Evidence for coupling of membrane targeting and function of the signal recognition particle (SRP) receptor FtsY

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Recent studies have indicated that FtsY, the signal recognition particle receptor of *Escherichia coli*, plays a central role in membrane protein biogenesis. For proper function, FtsY must be targeted to the membrane, but its membrane-targeting pathway is unknown. We investigated the relationship between targeting and function of FtsY *in vivo*, by separating its catalytic domain (NG) from its putative targeting domain (A) by three means: expression of split *ftsY*, insertion of various spacers between A and NG, and separation of A and NG by *in vivo* proteolysis. Proteolytic separation of A and NG does not abolish function, whereas separation by long linkers or expression of split *ftsY* is detrimental. We propose that proteolytic cleavage of FtsY occurs after completion of co-translational targeting and assembly of NG. In contrast, separation by other means may interrupt proper synchronization of co-translational targeting and membrane assembly of NG. The co-translational interaction of FtsY with the membrane was confirmed by *in vitro* experiments.

INTRODUCTION

FtsY is an essential component of the *Escherichia coli* signal recognition particle-like protein (SRP) targeting machinery (Luirink *et al.*, 1994; Seluanov and Bibi, 1997), and it is homologous to the α subunit of the mammalian SRP receptor (SR α) (Bernstein *et al.*, 1989; Romisch *et al.*, 1989). FtsY associates with the cytoplasmic membrane even though it has no transmembrane

domains, and the nature of this interaction is not yet understood fully. Structural and functional analysis of FtsY revealed that it consists of two major domains (de Leeuw *et al.*, 1997; Montoya *et al.*, 1997; Zelazny *et al.*, 1997). The N-terminal acidic (A) domain, consisting of 198 amino acids, is probably essential for targeting the C-terminal catalytic (NG) domain, consisting of 299 amino acids, to the membrane (Powers and Walter, 1997; Zelazny *et al.*, 1997). In addition to its role as an SRP receptor, it was proposed that FtsY might participate in additional steps during the biosynthesis of membrane proteins in *E. coli* (Valent *et al.*, 1998; Arvidson *et al.*, 1999), such as during transfer of the ribosome-nascent chain (RNC) complex to the integral membrane components of the Sec machinery (Valent *et al.*, 1998).

A model for co-translational membrane targeting of FtsY itself was recently proposed (Herskovits *et al.*, 2000). This model is based on the observation that although the NG domain is inactive when expressed alone (without the A domain), it is functional when fused to polypeptides derived from the membrane protein LacY (Zelazny *et al.*, 1997). LacY, the lactose permease of *E. coli*, is co-translationally targeted to the membrane (Macfarlane and Muller, 1995; Seluanov and Bibi, 1997). In addition, sequence analysis identified FtsY homologs in *actinomyces* that are, in contrast to *E. coli* FtsY, integral membrane proteins (Bibi *et al.*, 2001). In this regard, FtsY and the membrane-bound NG hybrids seem to behave similarly to mammalian SR α , which assembles co-translationally on its membrane attachment site

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(Young and Andrews, 1996). Here we studied FtsY biosynthesis and tested the possibility that a co-translational targeting pathway is important for its proper function (Herskovits *et al.*, 2000).

Demonstrating an obligatory co-translational membrane-targeting pathway *in vivo* is not yet achievable practically. Here we describe methods that approach this goal considerably. In order to distinguish between co- and post-translational targeting and assembly of FtsY *in vivo*, we separated the catalytic NG domain from the membrane targeting A domain by three means: (i) insertion of polypeptide spacers between A and NG; (ii) co-expression of separated A and NG domains; and (iii) insertion of a specific protease recognition site between A and NG and *in vivo* proteolysis. Finally, the ability of FtsY-RNCs to interact with inverted vesicles was examined *in vitro*. The results of these studies support the model where co-translational targeting of FtsY is important for its function.

RESULTS AND DISCUSSION

FtsY tolerates only short spacers between the A and NG domains

Previous studies have indicated that the A domain of FtsY acts in targeting (and maybe also attachment) of the catalytic NG domain to the membrane (de Leeuw *et al.*, 1997, 2000; Powers and Walter, 1997; Zelazny *et al.*, 1997; Millman and Andrews, 1999). In addition, it was hypothesized that the NG domain of FtsY might be targeted to the membrane during translation, similar to the NG domain of its mammalian homologue, SR α (Young and Andrews, 1996). If the NG domain of FtsY assembles co-translationally on its membrane target, it is reasonable to suggest that NG should be translated immediately after the FtsY-RNC exposing the A domain has reached the membrane (see *in vitro* section below). Therefore, hydrophilic spacers between the A and NG domains may cause a delay in translation of NG, may localize NG in a distance from the membrane, and thus prevent proper co-translational assembly. It is important to note that NG can tolerate non-native N-termini, indicating that the A domain is not directly involved in NG assembly (Zelazny *et al.*, 1997). Following this rationale, linkers of different lengths were inserted at the junction between the A and NG domains of wild-type FtsY and into the fusion joint of a membrane protein–NG hybrid, in which NG is placed after the sixth transmembrane domain of LacY (N6) (Zelazny *et al.*, 1997). DNA segments encoding the 100-amino acid-long biotin acceptor domain (BAD) from *Klebsiella pneumoniae* (Cronan, 1990), its 35-amino acid-long C-terminally truncated version or a random 7-amino acid-long linker, were inserted into the coding region of *ftsY*, between A and NG (Figure 1A). These constructs were named A-100aa-NG, A-35aa-NG and A-7aa-NG. In order to generate integral membrane protein constructs, the A domains of hybrids A-7aa-NG, A-35aa-NG and A-100aa-NG were replaced by N6 of LacY, thus generating N6-5aa-NG, N6-35aa-NG and N6-105aa-NG, respectively (Figure 1B). Plasmids were transformed into *E. coli* FJP10, which contains *ftsY* under regulation of the arabinose promoter. Since FtsY is essential for growth, it is possible to analyse the ability of the plasmid-encoded FtsY constructs to complement FtsY depletion, by monitoring the growth in arabinose-free media. In the case of the A-linker-NG constructs, only cells expressing *ftsY* or A-7aa-NG form colonies that are

comparable to wild type in the absence of arabinose (Figure 1C). Cells expressing A-35aa-NG form only small colonies and cells expressing A-100aa-NG are unable to grow on Luria–Bertani (LB) plates without arabinose. All LacY–NG-derived hybrids complement growth to some extent in the absence of arabinose, but size and number of colonies is inversely correlated to linker length (Figure 1D). Importantly, even with the long linker, hybrid N6-105aa-NG retains residual biological function, in contrast to A-100aa-NG, which is completely inactive, suggesting that the long linker does not abolish NG function in A-100aa-NG because of unrelated steric effects. Complementation was also assayed in liquid media, and the results are similar to those obtained with agar plates (data not shown). As shown by western blotting with anti-FtsY antibodies, all the A-linker-NG and N6-linker-NG hybrids were expressed similarly or better than the chromosomally encoded FtsY (Figure 1E). In addition to full-length products migrating according to their expected molecular weights, some breakdown products are also detected (Figure 1E, lanes 6–8). In addition, as tested by cell fractionation, all N6-linker-NG hybrids are found predominantly in the membrane fraction (data not shown), and cellular distribution of the A-linker-NG hybrids is similar to that of wild-type FtsY (Figure 1F). There are at least two possible reasons for the negative effect of the long linker insertions. First, functional interaction of NG with SRP requires a close proximity of NG with the membrane. Secondly, close proximity of NG with the membrane may be important for its proper co-translational assembly. If the latter is true, the linker may interrupt proper synchronization between targeting, translation and assembly of NG (Figure 5). To address these aspects we generated conditions where NG was separated from the A domain after targeting, using a specific protease.

Cleavage of FtsY *in vivo*

Experiments with split FtsY demonstrate that co-expression of the individual A and NG domains as separate polypeptides (Figure 2A) does not complement the growth defect of FtsY-depleted cells (Figure 2B). Since the NG domain alone complements when fused to an integral membrane protein (Zelazny *et al.*, 1997), we speculated that the A domain is only required for co-translational targeting of FtsY. If this is correct, the NG domain may be removed from A after targeting, without affecting FtsY function. If on the other hand FtsY is targeted post-translationally, proteolytic separation *in vivo* could prevent proper membrane targeting, assembly and function. Site-specific cleavage of FtsY *in vivo* was done with co-expressed tobacco etch virus (TEV) NIa protease. TEV protease is a cytoplasmic cysteine protease recognising a seven residue target sequence (Ehrmann *et al.*, 1997). A TEV protease target sequence (tevs) was inserted between the A and NG domains of FtsY (A-tevs-NG). Subsequently, the gene encoding a hybrid GST–TEV protease under the tight tetracycline promoter (*tet* p/o) was inserted into the same plasmid that encodes the A-tevs-NG construct. *Escherichia coli* FJP10 cells harbouring plasmid-encoded TEV protease or TEV+A-tevs-NG were induced with anhydrotetracycline (aTc) (for expression of TEV protease) after 2 h of arabinose depletion. The results demonstrate efficient induction of TEV protease expression with aTc (Figure 3A), and no full-length FtsY is detected in TEV protease induced cells, whereas a band corresponding to the NG domain is clearly accumulated (Figure 3B). Note that

Fig. 1. Effect of linker insertion on FtsY function. **(A and B)** Schematic representation of FtsY constructs. Linkers of different lengths (thin line) were inserted between the A domain of FtsY (black boxes) or N6 from LacY (black winding lines) and the NG domain of FtsY (grey boxes). **(C and D)** Constructs shown in **(A)** and **(B)**, respectively, were transformed into *E. coli* FJP10, which contains a chromosomal *ftsY* gene under the arabinose promoter. The activity of these constructs was analysed by the capacity of transformed FJP10 cells to form colonies on LB plates without arabinose. **(E)** *Escherichia coli* FJP10 cells harbouring the various FtsY constructs were grown without arabinose and induced by IPTG. Cultures were harvested, cells were sonicated and extracts (10 µg proteins) were analysed by western blotting with anti-NG antibodies. The proposed full-length forms of the proteins of interest are indicated by an asterisk to their left. **(F)** Cells expressing A-linker-NG were sonicated and membrane proteins (P) were separated from the soluble fractions (S) by ultracentrifugation. Samples (10 µg proteins) were then analysed as in **(E)**.

harvested cells were immediately treated with iodoacetamide to block proteolytic activity of TEV protease in cell extracts. Importantly, cells in which FtsY is cleaved by TEV protease grow normally, while FtsY-depleted cells are unable to grow (Figure 3C). Similar results were obtained when cells were plated immediately after transformation on LB-agar plates (Figure 3D). As shown in Figure 3E, the cleavage of FtsY occurs efficiently during growth. Notably, although almost no full-length FtsY is observed after 1 h of TEV protease induction, induced cells grow normally throughout the entire experiment (data not shown). Since NG expressed alone (Zelazny *et al.*, 1997) or with a

separated A domain (Figure 2) is unable to complement FtsY depletion, these results suggest that intact FtsY is required only during a very short period of time in order to enable proper co-translational assembly of NG on the membrane (Figure 5).

Interaction of FtsY translation intermediates with inner membranes *in vitro*

In order to test the model that the A domain of FtsY mediates co-translational targeting of NG to the membrane, the ability of stalled FtsY-RNCs to associate with the membrane was tested

Fig. 2. Expression and function of split FtsY. (A) *Escherichia coli* FJP10 cells harbouring the indicated FtsY constructs were grown in the presence (left panel) or absence (right panel) of arabinose and induced at mid-log phase ($OD_{600} = 0.3$) (left panel) or after 5 h of depletion (right panel) by IPTG for 1.5 h. Cultures were harvested, cells were sonicated, ultracentrifuged, and samples from the pellet (30 μ g proteins) (P) and the supernatant (15 μ g proteins) (S) were analysed by western blotting with purified anti-FtsY antibodies. (B) The activity of the deletion constructs of FtsY was analysed by the capacity of transformed FJP10 cells (left panel) to form colonies on LB plates without arabinose (right panel).

in vitro. We generated several *ftsY* deletions (Figure 4A) for production of RNCs. Translation was performed in the presence of inverted membrane vesicles (INVs), and the association between RNCs and INVs was assessed by two methods. First, after translation the isolated INVs were treated with 0.2 M Na_2CO_3 to remove weakly- and non-specifically-bound peripheral proteins and complexes from the membrane. After ultracentrifugation, pellet and supernatant fractions were separated by SDS-PAGE and analysed by autoradiography. The 59aa-RNC is found mainly in the soluble fraction (Figure 4B). Since ~30 amino acids are buried inside the ribosome, this observation indicates that exposure of the N-terminal 19 amino acids of FtsY from the ribosome is not sufficient for membrane association during translation. Even the 212aa-RNC, exposing ~180 amino acids of the A domain from the ribosome, exhibits only weak interaction with the membrane (Figure 4B). Interestingly however, exposure of the entire A domain of FtsY dramatically changes the distribution since most of the 234aa-RNC co-localizes with the membrane (Figure 4B). Similarly, the majority of the 412aa-RNC is also found in the membrane fraction. In order to exclude the possibility that the longer constructs may form aggregates that precipitate with the pellet, membranes were isolated by flotation in a stepwise density gradient. The top fractions containing membrane-associated FtsY-RNCs were isolated and compared with the soluble fractions by SDS-PAGE and autoradiography. There is a clear difference between 212aa-RNC that only partially interacts with the membrane and the 234aa- and 412aa-RNCs, which are predominantly found in the membrane fraction (Figure 4C). Finally, interactions between the various FtsY-RNCs and the membrane are relatively strong because they are not affected by treatment with carbonate (Figure 4B) or high salt (data not shown). These results

Fig. 3. Co-expression of cleavable FtsY (A-tevs-NG) and the tightly regulated TEV (GST-TEV) protease. (A and B) FJP10 cells harbouring A-tevs-NG with or without TEV protease were grown for 2 h in LB media without or with 0.5 mM IPTG for induction of A-tevs-NG. As indicated, TEV protease was induced by 100 ng/ml aTc for 2 h in one sample. Cells were harvested, sonicated and membrane fractions (P) were separated from the cytosolic fractions (S) by ultracentrifugation. Samples (20 μ g of protein) were analysed by western blotting with anti-NG antibodies (B). The TEV protease (A) was detected in total cell extracts (before ultracentrifugation) by anti-TEV protease antibodies. (C) Growth curves of FtsY-depleted *E. coli* FJP10 cells harbouring TEV protease or A-tevs-NG and TEV protease, with or without aTc. (D) FJP10 cells transformed with plasmids encoding the TEV protease, A-tevs-NG or both were plated immediately after transformation on the indicated plates (+/- arabinose). (E) *Escherichia coli* FJP10 cells harbouring co-expressing TEV protease and A-tevs-NG were grown for 3 h in LB media supplemented with 0.5 mM IPTG for induction of A-tevs-NG. The culture was split and TEV protease was induced in one sample by 100 ng/ml of aTc. Aliquots were harvested after 30 min, 1, 1.5 and 3 h after aTc treatment, sonicated and samples (30 μ g of total protein) were analysed by western blotting with anti-NG antibodies.

demonstrate the capability of FtsY to interact with the membrane co-translationally, and define the entire A domain as a crucial determinant for efficient membrane association.

Concluding remarks

In fractionation experiments, FtsY is found both as a peripheral membrane protein and a cytoplasmic protein (Luirink *et al.*, 1994). Although the soluble FtsY is able to support insertion of membrane proteins *in vitro* (Valent *et al.*, 1998), no function was attributed to cytoplasmic FtsY *in vivo*. Moreover, we have shown previously that, in the absence of soluble FtsY, integral membrane FtsY constructs function properly (Zelazny *et al.*, 1997). Therefore, we investigated here the targeting pathway of

the membrane bound FtsY *in vivo*, in cells devoid of a cytoplasmic form of this protein. Although it is practically impossible to prove unequivocally *in vivo* whether a certain protein must be targeted to the membrane during translation or not, we believe that collectively, our results with FtsY provide strong support for a model where it has to be targeted co-translationally for proper function. It was demonstrated previously that the biological activity of FtsY is mediated by its NG domain

provided it is targeted to the cytoplasmic membrane (Powers and Walter, 1997; Zelazny *et al.*, 1997). Here we show that targeting itself is not sufficient for proper function. The distance between NG and its targeting domain is also important. If co-translational assembly of NG on the membrane is a prerequisite for proper function, long linker insertions might be detrimental (Figure 5A). However, the linker insertion experiments do not rule out alternative explanations such as the linker might affect NG function simply by increasing the distance between NG and the membrane. The results of *in vivo* proteolysis make this explanation unlikely because complete detachment of NG from A does not affect function. A TEV protease site between A and NG is expected to remain buried inside the ribosome until the targeting step is completed (Figure 5B). Therefore, cleavage of FtsY should not occur during targeting. Alternatively during post-translational targeting, FtsY could be protected from cleavage by chaperones or as a specific folding intermediate. However, no protected cytoplasmic form of FtsY can be detected in the cytoplasmic fractions of cells co-expressing A-tevs-NG and TEV protease (Figure 3). After co-translational targeting and interaction of the newly synthesized NG domain with its putative membrane target, the TEV protease site becomes accessible to protease, which then separates A from NG (Figure 5). Accordingly we propose that after targeting, the covalent contact between A and NG is not required for proper function. Intact FtsY is only required (as shown in Figure 2) during translation and targeting. Therefore we hypothesize that FtsY is targeted to the membrane during translation and that this mode of targeting is essential for its biological activity (Herskovits and Bibi, 2000). The signals and cellular factors that direct co-translational targeting of FtsY to the inner membrane are currently being investigated by genetic and biochemical means.

METHODS

Materials. Antibodies to the NG domain of FtsY (anti-NG antibodies) were directed against synthetic peptides corresponding

Fig. 4. *In vitro* synthesis and membrane association of truncated FtsY-RNCs. (A) Schematic representation of the truncated FtsY nascent chain products. (B) After *in vitro* translation in the presence of INVs, the vesicles were treated by sodium carbonate and isolated by ultracentrifugation. Pellet (P) and supernatant (S) fractions were subjected to SDS-PAGE and autoradiography. (C) After *in vitro* translation in the presence of INVs, the samples were subjected to flotation analysis in high salt (0.5 M NaCl). Fraction 4 of each sample contains INVs. Fraction 1 of each sample contains soluble proteins and aggregates.

Fig. 5. Illustration of the possible effects of linker insertion and TEV protease cleavage. (A) Model for the effect of linker insertion. Left panel, co-translational targeting and assembly of NG in the context of wild-type FtsY. Right panel, targeting of A-100aa-NG. (B) Model for the effect of FtsY cleavage by the TEV protease after targeting. For detailed descriptions of the models see text.

to exposed segments according to the X-ray structure of the NG domain (Montoya *et al.*, 1997). The following peptides were chosen: (i) cys-his-ser-lys-leu-met-glu-glu-leu-lys-lys-ile-val-arg-val-met-lys-lys-leu-aspartic-acid (for preparation of antibody anti-FtsY#213), and cys-ala-gln-his-thr-gly-ala-aspartic-acid-ser-val-ile-phe-aspartic-acid-ile-gln-ala-ala-lys (for preparation of antibody anti-FtsY#211). Polyclonal anti-FtsY antibodies were prepared against purified His₆-tagged FtsY. Purified protein was used for affinity purification of anti-FtsY antibodies using AminoLink agarose (Pierce). Goat-anti-rabbit antibodies conjugated to horseradish peroxidase (HRP) were from Jackson ImmunoResearch Laboratories. Streptavidin-conjugated HRP was from Pierce.

Escherichia coli strains. *Escherichia coli* HB101 [*hsdS20* (*r-B*, *m-B*), *recA13*, *ara-14*, *proA2*, *lacY1*, *galk2*, *rpsL20* (*Smr*), *xyl-5*, *mtl-1*, *supE44*, λ -*F*] was used for propagation and preparation of various plasmid constructs. *Escherichia coli* FJP10 [MC4100, *ara+*, *ftsY*::*kan1*; *attB*::*ParaBAD-ftsY*⁺, *R6Kori*; *cam*^R; *pFtsEX-Sp* (*ftsE*⁺ *X*⁺, *spc*^R *p15Aori*); *recA*::*Tn10*], in which the chromosomal *ftsY* gene was replaced by *ftsY* under control of the *araB* promoter, was used for FtsY-depletion experiments (for the detailed construction of FJP10, see Supplementary data available at *EMBO reports* Online).

Construction of *ftsY* mutants. The following plasmids were used in this study (for the detailed construction of the plasmids see Supplementary data). (i) Plasmids encoding A-linker-NG hybrids include pT/A-7aa-NG, pT/A-35aa-NG and pT/A-100aa-NG. In addition, the A domain coding sequence in each of these plasmids was replaced by a DNA fragment encoding N6 of LacY (the N-terminal six transmembrane segments), thus constructing pT/N6-5aa-NG, pT/N6-35aa-NG and pT/N6-105aa-NG. (ii) Plasmids encoding the A domain of FtsY (pGEX-2T-A), the NG domain (pGEX-2T-NG) or both (pGEX-2T-NG+A). (iii) Plasmids encoding the A-TEV protease site-NG hybrid (pT/A-tevs-NG), or TEV protease (pT7-5/TEV+tetR) or both (pT7-5/TEV+tetR+A-tevs-NG). (iv) Plasmids constructed for *in vitro* studies.

Growth experiments. Cultures were grown at 37°C in LB medium supplemented with the antibiotics ampicillin (100 µg/ml), chloramphenicol (30 µg/ml), kanamycin (30 µg/ml) and spectinomycin (50 µg/ml) when necessary, and with the indicated inducers aTc (100 ng/ml), IPTG (0.05–0.5 mM) and L-arabinose (0.2%). For LB-agar plate tests, competent cells were transformed with the test constructs. After phenotypic expression for 1 h at 37°C, cells were collected by centrifugation (3 min, 13 000 r.p.m.) and resuspended in 100 µl of LB with or without inducers. The cells were then plated on LB-agar plates containing the appropriate antibiotics with or without inducers as indicated.

Cell fractionation and western blotting. In cultures expressing TEV protease, the protease was inactivated by addition of iodoacetamide (100 mM) prior to and after harvesting, and total protein synthesis was stopped by addition of chloramphenicol (150 µg/ml). Membranes and cytosolic fraction preparation and immunoblotting were performed as described previously (Zelazny *et al.*, 1997) with the following antibodies and dilutions: anti-NG, 1:8000; purified anti-FtsY antibodies, 1:200; anti-TEV protease antibodies, 1:18 000.

Targeting of FtsY RNCs to inverted vesicles. Linearized plasmids (see Supplementary data) were used for *in vitro* coupled transcription-translation experiments as described previously

(Valent *et al.*, 1997), using a kit obtained from Promega. The *in vitro* synthesis was carried out in the presence of INVs prepared from *E. coli* MC4100 (Valent *et al.*, 1997) (1.25 mg/ml protein) at 25°C for 20 min in the presence of [³⁵S]methionine. Three minutes after initiation, aurintricarboxylic acid (1 mM) was added to inhibit further initiation of translation, resulting in accumulation of RNCs harbouring relatively homogeneous nascent chain lengths. The translation was stopped by the addition of 3 mM chloramphenicol. Association of RNCs with the membrane was verified by treatment with 0.2 M Na₂CO₃ pH 11.3 for 10 min on ice. Integral membranes were then isolated by ultracentrifugation at 57 000 r.p.m. for 10 min (using a TLA100 rotor, Beckman). In addition, since Na₂CO₃ treatment might cause formation of aggregates, further isolation of the membranes was accomplished by flotation (de Leeuw *et al.*, 1997), using an Optiprep density gradient.

Supplementary data. Supplementary data are available at *EMBO reports* Online.

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