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The *In Vitro* Translocation of *Escherichia coli* Ribose-binding Protein via Various Targeting Routes

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The translocation of ribose-binding protein (RBP) into the inverted membrane vesicles (IMV) of *Escherichia coli* and eukaryotic microsomes was studied using the *in vitro* translation/translocation system. It was found that RBP was translocated into heterologous eukaryotic microsomes co-translationally, as well as post-translationally. However, RBP was translocated only post-translationally into IMV. Degradation fragments of RBP with the molar mass of 14 and 16 kDa were produced during the translocation into IMV. However, the amount of the degradation products decreased and the mature form of RBP appeared in the presence of phenylmethylsulfonyl fluoride (PMSF). PMSF and GTP accelerated the translocation of RBP. It was also found that SecB enhanced the post-translational translocation of RBP. It appears that RBP is translocated via at least two targeting paths.

Keywords: Ribose-binding protein, *In vitro* translocation, Protein targeting, GTP, SecB

Introduction

The binding proteins, present in the periplasmic space of gram-negative bacteria, are involved in the active transport and chemotaxis of various substrates. They are synthesized as precursors with signal peptides in the cytoplasm, exported into the periplasm and then processed to mature forms. Many components of the export machinery are involved in these steps. Although exact targeting routes for the periplasmic and outer membrane proteins in prokaryote have not been elucidated, SecB and fifty-four homologues (Ffh) are known to be the cytoplasmic targeting factors, which deliver preproteins to the SecA-SecYEG complex, the integral membrane translocation machinery (Wolin, 1994).

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Protein targeting to the *E. coli* cytoplasmic membrane can occur via the Sec pathway, or Ffh pathway. The Sec pathway uses a cytoplasmic chaperon, SecB that binds to the precursor proteins post-translationally, or at a late co-translational stage (Kumamoto and Francetic, 1993). The SecB is the only chaperon involved in the translocation process. It prevents misfolding and aggregation of precursor proteins, as well as their binding to nonproductive sites of the membrane (Hartl *et al.*, 1990; Lecker *et al.*, 1990). The SecB-precursor protein complex is delivered to the cytoplasmic membrane where the activated membrane bound SecA, a translocation ATPase, mediates the translocation of the precursor protein through the SecYEG translocon by ATP-driven cycles of insertion and extraction (Economou and Wickner, 1994; Economou *et al.*, 1995). On the other hand, several secreted and membrane proteins bind to the 4.5S RNA-48Kda Ffh complex (Duong *et al.*, 1997). The complex has GTPase activity (Powers and Wickner, 1997), and this Ffh pathway resembles the protein targeting the eukaryotic endoplasmic reticulum membrane.

The ribose-binding protein (RBP), one of the periplasmic binding proteins in *E. coli*, has been the subject of extensive studies in many laboratories (Phillips and Silhavy, 1992; Luirink *et al.*, 1994). However, the factor, which targets the precursor RBP to the membrane translocation site in a translocation-competent conformation, is unknown. Although various aspects of the translocation of RBP across the *E. coli* membrane were studied (Yi *et al.*, 1994; Chi *et al.*, 1995; Lee *et al.*, 1996; Ahn and Kim, 1996, 1998; Park *et al.*, 1997; Song and Kim, 1997), one important aspect of the translocation, the targeting steps, has not been resolved. The targeting studies reported so far give only ambiguous results as to the question of whether the RBP translocation is Ffh-dependent (Phillips and Silhavy, 1992; Luirink *et al.*, 1994), or SecB-dependent (Kumamoto, 1989; Kim *et al.*, 1992; Francetic and Kumamoto, 1996). In this study, we show that the RBP translocation is enhanced by phenylmethylsulfonyl fluoride (PMSF), GTP and SecB in the *in vitro* translation/translocation system. Therefore, it would exist in at least two

targeting paths for RBP translocation; one through the Ffh and the other through SecB. Also, an unknown proteolytic enzyme affects the process.

Materials and Methods

Materials The plasmid coding for RBP with the T7 promoter, pTS122, was provided by Professor C. Park of the Korea Advanced Institute of Science and Technology. It was amplified in *E. coli* XL1-Blue and purified using a CsCl gradient (Maniatis *et al.*, 1982). Canine pancreatic microsomal membranes and the *E. coli* T7 S30 extract system were purchased from Promega (Madison, WI, USA).

SecA protein was purified from a SecA-overproducing strain (RR1/pMAN400), as described previously (Kawasaki *et al.*, 1989). The SecB protein was also purified from a SecB-overproducing strain (BL21(DE3)/pJW25) following the method of Weiss *et al.* (1988). Antibodies against these proteins were obtained from immunized antisera of rabbits by passing through a protein A affinity column.

IMV for *in vitro* translocation were prepared from CP626 (MC4100 *flhD*$^+$ *rbsB102*::Tn*10*), a derivative strain of *E. coli* K-12, as described previously (Yamada *et al.*, 1989). The amount of membrane vesicles was expressed in terms of protein content, which was determined according to the method of Park *et al.* (1999).

Immunoblot analysis of *E. coli* T7 S30 extract Proteins from the *E. coli* T7 S30 extract were subjected to 12% SDS-PAGE, and subsequently blotted on nitrocellulose. SecA, SecB and Ffh were detected with the ECL Western blotting system (Amersham), as described by Choi and Rhee (1998).

Assay of *in vitro* translation/translocation of RBP *In vitro* translation of RBP was performed with the *E. coli* T7 S30 extract system in the presence of [^{35}S] methionine and Rnasin, as described by the manufacturer. The translocation reaction was carried out as described previously (DeVrije *et al.*, 1987; Jeoung and Yu, 1999). For the translocation into eukaryotic microsomes, 2 µl of microsomes were added to 10 µl of the translation system. For the translocation into bacterial IMV, 10 µg of IMV was added to each translocation mixture containing 5 µl of the ^{35}S-labeled RBP-translation mixture, 50 mM Tris-HCl (pH 7.9), mM KCl, and 2.5 mM $MgCl_2$, the final volume being 25 ml. The mixture of 5 mM PMSF and 10 mM each of ATP, NADH, or succinate were added to determine the effects of these components. The translocation of RBP was monitored by determining the amount of translocated RBP that is protected from the external proteinase K. Samples from the translocation reaction mixture were incubated in a final 1 M of GdnHCl for 2 hours; then exposed to externally added proteinase K for 25 min at room temperature to digest RBP, which had not been translocated into the vesicles. Any further translocation during the two hours of incubation in the 1 M GdnHCl was not observed.

To study the effect of PMSF and GTP on the RBP translocation, small molecules were removed in advance from the translational mixture with gel filtration using the pre-packed desalting column equilibrated with 50 mM potassium phosphate (pH 7.5). The void volume fraction was used as a substrate. Either 5 mM of GTP or PMSF was added to each reaction mixture. The extent of RBP translocation was examined at 0, 1, 2, 5, 10, and 40 min.

To investigate the effect of SecB on the RBP translocation into

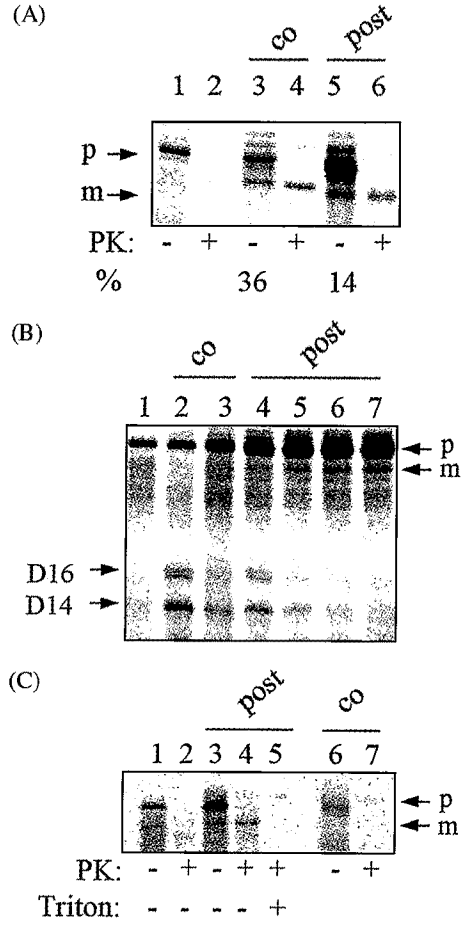

Fig. 1. Translocation of RBP into eukaryotic microsomes and *E. coli* IMV. (A) Microsomes were added co-translationally (lanes 3 and 4) and post-translationally (lanes 5 and 6). Lanes 1 and 2 are for the control without microsomes. Samples for lanes 2, 4 and 6 were treated to the translocation mixture with GdnHCl, and then with proteinase K. (B) IMVs were added co-translationally (lane 2 and 3) and post-translationally (lane 4-7). PMSF (5mM) was added co-translationally (lane 3) and post-translationally (lane 5). Energy sources containing ATP, NADH, and succinate were added post-translationally (lane 6). Both PMSF and energy sources were added post-translationally (lane 7). Lane 1 is a control without IMV. (C) Samples for lanes 1, 3 and 6 correspond to lanes 1, 4, and 2 of panel A, respectively. Lanes 2, 4 and 7 are the samples of lanes 1, 3 and 6, respectively, which proteinase K was treated as described in "Materials and Methods". The sample for lane 5 was treated with triton X-100, GdnHCl, and proteinase K for post-translational translocation. Co and post indicate co-translational and post-translational, respectively. The positions of the precursor (p) and mature (m) forms of RBP are indicated. The % is the percentage of processing, which was estimated from the band intensities of precursor and mature forms.

IMV, the translation mixture of ^{35}S-labeled RBP was incubated in the final concentration of 1 M GdnHCl for 2 hours and diluted 10-fold into each reaction mixture containing IMV, SecA (2 mg), various concentrations of SecB, 10 mM of ATP, 10 mM of NADH and 10 mM of succinic acid. Anti-SecB immunoglobulin G was also added for control. All samples were precipitated with 10% TCA, analyzed on 12% or 13.5% SDS-polyacrylamide gels and exposed to a PhosphorImaging plate for visualization on a Fuji BAS-1500 PhosphorImaging system. The percentage of processing to mature RBP caused by the signal peptidase was estimated from the band intensities. The band intensity of the mature RBP was corrected by multiplying 7/4 because the numbers of methionine residues in the precursor and mature form of RBP are 7 and 4, respectively.

Results and Discussion

Fig. 1A shows that RBP is translocated into eukaryotic microsomes co-translationally, as well as post-translationally. Proteinase K was externally added under the unfolding condition of RBP to confirm the translocation. Precursor RBP (pRBP) assumes a tightly folded structure in the native state, and protease treatment removes only the signal peptide unless it is unfolded (Park *et al.*, 1988). Therefore, proteinase K was added to digest all RBPs outside of the membrane vesicles after 1M GdnHCl was treated to reaction mixtures (Kim and Kim, 1996). The efficiency of co-translational translocation is more than twice that of post-translational translocation. The co- and post-translational translocations of RBP into microsomes indicate that soluble components of the bacterial export apparatus could interact with membrane components of eukaryotic secretion machinery. The ability of translocation in the heterologous system might be an example of the evolutionary convergency between prokaryotes and eukaryotes. It should be noticeable that the bacterial preprotein translated in the bacterial cell extract is translocated into eukaryotic microsomes, although there were some reports using heterologous *in vitro* system (Powers and Walter, 1997; Behrmann *et al.*, 1998).

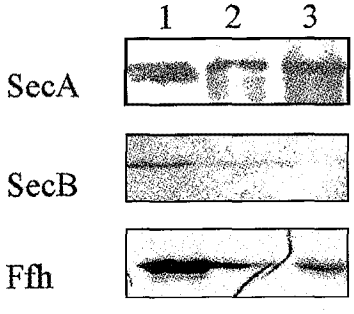

Fig. 2. The test for the existence of SecA, SecB and Ffh in the T7 S30 cell lysate for the *in vitro* translation. Immunoblot analysis was carried out to identify SecA, SecB and Ffh in the T7 S30 cell lysate, using an ECL detection kit. Lane 1 in each panel is the control protein, and lanes 2 and 3 represent the increasing amount of the added cell extract.

In contrast to the translocation into microsomes, RBP was translocated only post-translationally into IMV (shown in Figs. 1B and 1C.) Appreciable mature RBP was observed in the post-translational mode, but not in the co-translational mode. This post-translational translocation of RBP agreed with the *in vivo* translocation of RBP observed earlier (Randall, 1983). The translocation of RBP was also confirmed by a proteinase K protection assay under the unfolding condition of RBP (Fig. 1C). During this translocation process, 14 (D14) and 16 kDa (D16) fragments of RBP were observed (Fig. 1B, lane 2 and 4). These degradation products did not form in the presence of excessive PMSF during both the co-translational and post-translational translocation (Fig. 1B, lane 3 and 5). Some unknown serine protease appears to be present in the RBP translocation system. Degradation products did not form, however, the mature form was observed in the post-translational translocation in the presence of energy sources containing ATP, NADH and succinate (Fig. 1B, lane 6). It is possible that the increased translocation in the presence of

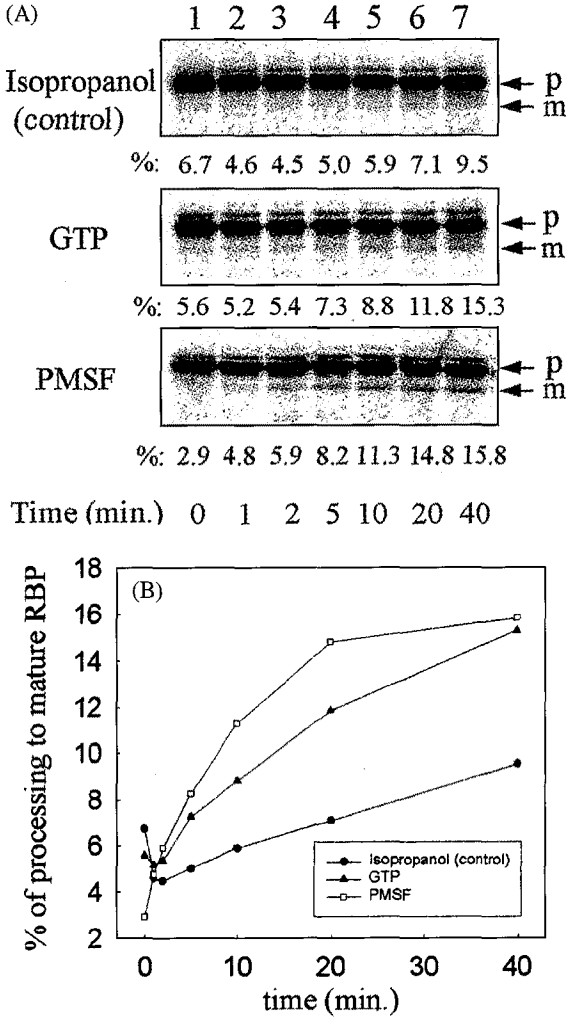

Fig. 3. Kinetics of the RBP translocation into IMV and the effects of GTP and PMSF. (A) The extent of RBP translocation was assayed at 0, 1, 2, 5, 10, and 40 min. (B) Diagram of panel A.

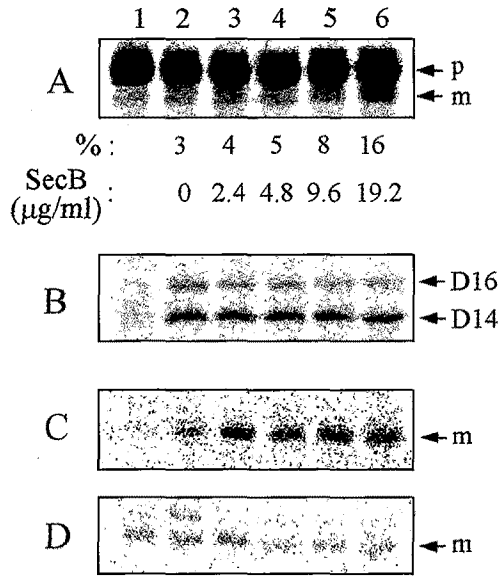

Fig. 4. Effect of SecB on the translocation process of RBP. (A) Lane 1 is a control without IMV. The quantities of SecB added in the final 25 μl of the translocation reaction are 0 μg (lane 2), 2.4 μg (lane 3), 4.8 μg (lane 4), 9.6 μg (lane 5), and 19.2 μg (lane 6). (B) The positions of degraded forms of RBP, 14 (D14) and 16 kDa (D16), are indicated at each sample of panel A. (C) Each sample of panel A was treated with GdnHCl and proteinase K (lane 1-6). (D) 14.5 μg of anti-SecB IgG was added to each sample of panel A (lane 1-6).

these energy sources may have prevented the formation of the fragments.

The possible presence of soluble factors such as SecB and Ffh, and SecA (a translocation ATPase) in the *E. coli* T7 S30 cell extract was checked with Western blot analyses using the antibodies against these factors. SecA and Ffh were found to be present (shown in Fig. 2), but SecB was not detected. We studied the effect of Ffh, which have GTPase activity (Philips and Silhavy, 1992), and SecB on the translocation of RBP as described later.

The effects of GTP as an energy source of Ffh, and PMSF as an inhibitor against serine protease, were analyzed kinetically. Each reaction mixture had the same amount of isopropanol in order to perform the kinetic studies under the same condition. The amount of mature RBP produced at 0, 1, 2, 5, 10, and 40 min after the initiation of the reaction are given in Fig. 3. PMSF and GTP enhanced the translocation of RBP, as compared to the control. It is expected that the effect of GTP on the RBP translocation is solely due to the GTPase activity of Ffh that is present in the T7 S30 cell extract.

SecB is known to interact only with partially folded or unfolded proteins (Randall, 1992), keeping them in translocation-competent forms (Randall *et al.*, 1990; Hardy and Randall, 1991). An unfolded translation mixture of RBP was diluted with 9 volumes of the translocation mixture to investigate the role of SecB in the translocation process of RBP. The translocation efficiency of unfolded RBP increased (Fig. 4A) and the D16, the heavier degradation form of RBP, gradually disappeared (Fig. 4B) as the amount of SecB was increased. A proteinase K protection assay was also carried out to confirm the translocation of RBP (Fig. 4C). Because RBP was not translocated in the presence of anti-SecB IgG (Fig. 4D), it is likely that SecB is involved in the translocation of RBP.

In conclusion, RBP is translocated via at least two targeting pathways involving either the Ffh or the SecB, and some unknown proteolytic enzyme might be involved in the RBP translocation.

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