A protein with sequence identity to Skp (FirA) supports protein translocation into plasma membrane vesicles of *Escherichia coli*

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We have purified to homogeneity a 15 kDa-protein from a ribosomal salt extract of *Escherichia coli* that compensates in vitro a defect of SecA but not of SecB. Removal of this protein from a cell-free transcription/translation system impairs translocation into plasma membrane vesicles of the precursors of LamB and to a lesser degree also of OmpA. These results suggest a role of the 15 kDa-protein in bacterial protein export. The NH_2-terminal 35 amino acids were found to be identical to those of the *skp (firA)* gene product, to which several putative functions have previously been attributed.

Protein export; Skp; FirA; Inside-out plasma membrane vesicle; SecA; Export factor

1. INTRODUCTION

A number of proteins have been identified both genetically and biochemically as being involved in protein export in *E. coli* (recently reviewed in [1]). The more intensively studied of these include SecA [1], SecB [1], SecY [1], trigger factor [1] GroEL and GroES [1,2].

Here we describe the purification and characterization of a soluble *E. coli* protein which we identified by its stimulating activity on protein transport into plasma membrane vesicles. This protein appears to be identical to a protein that has previously been reported to fulfill quite different functions such as (1) DNA-binding; (2) interaction with RNA-polymerase; (3) lipopolysaccharide-binding.

2. MATERIALS AND METHODS

2.1. *Purification of the 15 kDa-protein*

E. coli cells from 4–6 l cultures of strains MM18 [3] or MRE600 [4] were broken in a French pressure cell at 55 MPa and an S-30 was prepared [4]. Ribosomes from the S-30 were collected by centrifugation (Beckman Instruments, Ti 50.2 rotor; 45,000 rpm for 2.5 h), extracted in 25 ml buffer A (50 mM triethanolamine acetate (TeaOAc) pH 7.5, 15 mM $Mg(OAc)_2$, 1 mM DTT) containing 1 M KOAc and re-centrifuged. The ribosomal salt extract, dialyzed overnight against 3 × 1 liter buffer B (buffer A containing 50 mM KOAc), was applied to 15 ml packed DEAE-Sepharose CL-6B (Pharmacia) equilibrated with buffer B. The flow through was loaded onto 10 ml CM-Sepharose CL-6B (Pharmacia) equilibrated with buffer B, and bound proteins were eluted with buffer C (buffer A containing 220 mM KOAc). The eluate was concentrated by centrifugal ultrafiltration and further resolved on a Sephacryl S-300 (Pharmacia)-column (85 × 1.6 cm) using buffer B. Eluted proteins were identified by SDS-PAGE. Fractions containing most of the 15 kDa-protein were pooled, concentrated by centrifugal ultrafiltration and diluted 40-fold into pH 9.5-buffer (50 mM Tris base, 50 mM KOAc, 15 mM $Mg(OAc)_2$, 1 mM DTT, adjusted to pH 9.5 at 8°C with acetic acid). The protein solution was applied to 10 ml CM-Sepharose CL-6B equilibrated at pH 9.5 and proteins were eluted using a 50–300 mM K^+-gradient prepared in pH 9.5-buffer.

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Abbreviations: INV, inverted plasma membrane vesicles; K-INV, salt-extracted INV.

2.2. *Various methods*

Published procedures were employed for quantitative determination of radioactivity present in bands on SDS-gels [5], in vitro synthesis and translocation of LamB [6], preparation of an anti-15 kDa protein IgG column [7], and removal of the 15 kDa protein from an S-135 [8].

3. RESULTS AND DISCUSSION

We have previously shown that the precursor of the outer membrane protein LamB is translocated into plasma membrane vesicles with considerably reduced efficiency if the *E. coli* cell extract used for in vitro synthesis (S-135) is prepared from SecA-deficient mutant cells [6]. This translocation defect can be overcome by supplementing the SecA-protein [6]. Unexpectedly, SecA-free subfractions derived from a ribosomal salt extract also restored translocation to *secA*-mutant S-135s. This finding is illustrated in Fig. 1.

A ribosomal salt extract prepared from *secA* wild-type cells was subfractionated as detailed in Materials and Methods. Fig. 1A shows the protein pattern resulting from SDS-PAGE of fractions collected from the Sephacryl column (lanes 1 to 7). Aliquots of the fractions 2–7 were added to *secA* mutant-derived, cell-free translocation reactions. Fig. 1B, C shows ^{35}S-labeled translation/translocation products of LamB resolved by SDS-PAGE and visualized by fluorography. Translocation into salt-extracted, in-

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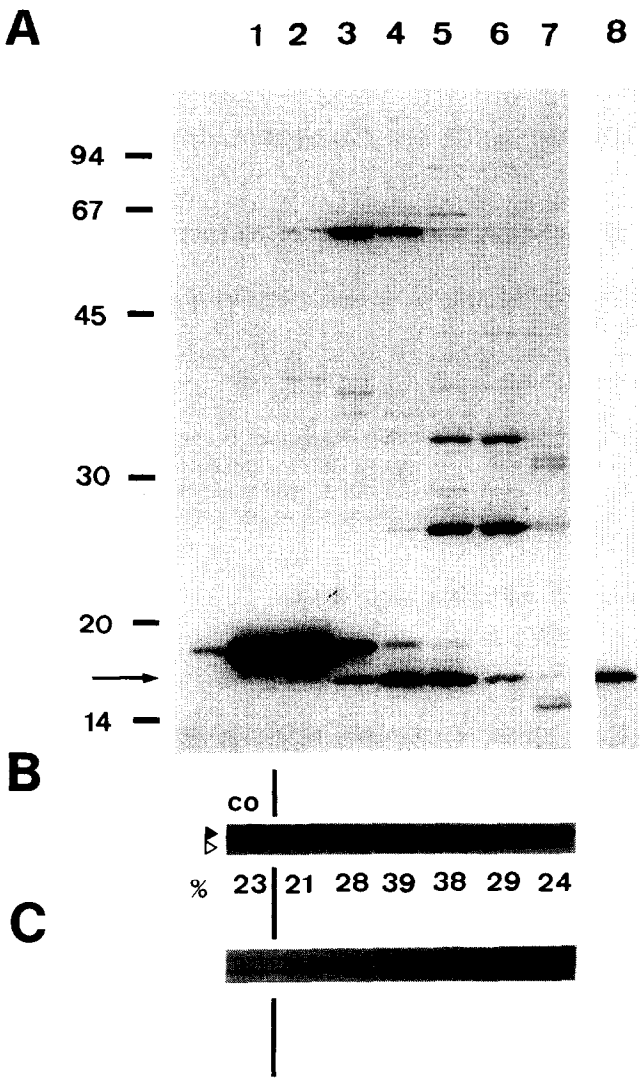

Fig. 1. Purification by gel filtration of an activity capable of compensating the SecA-defect in vitro. A ribosomal salt extract was fractionated as described in the text. Aliquots of fractions (lanes 1–7) eluting from the Sephacryl S-300 column were analyzed as follows. (A) SDS-PAGE and staining with Coomassie blue. Lane 8 shows the purified 15 kDa protein after re-chromatography on CM-Sepharose at pH 9.5. Migration of molecular weight standards is indicated in kDa. The arrow points to the 15 kDa protein. (B) Influence of added fractions on the proteolytic processing of preLamB (►) to LamB (▷) by cotranslationally added K-INV. PreLamB was synthesized by an S-135 prepared from the *secA*-mutant strain MM52 [3] grown at 42°C [6]. The K-INV were derived from a *secA*$^+$-strain. CO represents a control reaction to which elution buffer was added. The percentage processing, calculated as [cpm in LamB/(cpm in preLamB + cpm in LamB)] × 100, is indicated. (C) The portions of LamB-species shown in panel B, that are resistant to proteinase K [6].

verted membrane vesicles (K-INV) is indicated by the proteolytic conversion of preLamB to mature LamB (Fig. 1B, closed and open arrowhead, respectively) and by the protease resistance acquired after translocation into the vesicles (Fig. 1C). Quantitation of the proteolytic processing is indicated below panel B. The low processing and translocation of preLamB into K-INV, inherent in the *secA*-mutant system (Fig. 1B and C, co), are considerably stimulated by the two fractions shown in lanes 4 and 5. These fractions coincide with the peak elution of a 15 kDa protein (Fig. 1A, arrow).

The translocation-stimulating activity further copurified with the 15 kDa protein during subsequent chromatography on CM-Sepharose at pH 9.5, yielding a homogenous preparation of this protein (Fig. 1A, lane 8). On isoelectric focusing the 15 kDa protein turned out to be a basic protein with an isoelectric point of approximately 10 (not shown).

To confirm that the 15 kDa protein, which can at least partially substitute for the 100 kDa SecA, and SecA are in fact unrelated proteins, their antigenic crossreactivities were examined. Polyclonal antibodies against the purified 15 kDa protein were raised in mice. They are largely monospecific as shown by immunoblotting of several crude subcellular fractions (Fig. 2). As expected, the antibodies recognize the purified 15 kDa protein (lane 4) but do not crossreact with SecA (lane 7). Vice versa, anti-SecA-antibodies do not recognize the 15 kDa protein (not shown). Moreover, 15 kDa protein cross-reacting material is detected in S-135s containing both wild-type- (lanes 8 and 10) and mutant-SecA (lane 9). This result rules out the possibility that the 15 kDa protein stimulates translocation in SecA-deficient S-135s because it is absent from these extracts.

The antibodies were further used to examine the distribution of the 15 kDa antigen among several subcellular fractions. It is a soluble protein found in the postribosomal supernatant (Fig. 2, lane 1), the ribosomal fraction (lane 2), the ribosomal salt extract (lane 3) and, to a lesser degree, associated with the plasma membranes (INV, lane 5), from which it can be removed by high-salt treatment (K-INV, lane 6).

We then addressed whether manifestation of the stimulating activity of the 15 kDa protein on the translocation of preLamB into K-INV was dependent

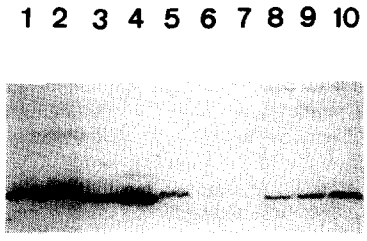

Fig. 2. The 15 kDa-protein is not antigenically related to SecA and is found in various subcellular fractions. Immunoblotting using polyclonal anti-15 kDa-antibodies was carried out for the following subcellular fractions of *E. coli*. Lane 1: 5 μl of a postribosomal supernatant (S-150); 2: equivalent amount of ribosomes; 3: equivalent amount of ribosomal salt extract; 4: 2 μg of pure 15 kDa-protein; 5: 6 μl of INV; 6: 8 μl of K-INV; 7: 0.3 μg of pure SecA (generous gift from Dr. William Wickner); 8: 4 μl of S-135 from strain MM52 grown at 30°C (*secA*$^+$); 9: 4 μl of S-135 from strain MM52 grown at 42°C (*secA*$^-$); 10: 4 μl of S-135 from strain MZ9 [4] (*secA*$^+$).

on a reduction in the translocation capacity of the cell-free system. Whereas the purified 15 kDa-protein clearly compensates in part for a SecA-deficiency (Fig. 3, lanes 1 and 2) it does not compensate for a lack of SecB (lanes 3 and 4). Furthermore, addition of the 15 kDa-protein also increases the translocation efficiency of a *secA* and *secB* wild-type cell-free system (not shown), albeit to a lesser degree than in the *secA*-mutant system.

To demonstrate an involvement of the 15 kDa-protein in protein export independent of the level of SecA, anti-15 kDa-protein-IgGs were cross-linked to protein A-Sepharose and this matrix was used to remove the 15 kDa-protein from a SecA-containing S-135. A control (mock-depleted) S-135 was prepared using a matrix to which preimmune IgGs had been coupled. As demonstrated in Fig. 3, lane 7, removal of the 15 kDa-protein reduces processing of preLamB by K-INV when compared to the mock-treated control (lane 6) and the untreated sample (lane 5). Quantitation of the data obtained in 6 independent experiments reveals an average reduction of processing by 39% ± 6 (with the translocation efficiency of the mock-treated control being set at 100%). As expected, addition of the 15 kDa protein partially reverses this effect, regaining 74 ± 9% of the translocation efficiency (not shown). Moreover, a decrease in processing due to a removal of the 15 kDa-protein was not restricted to LamB. An S-135 depleted of the 15 kDa protein also gives rise to a diminished processing (80%) of the precursor of OmpA (not shown).

When the purified 15 kDa protein was subjected to automated Edman degradation the following sequence of the 35 NH_2-terminal amino acid residues was obtained: Ala-Asp-Lys-Ile-Ala-Ile-Val-Asn-Met-Gly-Ser-Leu-Phe-Gln-Gln-Val-Ala-Gln-Lys-Thr-Gly-Val-Ser-Asn-Thr-Leu-Glu-Asn-Glu-Phe-Lys-Gly-Arg-Ala-Ser. This sequence is identical to the first 35 residues of the Skp (FirA)-protein [9–11]) of *E. coli*, and shows over 90% sequence identity to OmpH of *Salmonella typhimurium* [12].

The *firA*-locus was originally defined by ts-

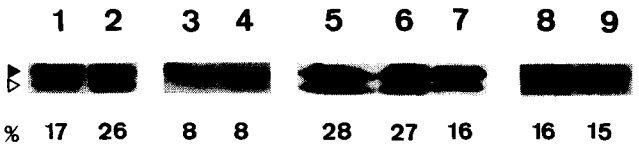

Fig. 3. Involvement of the 15 kDa-protein in translocation of preLamB into K-INV. PreLamB was synthesized in vitro in the presence of K-INV using and S-135 from the *secA*-mutant MM52 (lanes 1,2,8,9), from the *secB*-mutant CK1953 [21] (lanes 3 and 4), or from the *secA*$^+$-strain MZ9, (lane 5). The reactions shown in lanes 6 and 7 were directed by an MZ9-derived S-135 that had been passed over a preimmune (lane 6) or an anti-15 kDa-protein (lane 7) immunoaffinity column. Processing of preLamB to LamB was quantitated and is indicated in %. Reactions shown in lanes 2 and 4 had received 0.5 μg of purified 15 kDa protein each; that of lane 9 0.125 μg histone H2B (Sigma).

mutations that both render RNA synthesis thermosensitive and eliminate resistance to rifampicin, which is conferred by mutations of the RNA polymerase β gene [13]. The gene product of *firA* displaying histone-like properties (designated HLP-I) was proposed to interact directly with RNA polymerase [14]. Subsequently, FirA was shown to be identical [15] to the 17 kDa-protein (Skp) which had been identified and purified as constituent of the *E. coli* nucleoid [9]. The *firA* gene maps at the 4 min region on the chromosome and is located immediately upstream of genes that encode proteins involved in lipid A biosynthesis [10].

How can these properties of Skp (FirA) be related to our finding of an involvement in protein export? (1) The excess of positively charged amino acids explains well why Skp (FirA) sticks to polyanions such as DNA and ribosomes. However, it is unlikely that the cationic character of Skp (FirA) is the basis of its functioning in protein export. A different cationic protein, eukaryotic histone H2B, whose amino acid composition resembles that of the more typical histone-like protein HU of *E. coli* [cf. 16], did not stimulate translocation into K-INV when preLamB was synthesized in a SecA-deficient cell-free system (Fig. 3, lanes 8 and 9). This result was obtained with the highest possible concentration of H2B not abolishing in vitro synthesis, which was in the same order of magnitude as the concentration of Skp (FirA) required for activity. (2) Skp (FirA) does not affect translocation by influencing the rate of synthesis. Such an interrelationship is suggested by a previous report describing the suppression of a SecA-defect in vivo by various measures slowing down protein synthesis [17]. Skp, however, does not influence transcription in vitro [9]. Moreover, determination of a time course of LamB synthesis in vitro revealed that the addition to a *secA*-mutant S-135 of the 15 kDa-protein we isolated does not change the amount of preLamB and LamB synthesized at any time point, but only increases the percentage of processed LamB (not shown).

Surprisingly, the nucleotide sequence of *skp* disclosed the existence of an NH_2-terminal signal sequence [8] which had been removed from the mature protein purified in this and other laboratories. The meaning of this finding is not clear [15] particularly because this protein is found in several different subcellular fractions.

Recently Skp (FirA) was found to be homologous to OmpH, a protein associated with the outer membrane of *S. typhimurium* [12,18]. OmpH in turn appears to be related to a lipopolysaccharide-binding protein detected in various *Enterobacteriaceae* [19]. It is conceivable that the soluble Skp (FirA), due to its basic net charge, sticks to the outer membrane, as well as to other cell costituents, upon breakage of the bacteria cells. On the other hand, the existence of a signal sequence appears to suggest a location in the cell envelope.

At the present time it is difficult to reconcile all the diverse properties of Skp (FirA) deduced from its association with distinct cellular components with the activity of a single protein. Clearly, the only functional analyses of this protein are those of the *firA*-mutants and the one described herein. Our results suggest a role of Skp (FirA) in the export of outer membrane proteins. If this function is of a more general nature like that of chaperonins [20], conferring translocation-competence by directly interacting with export proteins, it might even explain how Skp (FirA) could be active on either side of the plasma membrane of *E. coli*.

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Note added after submission of the manuscript: In contrast to previous reports [15] *firA* and *skp* have recently been found to be different gene loci and the Skp-protein does not complement the *firA*-phenotype (I. Dicker, personal communication). Thus, the sequence of the 15 kDa protein described herein, is identical only to that of the Skp-protein.

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