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SecA protein is directly involved in protein secretion in *Escherichia coli*

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A high-expression plasmid for the *secA* gene was constructed. The SecA protein was then overproduced in *E. coli* and purified. The purified SecA stimulated the in vitro translocation of a model secretory protein into inverted membrane vesicles pretreated with 4 M urea. Membrane vesicles from a $secA^{ts}$ mutant exhibited lower translocation activity, which was enhanced by SecA. These results indicate that SecA is directly involved in protein secretion across the cytoplasmic membrane.

Protein, SecA; Protein overproduction; Protein secretion; (*E. coli*)

1. INTRODUCTION

A temperature-sensitive(ts) *secA* mutation was first described by Oliver and Beckwith [1] to be defective in secretion across the cytoplasmic membrane of proteins of the outer membrane and the periplasmic space in *Escherichia coli*. The *secA* gene was mapped at 2.5 min on the *E. coli* chromosomal map [1] and its entire nucleotide sequence was determined [2]. However, the participation of SecA, the *secA* gene product, in protein secretion has not been well demonstrated biochemically.

Recently, we developed an in vitro system that exhibits efficient protein translocation into inverted membrane vesicles of *E. coli*. This was achieved by fractionation of the conventional inverted membrane vesicle preparation by means of sucrose gradient centrifugation [3] and by the use of a model protein possessing an uncleavable signal peptide [4]. The uncleavability prevents in vitro premature processing of the signal peptide, thereby enhancing the translocation efficiency. Using this in vitro translocation system, the requirements of energy and soluble factors for the translocation reaction were studied [3,4].

In the present work, using the in vitro translocation system, we demonstrated that SecA is directly involved in membrane translocation of secretory proteins.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

The *E. coli* K12 strains used were: MC4100 (F^-, *araD136 Δ(lac)U169 relA rpsL thi*) [5], MM52 (MC4100*secA*^{ts}) [1] and RR1 (*pro leu thi lacY hsdR endA rpsL20 aro-14 galK2 xyl-5 mtl-1 supE44*) [6]. Plasmid pK107 carries a gene that codes for an uncleavable signal peptide-containing OmpF-Lpp chimeric protein (uncleavable OmpF-Lpp) [4]. The gene is under the control of the SP6 promoter.

2.2. *Preparation of S150-2 extracts and inverted membrane vesicles*

Membrane-free S150-2 extracts were prepared from *E. coli* MC4100 or MM52 as described in [7]. Purified inverted membrane vesicles were prepared as described in [3].

2.3. *Urea treatment of membrane vesicles*

Membrane vesicles (40 μg protein per reaction mixture) were treated with 4 M urea and then with 50 μM DCCD (*N,N'*-dicyclohexylcarbodiimide) as described in [3] except that membrane vesicles were recovered by centrifugation at

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350000 × *g* for 45 min and suspended in 50 mM Tris-acetate (pH 7.8)/10% glycerol/8.5% sucrose. In control experiments, membrane vesicles were similarly treated in the absence of urea.

2.4. *Transcription, translation and translocation reactions*

Uncleavable OmpF-Lpp was used as the substrate for translocation experiments. In vitro transcription and translation in the presence of Tran^{35}S-label, a mixture of 70% [^{35}S]methionine and 20% [^{35}S]cysteine (1000 Ci/mmol, 1 Ci = 37 GBq), were carried out as described in [4]. The translocation reaction was carried out at 40°C and the translocated protein, which was proteinase K-resistant, was detected on SDS-polyacrylamide gels by fluorography as described in [7]. The amount of the radioactive protein was determined densitometrically with a Toyo DMU-33C.

2.5. *Purification of uncleavable OmpF-Lpp*

After in vitro translation, ^{35}S-labeled OmpF-Lpp was purified by immunoaffinity chromatography as described in [4] and dialyzed against 50 mM Tris-acetate (pH 7.8)/5 mM $MgSO_4$/2 mM dithiothreitol.

3. RESULTS AND DISCUSSION

3.1. *Construction of a plasmid carrying the* secA *gene*

An outline of the construction is presented in fig.1. A lambda phage clone, 15B10 [8], was used as the source of the *secA* gene. The existence of the *secA* gene in this clone was supported by the position of this gene on the *E. coli* chromosome map [1] and the restriction profiles of the relevant DNA regions [8–10]. The lefthand half of the synthetic oligonucleotide linker shown in fig.1 is the upstream of the initiation codon on expression vector pUSI2 [11] and the righthand half was the 5′-terminus of the *secA* coding region [2].

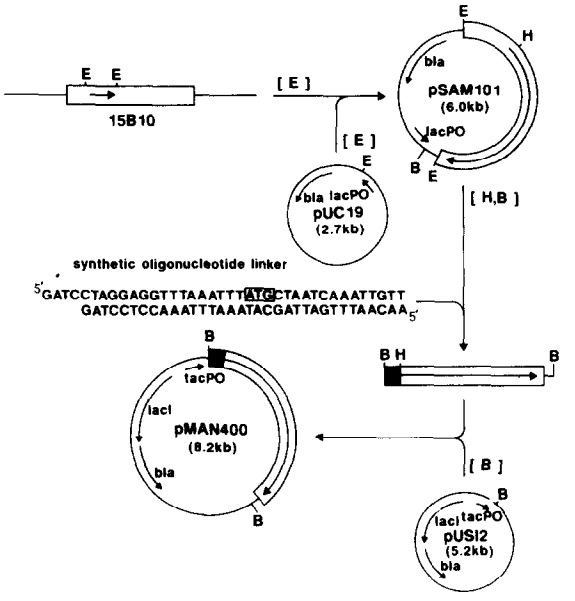

Fig.1. Construction of pMAN400 carrying the highly-expressible *secA* gene. The open box denotes chromosomal DNA. The arrow in the open box shows the coding region and the direction of transcription of the *secA* gene. The closed box denotes the synthetic oligonucleotide linker. The initiation codon ATG of the *secA* gene is boxed. The restriction endonucleases used are shown in parentheses with the following abbreviations: E, *Eco*RI; H, *Hpa*I; B, *Bam*HI. Cleavage sites are also shown.

3.2. *Overproduction and purification of SecA*

Upon induction with isopropyl-β-D-thiogalactoside (IPTG) of *E. coli* RR1 carrying the *secA* gene-containing plasmid, pMAN400, a 100 kDa protein was overproduced (fig.2, lanes 1–6). Based on its molecular mass and its immunoprecipitability with an anti-SecA antiserum (a generous gift from D.B. Oliver) (not shown), we conclude that this protein is SecA. Although SecA was reported to be a peripheral cytoplasmic membrane protein [12], the overproduced SecA was mainly localized in the cytosol (fig.2, lanes 1–6).

E. coli cells grown in 4 l of L-broth containing 1 mM IPTG were disrupted through a French pressure cell (8000 psi, 3 times), and the supernatant obtained on high-speed centrifugation (150000 × *g* for 2.5 h) was dialyzed against 10 mM Tris-acetate (pH 7.8)/14 mM Mg-acetate/60 mM K-acetate/1 mM dithiothreitol and subjected to ammonium sulfate fractionation. The 40–50% saturation fraction was dialyzed against the buffer and then chromatographed on a Sephacryl S-300 column (100 × 1.6 cm), but dithiothreitol was omitted. A fraction which essentially comprised pure SecA was obtained (fig.2).

3.3. *Translocation of uncleavable OmpF-Lpp with in vitro systems derived from wild-type and* secA^{ts} *cells*

In vitro translocation of uncleavable OmpF-Lpp took place efficiently when membrane vesicles and S-150 prepared from wild-type cells were used, whereas it was poor with those from *secA*^{ts} cells (fig.3). The activity in the latter case was, however, appreciably stimulated upon the addition of purified SecA. The experiment shown in fig.3 was performed at 40°C. Essentially the same results

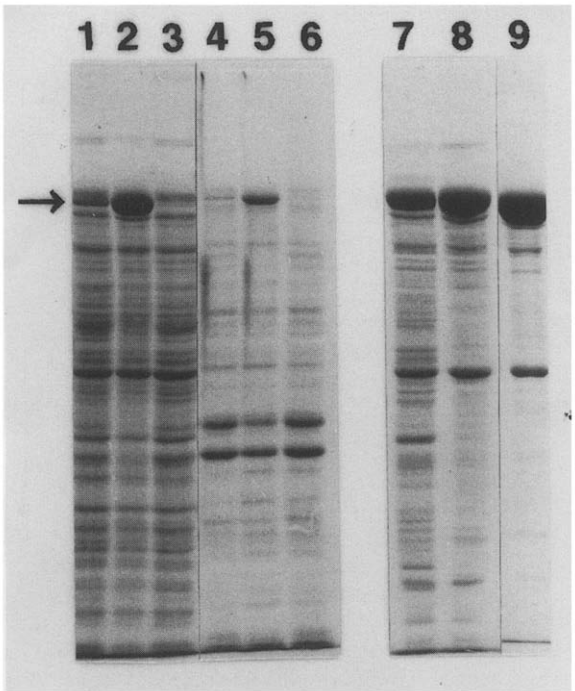

Fig.2. Overproduction and purification of SecA. *E. coli* RR1 was transformed with pMAN400, and then grown in the absence (lanes 1 and 4) and presence (lanes 2 and 5) of 3 mM IPTG. Equivalent amounts of the cytosol (lanes 1–3) and envelope (lanes 4–6) fractions were analyzed on SDS-polyacrylamide gels. Lanes 3 and 6 contained RR1, carrying pUSI2, grown in the presence of 3 mM IPTG. SecA in the cytosol fraction (lane 7) was then purified by ammonium sulfate fractionation (lane 8) and Sephacryl S-300 column chromatography (lane 9). The arrow indicates SecA.

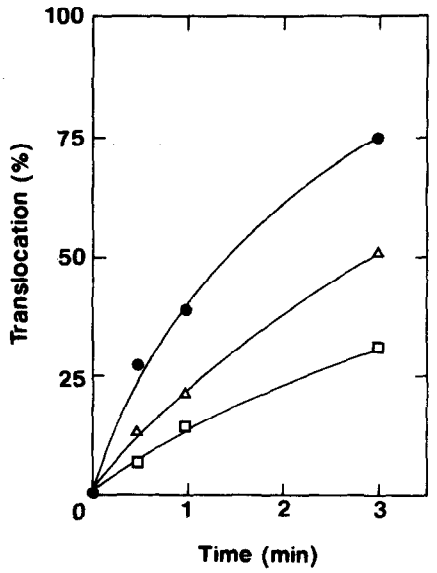

Fig.3. Time course of translocation of uncleavable OmpF-Lpp with in vitro assay systems derived from wild-type and *secA*^{ts} cells. Membrane vesicles (2.5 μg) and S150-2 (20 μg) prepared from wild-type (●) and *secA*^{ts} (□, △) cells were used for in vitro translocation of uncleavable OmpF-Lpp. (△) 0.25 μg of SecA was added. 100% translocation means that all of the substrate protein became resistant to proteinase K.

were obtained at lower temperatures (not shown), indicating that the in vivo ts phenotype was reflected only by the weak translocation activity in vitro.

3.4. *Purified SecA stimulates in vitro protein translocation*

SecA had no effect on translocation when membrane vesicles from wild-type cells were used (fig.4A). It was suggested that 6 M urea causes the depletion of SecA in the cytoplasmic membrane (Wickner, W., personal communication). 4 M urea treatment of membrane vesicles from wild-type cells resulted in an almost complete loss of translocation activity, which was restored by the addition of purified SecA (fig.4B). Since urea treatment results in the release of F_1-ATPase leading to the formation of a proton channel, vesicles thus treated were post-treated with 50 μM DCCD so that they could generate the proton-motive force required for the translocation reaction [3]. Although restoration of the activity with SecA was also observed after treatment with 6 M urea, the restoration was not as good as that in the case of 4 M urea (not shown). The same amount of S-150, as to protein (fig.4B), and a 20-fold excess of it (not shown) could not replace SecA at all as to the translocation restoration, suggesting that the restoration is specific to SecA.

The amount of SecA required for the restoration was then examined (fig.5). With membrane vesicles from wild-type cells, about 0.2 μg SecA/40 μg membrane protein was sufficient for full activity. When membrane vesicles from *secA*^{ts} cells were used, a larger amount of SecA was required for the maximum activation.

The reason for the requirement of excess SecA with urea-treated *secA*^{ts} membrane vesicles is not well known. The assembly of the mutant SecA in the membrane may be unusual, so that the protein cannot be extracted with 4 M urea, remaining in

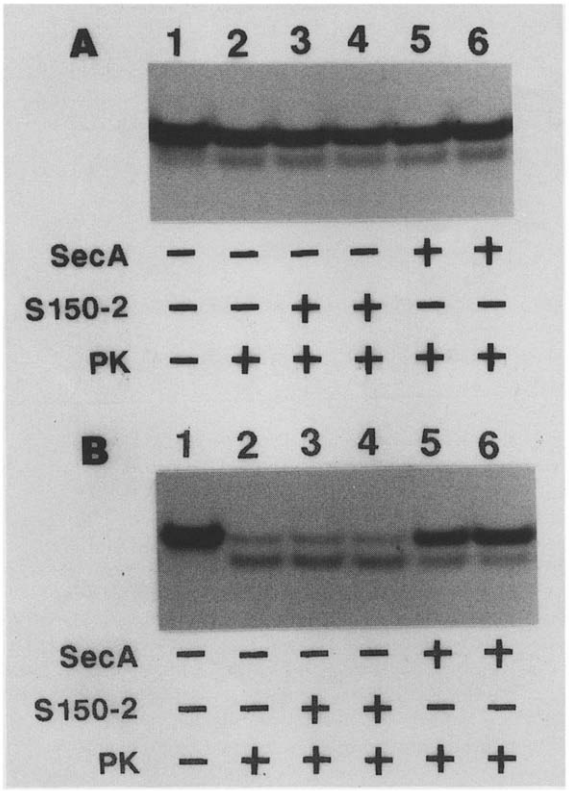

Fig.4. SecA stimulates the in vitro translocation into urea-treated membrane vesicles. Uncleavable OmpF-Lpp, purified on an immunoaffinity column, was translocated at 40°C for 10 min into urea-untreated (A) or urea-treated membrane vesicles (B) prepared from MC4100. The amounts of SecA added were 0.25 μg (lane 5) and 1.25 μg (lane 6), and those of S150-2 were 0.25 μg (lane 3) and 1.25 μg (lane 4). PK, proteinase K.

the membrane to compete with the externally added wild-type SecA. Studies on the mutant SecA may facilitate the understanding of SecA functions.

During the course of this study, we learned that D.B. Oliver's group also purified SecA and performed biochemical studies (personal communications).

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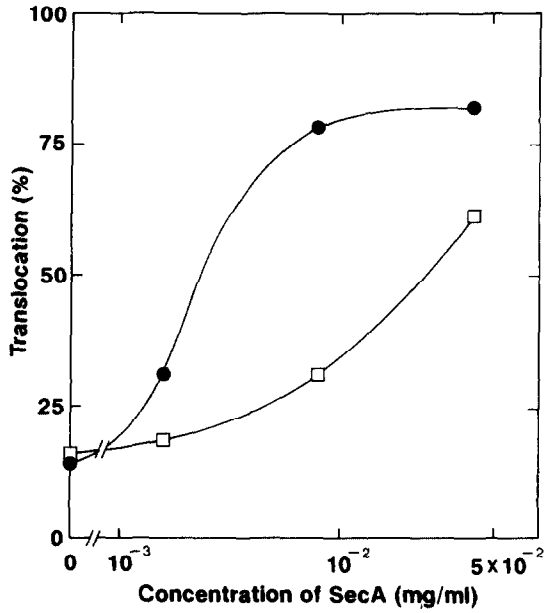

Fig.5. Effect of the SecA concentration on restoration of the translocation activity of urea-treated membrane vesicles. Urea-treated membrane vesicles were prepared from wild-type (●) and *secA*^{ts} (□) cells, and then translocation of the immunopurified uncleavable OmpF-Lpp was performed at 40°C for 10 min in the presence of the indicated concentrations of SecA. 100% translocation indicates the level of translocation with urea-untreated membrane vesicles.

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