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Conformational Change of *Escherichia coli* Signal Recognition Particle Ffh Is Affected by the Functionality of Signal Peptides of Ribose-Binding Protein

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We examined the effects of synthetic signal peptides, wild-type (WT) and export-defective mutant (MT) of ribose-binding protein, on the conformational changes of signal recognition particle 54 homologue (Ffh) in *Escherichia coli*. Upon interaction of Ffh with WT peptide, the intrinsic Tyr fluorescence, the transition temperature of thermal unfolding, and the GTPase activity of Ffh decreased in a peptide concentration-dependent manner, while the emission intensity of 8-anilinonaphthalene-1-sulfonic acid increased. In contrast, the secondary structure of the protein was not affected. Additionally, polarization of fluorescein-labeled WT increased upon association with Ffh. These results suggest that WT peptide induces the unfolded states of Ffh. The WT-mediated conformational change of Ffh was also revealed to be important in the interaction between SecA and Ffh. However, MT had marginal effect on these conformational changes suggesting that the *in vivo* functionality of signal peptide is important in the interaction with Ffh and concomitant structural change of the protein.

INTRODUCTION

In *Escherichia coli* (*E. coli*), newly formed proteins are targeted into the cytoplasmic membrane, periplasmic space or outer membrane by at least two different routes (de Gier et al., 1997). The signal recognition complex (SRP) complex composed of a SRP54 homologue (Ffh) and 7S RNA homologue (4.5S RNA) comprises one targeting route. Some evidences suggest that *E. coli* SRP interacts with the ribosome-bound nascent chain of several secretory and membrane proteins, preferentially nascent proteins possessing highly hydrophobic signal peptides (Koch et al., 1999; Scotti et al., 1999). Indeed, inner membrane integral proteins containing extremely hydrophobic signals are strongly dependent on SRP for proper membrane assembly (de Gier et al., 1996; 1998).

An alternative targeting pathway in *E. coli* operates through cytoplasmic SecB which binds with the mature region of precursor proteins such as maltose-binding protein at an early stage of translocation (Rapoport et al., 1996). These precursor proteins are "chaperoned" by forming a complex with SecB, the major chaperone for the export. The complex formation inhibits side-reactions such as aggregation and misfolding and aids preprotein binding to the membrane surface.

These two different targeting routes in *E. coli* converge at Sec translocase (Driessen and Nouwen, 2008), a central component of membrane-embedded translocation machinery, to which the precursor proteins bind. After this stage, the precursor proteins are translocated across the membrane through the translocase complex that is postulated to comprise an integral SecYEGD-FyajC complex and peripheral dimeric SecA ATPase (Economou, 1998). Signal peptide is also important for the recognition of the secretory protein by the SecA and integral SecYE protein complex.

The ribose-binding protein (RBP) of *E. coli* is a member of a family of binding proteins present in the periplasmic space of Gram-negative bacteria. These proteins are essential components of high-affinity osmotic shock-sensitive uptake systems for a wide variety of low molecular weight compounds such as sugars, amino acids, peptides, and other nutrients (Furlong, 1987; Sack et al., 1989). RBP serves as the initial receptor for membrane transporter of ribose with high affinity (Ames, 1986). RBP also functions as the initial receptor for chemotaxis toward the sugar by the interaction with a common chemotactic signal transducer, Trg (Bollinger et al., 1984). RBP translocation is independent of SecB protein (Collier et al., 1988; Martin et al., 1991) and follows the Ffh route (Patel and Austen, 1996). In spite of extensive studies of Ffh, however, information concerning the physical interaction between signal peptides and Ffh is still deficient.

In this study, we propose that functional signal peptide of RBP induces a destabilized structure of Ffh upon interaction, while the export-defective mutant peptide has marginal effect on the conformational change.

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MATERIALS AND METHODS

Materials

8-Anilinonaphthalene-1-sulfonic acid (ANS) and 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (1,5-IAEDANS) were purchased from Invitrogen (USA). Phospholipids were obtained from Avanti Polar Lipids (USA). RBP signal peptides, KRRnoW LamB signal peptide analogue, and N-terminally fluorescein-labeled peptides were synthesized by PepTron (Korea).

Methods

Synthesis and purification of peptides

The synthesized peptides were further purified by reverse phase high performance liquid chromatography using a 300 mm × 10 mm and 10 μm W-porex C8 column (Phenomenex, USA) as previously described (Chi et al., 1995; Yi et al., 1994). Elution was performed with a water-acetonitrile linear gradient containing 0.1% trifluoroacetic acid. Stock solutions were prepared by dissolving the lyophilized peptides in distilled water. The concentrations of stock solution were determined by quantitative amino acid analysis. Molecular weights of the purified peptide were confirmed using a Kratos Kompact matrix assisted laser desorption ionization II apparatus (Kratos Analytical, Japan). The amino acid sequences of the signal peptides are summarized in Table 1.

Liposome preparation

Liposomes were prepared as previously described (Ahn and Kim, 1998). Briefly, lipids were evaporated under a stream of argon gas. The dried lipid film was hydrated in 25 mM Tris-HCl (pH 7.4) containing 100 mM NaCl and 0.5 mM dithiothreitol (DTT) by vigorous vortexing and a brief sonication (30 s) in a bath-type sonicator. To obtain homogeneous large unilamellar vesicles, the dispersion was frozen and thawed five times and passed 20 times through two 100 nm pore size polycarbonate membranes in an Extruder Lipo-Fast (Avestin, Canada).

Preparation of Ffh and 4.5S RNA, and GTPase assay

Ffh protein was isolated from the overproducing *E. coli* strain JM109 harboring the *lac* repressor plasmid pDMI,1 and pDS12-48His6 (kindly provided by Dr. Henrich Lütcke of European Molecular Biology Laboratory, Heidelberg). Purification of Ffh was achieved as described previously (Lentzen et al., 1994). Overproduction and purification of 4.5S RNA was done as described previously (Miller et al., 1994). GTPase activity of Ffh was measured using an assay based on the absorption of unreacted GTP to charcoal (Miller et al., 1994). Briefly, after incubation of Ffh with each indicated amount of signal peptide at 25°C for 30 min, GTPase activity was measured by determining released ^{32}P-labeled phosphate from [γ-^{32}P]GTP. In all experiments, the complex of recombinant Ffh protein and 4.5 S RNA was used; the complex is hereafter referred to as Ffh.

SecA preparation and ATPase assay

SecA protein was purified from SecA-overproducing strain RR1/pMAN400 using a Cibacron Blue 3G Sepharose column as described previously (Ahn and Kim, 1996). To ascertain the functional activity of purified SecA, the ATPase activity was measured in 50 μl of assay buffer (50 mM HEPES, pH 7.0, 30 mM KCl, 30 mM NH_4Cl, 1 mM DTT, 4 mM ATP, and 20 μg/ml SecA) in the presence of *E. coli* phosphatidylethanolamime (*E. coli* PE)/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) (molar ratio of 7:3) large unilamellar vesicles (LUV) as described previously (Ahn and Kim, 1998). The enzyme-dependent release of phosphate was calculated by subtracting the phosphate released by hydrolysis of ATP in the sample without SecA.

Table 1. Amino acid sequences of the synthetic signal peptides

Peptide	Sequence
WT	MNMKKLAT**L**VSAVALSATVSANAMA
MT (L9P)[a]	MNMKKLAT**P**VSAVALSATVSANAMA
KRRnoW[b]	MMITLR**KRR**KLPLAVAVAAGVMSAQAMAC

[a]The precursor RBP with the mutant signal peptide (L9P) is not secreted into the periplasmic space (Iida et al., 1985). [b]KRRnoW has three additional positively charged amino acids (KRR) introduced into the N-terminal part of wild-type LamB signal peptide.

Labeling of proteins

Labeling of Ffh with 1,5-IAEDANS, a cysteine-specific fluorogenic probe, was performed as described previously (Jeganathan et al., 2006). The amount of bound 1,5-IAEDANS was determined as the absorption at 336 nm (ε_{336} = 6,100 M^{-1} cm^{-1}) (Hudson and Weber, 1973).

Fluorescence measurements

All fluorescence experiments to analyze conformational changes of Ffh were performed at 30°C. Fluorescence spectra were recorded with a Shimadzu RF-5301 PC spectrofluorometer equipped with a thermostat cuvette compartment. For the measurement of intrinsic fluorescence of Ffh, 0.6 μM Ffh dissolved in buffer composed of 20 mM HEPES, (pH 7.4), 100 mM NaCl, 0.1 mM DTT, and 0.1 mM Na-EDTA was incubated with increasing concentrations of signal peptides for 10 min, and the emission intensities were recorded in the range of 300-420 nm with an excitation wavelength of 292 nm. Changes in the exposure of hydrophobic regions on the Ffh upon interaction with signal peptides were monitored with fluorescence from 100 μM ANS, which was added to reaction samples. The fluorescence emission caused by excitation at 370 nm was measured between 420 and 600 nm. The thermal unfolding of Ffh was analyzed by following the decrease in fluorescence intensity at 326 nm (under an excitation wavelength of 280 nm) with a 0.5°C/min heating rate as described previously (Ahn et al., 2005). The fluorescence polarization of fluorescein-labeled peptides was measured using an 8100 Series 2 spectrofluorometer (SLM Aminco Instruments, USA) equipped in L format with Glan-Thompson calcite prism polarizers using a previously described method (Buchli et al., 2004). Fluorescein-labeled signal peptides (1.0 μM) were mixed with each indicated amount of Ffh protein. The sample was equilibrated at 30°C for 10 min before the measurement. The excitation and emission wavelength were 485 nm and 530 nm, respectively. In all fluorescence experiments, each measurement under the experimental condition was corrected for inner filter effect due to light scattering and absorption, as described elsewhere (Subbarao and MacDonald, 1993).

Fluorescence resonance energy transfer (FRET)

The energy transfer between Trp residues and 1,5-IAEDANS was measured in the emission range of 300-600 nm, with an excitation wavelength of 290 nm. The emission intensities at 490 nm were specifically selected to investigate the degree of energy transfer. SecA (0.4 μM) was mixed with the same concentration of 1,5-IAEDANS-labeled Ffh in the presence of 800

μM *E. coli* PE/POPG LUV prior to the addition of signal peptide to the desired concentration. After further incubation at 30°C for 20 min, FRET analysis was conducted.

Circular dichroism (*CD*)

CD spectra were obtained on a Model J-715 spectropolarimeter (Jasco, Japan) with a Model RTE-210 temperature controller (NesLab Instruments, USA). A 1.0 mm or 0.2 mm path length quartz cell was used for the measurements depending on the peptide concentrations. Ffh protein was suspended in a buffer solution (20 mM HEPES, pH 7.4, 100 mM NaCl, 0.1 mM dithiothreitol, and 0.1 mM Na-EDTA) with or without signal peptide, and the samples were incubated at 30°C for 10 min. The final spectra were the average of multiple scans, baseline corrected and smoothed by a noise reduction function.

Other methods

Protein concentrations were estimated with a bicinchoninic acid procedure (Pierce, USA) as described by the manufacturer. Peptide concentrations were determined using a fluorescamine assay (Castell et al., 1979). Data were analyzed by analysis of variance (ANOVA). In each case, the number of experiments is stated individually in the legend of each figure.

RESULTS AND DISCUSSION

Signal peptide-induced conformational change of Ffh

The environment of aromatic side groups can be studied using tryptophan (Trp) and tyrosine (Tyr) fluorescence as intrinsic probes. Ffh has two Tyr residues without Trp in its amino acid sequence. To analyze the structural change of Ffh upon association with signal peptides, the intrinsic fluorescence was measured at excitation wavelength of 280 nm (Fig. 1). The addition of wild-type (WT) signal peptide of RBP to Ffh solution resulted in reduction of approximately 30% in the fluorescence intensity with little shift in the maximum wavelength at the peptide/Ffh ratio (P/F, by a molar ratio) of 2.5. The experiment was repeated as increasing the concentration of peptide at fixed amount of Ffh as 0.6 μM. The emission intensities decreased and reached certain equilibrium at the ratio of approximately 1.5-2.0. These results suggest that WT peptide affects the conformation of Ffh and especially the polarity around Tyr residues in the protein although it could not be excluded that the peptide binding simply induces the fluorescence change without the structural change of Ffh. However, export-defective mutant (MT) signal peptide (Iida et al., 1985) had reduced effect on the fluorescence change, which the emission intensity was decreased by only 12%. To apply the functional signal peptide-induced conformational change of Ffh to other signal peptide of secretory protein, the experiment was repeated with KRRnoW, LamB signal sequence analogue (Zheng and Gierasch, 1997). As expected, the intrinsic emission fluorescence of Ffh was decreased with increasing the peptide concentration and showed similar intensities to those of WT.

To investigate the signal peptide-induced conformational change of Ffh in more detail, the extrinsic fluorophore ANS was used. When this probe binds to hydrophobic patches of a protein, the fluorescence intensity increases and the emission maximum shifts to shorter wavelength. Figure 2A shows that the emission fluorescence increased with increasing concentrations of WT peptide and the increase reached a plateau at a peptide/Ffh (P/F) ratio of around 2.0. In the presence of WT at the ratio of 2.7, the emission intensity was enhanced by about 6-fold as compared with that of Ffh alone as well as the maximum wavelength showed blue shifts (Figs. 2B and 2C). This

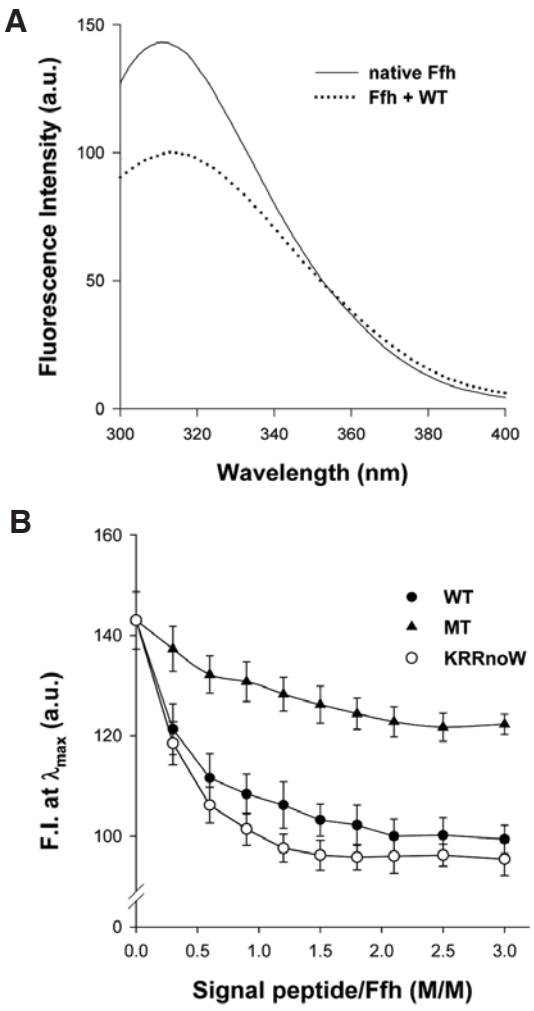

Fig. 1. Intrinsic Tyr fluorescence change of Ffh upon interaction with signal peptides. (A) The emission spectra of Ffh in the presence or absence of WT peptide under excitation wavelength of 280 nm. (B) For the interaction between Ffh and signal peptide, 0.6 μM Ffh was incubated with each indicated amount of signal peptide at 30°C for 10 min and the emission intensities were recorded at λ_{max}. The fluorescence intensities in the absence of signal peptide were set to constant value and other data points were plotted relative to this intensity. The abbreviation a.u. represents arbitrary unit.

ANS result suggests that additional hydrophobic regions of Ffh were exposed when the signal peptide was bound and that Ffh-WT complex assumes a more unfolded structure than that without the peptide. Additionally, this result may imply that a binding stoichiometry between WT and Ffh can be more than 1:1. In contrast, an export-defective mutant (MT) signal peptide showed reduced effect on the fluorescence change, in which the emission intensity was increased by approximately 3-fold. These ANS fluorescence results lead us to suggest that the *in vivo* functionality of RBP signal peptide is important in the conformational changes of Ffh. As a control experiment, when the experiment was performed with increasing the concentration of signal peptides only up to 10 μM in the absence of Ffh, no ANS binding was observed, consistent with an earlier study (Miller et al., 1994). To apply the functional signal peptide-induced conformational change of Ffh to other signal peptides of secretory

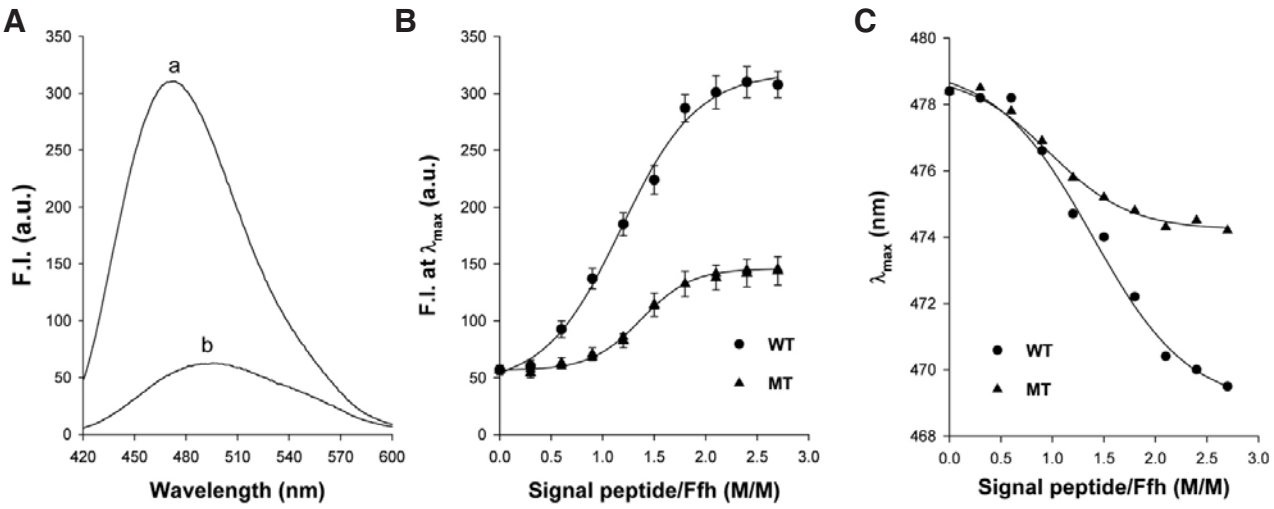

Fig. 2. Fluorescence of ANS bound to Ffh. Ffh (1.5 μM) was incubated with indicated amount of WT or MT peptide at 30°C for 20 min and then 100 μM ANS was added. (A) Fluorescence emission spectra of ANS bound to Ffh was also shown in the presence (a) or absence (b) of WT peptide at the P/F ratio of 2.7. (B) Wavelengths showing maximum emission fluorescence (λ_{max}) were plotted with increasing concentration of signal peptides. Data points represent mean ± S.E. of three independent experiments. (C) The emission intensities at λ_{max} were plotted as a function of peptide concentration. Data points represent the average values of two independent experiments. The abbreviation a.u. represents arbitrary unit.

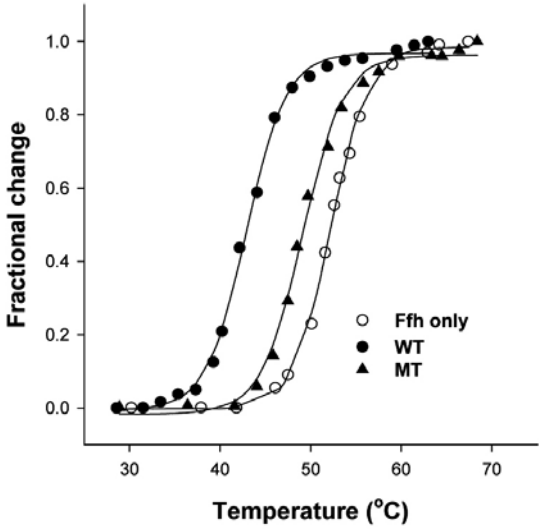

Fig. 3. Thermal unfolding of Ffh. The temperature-induced unfolding of 0.6 μM Ffh was conducted in the absence or presence of 1.2 μM signal peptides. Changes in the fluorescence intensity at 326 nm (under an excitation wavelength of 280 nm) were recorded as a function of temperature. The y-axis (fractional change) depicts relative fluorescence changes with the emission intensity of Ffh set to zero (0) and to one (1) at the initial and final temperature, respectively. Data points represent the average values of three independent experiments.

protein, the experiment was repeated with KRRnoW. However, the ANS binding experiment could not be analyzed because the peptide itself showed significant ANS binding even at concentrations below 10 μM (results not shown).

To investigate the compactness and the thermal stability of Ffh upon interaction with signal peptides, we performed the heat-induced unfolding experiment by following the decrease in fluorescence intensity at 326 nm with excitation at 280 nm, which is a maximal intrinsic fluorescence of Ffh. Native Ffh showed a transition temperature (T_m) near 52.2°C (Fig. 3; Table 2). However, in the presence of WT peptide at a P/F ratio of 2.0, the T_m of Ffh dramatically shifted to near 42.9°C. KRRnoW peptide also induced a similar T_m, approximately 42.5°C, at the same ratio. This result clearly demonstrated that WT (and KRRnoW) decreases the thermal stability of Ffh upon its binding and suggests that Ffh is converted into a more unfolded structure. In contrast, here again, the presence of MT brought about a small shift of T_m to near 49.1°C at the same P/F ratio, which is consistent with a reduced effect on the conformational change of Ffh when assayed by the ANS binding.

Table 2. Changes in the thermal unfolding transition temperature of Ffh induced by interaction with signal peptides

Peptide	Transition temperature (°C)
Ffh only	52.2 ± 1.2[a]
WT	42.9 ± 0.9
MT (L9P)	49.1 ± 1.3
KRRnoW	42.5 ± 1.1

[a]Data values represent mean ± S.E. of three independent experiments.

To obtain more insight into signal peptide-induced conformational changes in Ffh, secondary structure was analyzed by CD in the presence of each signal peptide. Figure 4 shows that the WT peptide induced only a marginal change in secondary structure of Ffh at a P/F ratio of 2.0. As a control experiment, we could not detect any remarkable ellipticity values of WT peptide alone up to 5.0 μM in aqueous solution and even in 50% trifluoroethanol as a secondary structure-inducing agent (results not shown). Therefore, we can assume that the CD signal resulted from solely Ffh protein. The MT peptide had no effect on the CD spectral change of Ffh (results not shown). However, we can not exclude the possibility that the secondary structure of Ffh can be changed upon interaction with signal

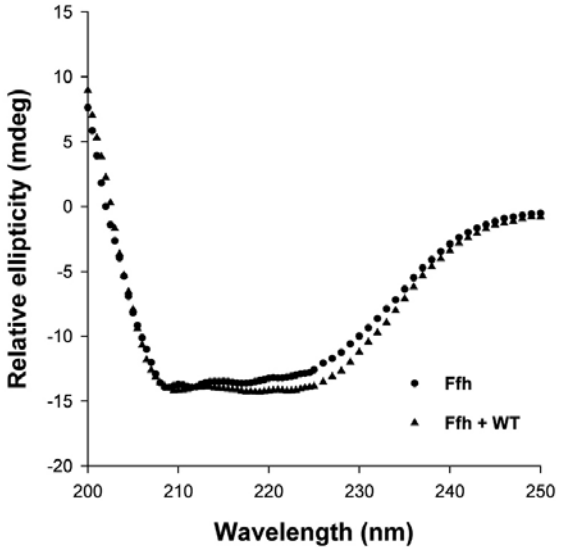

Fig. 4. CD spectra of Ffh. Ffh (0.8 μM) was suspended in buffer solution with 1.6 μM WT or without the peptide. After the samples were incubated at 30°C for 10 min and CD spectra were recorded. Relative ellipticity is expressed in the y-axis.

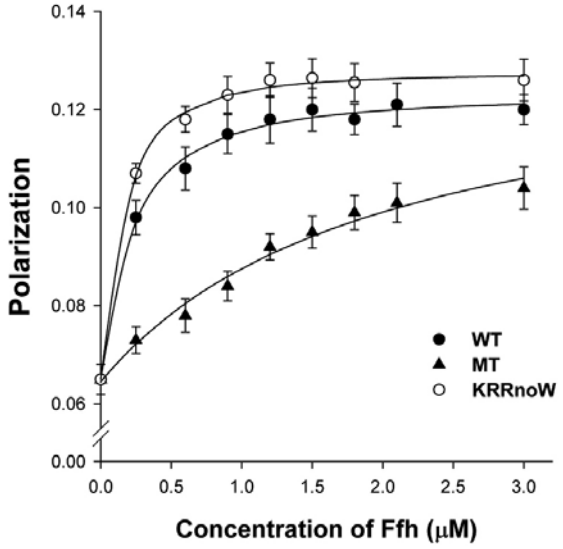

Fig. 5. Fluorescence polarization examinations. Changes in fluorescence polarization of 1.0 μM fluorescein-signal peptides were plotted as a function of Ffh concentration and calculated as described. Data points represent mean ± S.E. of three independent experiments.

peptide at higher P/F ratios, which was not tested in this study. Moreover, it was difficult to apply the CD experiment to other signal peptides because there is a probability that the signal peptides may have inherent notable secondary structures in CD analysis, and the interaction between Ffh and signal peptide affects the conformations of both. Actually, KRRnoW alone showed significant ellipticity values at the concentration used for the association with Ffh in contrast to RBP signal peptides. Considered together with the ANS fluorescence and the heat unfolding experiment, these results indicate that the WT peptide of RBP induces tertiary conformational changes of Ffh without significant secondary structural changes, and also imply that the peptide confers to the protein a more open structure upon interaction. Collectively, the present observations support the contention that Ffh is destabilized compared to native state by interaction with functional signal peptide. However, this possibility cannot be expanded generally to other signal peptides because the present study was not performed with other signal peptides in addition to RBP signal peptide and KRRnoW following the 'Ffh route' during their *in vivo* translocation.

Polarization of fluorescein-labeled signal peptides

The interaction of Ffh with signal peptides was investigated quantitatively using fluorescence polarization. The association of small peptides with higher molecular weight proteins greatly reduces their mobility. Hence, if a peptide labeled with a fluorescence probe binds to a protein, its polarization will increase. There was a dose-dependent increase in the polarization of WT peptide when the concentration of Ffh was increased (Fig. 5). KRRnoW peptide induced more increased polarization. This polarization result is also indicative of direct binding between Ffh and signal peptide. In contrast, when MT peptide was used, the polarization also increased in a peptide concentration-dependent manner, but overall values were significantly reduced compared to those of WT or KRRnoW and a plateau was not reached at high concentration of Ffh protein. The dissociation constants (K_d) for the interaction between Ffh and signal peptides were also calculated on the basis of the polarization and the intrinsic Tyr fluorescence data (Table 3). MT showed higher K_d values than those of WT by approximately 4-10 fold depending on its signal source. WT and KRRnoW peptides had similar K_d values each other. Consistently, all the results described up to now support the idea that the translocation functionality of RBP signal peptides plays important roles in the binding to Ffh and in the induction of conformational changes of the SRP protein.

Table 3. Dissociation constants (K_d) for the interaction between Ffh and signal peptides

Peptide	K_d (μM)
WT	0.212 ± 0.011[a] (**0.467 ± 0.032**)
MT (L9P)	2.098 ± 0.146 (**1.779 ± 0.135**)
KRRnoW	0.137 ± 0.008 (**0.435 ± 0.024**)

[a]Data values represent mean ± S.E. of three independent experiments. The dissociation constants were obtained by nonlinear regression of the polarization data (Fig. 5) assuming a binding stoichiometry of 1:1 between Ffh and signal peptides. The parentheses represent K_d values calculated by the intrinsic Tyr fluorescence data (Fig. 1B).

GTPase activity of Ffh upon interaction with signal peptides

Because the targeting process by SRP or Ffh is intimately coupled to a GTPase cycle, the GTPase activity of Ffh is influenced by signal peptides. The signal peptides of the precursor LamB proteins inhibit the GTPase activity of *E. coli* Ffh indicating the LamB protein being targeted via SRP route in part (Miller et al., 1994; Zheng and Gierasch, 1997). On the basis of these results, we measured the GTPase activity of Ffh upon interaction with signal peptides. To ascertain that the present assay was properly conducted, we utilized KRRnoW, which is known to inhibit the GTPase activity (Lentzen et al., 1994). The KRRnoW peptide had the most significant inhibition of the

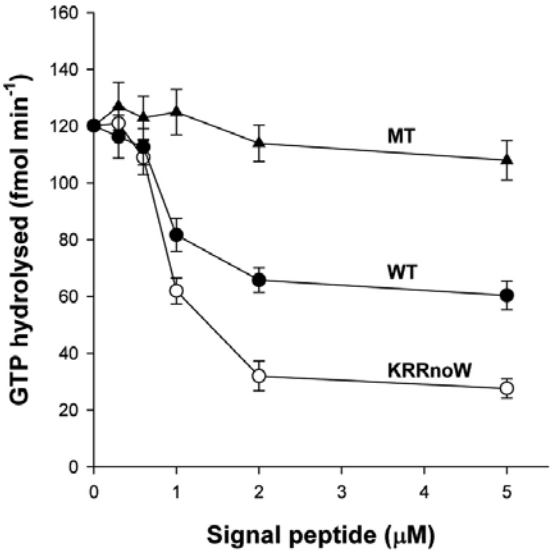

Fig. 6. Effect of signal peptides on the GTPase activity of Ffh. The GTPase assay was performed as described above. The enzyme-dependent release of phosphate from GTP was calculated by subtracting the release of phosphate in samples without the enzyme. Data points represent mean ± S.E. of three independent experiments.

GTPase activity followed by RBP WT peptide (Fig. 6). In contrast, the MT peptide had almost no effect on the activity. This result confirms that the wild type signal peptide of RBP firmly interacts with Ffh and follows the targeting route involving Ffh, while the mutant peptide interacts with the protein insignificantly.

Interaction between SecA and Ffh

Previous investigations have suggested that SecA and Ffh sequentially cooperate during the translocation of secretory proteins in *E. coli*, and that Ffh eventually converges at the translocase containing SecA in membranes (Driessen and Nouwen, 2008; Neumann-Haefelin et al., 2000; Valent et al., 1998). On the basis of these results, we tested the possibility that the RBP signal peptide could induce the physical association of membrane-bound SecA with Ffh. For this, we used FRET analysis between Trp residues in SecA (SecA has 7 Trp residues in its amino acid sequence) and 1,5-IAEDANS-labeled Ffh in the presence of a SecA/Ffh ratio of 1:1 with increasing the amount of signal peptides. The fluorescence intensity at 490 nm increased with increasing WT or KRRnoW concentrations (Fig. 7A). FRET reached a certain plateau at a peptide/Ffh ratio of approximately 2.0 with both peptides. In contrast, MT had little effect on the FRET. This result implies that Ffh actually interacts with SecA in membranes and that the WT peptide stimulates the physical interaction, although it has been suggested that SecA/SecB and Ffh constitute distinct targeting routes especially for inner membrane proteins (Koch et al., 1999; Scotti et al., 1999). However, even for Ffh-dependent membrane proteins, SecA is required for efficient translocation (Qi and Bernstein, 1999). Therefore, the present FRET results strengthen the view that the association between Ffh and membrane-SecA is both induced by the RBP signal peptide (and KRRnoW) and is required for targeting of the periplasmic protein.

As a control experiment, the FRET experiment was repeated between Ffh and soluble SecA in the absence of membrane components. The energy transfer values were significantly reduced even in the presence of WT peptide compared to those in the presence of membranes (Fig. 7B) suggesting that Ffh interacts with membrane-bound SecA aided by functional signal peptide. At present, however, it is unclear how 1,5-IAEDANS, a bulky fluorescent probe, exerts its influence in the conformation of Ffh and, thereby, the physical contact between both proteins. When CD spectra were acquired with native and 1,5-IAEDANS-labeled Ffh, the secondary structure (α-helix and β-sheet contents) was changed only marginally (results not shown).

Previously, it was reported that the WT and MT peptides of RBP have different secondary structures when determined by CD, with WT possessing higher and lower contents of α-helix and random coil, respectively, compared to those of MT (Ahn et al., 2002). On the basis of these results, we can conclude that the *in vivo* functionality of RBP signal peptide is important in inducing the conformational change of Ffh into an unfolded structure and the structural difference between WT and MT peptide may be determinant to exert effective interaction with Ffh. Additionally, it can be considered that this conformational change is consequently required for the interaction of Ffh with other translocation machinery such as FtsY (Miller et al., 1994) as well as SecA.

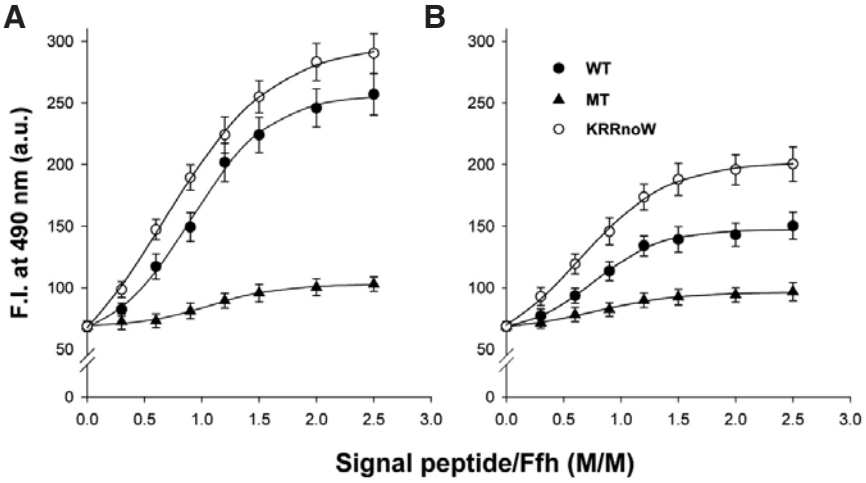

Fig. 7. FRET analysis between 1,5-IAEDANS fluorophore and Trp residues. (A) Ffh was labeled with 1,5-IAEDANS. The emission intensity at 490 nm was measured under an excitation wavelength of 290 nm to activate Trp fluorescence with increasing concentrations of WT (circle), KRRnoW (rectangle), or MT (triangle) at a Ffh/SecA ratio of 1:1 in the presence of *E. coli* PE/POPG membranes. (B) FRET was analyzed in the absence of membrane component using the same experimental conditions described in (A). In (A) and (B), the fluorescence intensities in the absence of signal peptide were set to constant value and other data points were plotted relative to this intensity. Data points represent mean ± S.E. of three independent experiments. The abbreviation a.u. represents arbitrary unit.

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