The *Escherichia coli* SRP and SecB targeting pathways converge at the translocon

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Two distinct protein targeting pathways can direct proteins to the *Escherichia coli* inner membrane. The Sec pathway involves the cytosolic chaperone SecB that binds to the mature region of pre-proteins. SecB targets the pre-protein to SecA that mediates pre-protein translocation through the SecYEG translocon. The SRP pathway is probably used primarily for the targeting and assembly of inner membrane proteins. It involves the signal recognition particle (SRP) that interacts with the hydrophobic targeting signal of nascent proteins. By using a protein cross-linking approach, we demonstrate here that the SRP pathway delivers nascent inner membrane proteins at the membrane. The SRP receptor FtsY, GTP and inner membranes are required for release of the nascent proteins from the SRP. Upon release of the SRP at the membrane, the targeted nascent proteins insert into a translocon that contains at least SecA, SecY and SecG. Hence, as appears to be the case for several other translocation systems, multiple targeting mechanisms deliver a variety of precursor proteins to a common membrane translocation complex of the *E. coli* inner membrane.

**Keywords:** protein targeting/SecA/SecY/signal recognition particle/translocon

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**Introduction**

In eukaryotes, protein targeting to the membrane of the endoplasmic reticulum (ER) is initiated by the cotranslational recognition of targeting signals by the signal recognition particle (SRP) (reviewed in Rapoport *et al.*, 1996). The SRP is a ribonucleoprotein particle composed of six proteins assembled on an RNA scaffold (7S RNA). Upon recognition of a targeting signal by the 54 kDa SRP subunit (SRP54), translational elongation of the nascent polypeptide chain is inhibited. The ribosome-bound nascent chain (RNC) in complex with SRP is then targeted to the membrane-associated α-subunit of the SRP receptor (SRα), and the RNC is subsequently released from the SRP. This targeting process requires the binding of GTP to both SRP54 and SRα (Connolly and Gilmore, 1989; Rapiejko and Gilmore, 1997). The released nascent chain enters the translocon, the ribosome makes a tight seal with the translocon, translation is resumed and the nascent chain inserts co-translationally into the aqueous translocation channel (Rapoport *et al.*, 1996; Johnson, 1997). The hydrolysis of GTP at SRP54 and SRα is required to dissociate the SRP–SRα complex and recycle these targeting factors (Connolly *et al.*, 1991).

Protein targeting to the *Escherichia coli* inner membrane can occur via the Sec pathway and the SRP pathway. The extensively studied Sec pathway uses a cytosolic chaperone, SecB, that binds post-translationally or at a late co-translational stage to the mature region of pre-proteins (Kumamoto and Francetic, 1993). The SecB–pre-protein complex is targeted to the membrane where SecA is activated for high-affinity recognition of SecB and pre-protein by binding to the membrane-embedded translocon (reviewed in Driessen *et al.*, 1998). SecB is released from the pre-protein as the ATPase SecA mediates post-translational translocation through the SecYEG translocon by ATP-driven cycles of insertion and de-insertion (Economou and Wickner, 1994; Economou *et al.*, 1995).

Although SecA is not an integral part of the *E. coli* translocon, it is considered part of the dynamic structure of the translocon at certain stages in the translocation process (Driessen *et al.*, 1998). In eukaryotes, SecA homologues have only been identified in chloroplasts (Nakai *et al.*, 1994; Yuan *et al.*, 1994). In contrast, the core structure of the mammalian and *E. coli* translocons appears to be conserved: both complexes are heterotrimeric, consisting of Sec61α, β and γ (Sec61 complex) and of SecY, SecE and SecG (SecYEG complex), respectively. SecE and Sec61γ, and especially SecY and Sec61α, share significant sequence similarity (Rapoport *et al.*, 1996). Sec61α is in close proximity to translocating proteins during co-translational translocation (High *et al.*, 1993; Mothes *et al.*, 1994). In *Saccharomyces cerevisiae*, the homologous Sec61 complex is involved in both co- and post-translational translocation. In addition, another trimeric translocon (Ssh1 complex) has been identified that probably functions exclusively in co-translational protein transport (Rapoport *et al.*, 1996; Wilkinson *et al.*, 1997).

The more recently discovered SRP pathway in *E. coli*, on the other hand, involves cytosolic factors that strongly resemble components involved in protein targeting to the eukaryotic ER membrane (Luirink and Dobberstein, 1994; Wolin, 1994). Thus, a small SRP has been identified that consists of a 4.5S RNA and a 48 kDa GTPase designated...
P48 (or Ffh for fifty-four homologue), which are homologous to the eukaryotic 7S RNA (Poritz et al., 1988) and SRP54 (Bernstein et al., 1989; Römisch et al., 1989), respectively. The SRP was shown in vitro to interact by virtue of P48 with RNCs of several secreted and membrane proteins, with a preference for substrates that expose particularly hydrophobic targeting signals (Valent et al., 1995, 1997). In agreement with these findings, recent evidence indicates that proteins equipped with strongly hydrophobic targeting signals (e.g. integral inner membrane proteins) are particularly dependent on the SRP for efficient membrane assembly in vivo (Mac Farlane and Müller, 1995; De Gier et al., 1996; Ulbrandt et al., 1997). Moreover, a putative SRP receptor (FtsY) has been identified based on sequence similarity with SRα (Bernstein et al., 1989; Römisch et al., 1989). FtsY interacts with the SRP in vitro in a GTP-dependent manner (Miller et al., 1994; Kusters et al., 1995) and is essential for the efficient secretion of certain proteins (Luirink et al., 1994) and the biogenesis of inner membrane proteins in vivo (Sehuano and Bibi, 1997).

Until now, a connection between the E.coli Sec and SRP pathways had not been established. In this study, we demonstrate that the two pathways merge at the inner membrane. Proteins targeted by the SRP are shown to interact co-translationally with the E.coli inner membrane. Release of the SRP from the RNC occurs at the membrane and requires both FtsY and GTP. After release from the SRP, the nascent chains insert into the membrane at a translocon that contains SecA, SecY and SecG.

Results

Short nascent inner membrane proteins associate with E.coli inner membranes

In a previous study (Valent et al., 1997), we have used an E.coli in vitro translation system in combination with bifunctional cross-linking reagents to investigate the molecular interactions of short nascent pre-secretory and membrane proteins in the cytosol. A direct interaction of the SRP with nascent polypeptides that expose particularly hydrophobic targeting signals was demonstrated, suggesting that inner membrane proteins are the primary physiological substrates of the E.coli SRP. In addition, the cytosolic chaperone trigger factor (TF) was found to interact with all nascent polypeptides long enough to protrude from the ribosome (Valent et al., 1995, 1997; Hesterkamp et al., 1996), indicating that TF has a general affinity for nascent polypeptides and is positioned near the nascent chain exit site on the E.coli ribosome.

In the present study, we investigated the later stages in SRP-mediated protein targeting and membrane insertion. First we examined whether short nascent membrane proteins are able to bind to import-competent E.coli inverted inner membrane vesicles (INVs) (De Vrije et al., 1987). As model targeting substrates we used leader peptidase I (Lep), a polytopic membrane protein that removes N-terminal signal peptides from exported proteins at the periplasmic side of the cytoplasmic membrane (reviewed in Dalbey, 1991), and FtsQ, a type II cytoplasmic membrane protein involved in cell division (Carson et al., 1991), which both interact with the SRP in vitro (Valent et al., 1995, 1997). In addition, Lep insertion has been shown to depend on the SRP in vivo (De Gier et al., 1996).

RNCs were prepared by translating truncated mRNAs in an E.coli membrane-free cell extract (Valent et al., 1997). The truncated mRNAs encode polypeptides of ~100 amino acids to allow optimal exposure of the N-terminal targeting sequence. The purified RNCs were incubated with INVs and subjected to flotation gradient analysis under high salt conditions. After centrifugation, four fractions were collected and analysed by SDS–PAGE and phosphor imaging. The top fraction contains floated membranes and targeted RNCs, whereas the untargeted RNCs remain in the bottom fractions. In Figure 1A, the percentage of membrane-associated (floated) RNCs is shown. Approximately 30% of the nascent Lep (101Lep) and FtsQ (108FtsQ) fractionated with the INVs, indicating efficient membrane association. These data indicate that nascent membrane proteins can interact with E.coli inner membranes in vitro.

We have demonstrated previously that the interaction of the SRP with nascent pre-secretory proteins correlates with the hydrophobicity of the exposed targeting sequence (Valent et al., 1995, 1997). To determine the effect of targeting sequence hydrophobicity on membrane association of nascent polypeptides, PhoA derivatives with mutated signal sequence core regions were used (Doud et al., 1993). A nascent PhoA construct (91PhoA) carrying a strongly hydrophobic signal sequence (9Leu, 1Ala) that interacts efficiently with the SRP (Valent et al., 1995, 1997) showed significant membrane association. In contrast, a 91PhoA construct exposing a moderately hydrophobic (4Leu, 6Ala) signal sequence that does not interact with the SRP (Valent et al., 1995, 1997) showed no significant membrane association (Figure 1A).

We conclude that the membrane association of short nascent pre-secretory and membrane proteins correlates with the hydrophobicity of the targeting signal and hence their ability to interact with the SRP. This suggests that the SRP plays a role in the targeting of these proteins.

Membrane-targeted nascent proteins interact with Sec translocon components

To probe the molecular environment of membrane-associated 108FtsQ, we used an unbiased cross-linking approach. Nascent polypeptides were incubated with INVs to allow targeting, and subsequently treated with the bifunctional cross-linking reagent disuccinimidyl suberate (DSS) (Figure 1B, lanes 1 and 2). DSS is the membrane-permeable analogue of BS3, a cross-linking reagent that we used previously to probe interactions of untargeted RNCs (Valent et al., 1997). After cross-linking, the samples were extracted with alkaline sodium carbonate buffer to separate integral membrane (Figure 1B, lane 3) from peripheral and soluble cross-linked complexes (Figure 1B, lane 4).

In the Na2CO3 pellet, two major 108FtsQ cross-linking adducts appeared at ~120 and ~41–44 kDa. The 120 kDa product was immunoprecipitated using anti-SecA (Figure 1A, lane 10), indicating that it represents a membrane-integral complex of the radiolabelled nascent FtsQ (12 kDa) and SecA (102 kDa). The fuzzy 42 kDa product was found exclusively in the Na2CO3-resistant fraction and immunoprecipitated with anti-SecY (Figure 1B, lane 9).
Cross-linking of nascent chains to Sec61α, the mammalian homologue of SecY (Rapoport et al., 1996), results in similarly smeared adducts (Laird and High, 1997). It should also be noted that although SecY has a predicted Mr of 49 kDa, it usually migrates as an ~35 kDa product upon SDS–PAGE (Akiyama and Ito, 1986). In addition, a faint cross-linking product of ~50 kDa was immunoprecipitated with anti-SecY (Figure 1B, lane 9) and anti-SecG (Figure 1B, lane 8) which presumably represents SecG cross-linked via SecY to a 108FtsQ–SecY adduct.

In the Na2CO3 supernatant, the cross-linking pattern was more complex (Figure 1B, lane 4). Immunoprecipitation revealed the presence of adducts to the SRP protein constituent P48 and to TF (Figure 1B, lanes 11 and 12) that have been observed previously with 108FtsQ synthesized in the absence of INVs (Valent et al., 1995, 1997). In addition, a fraction of the 108FtsQ–SecA complex was detected in the supernatant (Figure 1B, lane 16). This may represent cross-linking between 108FtsQ and the membrane-peripheral form of SecA, since it was not observed upon cross-linking in the absence of INVs (Valent et al., 1997). Almost all other radiolabelled products were lost upon purification of RNCs through a high salt sucrose cushion (Valent et al., 1997; also Figure 2A, compare lanes 2 and 3), indicating that these were not RNC-specific cross-linking products. No cross-linking adducts with integral membrane proteins SecY or SecG were detected in the Na2CO3 supernatant fraction (Figure 2B, lanes 14 and 15). A direct cross-linking between RNC and FtsY was not detected. Immunoprecipitation did not reveal any interactions of 108FtsQ with other targeting factors and chaperones known to interact with nascent polypeptides, such as DnaK/J and SecB (reviewed in Bukau et al., 1996). The cross-linking pattern obtained with 101Lep was remarkably similar to that observed with 108FtsQ (data not shown).

Our principal conclusion from this initial analysis is that nascent FtsQ and Lep form translocation intermediates that are in close proximity to the translocon components SecA, SecY and SecG. The simplest interpretation of these results is that the SRP mediates co-translational targeting to the translocon which is also used by proteins whose targeting is dependent on SecB.

The interaction of the SRP with nascent proteins has been shown to correlate with the hydrophobicity of the targeting sequence (Valent et al., 1995, 1997). This prompted us to examine the membrane interactions of mutant 91PhoA carrying signal sequences with incre-
SRP and SecB pathways converge at the translocon

Fig. 2. Requirements for the release of SRP from RNCs. (A) 108FtsQ RNCs were incubated in the presence or absence of 350 nM of rSRP. SRNCs were prepared by purifying an equal portion of rSRP-saturated RNCs through a high salt sucrose cushion. SRP–RNC interactions were monitored by cross-linking with DSS. Quenched samples were TCA-precipitated and analysed by SDS–PAGE. The RNC–P48 adduct (X-P48) is indicated on the left. The Mr of marker proteins are indicated on the right. (B) SRNCs were incubated for 5 min at 25°C with or without INVs (1.25 mg/ml protein), (mutant) FtsY (1 μM) or nucleotides (30 μM each), as indicated. After 5 min on ice, samples were treated with DSS and Na2CO3 as described in Materials and methods. The TCA-precipitated Na2CO3 supernatant is shown. The RNC–P48 adduct (X-P48) and RNC, P48 and FtsY containing adduct (arrow) are indicated on the left. The Mrs of marker proteins are indicated on the right.

SRP is released from RNCs at the membrane by FtsY which is in the GTP-bound state

The experiments described above demonstrated that membrane-inserted 108FtsQ RNCs are not associated with the SRP in contrast to untargeted RNCs (Figure 1B, compare lanes 5 and 11). This implies that the SRP is released before, or concomitant with, membrane insertion and association with the translocon. To investigate the requirements for dissociation of the SRP–RNC complex and membrane insertion, we used 108FtsQ since it exhibits strong cross-linking to P48 (Valent et al., 1995, 1997; Figure 1B, lane 11) and associates efficiently with SecA and SecY (Figure 1B, lanes 9 and 10). In order to analyse SRP binding directly (i.e. without immunoprecipitation), reconstituted E.coli SRP (rSRP) was added after translation to saturate RNCs with SRP. A substantial increase in cross-linking efficiency was observed (Figure 2A, compare lanes 1 and 2), indicating effective rSRP–RNC interaction. The rSRP–RNC complexes remained intact after purification over a high salt sucrose cushion, demonstrating the stability of the interaction (Figure 2A, lane 3; see also Valent et al., 1997). These purified complexes were designated SRNCs and used to study the requirements for release of the SRP from the RNCs.

By analogy with the eukaryotic system, the most likely candidate for a membrane receptor/release factor for the SRP is FtsY since it displays sequence similarity to the α-subunit of the mammalian SRP receptor (SRα) (Bernstein et al., 1989; Römisch et al., 1989) and interacts with rSRP in vitro in a GTP-dependent process (Miller et al., 1994; Kusters et al., 1995). For this reason, release of the SRP from 108FtsQ was studied by varying the presence of FtsY (Luirink et al., 1994) and nucleotides in the presence of wild-type INVs. FtsQ RNCs were saturated with rSRP and purified over a high salt sucrose cushion. These SRNCs were incubated in the presence or absence of INVs, FtsY and nucleotides (Figure 2B). Addition of INVs, FtsY and GTP was required for efficient loss of P48 cross-linking (Figure 2B, lanes 1–5 and 9). This indicates an FtsY-mediated dissociation of the SRP from the nascent chain at the membrane. GDP could not substitute for GTP in this reaction (Figure 2B, lane 6), reminiscent of the requirement for GTP in the analogous reaction in the mammalian system (Connolly and Gilmore, 1989).

To examine whether binding of GTP to FtsY is required for the release of the SRP from the RNCs, a mutant FtsY was used that is unable to bind GTP because of a point mutation in G4, the fourth consensus region for GTP binding (Kusters et al., 1995). This mutant, FtsY A449,
did not mediate the release of SRP from the nascent chain (Figure 2B, lane 12), indicating that binding of GTP to at least FtsY is a prerequisite for the release of SRP from the nascent polypeptide.

Hydrolysis of GTP was not required for the release of the SRP from RNCs. When using wild-type FtsY in the presence of the non-hydrolysable GTP analogue GMP-PNP, release of the SRP from RNCs was even more pronounced than when GTP was used (Figure 2B, compare lanes 5 and 7). GTP hydrolysis is essential for the release of the SRP from FtsY in vitro (Miller et al., 1994). It is possible that GMP-PNP locks the SRP in an SRP–FtsY complex, thus preventing SRP from undergoing cycles of nascent chain binding and release. This would lower the amount of SRP available to re-associate with the RNCs. AMP-PNP, a non-hydrolysable ATP analogue, was completely inactive in SRP release (Figure 2B, lane 8), showing the nucleotide specificity of the reaction. In the absence of added FtsY, only a slight release was observed (Figure 2B, compare lanes 5 and 7 with 9 and 11), reflecting the activity of the endogenous FtsY present in the INVs.

FtsY differs from SRα in that it is located not only in the target membrane but also in the cytosol (Luirink et al., 1997). It is thought that FtsY is only functional when bound to the inner membrane (Zelazny et al., 1997). Interestingly, in the absence of INVs (Figure 2B, lane 2), a soluble high molecular weight complex was observed (Figure 2B, arrow) that contains both P48 and FtsY since it was not detected when the SRP was released efficiently from the RNCs (Figure 2B, lanes 4 and 7) or when FtsY was not included (Figure 2B, lanes 9–11). This conclusion was verified by immunoprecipitation using antibodies directed against both P48 and FtsY (data not shown) and suggests that FtsY is able to interact with RNC–SRP complexes in the cytosol, independently of the presence of INVs. In the presence of FtsY A449 (Figure 2B, lane 12) or the absence of GTP (Figure 2B, lane 3), the same complex was formed (Figure 2B, arrow), suggesting that the binding of FtsY to the SRP–RNC complex also does not require GTP.

Taken together, we conclude from these results that cytosolic FtsY binds to the SRP which is associated with RNCs in a process that does not depend on nucleotides. Release of the SRP from the nascent chain occurs at the membrane and requires the binding of GTP to at least FtsY.

Following release of SRP, the nascent chain enters the translocon

Thus far, we have shown that nascent polypeptides exposing particularly hydrophobic targeting sequences interact with SRP in the cytosol and with the translocon components SecA and SecY in the membrane. Together with the observation that RNCs are only released from the SRP in the presence of GTP, FtsY and INVs, this suggests that the SRP and FtsY target RNCs to the membrane where the SRP is dissociated from the RNC, allowing the latter to interact with the translocon.

To study the putative transfer of the nascent chain from the SRP to the translocon in more detail, we investigated whether the requirements for release of the SRP and association with the translocon are related. SRNCs were incubated with INVs in the presence or absence of FtsY and nucleotides, cross-linked and subjected to Na2CO3 extraction. In the absence of both FtsY and nucleotides, strong cross-linking is observed to P48 in the supernatant (Figure 3A, lane 1) and pellet fractions (Figure 3B, lane 9), whereas cross-linking to SecA (Figure 3B, lane 5) and SecY (Figure 3B, lane 13) is weak, indicating inefficient dissociation of SRP from 108FtsQ and minimal association with the translocon. At the other end of the spectrum, efficient release of the SRP in the presence of FtsY and GTP (Figure 3A, lanes 3 and 4, and B, lanes 11 and 12) is accompanied by an increase in cross-linking to both SecA (Figure 3B, lanes 7 and 8) and SecY (Figure 3B, lanes 15 and 16), indicating transfer of the released nascent chains to the translocon. The intermediate release of SRP observed in the absence of added FtsY (Figure 3A, lane 2 and see above) results in intermediate association with SecA and SecY (Figure 3B, lanes 6 and 14). These data were also quantified and are presented graphically in Figure 3C. Consistent with these observations, flotation gradient analysis revealed that conditions resulting in dissociation of the SRP–RNC complex (presence of FtsY and GTP) induced efficient co-localization of 108FtsQ with the membrane (data not shown).

These results strongly suggest that the nascent 108FtsQ is transferred to the membrane-embedded translocon upon GTP-dependent release from the SRP by FtsY.

Discussion

Recent evidence indicates that the E.coli SRP and its putative receptor FtsY fulfil essential functions in the targeting and membrane assembly of inner membrane proteins (reviewed in De Gier et al., 1997). Overproduction of several inner membrane proteins reduced the cell viability of a strain in which the SRP level is depressed artificially, indicating titration of the essential SRP (Ulbradt et al., 1997). Moreover, depletion of the SRP components, 4.5S RNA and P48, resulted in impaired integration of several membrane proteins (Mac Farlane and Müller, 1995; De Gier et al., 1996; Ulbradt et al., 1997). Finally, we have demonstrated previously by in vitro cross-linking that the E.coli SRP interacts with short nascent polypeptides that carry particularly hydrophobic targeting sequences like inner membrane proteins usually do. Until now, the mechanism by which these SRP-dependent proteins are delivered at the inner membrane has remained obscure.

In this report, we describe the use of a cross-linking approach to elucidate late stages in SRP-mediated protein targeting in E.coli. As substrate proteins we used FtsQ and Lep, E.coli inner membrane proteins that both interact efficiently with the SRP in vitro (Valent et al., 1995, 1997) but differ in the membrane orientation of their first transmembrane segments (Wolfe et al., 1983; Carson et al., 1991). Furthermore, Lep has been shown to depend on the SRP for efficient membrane assembly in vivo (De Gier et al., 1996). Nascent N-terminal FtsQ and Lep fragments of ~100 amino acids were prepared by in vitro translation of truncated mRNA in a cell-free E.coli extract and supplemented with INVs. As a result, efficient membrane association of the nascent polypeptides was observed. Interestingly, upon release from the SRP–FtsY complex, the targeted nascent chains were found to be exclusively...
SRP and SecB pathways converge at the translocon

Fig. 3. Transfer of RNCs from SRP to translocon. Experimental details are the same as those in the legend to Figure 2B, except that all incubations are done in the presence of INVs. (A) The TCA-precipitated Na₂CO₃ supernatant is shown. The RNC–SecA adduct (X-SecA) and RNC–P48 adduct (X-P48) are indicated on the left. The Mr of marker proteins are indicated on the right. (B) The Na₂CO₃ pellet is shown, and immunoprecipitated (IP) cross-linking adducts are indicated (arrow). The Mrs of marker proteins are indicated on the right. (C) Quantitation of the data presented in (A) and (B). Maximum cross-linking to each indicated protein is set at 100%.

in close proximity to the integral translocon components SecA, SecY and SecG. This is the first time that a link between the SRP and Sec targeting pathways has been demonstrated.

The ability of nascent FtsQ and Lep to interact efficiently with INVs (Figure 1A) suggests that membrane association of these proteins can occur during translation in vivo. Membrane association appears to depend on the context of the ribosome: when the nascent chains were released from the ribosome by puromycin prior to the addition of INVs, membrane association did not occur (data not shown), most likely because the interaction with the SRP is lost (Valent et al., 1997). These data are consistent with the compulsory co-translational mode of protein targeting to dog pancreas microsomes by E.coli SRP and FtsY in a heterologous in vitro targeting assay (Powers and Walter, 1997). However, they do not necessarily imply that translation and translocation are tightly coupled for these proteins. So far, there is no indication that the SRP affects translation upon its interaction with nascent chains. In fact, such a role seems unlikely for the E.coli SRP given its lack of SRP9- and SRP14-like protein subunits that are essential for the translation arrest function of its eukaryotic counterpart (Siegel and Walter, 1988). It should be noted that in prokaryotes, translation arrest may not be required for SRP functioning due to the short traffic distances and fast translocation rates (Pugsley, 1993). It is conceivable that the E.coli SRP pathway just accelerates membrane association of nascent chains and their delivery at the translocon. It remains to be established whether E.coli ribosomes also contribute to the association with the membrane in a similar way to eukaryotic ribosomes that have affinity for the Sec61 complex (Kalies et al., 1994; Jungnickel and Rapoport, 1995).

It is of interest that SecA is in close proximity to nascent FtsQ and Lep after SRP-mediated targeting. SecA is considered the molecular motor of post-translational translocation in E.coli that drives the stepwise transfer of the pre-protein by ATP-dependent cycles of membrane insertion and de-insertion (Driessen et al., 1998). SecA and SecY have been found juxtaposed to a translocation intermediate of pro-OmpA that depends on SecB for efficient post-translational translocation (Joly and Wickner, 1993). It remains to be established whether the interaction of nascent FtsQ and Lep with SecA that we observe is functional in the sense that SecA generates the energy for...
membrane insertion (in the absence of a tight coupling between translation and translocation; see above) or plays a role in the transfer of the nascent chain to the core translocon component SecY. At present, we cannot exclude the possibility that SecA is close to the nascent chain only as a result of its association with the translocon. However, we consider this possibility unlikely given the affinity of SecA for the signal sequence, an interaction that depends on both the N-terminal charged region and the hydrophobic core region (Akita et al., 1990; Hikita and Mizushima, 1992). The elucidation of the exact requirements for membrane insertion awaits in vitro reconstitution of the entire SRP pathway.

Nascent membrane-inserted FtsQ and Lep were also found juxtaposed to the core translocon component SecY, reminiscent of the interaction of nascent type I and II ER membrane proteins with Sec61α (High et al., 1991b; High and Stirling, 1993; Do et al., 1996; Laird and High, 1997). A direct interaction between SecY and the targeting signal would be consistent with the proposed signal sequence proof-reading activity of SecY (Driessen et al., 1998). In contrast, in vivo studies concerning the Sec dependency of the translocation of the N-terminus of Lep indicated that this event can occur independently of SecA and SecY (Lee et al., 1992). However, in these studies, conditional Sec strains were used in which the Sec function cannot be eliminated completely. It is possible that a low level of SecA or SecY is sufficient for the translocation of the N-terminal domain, which is shorter and uses a more hydrophobic targeting signal than the Sec-dependent C-terminal domain (Wolfe et al., 1985).

Finally, SecG was found to be in the vicinity of the targeted nascent chains. This is probably due to its interaction with SecY (Homma et al., 1997), since a direct interaction with neither FtsQ nor Lep nascent chains could be demonstrated. Interestingly, Sec61β, which is similar in size and hydrophobicity to SecG (Rapoport et al., 1996), has been cross-linked directly to nascent ER membrane proteins (Laird and High, 1997).

Our cross-linking data unambiguously show that release of nascent polypeptides from the SRP and their association with the translocon are linked. The SRαx homologue FtsY is found to be essential for the release of the SRP from the nascent chain in a process that depends on binding of GTP to FtsY, analogous to the SRα-dependent dissociation of the eukaryotic SRP from the targeting signal (Connelly and Gilmore, 1989). The present data form the first biochemical definition of the function of FtsY in the SRP pathway and once again underline the strong conservation of the basic mechanism of SRP-mediated protein targeting throughout evolution (Althoff et al., 1994; Wolin, 1994). On the basis of in vitro binding studies (Powers and Walter, 1995) and analogy with the eukaryotic system (Connelly et al., 1991), hydrolysis of GTP at both P48 and FtsY is assumed to be required for the dissociation of the SRP–FtsY complex.

FtsY is located in both the cytoplasm and inner membrane of E.coli (Luirink et al., 1994). Release of the SRP from the nascent chains occurred at the membrane but, surprisingly, soluble FtsY was able to associate with RNC–SRP complexes in the absence of membranes and nucleotides. Possibly, in these complexes, FtsY has a direct targeting function. This may increase the efficiency or fidelity of the targeting reaction. However, it is not needed per se, since a hybrid FtsY that is permanently anchored in the inner membrane via a hydrophobic anchor sequence is able to complement the loss of FtsY in vivo (Zelazny et al., 1997). Association of soluble FtsY with the membrane may involve different distinct binding sites, but components involved have not yet been identified (De Leeuw et al., 1997).

In conclusion, based on the results of this study and the published data discussed, we propose that in E.coli the SRP and Sec targeting pathways function in parallel and probably converge at the same translocon in vivo (Figure 4). However, it remains possible that the SRP and Sec pathways deliver proteins at translocons that differ in their exact composition but share common core elements as has been observed in S.cerevisiae (Rapoport et al., 1996; Wilkinson et al., 1997). For specific nascent chains, the choice between the targeting routes is determined primarily by the hydrophobicity of the N-terminal targeting sequence (Valent et al., 1995, 1997). Thus, most inner membrane proteins follow the SRP route which offers the advantage of co-translational membrane association to avoid the exposure of aggregation-prone hydrophobic transmembrane segments in the cytosol. In addition, certain secreted proteins with relatively hydro-
phobic signal sequences, such as β-lactamase, may use this pathway preferentially (Phillips and Silhavy, 1992; Luirink et al., 1994). Other pre-proteins interact with SecB which binds to the mature protein domain and delivers it to SecA via a direct, probably electrostatic, interaction (Driessen et al., 1998). Partial overlap in substrate specificity of the two pathways may offer flexibility to the targeting process.

Materials and methods

Materials

Restriction enzymes and RNasin were from Boehringer Mannheim GmbH (Mannheim, Germany). Megashortscript T7 transcription kit was from Ambion Inc. (Austin, TX). Puromycin and nucleotides were supplied by Sigma Chemical Co. (St. Louis, MO). [35S]Methionine was from Amersham International (Buckinghamshire, UK). OptiPrep (60% solution) was from Nycomed Pharma AS (Oslo, Norway). DSS was from Pierce (Rockford, IL). Oligonucleotides were purchased from Isogen Bioscience BV (Maarsen, The Netherlands).

Strains and plasmid constructs

Strain MC4100 was used to obtain translation lysates and INVs (both prepared as described in De Vrije et al., 1993). Strain Top10 F’ was used to maintain the plasmid constructs pC4Meth94Lep, pC4Meth101FtsQ and pC4Meth84PhoA-WT and mutant derivatives (Valent et al., 1997).

In vitro transcription, translation, targeting and cross-linking

Plasmids were linearized with HindIII and transcribed using T7 polynu- merase. The resulting truncated mRNAs coding for the N-terminal region of the proteins were translated for 20 min at 25°C in an E.coli in vitro translation system (Valent et al., 1997).

To allow targeting, RNCs were incubated with INVs (0.25 mg/ml) for 5 min at 25°C and subsequently incubated on ice for 5 min. Cross-linking was induced with 2 mM DSS for 10 min at 25°C and quenched at 0°C by adding 1/10 volume of quencher buffer (1 M glycine, 100 mM NaHCO₃, pH 8.5).

To separate integral membrane from soluble and peripheral cross-linked complexes, samples were treated with 0.18 M Na₂CO₃ (pH 11.3) for 15 min on ice. The membrane fractions containing integral membrane proteins were collected by ultracentrifugation (10 min, 115 000 μg) and resuspended in RN buffer [100 mM KOAc, 5 mM Mg(OAc)₂, 50 mM HEPES–KOH pH 7.9]. Both pellet and supernatant fractions were analyzed by SDS–PAGE.

Flotation gradient analysis

Translation reactions (50 μl) were incubated with INVs (1.25 mg/ml protein) for 5 min at 25°C and subsequently incubated on ice for 5 min. Cross-linking was induced with 2 mM DSS for 10 min at 25°C and quenched at 0°C by adding 1/10 volume of quencher buffer (1 M glycine, 100 mM NaHCO₃, pH 8.5).

To separate integral membrane from soluble and peripheral cross-linked complexes, samples were treated with 0.18 M Na₂CO₃ (pH 11.3) for 15 min on ice. The membrane fractions containing integral membrane proteins were collected by ultracentrifugation (10 min, 115 000 μg) and resuspended in RN buffer [100 mM KOAc, 5 mM Mg(OAc)₂, 50 mM HEPES–KOH pH 7.9]. Both pellet and supernatant fractions were either Triton X-100 detergent or Tricine–SDS–PAGE bands and subsequently incubated on ice for 5 min. Cross-linking was induced with 2 mM DSS for 10 min at 25°C and quenched at 0°C by adding 1/10 volume of quencher buffer (1 M glycine, 100 mM NaHCO₃, pH 8.5).

To separate integral membrane from soluble and peripheral cross-linked complexes, samples were treated with 0.18 M Na₂CO₃ (pH 11.3) for 15 min on ice. The membrane fractions containing integral membrane proteins were collected by ultracentrifugation (10 min, 115 000 μg) and resuspended in RN buffer [100 mM KOAc, 5 mM Mg(OAc)₂, 50 mM HEPES–KOH pH 7.9]. Both pellet and supernatant fractions were either Triton X-100 detergent or Tricine–SDS–PAGE bands and subsequently incubated on ice for 5 min. Cross-linking was induced with 2 mM DSS for 10 min at 25°C and quenched at 0°C by adding 1/10 volume of quencher buffer (1 M glycine, 100 mM NaHCO₃, pH 8.5).

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Reconstitution of SRP and SecB complex

Equimolar amounts of purified 4.5S RNA and F48His6 (Lentzen et al., 1994) were incubated in reconstitution buffer (125 mM NH₄Cl, 12.5 mM MgCl₂, 25 μM EDTA, 0.5 M KOAc, 25% glycerol, 25 mM HEPES– KOH pH 7.5) for 10 min at room temperature to allow complex formation. Samples were chilled on ice and applied to a 1.5 ml discontinuous 5–20% sucrose gradient in 20 mM HEPES–KOH (pH 7.5), 100 mM NH₄Cl, 10 mM MgCl₂ and 250 mM KOAc, and centrifuged for 5 h at 200 000 g. Fractions (100 μl) were taken from the top, and 5 μl of each fraction was examined by SDS–PAGE. The gel was first stained in RNA staining buffer [90 mM boric acid, 2.5 mM EDTA, 150 μg/ml ethidium bromide, 90 mM Tris (uncalibrated) pH 8.3] to visualize the 4.5S RNA, and subsequently with Coomasie Brilliant Blue to visualize F48His6. The 2–4 fractions containing the peak amounts of both components were pooled. The concentration of SRP was determined by measuring the absorbance at 260 nm (1 A₂₆₀ corresponds to 1.1 μM 4.5S RNA).

To allow SRP–RNC complex formation, 108FtsQ was produced and incubated for 5 min at 25°C with 350 nM of the reconstituted SRP which was the minimal saturating concentration in our assay as established by quantitative immunoblotting (data not shown). Samples were chilled on ice, and SRP–RNC complexes were purified from the translation mixture by centrifugation through a high salt sucrose cushion (High et al., 1991a). These purified complexes were designated SRNCs.

Sample analysis and quantification

All samples were analyzed on 12 or 15% SDS–polyacrylamide gels. Radiolabeled proteins were visualized by phosphor imaging using a Molecular Dynamics PhosphorImager 471 and quantified using the Imagequant quantification software from Molecular Dynamics.

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