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Elevated cytosolic concentrations of SecA compensate for a protein translocation defect in *Escherichia coli* cells with reduced levels of negatively charged phospholipids

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Cellular extracts from cells with reduced synthesis of negatively charged phospholipids were found to support in vitro translocation of the precursor of the outer membrane protein PhoE with increased efficiency. Analysis of these extracts revealed that they contain increased levels of SecA. SecA depletion resulted in a loss of the translocation stimulatory activity, which could be restored by re-addition of purified SecA. We conclude that elevated cytosolic levels of SecA counteract the reduction of translocation efficiency due to low levels of negatively charged phospholipids in the inner membrane.

Protein translocation; SecA; PhoE; Bacterial membrane

1. INTRODUCTION

Several studies have revealed that negatively charged phospholipids in the *E. coli* inner membrane are important for the export of proteins out of the cytoplasm [1,2]. In these studies lipid biosynthetic mutants were applied [3,4] which have reduced levels of the major negatively charged lipid phosphatidylglycerol (PG). Protein translocation across the PG-depleted inner membrane was inhibited [11]. Re-introduction of negatively charged phospholipids into the membrane resulted in complete restoration of translocation [5], which indicates the direct involvement of this class of lipids in the translocation process.

In one study [1] it was noted that protein translocation was 2- to 3-fold enhanced when the in vitro translation–translocation reaction was carried out in the presence of a cell extract derived from the PG^- mutant instead of an extract from a wild-type strain [1]. This led to the suggestion that a factor present in the cytosol of the PG^- strain compensates for the loss of translocation efficiency.

In this study we used an *E. coli* strain with inducible synthesis of PG to investigate whether the translocation-stimulatory activity present in the cytosol is coupled to the PG content of the inner membrane. The cytosolic factor responsible for stimulation of translocation was identified as SecA, which supports the finding that SecA–lipid interactions are important in protein translocation [2].

Abbreviations: PG, phosphatidylglycerol; IPTG, isopropyl 1-thio-β-D-galactopyranoside.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and growth conditions*

E. coli HDL 11 [5] was grown at 37°C in L-Broth supplemented with chloramphenicol (20 μg/ml), kanamycin (50 μg/ml), tetracyclin (10 μg/ml) and the appropriate concentrations of isopropylthiogalactoside (IPTG, Sigma). *E. coli* MRE 600 [6] was grown at 37°C in yeast broth.

2.2. *Preparation of cell extracts and inverted inner membrane vesicles*

Strain HDL 11 was grown at 0, 10, 20, 30 or 50 μM IPTG which resulted in negatively charged phospholipid levels of 9.4, 14.5, 18.3, 20.2 and 20.4 mol%, respectively [5]. Inverted inner membrane vesicles and S- 135 cell extract were prepared basically as described [7]. The buffer used for the preparation of the extract was 40 mM Tris-acetate pH 8, 4 mM Mg-acetate, 28 mM K-acetate, 2 mM dithiothreitol. Inverted inner membrane vesicles with wild-type phospholipid composition were prepared from strain MRE 600. The protein content of the different fractions was determined by the BCA protein assay (Pierce) according to the manufacturer's instruction, using bovine serum albumine as a standard.

2.3. *Proteins and antisera*

PrePhoE [8], SecA [9] and SecB [10] proteins were isolated and purified as described. Antisera to SecA and SecB were a gift from H. de Cock [11]. ^{35}S-labeled protein A (278 Ci/mmol) was obtained from Amersham.

2.4. *In vitro translocation reactions*

In vitro translocation of prePhoE was basically carried out as described [8]. [^{35}S]prePhoE in 8 M urea was diluted 25-fold into a buffer (40 mM Tris-acetate, 4 mM Mg-acetate, 28 mM K-acetate, 2 mM dithiothreitol) supplemented with 4 mM ATP, inner membrane vesi-

cles (0.4 mg protein/ml) and S-135 cell extract or SecA as indicated. After an incubation of 20 min at 37°C, proteinase K (200 μg/ml) was added to degrade all of the non-translocated protein. This incubation was performed during 15 min at 37°C. Phenylmethylsulfonyl fluoride (2 mM) was added to inhibit the protease activity, and the samples were analyzed by SDS-PAGE and autoradiography. Translocation was determined by liquid scintillation counting of the rehydrated excised protein bands (precursor and mature together) of the dried gels. Translocation efficiency is expressed as the amount of translocated proteins relative to the total amount of precursor added per translocation reaction.

2.5. *Determination of SecA and SecB contents*

The contents of SecA in S-135 cell extracts, inner membrane preparations and total cells of strain HDL 11 as well as the SecB contents of HDL 11 S-135 cell extracts were determined by quantitative western blotting. From S-135 cell extracts, inner membrane vesicle preparations or total cell suspensions, 4, 35, or 14 μg protein, respectively, was applied to SDS-PAGE and electrophoretically transferred to nitrocellulose sheets. As standards 10 to 180 ng of purified SecA or SecB was applied. The blots were incubated in Tris-buffered saline (TBS, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl) supplemented with 3% w/v gelatin. After a 10 min wash in TTBS (TBS supplemented with 0.05% Tween 20) the nitrocellulose was incubated for 90 min with a polyclonal rabbit antiserum (directed against either SecA or SecB) which was 750-fold diluted into TBS supplemented with 1% w/v gelatine. The blots were washed for 10 min in TTBS and subsequently incubated with ^{125}I-labeled protein A (1 μCi) in 15 ml of TBS-1% gelatine for 1 h. After a 20 min wash in TTBS the blots were dried and an autoradiograph was made. The parts of the nitrocellulose corresponding to radioactive protein bands were cut out and processed for liquid scintillation counting. The radioactivity and the amount of protein present in the standards were established to have a linear relation, allowing an accurate determination of the SecA and SecB concentrations in the samples (average relative error < 10%).

2.6. *SecA depletion of S-135 cellular extracts*

In order to obtain protein A-Sepharose coupled to antibodies directed against SecA, 10 mg of protein A-Sepharose 4B (Pharmacia) in 360 μl of 30 mM Tris-HCl pH 8.6, 150 mM NaCl was incubated for 1 h at room temperature with 40 μl of anti-SecA antiserum. The suspension was centrifuged for 3 min at 16,000 × *g* and the Sepharose-containing pellet was washed twice with the same buffer used for cell extract preparation. To remove SecA from the extracts, the activated Sepharose was added to 100 μl S-135 extract and incubated for 2 h at room temperature. The suspension was centrifuged for 5 min at 16,000 × *g* and the supernatant was used as SecA-depleted extract after determination of the residual SecA content which amounted less than 0.1 mg/g.

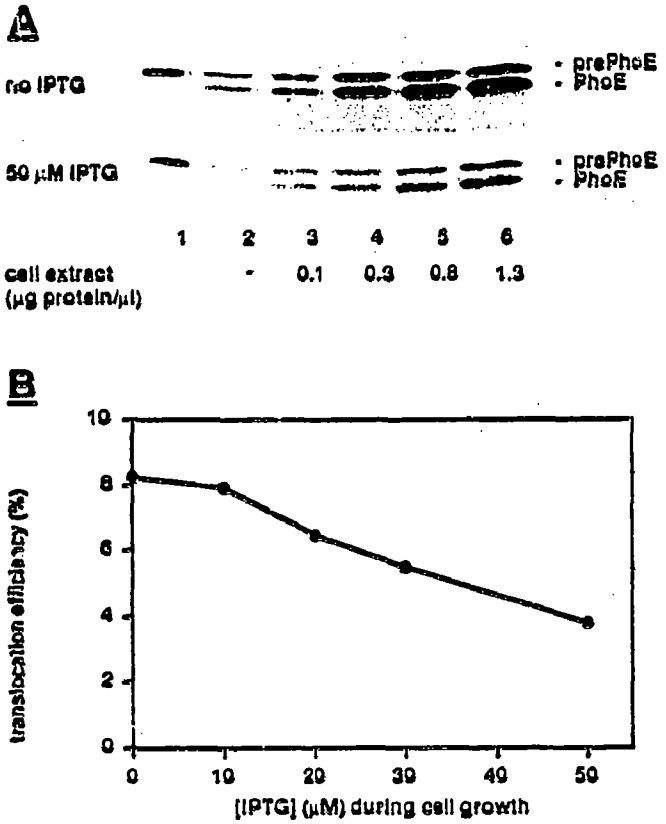

Fig. 1. Enhanced stimulation of prePhoE translocation in the presence of cell extract from cells with low PG contents. Panel A lanes 1 show a [^{35}S]prePhoE reference. Lanes 2–6 show precursor and mature PhoE bands, translocated (protected from proteolytic digestion) across MRE600 inner membrane vesicles in 25 μl in vitro translocation reactions containing increasing amounts of cell extract from HDL11 cells with either low PG contents (upper gel segment, no IPTG) or wild-type levels of PG (lower gel segment, 50 μM IPTG). Panel B, prePhoE translocation was assayed in 25 μl translocation mixtures, containing 0.8 μg/ml cellular extract from HDL11 cells grown at the indicated concentrations of IPTG. Translocation efficiency is expressed as the amount of translocated precursor and mature PhoE bands relative to the total amount of added prePhoE per translocation reaction.

3. RESULTS AND DISCUSSION

3.1. *Extracts from* E. coli *cells with reduced PG content have an increased capacity to support in vitro protein translocation*

S-135 cellular extracts were prepared from *E. coli* HDL 11 grown in the absence or presence of IPTG which induces the synthesis of negatively charged phospholipids in this strain. Different amounts of cell extract from these cells were added to a translocation system basically consisting of purified [^{35}S]prePhoE, inner membrane vesicles from wild-type strain MRE 600 and ATP. Fig. 1, panel A (lanes 2–6) shows that an increase of the concentration of cell extract in the translocation mixture results in enhanced translocation of precursor and mature PhoE protein. Interestingly the extract from cells with low PG content (the upper gel segment, no IPTG) is more powerful in stimulation of translocation than the extract from cells with a wild type phospholipid composition (the lower gel segment, 50 μM IPTG). The translocation efficiency increased from 1.6%, when no extract was added, to 10.3% and 5.0% when cell extracts were added from cells grown without or with 50 μM IPTG, respectively. This result indicates that the extract from cells with low PG contents contains (an elevated level of) a translocation-stimulatory factor and supports the original observation by De Vrije et al. [1] which was made using an in vitro synthesized precursor.

To investigate the relationship between the translocation stimulatory capacity and the synthesis of negatively

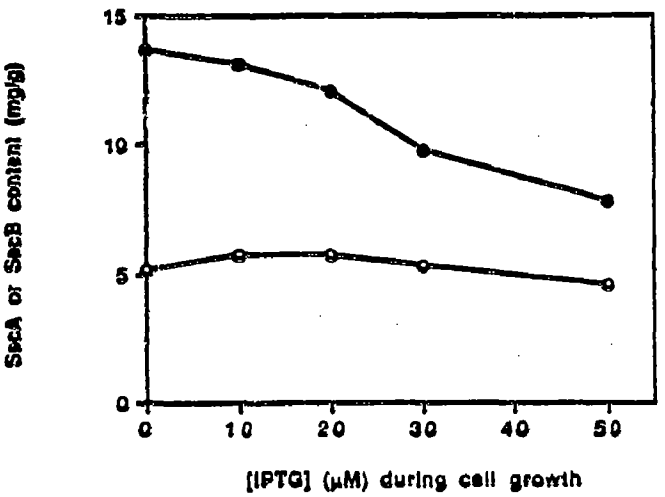

Fig. 2. Quantitative analysis of SecA (●) and SecB (○) contents of cell extracts from strain HDL11 with different levels of PG. S-135 cell extracts from HDL11 cells grown at 0, 10, 20, 30, or 50 μM IPTG were analyzed. SecA and SecB concentrations are expressed in mg per g total protein.

charged phospholipids, cell extracts were prepared from HDL11 cells grown at 5 different concentrations of IPTG. As shown in panel B, the translocation efficiency gradually increased from 3.8 to 8.3% when the used lysates were from cells grown at decreasing concentrations of IPTG (decreasing the level of negatively charged phospholipids). This indicates that a translocation stimulatory factor in the cytosol counteracts the translocation defect caused by low levels of negatively charged phospholipids in the inner membrane.

3.2. *Cells with reduced PG levels have increased cytosolic SecA concentrations*

Next, we attempted to identify the stimulatory factor. As we have reported previously [8], efficient translocation of purified prePhoE can be accomplished in a defined in vitro system containing either an S-135 cellular extract or purified SecA and SecB proteins. Therefore the concentration of these two proteins in the different lysates were determined by means of quantitative Western blotting. Fig. 2 shows that with increasing inducer concentrations the concentration of SecA decreases twofold whereas the SecB concentration in the cellular extract is not significantly affected.

This strongly suggests that SecA causes the stimulation of translocation observed in the translocation experiment shown in Fig. 1, panel B since the SecA concentration in the reaction mixture amounted 108, 103, 95, 76 and 61 nM when extracts from cells grown at 0, 10, 20, 30, or 50 μM IPTG, respectively, were added. These SecA levels fit into the concentration range which has been shown to be effective in the in vitro translocation reaction [8].

3.3. *Increased cytosolic SecA concentrations are responsible for stimulation of translocation*

In order to determine whether the stimulatory effect is indeed due to different SecA concentrations in the cell extracts, we depleted the 5 different extracts for SecA by means of immunoprecipitation and assayed their activity in the in vitro translocation assay. As shown in Fig. 3, after depletion all extracts largely lost their capacity to support translocation (1.7% translocation efficiency at 0 μM additional SecA). That this was the direct result of SecA depletion was demonstrated by addition of purified SecA to the depleted extracts in the translocation mixture resulting in a concentration-dependent increase of translocation which was the same for all of the different extracts, despite their original difference in translocation stimulatory activity. The depletion procedure alone (see section 2) had no effect on the translocation activity of the extracts since after a blank depletion, in which pre-immuneserum was used, the extracts retained full activity in translocation (not shown).

Since it has been reported that SecA has strong and functional interaction with acidic phospholipids [2,9,12,13] we asked whether the elevated SecA levels in the cytosolic fractions from cells with low PG contents are only the result of a shift in cellular localisation, from the inner membrane to the cytoplasm or result from an increase in total SecA production. Therefore we analyzed the SecA contents of inner membranes and complete cells derived from cultures grown in the absence or presence (50 μM) of IPTG. In agreement with localization studies from Cabelli et al. [12], we found a decrease of the SecA content of the inner membrane from 2.7 to 1.3 mg/g as the PG levels decreased. In total cells, however, the SecA concentration was increased from 3.5 to 4.7 mg/g, which indicates that the different SecA levels found in the cytosolic fraction of the cells are due to a change in both cellular localisation and total SecA concentration.

SecA is involved in many different stages of protein export [2,14] and interacts with almost all factors involved in the process [13,15,16]. Several models on the role of SecA in translocation have been proposed [14,17]. It is commonly believed that the major mode of action of SecA is at the plasma membrane. Insertion of SecA into the membrane bilayer has been suggested [17]. Two recent observations support this idea: SecA undergoes a conformational change when interacting with liposomes containing negatively charged phospholipids [18] and is able to insert into a PG monolayer in a nucleotide-modulated manner [9]. In the in vivo situation this insertion could be accompanied by a (partial) movement of the precursor into the membrane.

The synthesis of SecA is supposed to be regulated by autogenous repression; overproduction leads to a reduction of translation [19]. The translation of SecA has

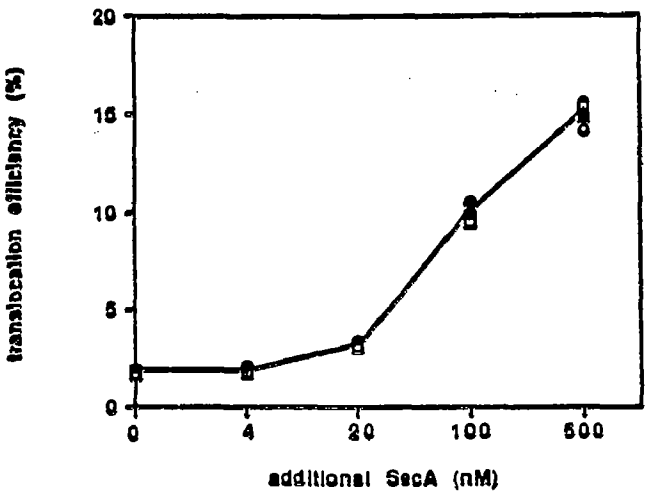

Fig. 3. PrePhoE translocation was assayed in an in vitro translocation reaction which contained (0.8 μg/μl) cell extract from HDL11 cells grown at 0 (●), 10 (○), 20 (■), 30 (□) or 50 μM IPTG (+) which were depleted from SecA, and the indicated concentrations of SecA.

been demonstrated to be coupled to the export status of the cell [20,21]. Translocation blocks resulting from overproduction of an export incompetent precursor [20] or mutations in the genes encoding membrane-embedded components of the translocation machinery (SecY, D and E) [17,21] form a derepression signal for the synthesis of SecA. Remarkably, a translocation defect in the cytosol due to a SecB null mutation does not trigger the SecA production [21]. This suggests that SecA translation is specifically derepressed by export defects located at the membrane [17], which includes low levels of negatively charged phospholipids.

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