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Cytosolic factor purified from *Escherichia coli* is necessary and sufficient for the export of a preprotein and is a homotetramer of SecB

(translation/wheat germ system/inverted vesicles/posttranslational translocation)

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ABSTRACT **We have purified to homogeneity a cytosolic factor from *Escherichia coli* that is required for the translocation of a preprotein into inverted vesicles of the *E. coli* plasma membrane. The preprotein used is a precursor of mutant maltose-binding protein. This mutant contains alterations of the carboxyl terminus. Unlike the precursor for wild-type maltose-binding protein, the mutant precursor does not acquire a protease-resistant conformation after synthesis and retains posttranslational translocation competence. The purified cytosolic factor, added posttranslationally, is necessary and sufficient to yield virtually 100% translocation of the mutant precursor into inverted vesicles. The purified factor amounts to 0.08% of the cytosolic proteins and is a 64-kDa tetramer consisting of four identical 16-kDa subunits. Amino-terminal sequence analysis revealed that it is identical to the *secB* gene product. The purified SecB homotetramer is part of a larger 150-kDa complex that represents the "export" factor activity. During purification, the export factor activity dissociates into a 64-kDa SecB homotetramer and unidentified component(s). For the posttranslational integration of another preprotein, the precursor for the *lamB* gene product, into inverted vesicles, the 64-kDa SecB homotetramer is also required but additional factor(s) makes integration more efficient.**

We have described (1) a heterologous cell-free translation/translocation system in which the translocation of a preprotein across the *Escherichia coli* plasma membrane could occur posttranslationally, with nearly 100% efficiency, but was strictly dependent on an *E. coli* cytosolic factor. The model preprotein used in this system was a mutant of the precursor for the maltose-binding protein (preMBP) that contained alterations in its carboxyl terminus and was, therefore, termed preMBP* (1). Whereas wild-type preMBP is known, within minutes after its synthesis, to fold into a proteinase K-resistant structure (comprising its mature portion) accompanied by loss of translocation competence (2, 3), preMBP* does not fold into a proteinase K-resistant structure and retains translocation competence (1). Thus, when preMBP* is synthesized in a wheat-germ cell-free system, it can be translocated posttranslationally and with virtually 100% efficiency into inverted vesicles (INV) derived from the *E. coli* plasma membrane, provided that appropriate amounts of *E. coli* postribosomal supernatant (PRS) are included in the posttranslational translocation reaction (1). The activity of the *E. coli* PRS required for the posttranslational translocation into INV of a preprotein synthesized in the wheat-germ cell-free system has been shown to be identical to the "export" factor activity (4) in a homologous but highly subfractionated *E. coli* translation/translocation system (1).

In this paper we describe the purification of a component of the export factor activity and show that it represents the product of the *secB* gene, identified by Kumamoto and Beckwith (5).

MATERIALS AND METHODS

***E. coli* Cell Extract.** An S30 fraction and the PRS were prepared according to Müller and Blobel (6) from the *E. coli* strain MRE600.

Purification of SecB. *Ammonium sulfate precipitation.* Saturated ammonium sulfate solution was added to PRS (40 ml) to a concentration of 40% and the mixture was rotated for 1 hr at room temperature. After centrifugation for 10 min at 8000 × *g*, the precipitated proteins were resuspended in 40 ml of 40% saturated ammonium sulfate and collected again by centrifugation. The pellet was dissolved in 6 ml of buffer A [10 mM triethanolamine acetate, pH 7.5/60 mM KOAc/14 mM $Mg(OAc)_2$/1 mM dithiothreitol] and dialyzed against buffer A.

Gel filtration on Sephacryl S-400. The dialyzed material from the ammonium sulfate precipitation was loaded at a flow rate of 15 ml/hr onto a Sephacryl S-400 column (2.5 × 100 cm; Pharmacia) that had been equilibrated with buffer A. Fractions of 6.5 ml were collected; a 5-μl aliquot of the dialyzed ammonium sulfate precipitate or a 15-μl aliquot of each fraction was analyzed by $NaDodSO_4$/PAGE followed by Coomassie blue staining for its protein profile, and a 1-μl aliquot of the dialyzed ammonium sulfate precipitate or a 3-μl aliquot of each fraction was analyzed for activity.

Hydroxylapatite column chromatography. The peak-activity fractions of the Sephacryl S-400 column were pooled and loaded on a hydroxylapatite column (1.5 × 6 cm; Bio-Rad) that had been equilibrated with buffer B [10 mM sodium phosphate, pH 6.8/10 mM triethanolamine acetate, pH 7.5/60 mM KOAc/14 mM $Mg(OAc)_2$/1 mM dithiothreitol]. The column was eluted with step gradients (100 ml per step) of buffer B containing 10 mM, 25 mM, 55 mM, 100 mM, or 200 mM sodium phosphate. A 2-ml aliquot of each fraction was concentrated to 50 μl with a Centricon 10 (Amicon) and a 10-μl aliquot of each concentrated fraction was analyzed by $NaDodSO_4$/PAGE followed by Coomassie blue staining or a 0.5-μl aliquot was assayed for activity.

DEAE-Sepharose CL-6B ion-exchange column chromatography I. The 55 mM sodium phosphate eluate from the hydroxylapatite column was loaded on a DEAE-Sepharose CL-6B column (1.5 × 6 cm; Pharmacia) that had been equilibrated with buffer A. The column was washed with 100

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Abbreviations: INV, inverted vesicles derived from the *E. coli* plasma membrane; preMBP, precursor of the maltose-binding protein; preMBP*, precursor of the mutant maltose-binding protein; PRS, postribosomal supernatant; preLamB, precursor of the *lamB* gene product.

ml of buffer A and material was eluted with two steps: first with 100 ml of buffer A containing 300 mM KOAc and then with 100 ml of buffer A containing 600 mM KOAc. A 2-ml aliquot of each fraction was concentrated to 50 μl as described above and a 10-μl aliquot of each concentrated fraction was analyzed by $NaDodSO_4$/PAGE followed by Coomassie blue staining, or a 0.5-μl aliquot was assayed for activity.

DEAE-Sepharose CL-6B ion-exchange column chromatography II. The 600 mM KOAc eluate from the first DEAE-Sepharose CL-6B column was diluted by adding 2 vol of ice-cold H_2O, and the mixture was loaded onto a second DEAE-Sepharose CL-6B column (1 × 2.5 cm) that had been equilibrated with buffer A. The column was washed with buffer A and material was eluted by step gradients (20 ml per step) of buffer A containing 300 mM, 350 mM, 400 mM, 450 mM, 500 mM, or 600 mM KOAc. A 2-ml aliquot of each fraction was concentrated to 50 μl and a 4-μl aliquot of each concentrated fraction was analyzed by $NaDodSO_4$/PAGE followed by Coomassie blue staining, or a 0.1-μl aliquot was assayed for activity.

Gel filtration on Sephacryl S-300. The 500 mM KOAc eluate from the DEAE-Sepharose CL-6B column II was concentrated to 1 ml by Immersible CX-10 ultrafilter (Millipore) and loaded at a flow rate of 3 ml/hr onto a Sephacryl S-300 column (1.5 × 120 cm; Pharmacia) that had been equilibrated with buffer A. Fractions of 1.3 ml were collected and a 15-μl aliquot of each fraction was analyzed by $NaDodSO_4$/PAGE followed by Coomassie blue staining, or a 1.5-μl aliquot was assayed for activity.

Translocation Assay. mRNA (500 ng) for preMBP* or the precursor for the *lamB* gene product (preLamB) was translated in 25 μl of wheat-germ cell-free translation system as described (7). After completion of translation a PRS was prepared in a Beckman Airfuge, rotor A 100/18, by centrifuging the translation mixture for 30 min at 135,000 × *g*. PRS (23 μl) was supplemented with 5 mM ATP, 8 mM creatine phosphate, and creatine phosphokinase at 40 μg/ml and incubated with 25 μl of buffer A containing *E. coli* PRS or fractions of *E. coli* PRS in the presence of high-salt-washed INV (0.5 A_{280} unit/ml) in a total volume of 50 μl for 60 min at 25°C. After incubation, proteinase K digestion was carried out as described (1). Only protease-resistant chains were shown.

$NaDodSO_4$/PAGE. For $NaDodSO_4$/PAGE, 10–15% polyacrylamide gels were used in all cases.

Definition of Activity Unit. One unit of activity is defined as the amount of material that yields 50% translocation of preMBP* across INV in a 50-μl translocation assay. Percent translocation was determined as described (1).

RESULTS

The purification of export factor activity from *E. coli* PRS was monitored by $NaDodSO_4$/PAGE followed by Coomassie blue staining (see Figs. 1–4 *Upper*) and by activity assays (see Figs. 1–4 *Lower*) in the heterologous translation/translocation system (1). In this system, mRNA for preMBP* is first translated in a wheat-germ cell-free system. A PRS is then prepared and subsequently incubated with INV. Translocation is strictly dependent on the presence of *E. coli* PRS in the posttranslational translocation reaction. The efficiency of translocation is proportional to the amount of *E. coli* PRS added, being virtually 100% at a saturating concentration of PRS (1).

As a first step toward purification we found that 40% saturated ammonium sulfate precipitated 90% of the activity (data not shown; however, see Table 1).

After resuspension and dialysis, the ammonium sulfate-precipitated material was then subjected to sieving on Sephacryl S-400 (Fig. 1). The activity was recovered in fractions that, based on marker proteins, suggested a molecular mass

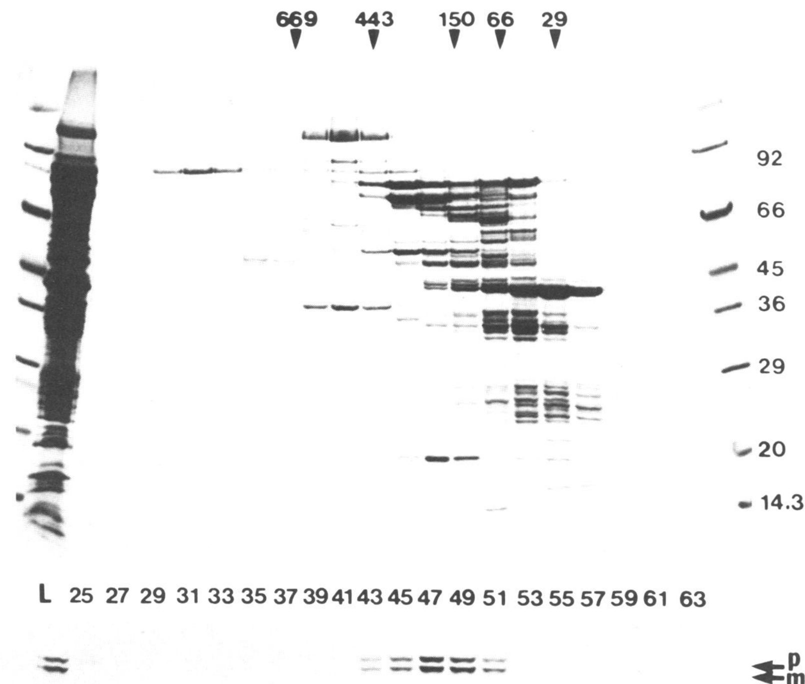

FIG. 1. Gel filtration of ammonium sulfate-fractionated PRS on Sephacryl S-400. (*Upper*) Analysis of fractions by $NaDodSO_4$/PAGE and staining of the gel with Coomassie blue. (*Lower*) Activity assay of fractions. L, dialyzed ammonium sulfate precipitate; numbers above the *Upper* panel indicate the molecular mass of marker proteins [thyroglobulin (669 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa)] run separately on the same column; numbers on the right of the *Upper* panel indicate masses of marker proteins in kDa; numbers between the two panels indicate column fractions that were analyzed. p and m, Precursor and mature form of MBP*, respectively.

of ≈150 kDa, confirming previous data (8) and being consistent with the estimated sedimentation rate of export factor at ≈7 S (1).

The activity peak fractions after sieving were pooled and loaded on a hydroxylapatite column. About 80% of the activity loaded on the column was eluted at 55 mM phosphate buffer (Fig. 2*A* and Table 1).

The active fraction was then subjected to two cycles of anion-exchange chromatography on DEAE-Sepharose CL-6B. Most of the protein eluted at up to 300 mM salt whereas the activity was eluted by a subsequent 600 mM salt step (Fig. 2*B* and Table 1). Rechromatography of the active fraction and elution in steps between 300 and 600 mM salt showed that most of the activity was eluted between 450 and 500 mM salt (Fig. 2*C* and Table 1).

Finally, after concentration, the active fraction was subjected again to sieving, this time on Sephacryl S-300 (Fig. 3 and Table 1). The activity was now found in fractions with a molecular mass of ≈64 kDa that contained a single Coomassie blue-stained polypeptide of an estimated molecular mass of 16 kDa (Fig. 3). These data suggested that the purified activity resides in a tetramer consisting of four 16-kDa polypeptides. Moreover, the molecular mass of 64 kDa for the purified tetramer (Fig. 3) and the molecular mass of 150 kDa for export factor (Fig. 1) suggested that the export factor is a complex that consists of the purified tetramer and some additional component(s) that were dissociated from this complex during hydroxylapatite and/or DEAE-Sepharose CL-6B chromatography. The dissociated component(s) is apparently not required for the translocation of preMBP*.

When the efficiency of translocation of preMBP* and preLamB was compared at various steps of the purification (Fig. 4), there was a significant loss (from 30% to 15%) in the translocation of preLamB after hydroxylapatite chromatography, indicating that another factor(s) may help in integrating preLamB into the plasma membrane [LamB is first integrated into the plasma membrane and then transported to the outer membrane (9)].

Table 1. Purification of translocation-stimulating activity

Step	Total protein, mg	Total activity, units	Specific activity, units/mg	Fold purification	Activity recovery, %
PRS	1000.0	40,000	40	1.0	100.0
$(NH_4)_2SO_4$	108.0	36,000	333	8.3	90.0
S-400	30.0	33,300	1,110	27.8	83.3
HA	14.5	26,400	1,820	45.5	66.0
DEAE (I)	1.3	25,000	19,200	480.0	62.5
DEAE (II)	0.6	17,100	28,500	712.5	42.8
S-300	0.18	9,000	50,000	1250.0	22.5

S-400, Sephacryl S-400; HA, hydroxylapatite; DEAE I and II, DEAE-Sepharose CL-6B chromatography I and II, respectively; S-300, Sephacryl S-300.

The purification summary data in Table 1 indicate that the purified tetramer presents 0.08% of the proteins in the *E. coli* PRS.

We also determined the amino-terminal sequence of the 16-kDa polypeptide using the activity peak fraction after sieving on Sephacryl S-300 (Fig. 3). The sequence data for the amino-terminal 34 residues is shown in Fig. 5. From the yield of the consecutive Edman degradations relative to the starting material, we estimate that >90% of the starting material was analyzed. This together with the fact that there was only one residue at each step indicated that all four 16-kDa polypeptide chains are identical. Thus the 64-kDa complex is a homotetramer.

Comparison of our partial amino acid sequence data (Fig. 5) with the amino acid sequence deduced from the DNA sequence of the *secB* gene that gives a protein with a calculated molecular weight of 16,649 (10) shows complete identity. Thus we conclude that the 16-kDa polypeptide forms a homotetramer that is required for the posttranslational translocation of preMBP* into *E. coli* INV, after synthesis in the wheat-germ system and that this protein is the product of the *secB* gene.

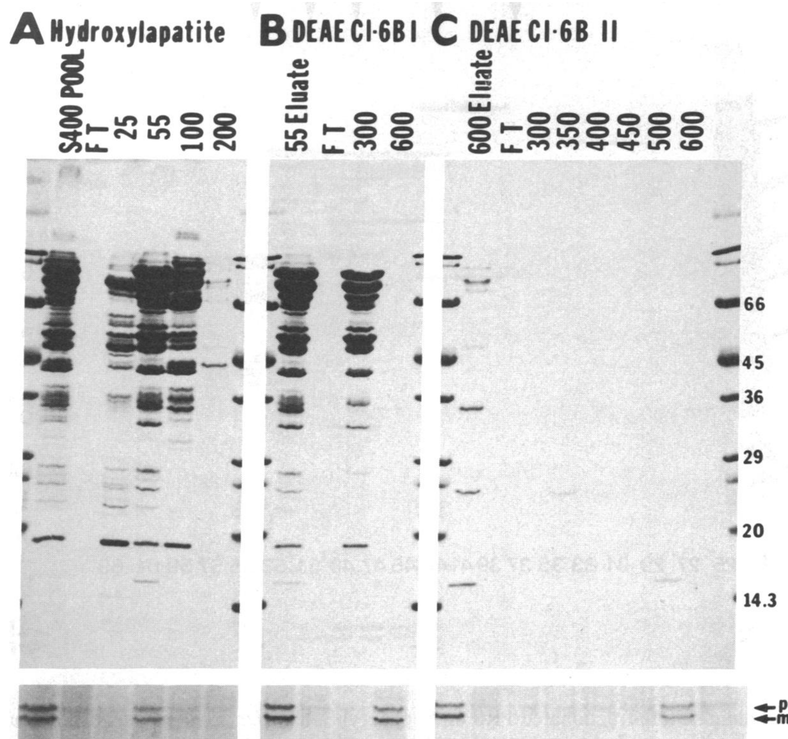

FIG. 2. Chromatography on hydroxylapatite and on DEAE-Sepharose CL-6B and rechromatography on DEAE-Sepharose CL-6B. (*Upper* and *Lower*) As in Fig. 1. FT, fraction; 55 eluate, 55 mM sodium phosphate eluate; 600 eluate, 600 mM sodium phosphate eluate. Other numbers refer to fractions.

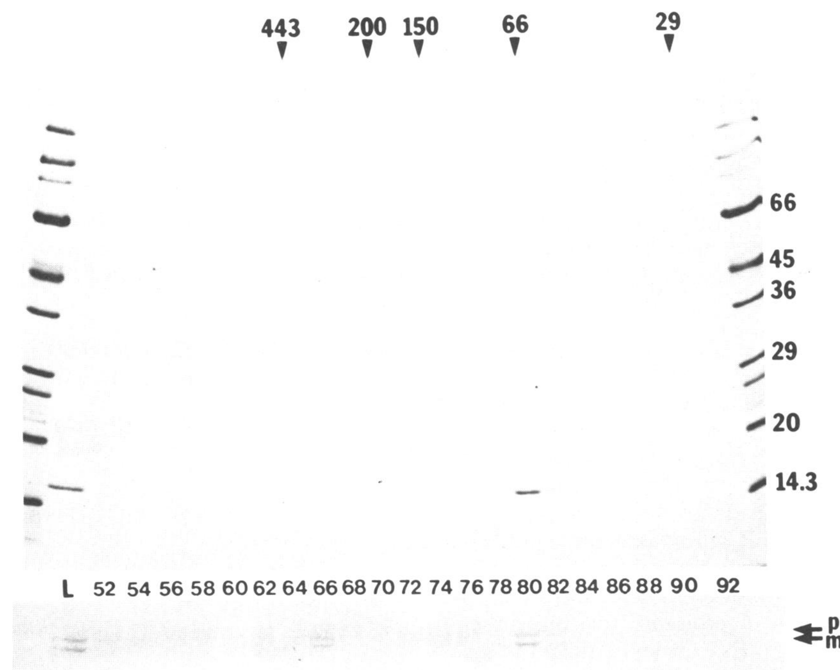

FIG. 3. Gel filtration on Sephacryl S-300. (*Upper* and *Lower*) As in Fig. 1. Numbers to the left of the *Upper* panel indicate masses of marker proteins in kDa; numbers between the two panels indicate those column fractions that were analyzed. The proteinase K-resistant bands seen in fraction 66 could not be detected when this fraction was assayed again and therefore represent an artifact.

DISCUSSION

We have purified a component of the ≈150-kDa export factor activity (4, 8) that is required for posttranslational translocation of preMBP* into INV (1). The purified component is a 64-kDa protein that consists of four identical 16-kDa subunits. Amino-terminal sequence analysis of the 16-kDa subunit (Fig. 5) established that it is the product of the *secB* gene that was identified (5) and sequenced (10) by Kumamoto and colleagues. Thus, genetic (5, 10) and biochemical (1, 4, 8) approaches have independently identified the same protein.

Independently, Weiss *et al.* (11) and Kumamoto *et al.* (12) have purified SecB from bacterial strains that contained the *secB* gene in an inducible multicopy plasmid. Under these conditions purification of the protein, which amounted to 30% of the cytoplasmic protein (11), obviously required fewer steps than the purification of the chromosomal gene expressed SecB, that constituted only 0.08% of the cytosolic proteins (Table 1). Nevertheless, a comparison of our data with those of Weiss *et al.* (11) suggests some interesting differences. Our purified SecB was not blocked at the amino terminus, allowing us to determine the sequence of 34 amino-terminal residues by consecutive Edman degradation (Fig. 5). In contrast, Weiss *et al.* (11) reported that their purified SecB was blocked to Edman degradation, suggesting that the amino terminus was modified. If this, and perhaps other modifications, occurred *in vivo*, as a result of overproduction, the purified protein may differ also in its functional properties from those of SecB purified from a nonoverproducing strain. In fact, Weiss *et al.* (11) reported that purified SecB when added to a translation/translocation system from SecB$^-$ cells stimulated translocation from a base level of 7% to only 19%, far short of the 71% translocation observed in a translation/translocation system from SecB$^+$ cells. One of several explanations [see Weiss *et al.* (11)] is that overproduced SecB loses competence in one of several functional sites, due to modification of this site as a result of overproduction. Thus, although purification from overproducing strains is convenient and useful for certain purposes (e.g., antibody production) a detailed functional analysis of the

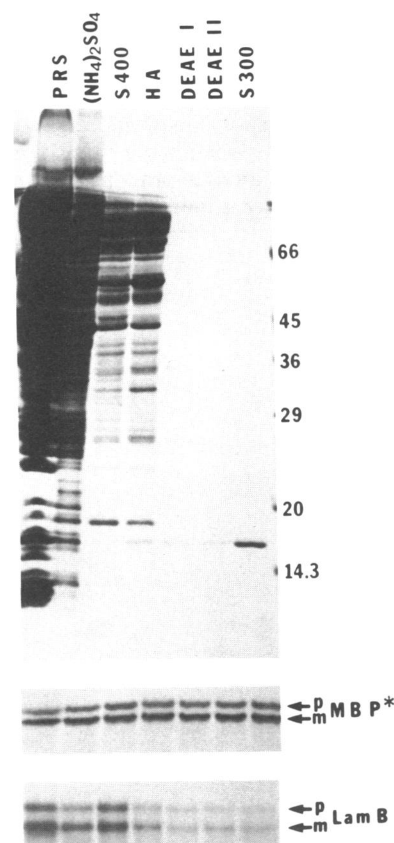

FIG. 4. Summary of purification procedure. (*Upper*) As in Fig. 1. (*Lower*) Corresponding activity assays using preMBP* or preLamB. (*Upper*) Amounts of protein analyzed: PRS, 500 μg; $(NH_4)_2SO_4$, 120 μg; Sephacryl S-400 (S400), 60 μg; hydroxylapatite, 58 μg; DEAE-Sepharose CL-6B chromatography (DEAE I), 6 μg; DEAE-Sepharose CL-6B chromatography II (DEAE II), 3 μg; Sephacryl S-300 (S300), 2 μg. (*Lower*) Amounts of proteins (in μg) of the corresponding fractions analyzed: 125, 13, 5, 3, 0.4, 0.2, 0.1, respectively.

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1               5                   10                  15
Ser-Glu-Gln-Asn-Asn-Thr-Glu-Met-Thr-Phe-Gln-Ile-Gln-Arg-Ile-

                    20                  25
Tyr-Thr-Lys-Asp-Ile-Ser-Phe-Glu-Ala-Pro-Asn-Ala[a]-Pro[a]-His[a]

30
Val[a]-Phe[a]-Gln[a]-Lys[a]-Asp[a]
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FIG. 5. Amino-terminal sequence of the Sephacryl S-300 activity peak. Protein (0.3 μg) was subjected to 34 cycles of automated Edman degradation. Residues marked with a superscript a are considered tentative because the phenylthiohydantoin-derivative HPLC peak area was not at least 50% greater than the analogous peak from the previous cycle. Except for a missing amino-terminal methionine, the above sequence is identical to the amino acid sequence of SecB deduced from the DNA sequence (10).

protein might require purification from a nonoverproducing strain.

Our data suggest that the 64-kDa homotetrameric SecB is only part of the export factor activity. In crude PRS, as well as in the precipitate from a 40% $(NH_4)_2SO_4$ fractionation of PRS, the 64-kDa homotetrameric SecB behaves like a 150-kDa complex (ref. 8, and Fig. 1). After hydroxylapatite and/or DEAE-Sepharose CL-6B chromatography, the 64-kDa complex is observed. Posttranslational translocation of preMBP* requires only the 64-kDa SecB complex, whereas posttranslational translocation of preLamB (or, more precisely, integration of LamB into INV) is less efficient in the presence of saturating concentrations of SecB homotetramer (Fig. 5), suggesting that additional factor(s) can stimulate this process. These findings point to the importance of choice regarding the nature of the preproteins studied in cell-free translocation systems. If it were not for preMBP*, we would not have been able to purify the SecB homotetramer as an entity necessary and sufficient for posttranslational translocation of preproteins, such as preMBP*.

In summary, the purified 64-kDa SecB homotetramer that is part of the 150-kDa export factor appears to be necessary and sufficient for the translocation of preMBP* into INV. For the translocation (integration) of other preproteins additional component(s) may exert stimulating effects. Data to be presented elsewhere suggest that the SecB tetramer functions in signal-sequence recognition.

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