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


Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel, 1Department of Molecular Microbiology, Vrije University, 1081HV Amsterdam, The Netherlands, 2Cardiff School of Biosciences, Cardiff University, Cardiff CF10 3US, UK, 3Department of Microbiology, Iowa State University, 207 Science I Building, Ames, IA 50011, 4Department of Biology, College of William and Mary, Williamsburg, VA 23187, USA and 5Department of Molecular Life Science, Tokai University School of Medicine, 259-1193 Isehara, Japan

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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 membrane targeting of FtsY. 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 (SRα) (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 polyproteins 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 actinomycetes 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 SRα, which assembles co-translationally on its membrane attachment site.
(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, SRt (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) Nla protease. TEV protease is a cytoplasmic cysteine protease recognizing 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
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.
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 Na₂CO₃ 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 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 to

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.
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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-lys-lys-ile-val-arg-val-met-lys-lys-lye-aasp-val (for preparation of antibody anti-FtsY, see Supplementary data) were 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 [hslS20 (r-b, m-B), recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Smr), xyl-5, met-1, supE44, λ-A] was used for propagation and preparation of various plasmid constructs. Escherichia coli FLP10 [MC4100, ara+, ftsY::kan1; attB:ParaBAD-ftsY, R6Kori; camR, pFtSEX-p (ftsYE*, spcB 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 FLP10, 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-N, pT/A-35aa-N and pT/A-100aa-N. In addition, the A domain coding sequence in each of these plasmids was replaced by a DNA fragment encoding Nδ of LacY (the N-terminal six transmembrane segments), thus constructing pT/N6-5aa-N, pT/N6-35aa-N and pT/N6-105aa-N. (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-TEV-NG), or TEV protease (pT7-5/TEV+telR) or both (pT7-5/TEV+telR+A-TEV-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 1Ac (100 ng/ml), IPTG (0.05–0.5 mM) and L-arabinose (0.2%). For LB–agar plate tests, competent cells were treated 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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REFERENCES


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