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YidC-dependent translocation of green fluorescence protein fused to the FliP cleavable signal peptide

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Abstract

Escherichia coli FliP is a rare bacterial polytopic membrane protein synthesized with a cleavable, highly hydrophobic signal peptide. More hydrophilic Tat-dependent or Sec-dependent signal peptide is functionally capable of substituting for the FliP signal peptide, but a signal anchor of inner membrane protein fails to do so. To assess the intrinsic characteristics of the FliP signal peptide in mediating protein translocation, we fused it to green fluorescence protein and observed that the translocation of the chimera (FliPss–GFP) was dependent of Ffh, SecA, SecY and SecD. In addition, we showed for the first time the involvement of YidC in protein translocation across the inner membrane.

Keywords: Signal peptide; Inner membrane protein; GFP, Sec translocase, YidC

1. Introduction

In *Escherichia coli* there are multiple pathways that are involved in directing proteins to the inner membrane. Most presecretory proteins are synthesized with a cleavable signal peptide at their N-terminus and targeted, post-translationally or at a late co-translational stage, by SecB and SecA in unfolded conformation to the inner membrane SecYEG translocase [1]. A small group of periplasmic proteins carrying twin-arginine signal peptides are targeted post-translationally in folded conformation to the distinct TatB–TatC complex by unknown, or independently of cytoplasmic factors [2]. In contrast to their eucaryotic counterpart, most bacterial inner membrane proteins (IMP) do not contain cleavable signal peptide and their N-terminal transmembrane segment (TM, also called signal anchor) serves as the membrane targeting signal. The IMPs are targeted in a co-translational manner to the SecYEG translocase by the signal recognition particle (SRP) and its receptor FtsY [3]. Translocation of large periplasmic domains of IMPs seems to require the assistance of SecA [4,5]. Therefore, the SRP and SecB–SecA targeting pathways converge at the SecYEG complex which translocates IMPs laterally into the inner membrane, or the presecretory proteins linearly across the inner membranes [1,3]. In contrast to most IMPs, some small phage coat proteins were originally reported to insert spontaneously into the membrane, i.e., independently of the Sec-translocase and the SRP targeting pathway [6]. Recently, YidC was identified as a factor that assists the integration of both Sec-dependent and Sec-independent IMPs [7–10]. In addition, it is specifically used for the insertion of membrane proteins and not for the translocation of exported proteins [9].

FliP is a component of the flagellar export apparatus [11,12]. Unlike most bacterial IMPs, which do not contain cleavable signal peptide [13,14], FliP is synthesized as a precursor with a cleavable N-terminal signal peptide [11,12]. In general, IMPs are co-translocationally targeted by the SRP pathway to the membrane and *E. coli* SRP binds preferentially to particularly hydrophobic targeting signals present in the N-terminus of nascent IMPs [3]. The mean hydrophobicity of the FliP signal peptide is 1.81, which was lower than those of SRP-specific targeting signals of integral membrane proteins FtsQ (2.23) and MtlII (2.08), but higher than those of the Sec-signal peptides of OmpA (1.65) and MalE (1.63), or the Tat-signal peptide of TorA (1.52). Recently, we reported that two altered FliPs with more hydrophilic Tat- or Sec-

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dependent signal peptides were both able to restore the motility of the Δ*fliP* mutant [15]. Therefore, the Tat- and the Sec-dependent signal peptides seemed to be compatible with the FliP function. Moreover, deletion of the FliP signal peptide or replacing it with the transmembrane segment of MotA severely impaired the FliP function [15]. Together these results showed that a cleavable signal peptide is required for the full function of FliP. In this study, to further analyze the intrinsic characteristics of the FliP signal peptide we constructed a peculiar model protein FliPss–GFP and studied its translocation under different genetic backgrounds.

2. Materials and methods

2.1. Bacterial strains and media

E. coli strains MC4100A (F' *lac*Δ(*lacZYA-argF*)U169 *araD139 rpsL150 thi flbB5301 deoC7 ptsF25 relA1 ara*$^+$), MCMTAA (as MC4100A, *tatB*::Kan), B1LK0A (as MC4100A, Δ*tatC*) and CU164A (as MC4100A *secY39cs, zhd-33*::Tn10, *ara*$^+$) were described by Santini et al. [16] and Ize et al. [17]. Other strains used include DH5α (F$^-$ φ80 *lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR endA1 recA1 hsdR17* (r_k^-, m_k^+) *supE44 thi-1 gyrA96 relA1 phoA*), HTP406 (as MC4100, Δ*ara714 pheA3141::*Tn*10Kan ffh-87*) [18], MM52 (as MC4100 *secAts51*) [19], KJ173 (*secD29cs phoR zaj::*Tn*10 araD139* Δ(*ara-leu*)7679 Δ(*lac*)X74 *galE galK rpsL150 thi*) [20], MC1060 (F$^-$ Δ(*codB-lacI*)3 *galK16 galE15 LAM$^-$ e14$^-$ mcrA0 relA1 rpsL150*(StrR) *spoT1 mcrB1 hsdR2*) [21] and MYC-cs (as MC1060, *yidCcs*) [21]. Plasmids used were pBAD24 [22], pTorAss–GFP (pRR–GFP) [16] and pgfpmut3 [23].

E. coli strains were routinely grown at 37 °C in Luria–Bertani (LB) medium or on LB plates [24]. As required, glucose (0.2%), arabinose (0.2%), maltose (0.2%), and ampicillin (100 μg/ml) were added to the medium. Pre-cultures were grown from single colonies and used at 100-fold dilutions for inoculation of the experimental cultures.

2.2. Cellular fractionation, electrophoresis and immunological procedures

Periplasm, spheroplasts, membranes and cytoplasmic fractions were prepared by lysozyme/EDTA/cold osmoshock and ultracentrifugation as described previously [25,26]. To extract peripherally bound membrane proteins, the membrane was washed with 6 M urea. To further separate membrane proteins from aggregates, the membrane fractions were solubilized by 4% Triton X-100 in 40 mM Tris–HCl (pH 7.6) and centrifuged using an Airfuge at 30 psi for 10 min or ultracentrifugation at 160,000 × *g* for 90 min. Protease inhibitor COMPLETE (Roche) was added at the maximal recommended concentration in all buffers used. To study impact of various mutations on the FliPss–GFP processing, bacteria were incubated in LB–ampicillin–glucose (0.2%) media at permissive temperatures (37 °C for cold-sensitive and 30 °C for thermo-sensitive strains, respectively) until early exponential phase. The cultures were put on ice for 3 min, then to 24 °C for 1 h for the cold-sensitive mutants or at 42 °C for 1 h for the thermo-sensitive mutants. The cells were harvested by centrifugation, washed once with LB, and resuspended in LB–ampicillin–arabinose media and incubated for 1 h at non-permissive temperatures (24 °C for cold-sensitive and 42 °C thermo-sensitive strains). Cellular fractions were then prepared. Protein samples were separated by polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% SDS on 15% acrylamide gels. The immunoblot was performed by using an ECL$^+$ blotting system according to the manufacturer's instructions (Amersham Biosciences).

Pulse-chase labeling and immuno-precipitation experiments were performed as described in [27]. The samples were analyzed by 12.5% SDS-PAGE. Radioactive signals on dried gels were detected by Storm 820-PhosphorImager and quantified by Kodak image analysis software.

2.3. Cloning of FliPss–GFP fusion

The 81-base pair-fragment, encoding the signal peptide and the first six residues of the mature moiety of FliP, was amplified from MC4100A by PCR using primers FliPE-F (5′-ccg gaa ttc acc atg cgt cgt tta ttg tct gtc gca cc-3′) and R-ssFliPNh8 (5′-acc gct agc ggt gat acc cgg cag ttg cgc-3′) in the Expand High Fidelity PCR System according to the manufacturer's instructions (Roche). The amplified fragment was purified, digested by *Eco*RI and *Nhe*I and cloned into the corresponding sites of the plasmid pRR–GFP. The gene fusion *fliPss–gfp* in the resulting plasmid pFliPss–GFP was confirmed by DNA sequencing.

3. Results and discussion

3.1. Translocation of the FliPss–GFP fusion protein is dependent of Ffh, SecA, SecD and SecY

In general, highly hydrophobic signal anchor is essential for SRP-mediated, co-translational membrane insertion of polytopic IMPs [6]. Intriguingly, a cleavable signal peptide is required for the full function of the IMP FliP [15]. In addition, the FliP signal peptide is less hydrophobic than the SRP-specific signal anchors, and more hydrophilic Tat-dependent or Sec-dependent signal peptide can replace the native FliP signal peptide without impairing the FliP function [15]. To further analyze the membrane-targeting characteristics of the FliP signal peptide, we constructed a peculiar model protein FliPss–GFP and studied its translocation under different genetic backgrounds.

Cellular fractions were prepared from the wild type strain and the *secYcs24* mutant carrying the plasmid pFliPss–GFP and were analyzed by immunoblot using antisera against GFP (Fig. 1A1). The absence of maltose binding protein (MBP)