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Co-translational protein targeting catalyzed by the *Escherichia coli* signal recognition particle and its receptor

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The Ffh-4.5S ribonucleoprotein particle (RNP) and FtsY from *Escherichia coli* are homologous to essential components of the mammalian signal recognition particle (SRP) and SRP receptor, respectively. The ability of these *E.coli* components to function in a bona fide co-translational targeting pathway remains unclear. Here we demonstrate that the Ffh-4.5S RNP and FtsY can efficiently replace their mammalian counterparts in targeting nascent secretory proteins to microsomal membranes *in vitro*. Targeting in the heterologous system requires a hydrophobic signal sequence, utilizes GTP and, moreover, occurs co-translationally. Unlike mammalian SRP, however, the Ffh-4.5S RNP is unable to arrest translational elongation, which results in a narrow time window for the ribosome nascent chain to interact productively with the membrane-bound translocation machinery. The highly negatively charged N-terminal domain of FtsY, which is a conserved feature among prokaryotic SRP receptor homologs, is important for translocation and acts to localize the protein to the membrane. Our data illustrate the extreme functional conservation between prokaryotic and eukaryotic SRP and SRP receptors and suggest that the basic mechanism of co-translational protein targeting is conserved between bacteria and mammals.
Keywords: co-translational/functional conservation/ protein targeting/signal recognition particle/SRP receptor/translocation

Introduction

In eukaryotic cells, the first step of protein translocation across the endoplasmic reticulum (ER) involves the co-translational recognition of signal sequences by the 54K subunit of the signal recognition particle (SRP), a cytosolic ribonucleoprotein particle (RNP) composed of six proteins and a single RNA (Walter and Johnson, 1994). Following signal sequence recognition, SRP induces a transient pause or arrest in translational elongation. The resulting ribosome–nascent chain–SRP complex is then targeted to the ER membrane by interaction of SRP54 with the α subunit of the SRP receptor (SRα), a heterodimeric membrane protein. Following this interaction, the ribosome–nascent chain is released by SRP and becomes associated with the membrane-bound translocation machinery, where translation resumes concomitant with passage of the nascent protein into the ER lumen.

Two proteins from *Escherichia coli*, Ffh and FtsY, are homologous to SRP54 and SRα, respectively (Bernstein *et al*., 1989; Römisch *et al*., 1989). In addition, Ffh is a subunit of an RNP in combination with 4.5S RNA, which resembles the SRP54 binding domain in mammalian SRP RNA (Poritz *et al*., 1990; Ribes *et al*., 1990). The discovery of these prokaryotic components led to the proposal that a co-translational targeting pathway exists in *E.coli*. This proposal is supported by the fact that the Ffh-4.5S RNP and FtsY mimic several properties of mammalian SRP and its receptor, both *in vitro* (Miller *et al*., 1994; Hauser *et al*., 1995) and *in vivo* (Poritz *et al*., 1990; Phillips and Silhavy, 1992; Luirink *et al*., 1994). In contrast, it has been argued that these components do not function co-translationally, but rather have a role in protein synthesis (Brown, 1989), or participate in a post-translational targeting route or in protein folding (Hauser *et al*., 1995; Valent *et al*., 1995; Bukau *et al*., 1996). The existence of such contrasting views reflects the fact that it has yet to be determined whether these components in fact initiate co-translational translocation.

Previous studies have shown that Ffh, either in place of SRP54 in the form of a chimeric SRP or in combination with 4.5S RNA, can interact with ribosome–nascent chains in a signal sequence-dependent manner (Bernstein *et al*., 1993; Hauser *et al*., 1995). However, these nascent chains cannot be targeted to microsomal membranes, possibly because Ffh cannot functionally interact with SRα. In this study, we tested this possibility directly by asking whether the Ffh-4.5S RNP can target secretory proteins, *in vitro*, under conditions where SRα was replaced by FtsY. Remarkably, we observed that these bacterial components can indeed promote the efficient and accurate co-translational targeting of signal sequence-bearing nascent chains.

Results

Targeting of stable ribosome–nascent chains by the Ffh-4.5S RNP and FtsY

Mild proteolysis of microsomal membranes has been shown quantitatively to remove the large cytoplasmic domain of SRα that interacts with SRP54 and abolish the translocation activity of these membranes (Walter *et al*., 1979; Gilmore *et al*., 1982b; Meyer *et al*., 1982). This activity can be restored by re-addition of either full-length SRα (Andrews *et al*., 1989) or the soluble fragment of this protein [termed SRα(frag)], generated by proteolysis (Gilmore *et al*., 1982b), demonstrating that SRα is the only membrane-associated component required for translocation that is removed by protease treatment.

Salt-washed microsomes (KRMs) were treated briefly with trypsin and the resulting membranes (TKRMs) were used in translocation reactions supplemented with either purified SRα(frag) or full-length FtsY. As a model targeting

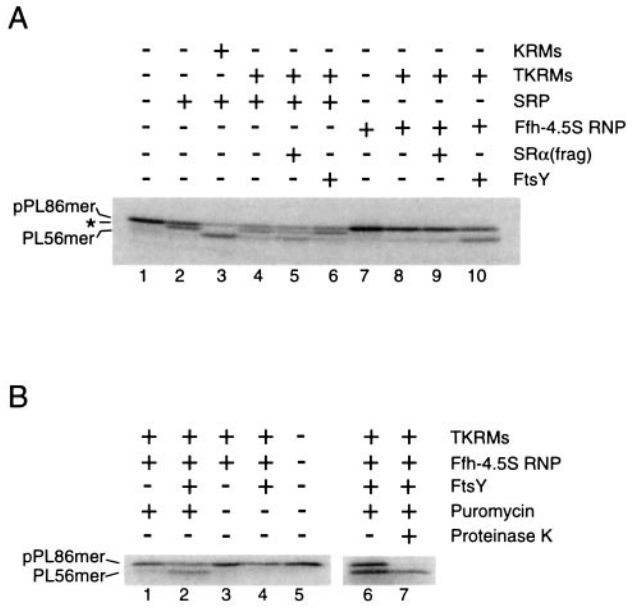

Fig. 1. Translocation of elongation-arrested nascent chains catalyzed by the Ffh-4.5S RNP and FtsY. (**A**) ^{35}S-labeled pPL86mer nascent chains were synthesized in the presence of the indicated components, followed by addition of puromycin and incubation at 37°C to allow translocation of targeted nascent chains. Samples were subsequently analyzed by SDS–PAGE on 10–15% gradient gels, followed by PhosphorImager analysis. pPL86mer and pPL56mer indicate the signal sequence uncleaved and cleaved forms of this substrate, respectively. The asterisk denotes an elongation-arrested form of pPL86mer that is observed in the presence of mammalian SRP. (**B**) Control reactions demonstrating that appearance of pPL56mer in reactions catalyzed by the Ffh-4.5S RNP and FtsY requires puromycin addition and that this fragment is protected from subsequent digestion with proteinase K.

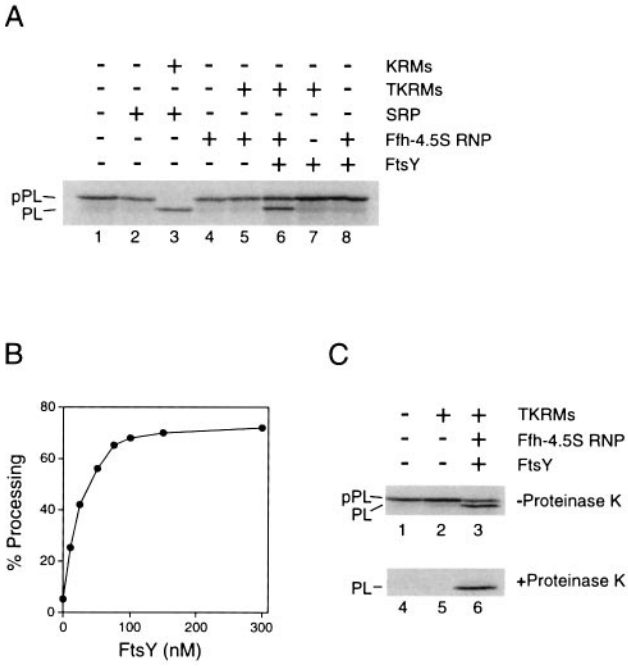

Fig. 2. Translocation of full-length preprolactin catalyzed by the Ffh-4.5S RNP and FtsY. (**A**) pPL and PL indicate the precursor and signal sequence-cleaved form of prolactin, respectively. (**B**) Processing of pPL as a function of FtsY concentration. Translocation reactions contained TKRMs and the Ffh-4.5S RNP, in addition to the indicated amounts of FtsY protein. (**C**) Protease protection of the signal sequence-cleaved form of prolactin. One-half of each translocation reaction was treated with proteinase K (lanes 4–6) or left untreated (lanes 1–3).

substrate, we used the N-terminal 86 amino acid residues of preprolactin (pPL86mer), synthesized from a truncated mRNA, to produce a stable ribosome–nascent chain complex (Connolly and Gilmore, 1986). Following targeting, nascent chains were released from the ribosome by treatment with puromycin and their subsequent translocation was monitored by signal sequence cleavage (Figure 1).

No significant translocation activity was observed in the presence of TKRMs and the Ffh-4.5S RNP alone (Figure 1A, lane 8). Remarkably, when FtsY was added, however, ~50% of pPL86mer was translocated (Figure 1A, lane 10). The reaction was specific for FtsY, as no processing was observed when SRα(frag) was added instead to reactions containing the Ffh-4.5S RNP (Figure 1A, lane 9). Similarly, SRα(frag), but not FtsY, stimulated translocation in reactions containing both TKRMs and SRP (Figure 1A, compare lanes 5 and 6). Both Ffh and 4.5S RNA were required for targeting, as no processing was observed when either component was added alone (data not shown). Control experiments confirmed that the appearance of processed pPL86mer nascent chains was dependent upon addition of puromycin (Figure 1B, compare lanes 2 and 4). In addition, these processed nascent chains were protected from protease digestion, confirming that they had been translocated into the ER lumen (Figure 1B, lane 7). From these results we conclude that the Ffh-4.5S RNP can in fact promote the targeting and translocation of a nascent secretory protein in the presence of its cognate receptor.

Translocation of full-length preproteins

To extend the above observations, we next asked whether the bacterial components could catalyze translocation of full-length preprolactin (pPL), that is, under conditions where targeting was coupled to ongoing protein synthesis. Indeed, in the presence of both the Ffh-4.5S RNP and FtsY, significant translocation of pPL was observed (~50% processing at 50 nM FtsY) (Figure 2A, lane 6 and Figure 2B); this activity reached a plateau of ~75% processing at a concentration of 100 nM FtsY (Figure 2B). As was demonstrated above for pPL86mer nascent chains, the reaction was specific for the Ffh-4.5S RNP, as no processing was observed when mammalian SRP was added instead to reactions containing FtsY (data not shown). In addition, the processed form of prolactin was protected from protease digestion (Figure 2C).

The generality of these results was confirmed by the fact that another preprotein, β-lactamase (Bla), was also translocated efficiently (~50% processing) in the presence of the Ffh-4.5S RNP and FtsY (Figure 3A, left panel). Virtually no processing of yeast α-factor (pp-αF) was observed in the presence of the *E.coli* components, however, in contrast to its efficient targeting by mammalian SRP (Figure 3A, right panel). As pp-αF contains an only moderately hydrophobic signal sequence (Ng *et al*., 1996), this difference suggests that Ffh may have a more strict dependency on signal sequence hydrophobicity. This situation would be similar to that in yeast, where pp-αF is not recognized efficiently by SRP and is instead targeted post-translationally (Ng *et al*., 1996). The requirement for a hydrophobic signal sequence in Ffh-dependent targeting

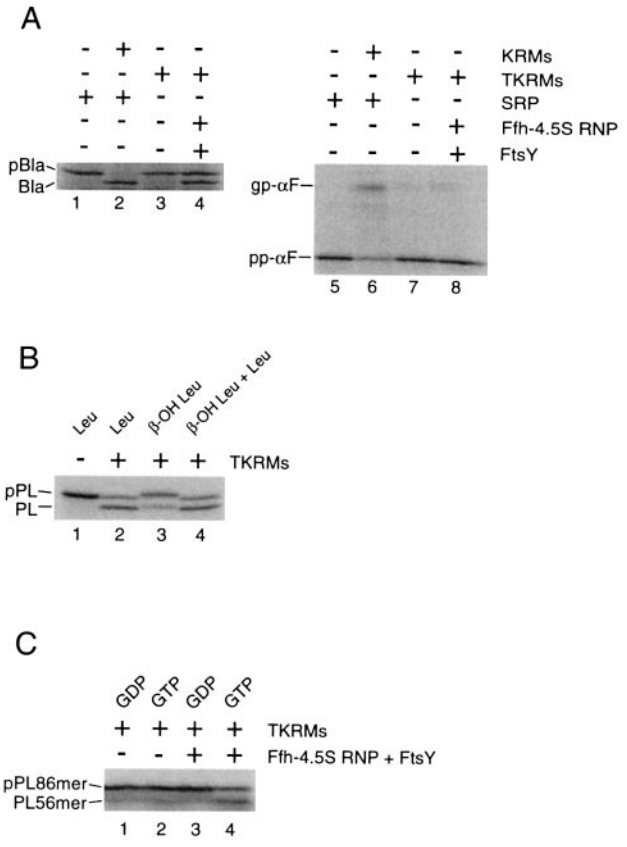

Fig. 3. (**A**) Left panel: targeting of β-lactamase, where pBla and Bla indicate the full-length and processed forms of this protein, respectively. Right panel: targeting of α-factor, where pp-αF denotes the untranslocated form and gp-αF indicates the signal sequence-cleaved and glycosylated form of this protein. (**B**) Incorporation of β-OH leucine into preprolactin. Nascent chains were synthesized in the presence of 12.5 mM β-OH leucine, 2.5 mM leucine, or both, as indicated. In addition, the Ffh-4.5S RNP and FtsY were present in lanes 1–4. (**C**) Testing the GTP requirement for translocation. pPL86mer and PL56mer indicate the unprocessed and signal sequence-cleaved form of this substrate, respectively.

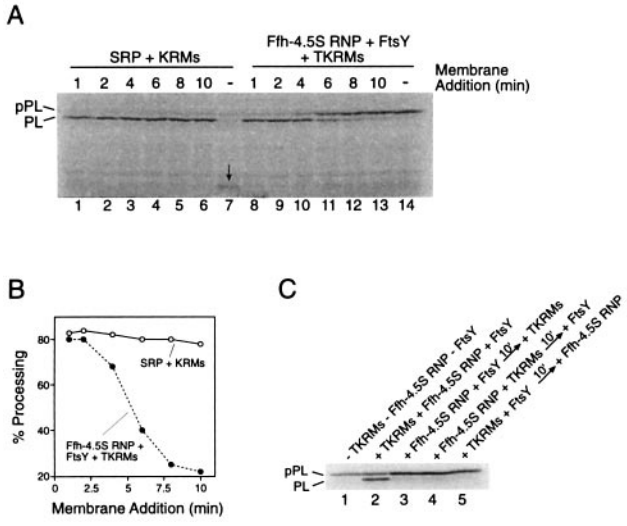

Fig. 4. Targeting of nascent chains by the Ffh-4.5S RNP and FtsY occurs co-translationally. (**A**) Membranes (KRMs or TKRMs, as indicated) were added at different times to synchronized translation reactions containing either SRP (lanes 1–7) or the Ffh-4.5S RNP and FtsY (lanes 8–14). A minus sign indicates that membranes were omitted from that sample. The arrow in lane 7 indicates the arrested fragment of pPL, which occurs when membranes are omitted from reactions containing SRP. Note that a corresponding fragment is not observed in reactions containing the 4.5S RNP (lane 14). (**B**) Quantitation of data from (A). (**C**) Order of addition experiment. Synchronized translation reactions were initiated in the presence of the components indicated. After 10 min, the missing component was added (lanes 3–5) and translation was allowed to continue for an additional 20 min.

was confirmed by the fact that incorporation of β-hydroxy leucine (β-OH Leu), a polar amino acid analog (Walter *et al*., 1981), into the signal sequence of pPL resulted in a substrate that was less efficiently translocated (Figure 3B, lane 3). When L-Leu was added during the reaction in addition to β-OH Leu, the β-OH Leu was competed out and Ffh-4.5S RNP-dependent translocation was fully restored (Figure 3B, lane 4).

Targeting by the Ffh-4.5S RNP and FtsY requires GTP

Targeting requires GTP (Connolly and Gilmore, 1986) and, like their mammalian counterparts, both Ffh and FtsY contain GTPase domains, the X-ray structures of which have been determined recently (Freymann *et al*., 1997; Montoya *et al*., 1997). Furthermore, these proteins interact stably in their GTP-bound states (Miller *et al*., 1994) and act as reciprocal GTPase-activating proteins (GAPs) for one another (Powers and Walter, 1995). We therefore determined the nucleotide specificity of the translocation reaction catalyzed by these components, using pPL86mer nascent chains as a substrate. Ribosome–nascent chains were purified from exogenous nucleotides and combined with TKRMs, the Ffh-4.5S RNP and FtsY. As expected, signal sequence processing was observed only when GTP, but not GDP, was additionally present (Figure 3C).

Targeting by E.coli SRP and SRP receptor occurs co-translationally

The functional similarities between the prokaryotic and eukaryotic components, observed in the preceding experiments, strongly suggested that the Ffh-4.5S RNP and FtsY also targeted preproteins co-translationally. We tested this directly by adding membranes at various times following initiation of pPL synthesis in a synchronized translation reaction (Figure 4A and B). We observed that the Ffh-4.5S RNP-catalyzed translocation was critically dependent on the time of membrane addition, falling off sharply after a few minutes and approaching background levels after 10 minutes. Order of addition experiments confirmed that each of the components, Ffh-4.5S RNP, FtsY and TKRMs, needed to be present at the start of the reaction in order for targeting to occur (Figure 4C). In contrast, reactions using mammalian SRP showed no significant change in the amount of processed prolactin during the course of the experiment, consistent with its ability to arrest translation and thereby maintain the nascent chain in a translocation-competent state (Walter and Blobel, 1981) (Figure 4A and B).

In this experiment, the ability of SRP to arrest translational elongation was demonstrated directly in reactions that never received membranes; here no full-length preprolactin was observed but rather a much smaller arrested fragment was produced (Figure 4A, lane 7, arrow). In contrast, no such fragment was observed in corresponding

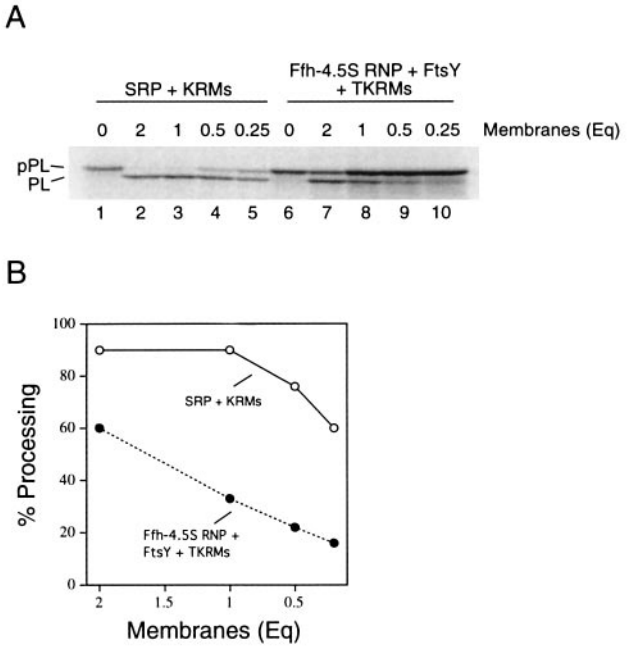

Fig. 5. Efficiency of translocation as a function of membrane concentration. (**A**) Standard translocation reactions contained the components indicated, including the number of membrane equivalents used. (**B**) Quantitation of data from (A).

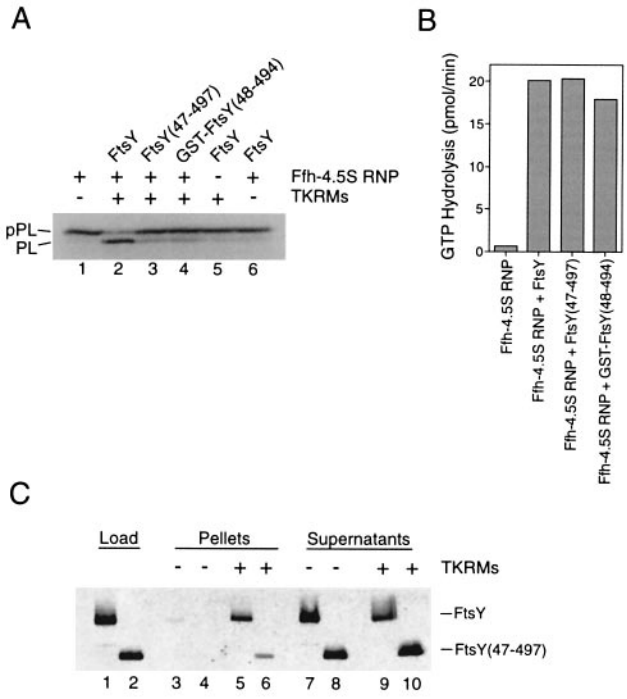

Fig. 6. Importance of the N-terminal domain of FtsY in targeting. (**A**) Translocation of pPL catalyzed by the Ffh-4.5S RNP and different forms of FtsY. (**B**) GTP hydrolysis activity of the different forms of FtsY in the presence of the Ffh-4.5S RNP. The FtsY proteins displayed no significant GTPase activity by themselves. (**C**) Membrane association of FtsY versus FtsY(47-497). TKRMs were incubated with FtsY (even lanes) or FtsY(47-497) (odd lanes) and subsequently pelleted through a sucrose cushion to separate bound (Pellets) from unbound (Supernatants) FtsY proteins. Western blot analysis was then carried out on the samples using a polyclonal antiserum raised against FtsY(47-497).

reactions containing the Ffh-4.5 S RNP and only full-length pPL was produced (Figure 4A, lane 14). This difference in behavior between the Ffh-4.5S RNP and mammalian SRP is consistent with the fact that the *E.coli* particle is missing the domain comprising the 5′ and 3′ ends of mammalian SRP RNA that is required for translational arrest (Siegel and Walter, 1985).

In a second approach, we reasoned that translocation catalyzed by the Ffh-4.5S RNP and FtsY should demonstrate a dependency on membrane concentration, because the efficiency of targeting within a given time window should vary with the number of available translocation sites. Indeed, we observed that as the concentration of membranes was reduced, translocation of pPL was significantly attenuated in reactions catalyzed by the bacterial components (Figure 5A and B). In contrast, membrane dilution had a less severe effect in reactions using mammalian SRP (Figure 5A and B). These results thus affirm the above conclusion that the Ffh-4.5S RNP targets nascent chains co-translationally, and that a narrow time window exists for the ribosome–nascent chain complex to interact productively with translocation sites in the membrane.

The N-terminal domain of FtsY is required for targeting

Our previous studies of FtsY function employed a version of this protein where residues 48–494 were fused to the C-terminus of GST (Miller *et al*., 1994; Powers and Walter, 1995). Interestingly, we found that this fusion protein was unable to substitute for full-length FtsY in promoting efficient translocation of pPL (Figure 6A, lane 4). This difference was not due to the presence of the GST moiety, however, since simply the removal of the first 46 residues of FtsY caused a dramatic reduction in translocation efficiency (Figure 6A, lane 3). This truncated protein, termed FtsY(47-497), exhibited a stimulated GTPase activity in the presence of the Ffh-4.5S RNP that was indistinguishable from full-length FtsY (Figure 6B), indicating that an impaired interaction with the particle is not likely to account for the observed loss of translocation efficiency. Instead, we found a significant difference in the ability of these proteins to interact with microsomal membranes; thus, whereas >30% of full-length FtsY associated with TKRMs in a sedimentation assay, <5% of FtsY(47-497) was found associated with these membranes (Figure 6C, compare lanes 5 and 6). Increased translocation activity (~20% processing) was observed at high concentrations (300 nM) of FtsY(47-497) (data not shown), suggesting that the N-terminal domain of FtsY acts to increase the local concentration of receptor at the membrane but may not play a direct and indispensable role in targeting. These observations are thus reminiscent of SRα, which is also anchored to the membrane by its N-terminal domain, yet can function less efficiently as a soluble fragment (Andrews *et al*., 1989).

Discussion

We have demonstrated that the Ffh-4.5S RNP and FtsY can function directly in protein targeting. That the prokaryotic components can efficiently replace both mammalian SRP and SRP receptor in the eukaryotic *in vitro* system is very remarkable. In particular, these results suggest that the molecular determinants through which these components

interact with both the ribosome and the membrane-bound translocation apparatus must be highly conserved throughout evolution. Such interactions have been postulated to govern the co-translational mode of protein targeting. To date, however, the putative interacting surfaces of SRP and the ribosome and of SRP receptor and the translocon have not been defined experimentally. Our data suggest that phylogenetic comparisons may provide invaluable tools in this quest. It remains possible, however, that direct interactions between the Ffh-4.5S RNP and FtsY with the ribosome and translocon are dispensable. According to this scenario, the Ffh-4.5S RNP and FtsY would form a more autonomously functioning targeting apparatus, Ffh interacting exclusively with signal sequences and FtsY targeting the ribosome–nascent chain–Ffh-4.5S RNP complex to the membrane without interacting directly with any translocon components. SRP and SRP receptor would then function simply to increase the local concentration of signal sequence-bearing nascent chains in the vicinity of the membrane surface. In either case, our results demonstrate that this simplified targeting system using these bacterial components contains the very essence of SRP and SRP receptor function. We note, however, that the β-subunit of the SRP receptor is still present in the TKRM fraction as it is much less sensitive to proteolysis than SRα. SRβ could, therefore, still play a functional role in the observed targeting reaction, now collaborating with FtsY instead of its normal partner SRα.

The ability of FtsY functionally to replace SRα in the heterologous system was unexpected because the bacterial protein lacks a recognizably similar N-terminal domain required for anchoring SRα to the membrane and for interactions with the β subunit of the SRP receptor (Young *et al*., 1995). FtsY has instead a smaller N-terminal domain which contains a high proportion of negatively charged residues (Gill and Salmond, 1990) and which is a conserved feature among prokaryotic SRα homologues (D.Freymann and P.Walter, unpublished results). We have found that this domain is required for efficient translocation and that it acts by increasing the association of FtsY with the membrane. Consistent with these results, Bibi and colleagues have demonstrated recently that the N-terminal domain of FtsY is required *in vivo* and that it facilitates binding of FtsY to the plasma membrane of *E.coli* (Zelazny *et al*., 1997). In addition, they have found that this domain can be replaced entirely by an unrelated membrane-spanning region, consistent with our conclusion that the N-terminal domain of FtsY serves primarily to increase the avidity of this protein for the membrane. The nature of the interaction between FtsY and the membrane remains to be explored.

Our results demonstrate unambiguously that that the Ffh-4.5S RNP and FtsY can work as functional analogues of SRP and SRP receptor in mediating co-translational protein targeting. These results suggest—but cannot prove because of the heterologous nature of our assay—that the prokaryotic components function similarly in *E.coli* cells. The existence of an SRP-dependent targeting pathway in *E.coli* has been met with skepticism, primarily because these components were never identified in genetic screens as secretory mutants (Brown, 1989; Bassford *et al*., 1991; Beckwith, 1991). Furthermore, mutation or depletion of these components results in only modest translocation defects for a limited number of secretory proteins, *in vivo* (Poritz *et al*., 1990; Phillips and Silhavy, 1992; Luirink *et al*., 1994). One possible explanation for these observations is that substrates which normally use SRP in *E.coli* can also be efficiently targeted via the well-characterized post-translational pathway involving the SecA and SecB proteins (Wickner *et al*., 1991). Alternatively, it is possible that *E.coli* proteins which possess a strict requirement for SRP have so far escaped detection. Indeed, recent genetic analyses have revealed that several polytopic transmembrane proteins require Ffh and FtsY for their proper membrane integration (Macfarlane and Müller, 1995; de Gier *et al*., 1996; Seluanov and Bibi, 1997; Ulbrandt *et al*., 1997). That these proteins often contain very hydrophobic membrane-spanning regions is consistent with the preference by Ffh for highly hydrophobic signal sequences (Valent *et al*., 1995) (Figure 3).

SRP and SRP receptor homologs are found in all cells examined to date, including representatives of the three kingdoms—eubacteria, archaea and eukaryotes (Walter and Johnson, 1994; Bult *et al*., 1996). In contrast, components mediating post-translational translocation are restricted, SecA and SecB to eubacteria, and the Sec62/63 complex to eukaryotes. The extreme evolutionary structural and functional conservation of SRP and the SRP receptor thus provides further support for the emerging view that co-translational protein targeting mediated by these components represents the ancestral mode of handling protein substrates prior to translocation, whereas post-translational translocation is likely to be of more recent evolutionary origin. As substrate proteins are fed into the translocon while emerging from the ribosome, co-translational translocation may be intrinsically less restrictive with regard to structural properties of substrate proteins, such as their tendency to aggregate or fold prematurely, that are detrimental to their translocation using a post-translational mechanism.

Finally, our results are of immense practical value as they affirm the use of the Ffh-4.5S RNP and FtsY as a model system for the study of SRP and SRP receptor function, *in vitro*. For example, now that the crystal structures of the GTPase domains of both Ffh and FtsY are known, we are in the position to test the effects of structure-predicted site-directed mutants in these components within a functional context.

Materials and methods

Preparation of messenger RNAs

Plasmid pSPBP4, containing the coding sequence for preprolactin, was linearized with *Eco*RI or *Pvu*II to generate the template for synthetic mRNAs encoding either full-length pPL or pPL86mer, respectively. Both mRNAs were prepared by *in vitro* transcription as described (Powers and Walter, 1996). Messenger RNAs encoding pBla and pp-αF were purchased from Promega.

Protein expression and purification

Full length FtsY and FtsY(47-497) was PCR-amplified from plasmid pDG1 (Gill and Salmond, 1990) using the specific upstream primers, 5′-GAT-AAC-CAT-GGC-GAA-AGA-AAA-AAA-ACG-3′ and 5′-GAT-AAC-CAT-GGC-CTC-TGA-ACA-AGC-C-3′, respectively, and a common downstream primer, 5′-GAT-AAA-GCT-TAT-CCT-CTC-GGG-C-3′. Both PCR products were digested with *Nco*I and *Hin*dIII and introduced into plasmid pET-22b-1. [This plasmid is a modified form of pET-22b(+) (Novagen) where the start codon and leader sequence preceding the polylinker cloning site were removed by digestion with *Nde*I and

*Msc*I, followed by filling in with Klenow fragment and ligation.] Proteins expressed from these constructs contained at their C-termini an additional five residues from the polylinker cloning site, followed by six histidine residues to facilitate purification by nickel-chelate chromatography. Both proteins were produced by overexpression in strain BL21(DE3) and purified to apparent homogeneity by column chromatography using first Q-Sepharose (Pharmacia) followed by Ni-NTA–Agarose (Qiagen). The Ffh-4.5S RNP and GST-FtsY(48-494) were prepared as described (Bernstein *et al*., 1993; Miller *et al*., 1994). The elastase fragment of SRα [SRα(frag)] was purified as described (Siegel and Walter, 1985).

In vitro translation and translocation assays

Salt-washed canine pancreatic microsomal membranes (KRMs) and trypsin-digested membranes (TKRMs) were prepared as described (Gilmore *et al*., 1982a; Walter and Blobel, 1983; Andrews *et al*., 1989). Western blot analysis demonstrated that the TKRMs lacked detectable SRα; however, we routinely observed ~5–10% background processing with these membranes, as has been documented previously (Rapiejko and Gilmore, 1992). Wheat germ translation reactions (10 µl) contained [^{35}S]methionine and were performed as described (Strub *et al*., 1991). Unless stated otherwise, reactions contained 0.5 µg mRNA, 2 equivalents (eq) (defined in Walter and Blobel, 1983) of membranes and 50 nM each of SRP, the Ffh-4.5S RNP and/or FtsY, where appropriate. For comparison, KRMs contain ~100 fmol of SRP receptor per equivalent (Tajima *et al*., 1986), which corresponded to a final concentration of 20 nM SRα in our experiments. In experiments using elongation-arrested pPL86mer nascent chains, samples were subsequently treated with 2 mM puromycin for 15 min at 37°C to allow release of nascent chains from the ribosome and their subsequent translocation across the ER membrane (Hauser *et al*., 1995). Synchronized translation reactions were initiated by addition of mRNA to preformed translation mixtures and were allowed to proceed for 1 min, followed by addition of the cap analog 7-methylguanosine-5′-monophosphate (7MeG) to a final concentration of 4 mM (Siegel and Walter, 1985). Where indicated, samples were treated with proteinase K (0.1 mg/ml) for 10 min at 25°C.

Nucleotide requirement for targeting

pPL86mer ribosome–nascent chains were synthesized and separated from small molecules by centrifugation over a sucrose cushion as described (Powers and Walter, 1996), except that the salt concentration was maintained at 150 mM KOAc to limit SRP-independent translocation (Lauring *et al*., 1995). Purified ribosome–nascent chains were incubated for 10 min at 25°C with TKRMs, the Ffh-4.5S RNP and FtsY, and 1 mM GTP or GDP. Samples were then treated with puromycin and incubated as described above. Control experiments confirmed that the appearance of processed PL56mer nascent chains was dependent upon addition of puromycin and was protected from digestion by proteinase K.

GTP hydrolysis assay

GTPase reactions were conducted by combining purified Ffh-4.5 RNP and FtsY (100 nM each) for 20 min at 25°C in buffer containing 50 mM TEA, pH 7.5, 2.5 mM $Mg(OAc)_2$, 150 mM KOAc, 0.01% Nikkol, 1 mM DTT and 250 µM GTP. The amount of GTP hydrolyzed during the reaction was determined by a sensitive phosphate-binding assay using the dye malachite green (Lanzetta *et al*., 1979; Fisher and Higgins, 1994) (D.Freymann and P.Walter, in preparation). This assay allowed us accurately to determine the GTPase activity of these proteins under conditions identical to those used in translocation reactions. Control reactions confirmed that rate of GTP hydrolysis was linear over the time course of the experiment.

Binding FtsY to microsomal membranes

Two eq of TKRMs were combined with 50 nM FtsY or FtsY(47-497) in 10 µl of buffer containing 50 mM TEA, pH 7.5, 2.5 mM $Mg(OAc)_2$, 150 mM KOAc, 1 mM DTT for 30 min at 25°C. Samples were then loaded onto a 75 µl cushion containing the same buffer plus 0.5 M sucrose and centrifuged at 70 000 r.p.m. in a TLA100 rotor for 15 min at 4°C. Supernatants were TCA-precipitated and samples analyzed by SDS–PAGE, followed by immunoblotting for FtsY.

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