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Differential Translocation of Protein Precursors across SecY-Deficient Membranes of *Escherichia coli*: SecY Is Not Obligatorily Required for Translocation of Certain Secretory Proteins In Vitro

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SecY, a component of the protein translocation system in *Escherichia coli*, was depleted at a nonpermissive temperature in a strain which had a temperature-sensitive polar effect on the expression of its *secY*. Membrane vesicles prepared from these cells, when grown at the nonpermissive temperature, contained about 5% SecY and similarly low levels of SecG. As expected, translocation of alkaline phosphatase precursors across these SecY-deficient membranes was severely impaired and appeared to be directly related to the decrease of SecY amounts. However, despite such a dramatic reduction in SecY and SecG levels, these membranes exhibited 50 to 70% of the wild-type translocation activity, including the processing of the signal peptide, of OmpA precursor (proOmpA). This translocation activity in SecY-deficient membranes was still SecA and ATP dependent and was not unique to proOmpA, as lipoprotein and lambda receptor protein precursors were also transported efficiently. Membranes that were reconstituted from these SecY-depleted membranes contained undetectable amounts of SecY yet were also shown to possess substantial translocation activity for proOmpA. These results indicate that the requirement of SecY for translocation is not obligatory for all secretory proteins and may depend on the nature of precursors. Consequently, it is unlikely that SecY is the essential core channel through which all precursors traverse across membranes; rather, SecY probably contributes to efficiency and specificity.

The mechanisms of protein secretion in bacteria have been studied extensively in the last decade. Genetic and physiological studies have established that several gene products of *Escherichia coli*, i.e., SecA, SecB, SecD, SecE, SecF, SecG, and SecY (PrlA), are involved in the signal peptide-dependent secretion pathway (the Sec pathway) for many cytoplasmic membrane proteins, the periplasmic protein, and outer membrane proteins (6, 7, 31, 37, 52, 56, 57). Biochemical studies with in vitro translocation systems (54, 65) have further established a requirement of ATP, which may be enhanced by the proton motive force (14, 15, 67) and have in general provided complementary evidence for the genetic studies (24, 25, 38).

The *secY* gene was originally identified as an allele of the defective signal sequence suppressor gene (*prlA*) (23). This gene codes for SecY, which initially was thought to be involved in some form of signal sequence interaction. Further analysis of several *prlA* suppressors indicated that SecY has a specific interaction with SecE, and together they possess some proofreading function that is important for the protein export process (7, 26, 27, 50). The *secY* gene is located within the *spc* ribosomal protein operon of *E. coli* and encodes a 49-kDa integral membrane protein, which has 10 putative transmembrane segments and both its N and C termini protruding into the cytoplasm (2, 13). The importance of SecY to protein export was also demonstrated by several point mutations in *secY*, leading to conditional translocation defects (4, 58). The conditional temperature defects of these SecY mutants, $SecY24^{ts}$ and $SecY39^{cs}$, have been confirmed in vitro (4, 24), and the SecY24 defect can be suppressed by SecA (25). In addition, the overproduction of PrlA4 relieves the maltose sensitivity caused by a high level of LamB::LacZ fusion proteins (6), suggesting that SecY plays an essential role in protein export. Moreover, SecY is homologous to Sec61, which has been shown to be a component of translocation channels in yeast and mammalian systems (57).

This essential nature of SecY in protein translocation is also supported by in vitro translocation systems involving proteoliposomes that had been reconstituted either by octylglucoside solubilization or by sonication. Use of these proteoliposomes showed active translocation only in the presence of SecYEG (1, 9, 29, 44–46, 62). Extensive work by several groups led to the mechanistic model of protein translocation proposed by Wickner and Leonard (67). The current model depicts the SecYEG complex as the essential translocation core channel in membranes (10, 19, 29), where in the periphery SecA hydrolyzes ATP (40) to insert and deinsert a 30-kDa domain and cycles on and off membranes during translocation (21, 67). The other integral membrane SecDFYajC complex (20, 60) is viewed as stabilizing the SecYEG complex and is involved in peptide release and proton motive force during protein translocation (3, 20, 22, 43). However, the reconstituted membranes that were obtained from cholate-solubilized wild-type membranes (and contained less than 1% SecY) were found to be still active in protein translocation (65, 66), suggesting that SecY may not be essential for protein translocation under these conditions. Moreover, it has also been shown recently that SecE is not obligatorily required for the translocation of certain proteins (68), and SecG is only conditionally required in vivo and in vitro (47). Thus, the roles of SecYEG as the core channel are still not certain.

To help resolve this conflict, we have used another approach

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to obtain SecY-deficient membranes. Native SecY-deficient membrane vesicles were isolated from SecY-depleted cells, which were obtained at a nonpermissive temperature by a polar amber mutation in an upstream ribosomal protein gene. Our data support the notion that SecY may be required for some protein precursors but is not essential for the translocation of a subset of precursor proteins in vitro.

MATERIALS AND METHODS

Strains and media. *E. coli* strains used in this study were the temperature-sensitive *rplO215* mutant KI200 (32), RNase I^- D10 (28), MC4100 (12), and the SecA temperature-sensitive mutant BL15.5 (11). Plasmid pYM140 containing the *lpp* (lipoprotein) gene (61) was used as the source of mRNA of proLpp. Cells were grown in LinA medium (18, 59) supplemented with 0.5% glucose.

Preparation of inverted or reconstituted membrane vesicles. Inverted membranes were prepared by using a French press and sucrose block gradients as described previously (59). SecY-deficient membranes were isolated from KI200 cells after growth ceased at 42°C upon a shift from 30°C, with appropriate dilutions of the same medium. Membrane reconstitution with sodium cholate was carried out as described previously (65), with some modifications. In general, native membranes were dissolved in solubilizing buffer (0.4 M sucrose, 1 M potassium acetate, 20 mM triethanolamine acetate [pH 7.5], 1.5 mM magnesium acetate, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.75% sodium cholate), incubated on ice for 60 min, and centrifuged at 90,000 rpm for 30 min in a Beckman TLA-100.2 rotor. The supernatant was dialyzed at room temperature against TK_{50} buffer (50 mM KCl, 10 mM Tris-HCl [pH 7.6]) for 6 h and then centrifuged as described above. The reconstituted membrane vesicles were resuspended in TK_{50} buffer.

Synthesis of ^{35}S-precursors. An *E. coli* cell-free translation system was used to synthesize ^{35}S-proOmpA and ^{35}S-proLpp as described previously (59). Usually, S30 isolated from D10 was used as the ribosome-containing extract. For SecA-free conditions, S30 isolated from BL15.5 was used (11). The posttranslational mixtures after centrifugation to remove ribosomes and endogenous membrane vesicles were used as the ^{35}S-precursor solution. To obtain purified ^{35}S-proOmpA, ^{35}S-precursor solution was passed through an anti-proOmpA immunoglobulin G-coupled carbonyl-diimidazole-agarose (Pierce) column equilibrated with 50 mM Tris HCl (pH 8.0) and 1 mM dithiothreitol. After being washed with the same buffer, ^{35}S-proOmpA was eluted by 1 M triethylamine (pH 11.7) and neutralized with 1 M KH_2PO_4 to pH 7.6. The syntheses of ^{35}S-proLamB and ^{35}S-proPhoA were carried out by using a coupled transcription-translation system as described previously (68).

In vitro protein translocation. A typical translocation reaction mixture (100 μl) contained 90 μl of ^{35}S-precursor solution, 1 μl of an ATP-regenerating stock (59), and the proper amount of membrane vesicles as indicated. After appropriate incubation, the reaction was terminated by chilling on ice and the reaction mixture was treated with proteinase K (0.1 mg/ml), unless otherwise indicated, on ice for 15 min. The digestion of proteinase K was stopped by addition of 0.8 ml of stop solution (10 mM Tris-HCl [pH 7.6], 0.5 M KCl, 10 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride). Sedimentation of membranes, analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and fluorography all were carried out as described previously (59). The proteinase K-resistant and membrane-cosedimented labeled proteins, both precursor and mature forms, are referred to as translocated proteins. All precursors and translocated proteins in the membrane vesicles are sensitive to proteinase K treatment in the presence of 1% Triton X-100.

Other methods. SDS-PAGE was carried out as described by Laemmli (39). For lipoprotein, a 15% gel containing 7.55 M urea was used as described elsewhere (61). Immunoblot analysis was performed as described previously (68). Typically, 10 μg of membranes was dissolved in 2× sample buffer, incubated at 37°C for 30 min, and then subjected to SDS-PAGE and immunoblotting. Antibodies against SecA, $SecD_{528–548}$, $SecE_{64–92}$, $SecG_{77–99}$, and $SecY_{347–362}$ were purified by using peptide-coupled Reacti-gel as instructed by the manufacturer (Pierce). Anti-$SecF_{222–247}$ antiserum was used directly in immunoblots with 1,000-fold dilution. The relative Sec protein amounts were assayed by immunoblotting with appropriate dilutions as described elsewhere (68). Protein amounts were determined by the Bradford method (8), using a Bio-Rad assay kit. Densitometric quantification was performed with the Protein Databases Inc. (Huntington Station, N.Y.) image analyzing system.

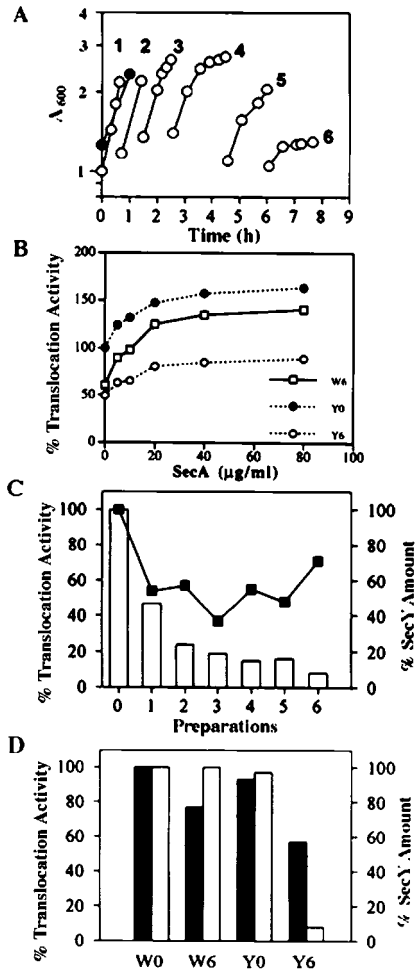

FIG. 1. SecY contents of membranes are not proportional to their translocation activities. (A) KI200 cells were grown at 30°C in LinA medium supplemented with 0.5% glucose. The 30°C culture was inoculated into the same medium and incubated at 30 and 42°C, so that the OD_{600} (optical density at 600 nm) reading reached 1.0. When the OD_{600} reached around 2, the whole 30°C culture was subjected to membrane preparation (close circles). Half of the 42°C culture was diluted with the same volume of 42°C-prewarmed medium and kept at 42°C. The other half was subjected to membrane preparation. These steps were repeated for 42°C culture until cell growth ceased as shown in panel A (open circles). The numbers represent the order of membrane preparations. (B) Wild-type MC4100 membranes were prepared from cells grown at 42°C for 6.5 h (W6). Y6, membranes prepared from 42°C-grown growth-ceased KI200 cells (A, preparation 6); and Y0, membranes from 30°C-grown KI200 cells. Translocation activities of membranes (50 μg/ml) were determined with D10 S30, proOmpA, and varied amounts of purified SecA at 37°C for 5 min as described in Materials and Methods. The translocation activity of Y0 membranes without additional SecA was set at 100%. (C) SecY amounts (open bars) of membranes prepared as for panel A, determined by immunoblotting as described in Materials and Methods and expressed relative to those of Y0 membranes. Translocation activities (closed squares) of these membranes were measured as for panel B, with addition of SecA (20 μg/ml), and were determined relative to activities of Y0 membranes. (D) Translocation activities at 37°C for 5 min (closed bars) and SecY amounts (open bars) of each membrane, determined as described for panels B and C. W0, membranes from MC4100 cells grown at 30°C, the data for which were taken as 100%.

RESULTS

SecY-deficient membranes are partially active in the translocation of proOmpA. *E. coli* KI200 with the *ts215* mutation in an MC4100 background (31, 32) was used to prepare SecY-deficient membrane vesicles. The *ts215* mutant, carrying a temperature-sensitive amber suppressor, has an amber mutation in the gene *rplO*, located upstream of *secY*, and exerts a polar effect on the expression of *secY*. KI200 cells were grown in LinA medium, and membrane vesicles were prepared from each generation after being shifted to 42°C (Fig. 1A). SecY amounts in the membranes prepared from KI200 cells grown at the nonpermissive temperature were decreased by generations, as detected by $SecY_{347–362}$ peptide antibodies (Fig. 1C).

When growth ceased, the membranes from these cells contained only about 5% of the SecY normally present within the membrane. The decreased contents of SecY were also verified by use of SecY C-terminal peptide antibodies. In our hands, it is very difficult to deplete SecY consistently to less than 5%, possibly because the *ts215* mutant has been known to revert easily (31). These SecY-deficient membranes (Y6) were tested for translocation activity with proOmpA to determine if the function of SecY is indeed essential for protein translocation in vitro. Figure 1B shows that the Y6 membranes still retained greater than 60% translocation activity over the wide range of SecA tested compared with membranes prepared from KI200 cells grown at 30°C (Y0). These results are unexpected since the *ts215* mutant has been known to accumulate precursor proteins in vivo at nonpermissive temperatures (31). As a potentially more suitable comparison, wild-type membranes were prepared from MC4100 cells that were grown at 30°C (W0) and then shifted to 42°C for 6.5 h (W6), precisely the same time period as for the Y6 preparation. (Since tetracycline was not added to the media for growth of all strains, MC4100 membranes were used as the wild-type control). The SecY amounts of W0 and W6 membranes were similar to those of Y0 membranes. However, Y6 membranes retained approximately 70% translocation activity compared with W6 membranes (Fig. 1D). Furthermore, even though the amounts of SecY from KI200 continued to decrease after one generation of growth at 42°C, there was no further loss of translocation activity for proOmpA (Fig. 1C). Thus, the OmpA translocation activity is not directly related to the further depletion of SecY within the membrane. Such disproportionalities in SecY amounts and translocation activities indicate strongly that SecY is not obligatorily required for OmpA translocation in vitro.

Characterization of translocation activity of SecY-deficient membranes. To gain some insight into the nature of SecY-deficient membranes, we examined the time course of translocation of proOmpA. Kinetic analysis at 30°C showed that the translocation rates of both SecY-containing (30°C-grown) and SecY-deficient membranes were constant up to 30 min (Fig. 2A). The SecY-deficient membranes retained around 60% translocation activity in comparison to the control membranes. Compared with wild-type W6 membranes that were prepared from cells grown at 42°C (Fig. 2B), SecY-deficient membranes retained almost 90% of their activity throughout the first 5 min of translocation at 37°C.

To investigate if the translocation of proOmpA across SecY-deficient membranes was still SecA and ATP dependent, purified nascent ^{35}S-proOmpA, which had been obtained from the translation mixture by passage through an antibody-affinity column (59), was used in the in vitro translocation assay. Figure 3A shows that translocations across both SecY-containing and SecY-deficient membranes were abolished in the absence of ATP (lanes 3 and 4), demonstrating the dependence on ATP in translocation for both membranes. Without the addition of exogenous SecA, both membranes showed weak translocation activities (Fig. 3A, lanes 2 and 5; 16 and 3%, respectively, relative to lane 4 with SecA), which were enhanced by the addition of SecA (lanes 4 and 7; 6- and 12-fold increases, respectively), reflecting the activity of membrane-bound SecA and the dependence of the whole process on SecA. Moreover, the SecY-deficient membranes were still capable of processing the protein precursors into their mature forms, like normal SecY-containing membranes (Fig. 3A, lanes 4 and 7). The SecA dependence of translocation across SecY-deficient membranes was further verified by the inhibition by azide (Fig. 3B). Like SecE-depleted membranes (68), SecY-deficient membranes were more sensitive to freeze-thawing and sodium azide than SecY-containing membranes. The 50% inhibitory concentrations of azide for SecY-deficient and SecY-containing membranes were 1.8 and 3.4 mM, respectively.

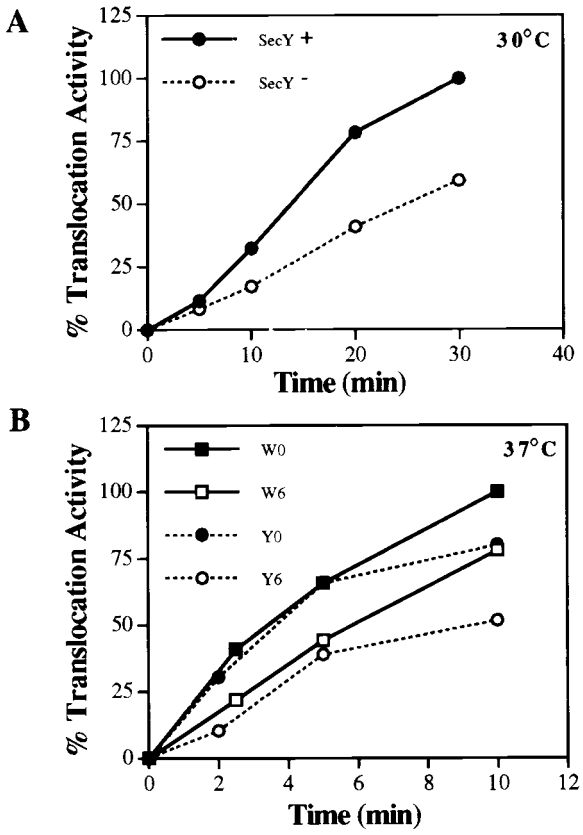

FIG. 2. Kinetics of translocation of proOmpA. (A) The translocation of proOmpA was carried out at 30°C for times indicated with SecA (20 μg/ml) and membranes (150 μg/ml) prepared from KI200 cells grown at 30°C (SecY$^+$) or 42°C growth-ceased cells (SecY$^-$). The translocation activity of SecY$^+$ membranes at 30 min was taken as 100%. (B) Translocation of proOmpA with membranes (50 μg/ml) and SecA (20 μg/ml) was carried out at 37°C for the indicated times. W0, W6, Y0, and Y6 represented the same membranes as described for Fig. 2. The translocation activity of W0 membranes at 10 min was taken as 100%.

Time kinetics data (Fig. 2) suggested that the amount of nascent precursors is not limiting for translocation. To address this point further, we carried out two types of saturation experiments as described in more detail elsewhere (68). First, over the fivefold range of amounts (50 to 250 μg/ml) of membranes used, the relative activity of SecY-containing and SecY-deficient membranes did not change significantly (data not shown). Second, the translocation capacity of SecY-deficient membranes was also determined by addition of a large amount of purified unlabeled (cold) competent proOmpA to compete newly translated ^{35}S-proOmpA. As shown in Fig. 3C, SecY-deficient membranes required 33 μg of cold proOmpA per ml to obtain 50% inhibition, in contrast to the 80, 69, and 62 μg/ml needed for similar inhibition of the W0, W6, and Y0 membranes, respectively. This finding indicates that the SecY-deficient membranes had a translocation capacity that was at least 41% of that of their SecY-containing counterparts and that the amounts of precursors used in the translocation reaction were not limiting. Moreover, levels of binding of proOmpA to SecY-deficient membranes were comparable to those of SecY-con-

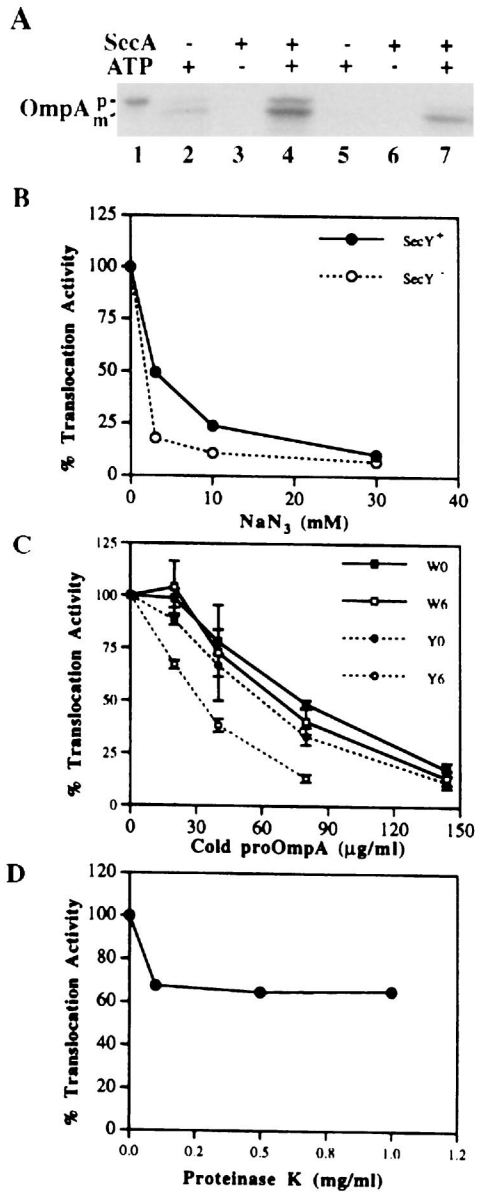

FIG. 3. Characterization of SecY-deficient membranes. (A) Translocation of purified nascent ^{35}S-proOmpA (lane 1) was carried out at 37°C for 15 min with membranes (300 μg/ml) used for Fig. 2A. SecA (10 μg/ml) and/or ATP (1 mM) were also added during translocation as indicated. Lane 1, 10% of total input precursors; lanes 2 to 4, SecY$^+$ membranes; lanes 5 to 7, SecY$^-$ membranes. See text for details. (B) Translocation of proOmpA was carried out at 37°C for 10 min with membranes (50 μg/ml) used for Fig. 2B and SecA (20 μg/ml) in the presence of different amounts of sodium azide as indicated. The activity without sodium azide was taken as 100% for each membrane preparation. Data are averages of two independent experiments. (C) Translocation of proOmpA was performed at 37°C for 5 min with membranes (50 μg/ml) used for Fig. 2B and SecA (20 μg/ml) in the presence of the indicated amounts of purified cold proOmpA. The activity without cold proOmpA was taken as 100% for each membrane preparation. Data are shown as means ± standard errors from three independent experiments. The error bars at 40 μg of proOmpA per ml were 5.9, 22.8, and 5.4% for W0, W6, and Y0 membranes, respectively. (D) Translocation of proOmpA was performed with 100 μg of SecY-deficient membranes per ml at 30°C for 30 min, and then the samples were treated with different concentrations of proteinase K on ice for 15 min. The OmpA and its precursor amounts without proteinase K were taken as 100%. p, precursor; m, mature form.

TABLE 1. Comparison of Sec protein amounts in SecY$^+$ and SecY$^-$ membranes

Membranes[a]	Relative level[b]					
	SecA	SecD	SecE	SecF	SecG	SecY
SecY$^+$	202 ± 39	103 ± 32	90 ± 13	107 ± 4	87 ± 6	85 ± 12
SecY$^-$	520 ± 72	86 ± 2	24 ± 7	24 ± 7	6 ± 0	5.5 ± 0.3

[a] SecY$^+$ and SecY$^-$ are membranes from KI200 cells grown at 30 and 42°C, respectively.

[b] For immunoblots of various Sec proteins, appropriate dilutions of membrane samples were carried out to obtain comparable levels for all tested proteins. Data are presented as means ± standard errors of four independent experiments except those for SecG, which are the averages of two experiments, and are relative to levels for MC4100 membranes grown at 37°C, which were set as 100%.

taining membranes (data not shown). To rule out the possibility that the apparent translocation was due to an insufficient amount of proteinase K added to the in vitro system, titration of proteinase K resistance was performed for each translocation assay. The OmpA amount that was resistant to proteinase K in the SecY-deficient membranes did not change significantly in the presence of proteinase K concentrations ranging from 0.1 to 1.0 mg/ml during the in vitro translocation assay (Fig. 3D). Similar results were obtained with wild-type membranes (data not shown). These experiments showed that SecY-deficient and SecY-containing membranes had similar translocation characteristics.

Effect of depletion of SecY on other Sec protein contents. SecY has been shown to interact with SecE and SecG (4, 7, 9, 10, 26, 27, 29) and to be stabilized by SecE and SecF (42, 55). An interaction between SecY, SecD, and SecF has also been reported (60). Moreover, depletion of SecE causes decreases of SecD, SecF, SecG, and SecY (68). Since these integral membrane Sec proteins all interact, SecY-deficient membranes were assayed for the presence of these other Sec proteins by immunoblotting. As expected, the depletion of SecY decreased the amounts of SecE and SecF to around 24% and of SecG to 6% (relative to wild-type MC4100 membranes) but maintained the SecD content at around 86% and increased the SecA content approximately fivefold (Table 1). Thus, SecY-deficient membranes still contained most of SecD and one-quarter of the amount of other Sec proteins (except for SecG), which might account for their ability to retain their translocation potential. It is interesting that SecY$^+$ membranes from KI200 cells, grown at 30°C, contained levels of SecA that were similar to those of MC4100 wild-type membranes from cells grown at 30°C but were twofold higher than levels in those grown at 37°C. Levels of Sec proteins other than SecA, however, remained constant, indicating that the amounts of other Sec proteins within the membranes of wild-type cells are independent of growth at 30 or 37°C. Thus, the twofold increase in the concentration of SecA in SecY$^+$ membranes is due to the growth of the cells at 30°C, although it is not clear why this is so.

Translocation of proLpp and proLamB across SecY-deficient membranes. To clarify whether the active translocation across SecY-deficient membranes was specific only to proOmpA transport, we also examined the effects of SecY deficiency on translocation of other secretory proteins. The translocation activity of SecY-deficient membranes for proLpp was found to be greater than 66% at 20 min of translocation at 30°C (Fig. 4A). For precursors of LamB, about 60% translocation activity was obtained after translocation for 5 min at 37°C in the presence or absence of SecB (Fig. 4B). Hence, the

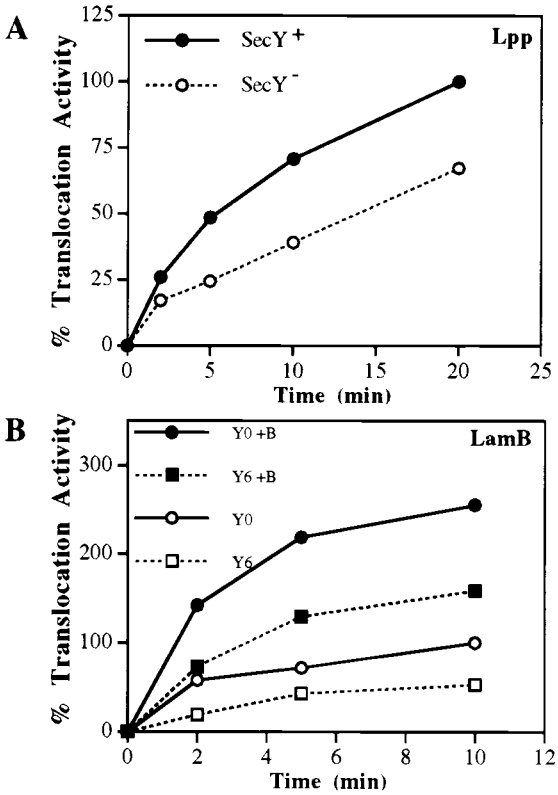

FIG. 4. Kinetics of active translocation of proLpp and proLamB across SecY-deficient membranes. (A) Translocation of proLpp was carried out at 30°C with membranes (100 μg/ml) prepared from 30°C (SecY$^+$)- and 42°C (SecY$^-$)-grown KI200 cells. The translocation activity of SecY$^+$ membranes at 20 min was taken as 100%. (B) Translocation of proLamB with membranes (50 μg/ml) used for Fig. 3B and SecA (20 μg/ml) was performed at 37°C in the presence (+B) or absence of SecB (10 μg/ml). Translocation activity of Y0 membranes at 10 min without SecB was taken as 100%.

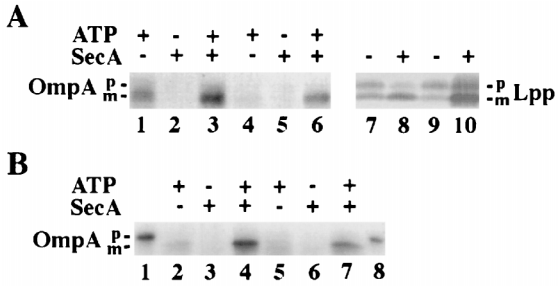

FIG. 5. Reconstituted membranes are active in translocation of proOmpA and proLpp. (A) D10 membranes were treated with cholate and then reconstituted to SecY-depleted vesicles (lanes 4 to 8) as described in Materials and Methods. The SecY-containing D10 membranes (lanes 1 to 3, 9 and 10) were treated in the same way without cholate. Translocation of proLpp with membranes (150 μg/ml) was carried out at 30°C for 30 min with or without SecA (10 μg/ml) as indicated (lanes 7 to 10). Translocation of purified ^{35}S-proOmpA was carried out with membranes (300 μg/ml) at 37°C for 15 min with or without SecA (10 μg/ml) and ATP (1 mM) as required (lanes 1 to 6). (B) SecY$^+$ (lanes 2 to 4) and SecY$^-$ (lanes 5 to 7) membranes used for Fig. 4A were reconstituted as described above. Translocation was carried out as described for Fig. 3A. Lanes 1 and 8, affinity column-purified proOmpA only, 10% of input. p, precursor; m, mature form.

active translocation of SecY-deficient membrane vesicles is not unique to transport of proOmpA across the membrane.

Translocation with SecY-deficient reconstituted membranes. It was previously reported that membrane vesicles reconstituted from cholate-solubilized membranes contained less than 1% SecY yet retained substantial translocation activity (65, 66). Using the same reconstitution method, we confirmed that the SecY content was reduced to less than 1% in the membranes reconstituted from membranes of the routinely used wild-type strain D10. We also found that these membranes had more than 60 and 45% of the activities of SecY$^+$ membranes in translocation of proOmpA and proLpp, respectively, a process that included the processing of their signal peptides (Fig. 5A, lanes 6 and 10). Furthermore, these translocations were verified to be also SecA and ATP dependent (Fig. 5A).

To deplete SecY to a negligible amount, SecY-depleted membranes were subjected to the same reconstitution after cholate solubilization. As expected, these reconstituted SecY-deficient membranes contained undetectable levels of SecY ($<<$1%, either full size or fragment) yet still retained more than 70% of proOmpA translocation activity (Fig. 5B, lanes 4 and 7), which again showed a requirement for SecA and ATP (Fig. 5B). Collectively, these reconstituted membranes were still substantially active in translocation of proOmpA and proLpp, even though they contained no detectable SecY, and had the ability to process both precursors to their mature forms.

Translocation of PhoA is directly related to the SecY amount. The translocation of proPhoA has been shown to be more sensitive than that of proOmpA to chemical disturbance (54) and was therefore examined in SecY-deficient membranes. Indeed, SecY-deficient membranes were severely defective in the translocation of proPhoA. These membranes showed only 14% translocation activity after translocation for 5 min (Fig. 6A). To characterize further the relationship of SecY amounts to PhoA translocation, different preparations of membranes were used after levels of SecY had been decreased by dilution of cell growth at 42°C. In marked contrast to the translocation of proOmpA (Fig. 1C), the translocation activities of proPhoA corresponded closely to the amounts of SecY in the membrane (Fig. 6B). These results are different from those obtained for OmpA, LamB, and Lpp precursor transport, indicating that the requirement of SecY for translocation can vary for export of different proteins.

DISCUSSION

As mentioned above, SecY is believed to be essential for protein export in *E. coli*, as defined by extensive genetic studies on *prlA* suppressors (6, 7, 23, 26, 27, 50) and *secY* mutants (4, 24, 58, 60) and vast biochemical experiments with reconstituted membranes (1, 9, 29, 44–46, 62). Consequently, SecY has generally been envisioned as the channel through which protein precursors are required to traverse (67). The importance of SecY was further supported by its evolutionary conservation: homologs of SecY have been found to be widespread in nature, being present in archaebacteria, eubacteria, plants, yeasts, and mammalian cells (53, 57, 63). However, early reports that membranes reconstituted from cholate-dissolved native membranes were SecY deficient (containing less than 1% SecY) yet active in translocation of a mutant form of the maltose binding protein precursor (65, 66) began to raise questions as to the nature of SecY in protein transport, even though that work has been generally ignored (67). We have shown here that inverted, native membrane vesicles containing about 5% of the normal levels of SecY exhibit reduced but still considerable translocation activities (around 50%) for proOmpA, proLpp,

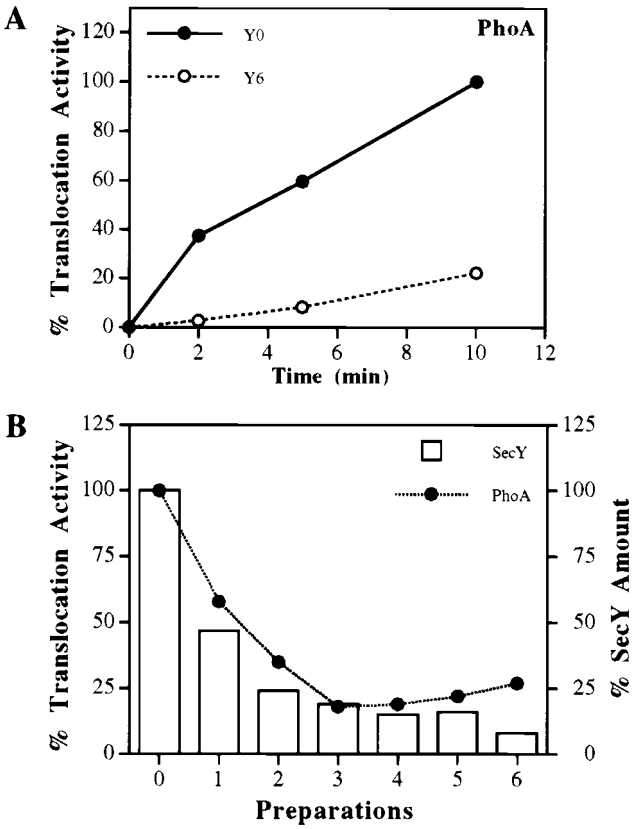

FIG. 6. SecY-dependent translocation of PhoA. (A) The translocation kinetics of proPhoA was assayed as for Fig. 4B. The translocation activity of Y0 membranes at 10 min was taken as 100%. (B) Relative translocation activities for PhoA at 37°C for 5 min and SecY amounts of different membranes used for Fig. 1 were determined as for panel A and Fig. 1C, respectively. Data are averages of two independent experiments.

and proLamB. Furthermore, we have also demonstrated that membrane vesicles reconstituted from these SecY-deficient membranes, which contain no detectable SecY (theoretically 1% of 5%), also show significant translocation activities for proOmpA and proLpp. Consequently, it is unlikely that the residual SecY in these SecY-deficient membranes is sufficient for the translocation activity observed, since the kinetics of translocation in the SecY-deficient and the SecY-containing membranes are similar and the retention of translocation activity is not related to any additional decrease in the amount of SecY (Fig. 1C). Such findings would argue strongly against the view that SecY is essential for translocation, at least for a subset of secretory proteins in vitro.

Notwithstanding these results, we did find that the translocation of proPhoA across SecY-deficient membranes is severely impaired, and the decrease in their translocation activity corresponds closely to the decrease of SecY amounts. Such findings suggest that a SecY dependence does exist for the translocation of this and possibly other proteins. For these proteins, SecY would be essential for their translocation. Even though no null mutant of *secY* has been reported, SecY is believed to be essential for cell growth since temperature-sensitive and cold-sensitive *secY* mutants have been found (4, 58) and the growth of KI200 cells, harboring a mutation that imposes a temperature sensitivity to the expression of SecY, ceases after a shift to the nonpermissive temperature (32). The apparent dependence on SecY for the translocation of proteins such as PhoA (and potentially other essential proteins) may explain this essential nature of SecY for cell growth, as revealed by extensive genetic studies (6, 7, 23). However, it is important to note that at nonpermissive temperatures when SecY24 is degraded (34), substantial translocation of OmpA and other protein precursors takes place, albeit less efficiently (34, 58), and the defect can be suppressed by SecA in vitro (24, 25). The results presented here show that SecY may be essential for the translocation of some, but not all, proteins. In this regard, one of the more compelling arguments for the indispensability of SecY within the Sec system is its high evolutionary conservation among a large number of diverse organisms (57). However, the bacterial signal recognition particle (each component, Ffh, FtsY, and 4.5S RNA, is essential for cell growth) is required for some targeting receptors, but is not used by all (64), and can replace the mammalian signal recognition particle for in vitro translocation (51). Even so, like SecY, each component is conserved throughout nature and is essential for the translocation in higher eukaryotic systems (57). On the other hand, SecA is essential for Sec-dependent protein translocation in bacteria (40), yet it has not been so readily conserved in the translocation system of higher organisms, even though the use of ATP hydrolysis as an energy source for translocation is retained (40, 57). Thus, our data do not necessarily conflict with the finding from in vivo genetic studies that SecY is essential for cell growth.

There are at least two alternative possibilities that may account for the partially active translocation of OmpA, LamB, and Lpp across SecY-deficient membranes. As discussed earlier (68), one possibility is that some other non-Sec proteins replace the function of SecY as an alternative bypass, such as PspA, a 26-kDa peripheral inner membrane protein, facilitates protein translocation by maintaining the proton motive force (36). However, it is unclear whether these proteins play any role, important or otherwise, in the actual translocation of proteins across SecY-deficient membranes. In addition, it would also be difficult to explain why the same membranes are not active for PhoA translocation. An alternative possibility is that some secretory proteins, by their nature, are less dependent on SecY for their passage through the membrane. Consequently, the residual Sec proteins that are present within the SecY-deficient membranes (which include almost 90% of SecD and an increased SecA complement) are somehow sufficient for translocation of these apparently SecY-independent protein precursors. Similar results have been obtained with SecE-depleted membranes, in which SecD, SecF, SecG, and SecY are all decreased to about 20% of their normal concentration and SecA is again increased, this time 16-fold (68). Moreover, these SecE-deficient membranes (which contain ~2% of the normal amounts of SecE) are also found to be still substantially active in the translocation of OmpA, LamB, and Lpp precursors. Considering the similarities between the results that we have obtained previously with the SecE-depleted membranes (68) and those with the SecY-depleted membranes that we have defined in this report, it is unlikely that SecYEG is the essential core channel through which all proteins traverse membranes. Moreover, the bindings of proOmpA to SecY-deficient and SecY-containing membranes are comparable (data not shown), indicating that binding of precursors to SecY is not an essential event, even though their interaction has been shown by cross-linking (33). It is important to note that in all of these deficient membranes, there are substantial amounts of other Sec proteins. Indeed, antibodies against some Sec proteins, but not SecY, were found to inhibit the translocation across SecY-deficient membranes (unpublished data), indicating the importance of the residual Sec proteins.

Integral SecA has been shown to be the active form for translocation (35, 66), and recent evidence indicates that some SecA may be permanently embedded within the membrane (16). Moreover, a membrane-specific 48-kDa fragment has been proposed to be part of the channel (17). Consequently, it is highly likely that SecA plays an important structural role within the translocation channel, beyond its established role as the ATP-dependent driving force of transport process. We propose, therefore, that SecA is the only indispensable component of the protein translocase and itself is the essential core channel, even though SecA alone may not be sufficient (16, 17, 66). The other Sec components, as a group, need only confer speed and efficiency to the process. It is worth emphasizing that in this context, individual components of the SecYEG complex may not be essential but can still play important and significant roles in protein translocation, much like the roles of the proton motive force in protein translocation (3, 49) and the roles of H^+-ATPase in generating a proton motive force or ATP synthesis (30, 41). In this view, the SecYEG complex, like the proton motive force, mechanistically is not essential for protein translocation but functionally is important in greatly enhancing the efficiency of the process.

The differential dependence of SecE (68) and SecY reported here differs from, but does not necessarily contradict, the prevailing views formulated by the extensive reconstitution studies which show essential dependence on SecYEG for translocation in proteoliposome systems (67). It is important to note that in such proteoliposome systems with purified components, only 15% of SecYEG is active and each active SecYEG site is only about 25% as active as native membrane as detected with proOmpA, and there is no signal peptide processing (5, 9). In comparison, more than 50% translocation activity of proOmpA with signal peptide processing (see also reference 65) was observed in the SecY (and SecE)-deficient membranes, despite the observations that SecG is virtually absent and the SecE content is only 24% in SecY-deficient membranes. Thus, the SecY-deficient (and SecE-deficient) membranes are as active, in kinetics and in extent of translocation, as SecYEG proteoliposomes for proOmpA translocation, if not more so. It is worth noting that the translocation of the proteins that have more stringent requirements, such as PhoA, may well require more than SecYEG in the proteoliposomes for activity (which has not been tested yet), as in the case of SecY-deficient membranes, while the less demanding proteins, such as proOmpA, may function in either of the less efficient translocation systems. Further work is needed to clarify this issue.

With regard to the SecYEG complex, while it is known that SecY, SecE, and SecG form the integral complex in stoichiometric amounts within the inner membrane of *E. coli* (19), it has been reported that there is a preferential interaction of SecG with SecE (48). In our work, however, it is only SecG, not SecE, that is reduced to levels comparable to those of SecY in the SecY-deficient membranes. The SecE concentration mirrors that of SecF, approximately 25%. In addition, the amounts of SecG and SecY are also found to be decreased in similar amounts (approximately 20%) in the SecE-depleted membranes, not nearly as low as the 2% levels for SecE (68). These data suggest that SecG may actually have a closer relationship with SecY within the inner membrane of *E. coli* than does SecE. Strains of *E. coli* which harbor a deletion within the *secG* locus have been shown to be cold sensitive (47). SecY-deficient membranes have considerably less SecG. However, only some preparations of these membranes, in our hands, demonstrated any cold sensitivity with respect to protein translocation in vitro. This finding might indicate that the cold-sensitive translocation that had been inferred for SecG deletion mutations (47) was more a function of the different cell physiological conditions under which the membranes were assembled than a consequence of any cold-sensitive deficiency in translocation.

It has been reported that SecYEG is the high-affinity receptor for SecA (19). However, in this and earlier studies, depletions of SecE (68) and SecY (65, 66), and depletions of both SecY and SecG, had little major effect on the transport of particular proteins across the membranes. On the contrary, such depletions have been shown to increase the amounts of SecA found in these membrane fractions. Consequently, the nature of SecA and SecYEG interactions requires further investigation.

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