic membrane protein MtlA, at least not within the first 189 amino acids including a 63 residue-long hydrophilic loop (Sugiyama *et al.*, 1991). Since this loop contains four lysine residues, binding of trigger factor would not have been missed using the amine-reactive cross-linker DSS. The same reasoning applies to other parts of nascent MtlA189 that contain lysine residues similarly located to those within nascent pOmpA191.

The individual targeting pathways of membrane and secreted proteins therefore have a 2-fold origin: (i) the presence of an N-terminal signal sequence with a high hydrophobic core concomitant with a lack of a trigger factor binding site results in the exclusive recognition of polytopic membrane proteins by Ffh (P48); (ii) a sequence-specific recognition of a nascent chain by trigger factor prevents binding of Ffh (P48) to secretory proteins containing an N-terminal signal sequence of low hydrophobicity, i.e. a weak binding site for Ffh (P48). Avoidance of interaction with SRP by trigger factor enables these proteins to bind to SecA/SecB late during or after

translation. Proteins like FtsQ and leader peptidase are recognized both by trigger factor and Ffh (P48) and consequently involve SRP and SecA in their targeting and assembly (Valent *et al.*, 1997, 1998). Because SecA and SecB were not found to bind to MtlA and obviously do not associate with cytosolic proteins, the recognition sites for both Sec proteins must differ from those for Ffh (P48) and trigger factor. The precise nature of the binding sites for SecA and SecB remains to be determined. Exactly how trigger factor interferes with the association of nascent secretory proteins with Ffh (P48) is also not known. A simple model would suggest a sterical vicinity of the trigger factor binding site to the signal sequence allowing for a displacement of a weakly bound Ffh (P48) as soon as the growing polypeptide chain exposes its docking site for trigger factor.

Materials and methods

Plasmids

The *ompA* gene present on plasmid pDMB (Behrmann *et al.*, 1998) was modified to contain a *SpeI* cleavage site changing Asn to Thr and Thr to Ser at positions +4 and +5 of the mature OmpA, and a *NcoI* site that led to the insertion of Val after the initiator Met of the signal sequence. It was finally subcloned behind the T7/*lacUV5* promoters of plasmid pKSM717 (Maneewannakul *et al.*, 1994) yielding plasmid p717OmpA. The *mtlA* gene was excised from pMtlII-9 (Koch *et al.*, 1999) and ligated into pKSM717. Subsequently, incorporation of a *BamHI* cleavage site upstream of *mtlA* allowed removal of 149 bp 5' to the ribosome-binding site of *mtlA* yielding plasmid p717MtlA-B. This plasmid served as template for the introduction of TAG stop codons at amino acid positions 27 (Phe), 29 (Ala), 32 (Ile), 37 (Phe) and 42 (Trp) within the first transmembrane helix of MtlA. This was achieved by a two-step PCR method involving three primers each (Dilsiz and Crabbe, 1994). All changes were verified by nucleotide sequence analysis.

Synthesis of RANCs

Proteins were synthesized from plasmids p717OmpA and p717MtlA-B by the reconstituted transcription/translation system of *E. coli* as described (Behrmann *et al.*, 1998) with the following modifications: the concentration of phosphoenolpyruvate was lowered to 5 mM and putrescin was added at 8 mM. If the final concentration of Mg^{2+} exceeded 6 mM, read through of stop codon mutants was observed. Triethanolamine acetate was replaced by HEPES–NaOH in order to avoid quenching of the *N*-hydroxysuccinimide ester groups present in the chemical cross-linkers used. For the synthesis of pOmpA125 and pOmpA191, reactions contained the $\beta 5$ and $\beta 8$ oligodeoxynucleotides described elsewhere at 120 μ g/ml (Behrmann *et al.*, 1998). Synthesis of MtlA189 was achieved by addition of 60 μ g/ml 5'-ACCGTGGTTAATGGCGTTGTT-3', that of MtlA130 by addition of 40 μ g/ml 5'-GATGCCTGCGGAGAAG-TTAT-3'. Synthesis of MtlA130 was enhanced by providing exogenous RNaseH (1 U/reaction). More distinct bands of nascent chains were obtained when the truncated mRNAs were protected against 10Sa RNA-mediated peptide tagging and release from the ribosome (Keiler *et al.*, 1996; Himeno *et al.*, 1997) by use of 10 μ g/ml 10Sa RNA anti-sense oligodeoxynucleotide (Hanes and Plückthun, 1997). If not continued by immunoprecipitation, samples were precipitated with 5% TCA and prepared for SDS–PAGE.

Isolation and treatment of RANCs

RANCs were collected by centrifugation in a TLA-100 Beckman rotor at 70 000 r.p.m. for 60 min at 4°C as described (Behrmann *et al.*, 1998) and resuspended in RANC buffer consisting of 40 mM HEPES–NaOH pH 7.5, 70 mM potassium acetate, 6 mM magnesium acetate, 8 mM putrescin, 0.1 mM spermidine, 5 mM phosphoenolpyruvate, 1 mM DTT. To obtain complexes between tRNA and pOmpA, isolated RANCs were resuspended in RANC buffer containing 8 M urea, incubated for 30 min on ice, and subsequently diluted 10-fold with RANC buffer for further incubations. Where applicable, isolated RANCs were incubated for 15 min at 37°C with INV and purified Sec and SRP proteins in the presence of 2.5 mM ATP, 8 mM creatine phosphate, 40 μ g/ml creatine

phosphokinase and 5 mM phosphoenolpyruvate. Final treatment with 0.8 mM puromycin was performed for 10 min at 37°C.

Cross-linking

For chemical cross-linking using sulfo-HSAB (Pierce), 5 µl of a 2 mg/ml solution of sulfo-HSAB freshly prepared in 100 mM HEPES-NaOH pH 7.5 were added to 25 µl reactions and incubated for 20 min at 37°C. Subsequently, samples were irradiated with UV light of $\lambda = 254$ nm for 30 min at room temperature. For chemical cross-linking using DSS (Pierce), 2.5 µl of a 25 mM solution of DSS prepared in DMSO were added to 25 µl reactions, incubated for 30 min at 25°C, and quenched by Tris-HCl pH 7.5 at a final concentration of 50 mM for 15 min at 25°C. Site-specific incorporation of Tmd-Phe followed previously described protocols for the preparation of Tmd-Phe-pdCpA, run-off transcription of *FokI*-linearized plasmid pEAsn encoding a tRNA^{Asn}-derived suppressor tRNA (Cload *et al.*, 1996), purification of suppressor tRNA and ligation with Tmd-Phe-pdCpA (Graf *et al.*, 1997). UV irradiation was performed as described above.

Miscellaneous

Gradient-purified, inside out plasma membrane vesicles were prepared and routinely salt-extracted as described (Behrmann *et al.*, 1998). Ffh (P48), FtsY, 4.5S RNA, SecA, SecB (Koch *et al.*, 1999) and T7 RNA polymerase from strain BL21 containing plasmid pAR1219 (obtained from Brookhaven National Laboratory, Upton, Long Island, NY) were prepared as described. Precipitation with CTAB has been detailed elsewhere (Behrmann *et al.*, 1998). Immunoprecipitation was performed on 4-fold—unless stated otherwise—scaled-up reactions using polyclonal rabbit antibodies (Schäfer *et al.*, 1999). In some of the experiments, immunoglobulins were covalently linked to the protein A-Sepharose matrix (Alconada *et al.*, 1995).

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References

- Alconada, A., Gärtner, F., Hönlinger, A., Kübrich, M. and Pfanner, N. (1995) Mitochondrial receptor complex from *Neurospora crassa* and *Saccharomyces cerevisiae*. *Methods Enzymol.*, **260**, 263–286.
- Behrmann, M., Koch, H.-G., Hengelage, T., Wieseler, B., Hoffschulte, H.K. and Müller, M. (1998) Requirements for the translocation of elongation-arrested, ribosome-associated OmpA across the plasma membrane of *Escherichia coli*. *J. Biol. Chem.*, **273**, 13898–13904.
- Bernstein, H.D., Poritz, M.A., Strub, K., Hoben, P.J., Brenner, S. and Walter, P. (1989) Model for signal sequence recognition from amino acid sequence of 54K subunit of signal recognition particle. *Nature*, **340**, 482–486.
- Bernstein, H.D., Zopf, D., Freymann, D.M. and Walter, P. (1993) Functional substitution of the signal recognition particle 54-kDa subunit by its *Escherichia coli* homolog. *Proc. Natl Acad. Sci. USA*, **90**, 5229–5233.
- Chun, S.Y. and Randall, L.L. (1994) *In vivo* studies of the role of SecA during protein export in *Escherichia coli*. *J. Bacteriol.*, **176**, 4197–4203.
- Cload, S.T., Liu, D.R., Froland, W.A. and Schultz, P.G. (1996) Development of improved tRNAs for *in vitro* biosynthesis of proteins containing unnatural amino acids. *Chem. Biol.*, **3**, 1033–1038.
- de Gier, J.W.L., Mansournia, P., Valent, Q.A., Phillips, G.J., Luijck, J. and von Heijne, G. (1996) Assembly of a cytoplasmic membrane protein in *Escherichia coli* is dependent on the signal recognition particle. *FEBS Lett.*, **399**, 307–309.
- de Gier, J.W.L., Scotti, P.A., Sääf, A., Valent, Q.A., Kuhn, A., Luijck, J. and von Heijne, G. (1998) Differential use of the signal recognition particle translocase targeting pathway for inner membrane protein assembly in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **95**, 14646–14651.
- Dilsiz, N. and Crabbe, M.J.C. (1994) A high-yield modification of mutation by overlap extension using three primers. *Anal. Biochem.*, **222**, 510–511.
- Graf, R., Brunner, J., Dobberstein, B. and Martoglio, B. (1997) Probing the molecular environment of proteins by site-specific photocrosslinking. In Celis, J.E. (ed.), *Cell Biology—A Laboratory Handbook*, 2nd edn. Academic Press, San Diego, CA, pp. 495–501.
- Guthrie, B. and Wickner, W. (1990) Trigger factor depletion or overproduction causes defective cell division but does not block protein export. *J. Bacteriol.*, **172**, 5555–5562.
- Hanes, J. and Plückthun, A. (1997) *In vitro* selection and evolution of functional proteins by using ribosome display. *Proc. Natl Acad. Sci. USA*, **94**, 4937–4942.
- Hauser, S., Bacher, G., Dobberstein, B. and Lütcke, H. (1995) A complex of the signal sequence binding protein and the SRP RNA promotes translocation of nascent proteins. *EMBO J.*, **14**, 5485–5493.
- Hesterkamp, T., Hauser, S., Lütcke, H. and Bukau, B. (1996) *Escherichia coli* trigger factor is a prolyl isomerase that associates with nascent polypeptide chains. *Proc. Natl Acad. Sci. USA*, **93**, 4437–4441.
- Himeno, H., Sato, M., Tadaki, T., Fukushima, M., Ushida, C. and Muto, A. (1997) *In vitro* trans translation mediated by alanine-charged 10Sa RNA. *J. Mol. Biol.*, **268**, 803–808.
- Ito, K. (1984) Identification of the secY (prlA) gene product involved in protein export in *Escherichia coli*. *Mol. Gen. Genet.*, **197**, 204–208.
- Keiler, K.C., Waller, P.R.H. and Sauer, R.T. (1996) Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. *Science*, **271**, 990–993.
- Koch, H.G., Hengelage, T., Neumann-Haefelin, C., MacFarlane, J., Hoffschulte, H.K., Schmiz, K.L., Mechler, B. and Müller, M. (1999) *In vitro* studies with purified components reveal signal recognition particle (SRP) and SecA/SecB as constituents of two independent protein-targeting pathways of *Escherichia coli*. *Mol. Biol. Cell*, **10**, 2163–2173.
- Kumamoto, C.A. and Francetić, O. (1993) Highly selective binding of nascent polypeptides by an *Escherichia coli* chaperone protein *in vivo*. *J. Bacteriol.*, **175**, 2184–2188.
- Luirink, J., ten Hagen-Jongman, C.M., van der Weijden, C.C., Oudega, B., High, S., Dobberstein, B. and Kusters, R. (1994) An alternative protein targeting pathway in *Escherichia coli*: studies on the role of FtsY. *EMBO J.*, **13**, 2289–2296.
- MacFarlane, J. and Müller, M. (1995) The functional integration of a topotypic membrane protein of *Escherichia coli* is dependent on the bacterial signal recognition particle. *Eur. J. Biochem.*, **233**, 766–771.
- Maneewannakul, S., Maneewannakul, K. and Ippen-Ihler, K. (1994) The pKSM710 vector cassette provides tightly regulated lac and T7lac promoters and strategies for manipulating N-terminal protein sequences. *Plasmid*, **31**, 300–307.
- Martoglio, B., Hofmann, M.W., Brunner, J. and Dobberstein, B. (1995) The protein-conducting channel in the membrane of the endoplasmic reticulum is open laterally toward the lipid bilayer. *Cell*, **81**, 207–214.
- Matlack, K.E.S., Plath, K., Misselwitz, B. and Rapoport, T.A. (1997) Protein transport by purified yeast Sec complex and Kar2p without membranes. *Science*, **277**, 938–941.
- Phillips, G.J. and Silhavy, T.J. (1992) The *E.coli* *ffh* gene is necessary for viability and efficient protein export. *Nature*, **359**, 744–746.
- Poritz, M.A., Strub, K. and Walter, P. (1988) Human SRP RNA and *E.coli* 4.5S RNA contain a highly homologous structural domain. *Cell*, **55**, 4–6.
- Poritz, M.A., Bernstein, H.D., Strub, K., Zopf, D., Wilhelm, H. and Walter, P. (1990) An *E.coli* ribonucleoprotein containing 4.5S RNA resembles mammalian signal recognition particle. *Science*, **250**, 1111–1117.
- Qi, H.Y. and Bernstein, H.D. (1999) SecA is required for the insertion of inner membrane proteins targeted by the *Escherichia coli* signal recognition particle. *J. Biol. Chem.*, **274**, 8993–8997.
- Randall, L.L., Topping, T.B., Hardy, S.J.S., Pavlov, M.Y., Freistoffer, D.V. and Ehrenberg, M. (1997) Binding of SecB to ribosome-bound polypeptides has the same characteristics as binding to full-length, denatured proteins. *Proc. Natl Acad. Sci. USA*, **94**, 802–807.
- Ribes, V., Römisch, K., Giner, A., Dobberstein, B. and Tollervey, D. (1990) *E.coli* 4.5S RNA is part of a ribonucleoprotein particle that has properties related to signal recognition particle. *Cell*, **63**, 591–600.
- Römisch, K., Webb, J., Herz, J., Prehn, S., Frank, R., Vingron, M. and Dobberstein, B. (1989) Homology of 54K protein of signal recognition particle, docking protein and two *E.coli* proteins with putative GTP-binding domains. *Nature*, **340**, 478–482.
- Schäfer, U., Beck, K. and Müller, M. (1999) Skp, a molecular chaperone of Gram-negative bacteria, is required for the formation of soluble

- periplasmic intermediates of outer membrane proteins. *J. Biol. Chem.*, **274**, 24567–24574.
- Scholz,C., Stoller,G., Zarnt,T., Fischer,G. and Schmid,F.X. (1997) Cooperation of enzymatic and chaperone functions of trigger factor in the catalysis of protein folding. *EMBO J.*, **16**, 54–58.
- Seluanov,A. and Bibi,E. (1997) FtsY, the prokaryotic signal recognition particle receptor homologue, is essential for biogenesis of membrane proteins. *J. Biol. Chem.*, **272**, 2053–2055.
- Stoller,G., Rücknagel,K.P., Nierhaus,K.H., Schmid,F.X., Fischer,G. and Rahfeld,J.U. (1995) A ribosome-associated peptidyl–prolyl *cis/trans* isomerase identified as the trigger factor. *EMBO J.*, **14**, 4939–4948.
- Struck,J.C.R., Toschka,H.Y., Specht,T. and Erdmann,V.A. (1988) Common structural features between eukaryotic 7SL RNAs, eubacterial 4.5S RNA and scRNA and archaeobacterial 7S RNA. *Nucleic Acids Res.*, **16**, 7740.
- Sugiyama,J.E., Mahmoodian,S. and Jacobson,G.R. (1991) Membrane topology analysis of *Escherichia coli* mannitol permease by using a nested-deletion method to create mtlA–phoA fusions. *Proc. Natl Acad. Sci. USA*, **88**, 9603–9607.
- Traxler,B. and Murphy,C. (1996) Insertion of the polytopic membrane protein MalF is dependent on the bacterial secretion machinery. *J. Biol. Chem.*, **271**, 12394–12400.
- Ulbrandt,N.D., Newitt,J.A. and Bernstein,H.D. (1997) The *E.coli* signal recognition particle is required for the insertion of a subset of inner membrane proteins. *Cell*, **88**, 187–196.
- Valent,Q.A., Kendall,D.A., High,S., Kusters,R., Oudega,B. and Lührink,J. (1995) Early events in preprotein recognition in *E.coli*: interaction of SRP and trigger factor with nascent polypeptides. *EMBO J.*, **14**, 5494–5505.
- Valent,Q.A., de Gier,J.W.L., von Heijne,G., Kendall,D.A., ten Hagen-Jongman,C., Oudega,B. and Lührink,J. (1997) Nascent membrane and presecretory proteins synthesized in *Escherichia coli* associate with signal recognition particle and trigger factor. *Mol. Microbiol.*, **25**, 53–64.
- Valent,Q.A., Scotti,P.A., High,S., de Gier,J.W.L., von Heijne,G., Lentzen,G., Wintermeyer,W., Oudega,B. and Lührink,J. (1998) The *Escherichia coli* SRP and SecB targeting pathways converge at the translocon. *EMBO J.*, **17**, 2504–2512.
- von Heijne,G. (1989) Control of topology and mode of assembly of a polytopic membrane protein by positively charged residues. *Nature*, **341**, 456–458.
- Werner,P.K., Saier,M.H., Jr and Müller,M. (1992) Membrane insertion of the mannitol permease of *Escherichia coli* occurs under conditions of impaired SecA function. *J. Biol. Chem.*, **267**, 24523–24532.
- Wolfe,P.B., Rice,M. and Wickner,W. (1985) Effects of two *sec* genes on protein assembly into the plasma membrane of *Escherichia coli*. *J. Biol. Chem.*, **260**, 1836–1841.
- Yamato,I. (1992) Membrane assembly of lactose permease of *Escherichia coli*. *J. Biochem.*, **111**, 444–450.

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