Biochimica et Biophysica Acta, 1065 (1991) 89–97

ADONIS 000527369100226C

BBAMEM 75248

SecY is an indispensable component of the protein secretory machinery of *Escherichia coli*

Ken-ichi Nishiyama, Yukihito Kabuyama, Jiro Akimaru, Shin-ichi Matsuyama, Hajime Tokuda and Shoji Mizushima

Institute of Applied Microbiology, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo (Japan)

(Received 16 January 1991)

Key words: SecY; Protein secretion; Reconstitution; OmpT; Anti-SecY antibody

Using a reconstitution system for protein translocation, the involvement of SecY in the translocation of secretory proteins across the cytoplasmic membrane of *Escherichia coli* was studied. Anti-SecY antibodies raised against the N- and C-terminal sequences prevented the functional reconstitution of the translocation system. Depletion of SecY from the solubilized membrane preparation was performed by treatment with anti-SecY IgG, followed by removal of IgG with protein A-agarose. The SecY-depleted preparation was inactive as to functional reconstitution. However, reconstitution with it was demonstrated in the presence of a protein fraction, which was released from the anti-SecY immunoprecipitate upon addition of the SecY fragment used to raise the antibody. Reconstitution with the SecY-depleted membrane fraction was also demonstrated in the presence of a purified SecY preparation. OmpT proteinase specifically cleaved SecY in the solubilized membrane preparation. The cleavage was accompanied by a decrease in the reconstituted activity. Based on these findings we conclude that SecY is an indispensable component of the secretory machinery.

Introduction

Several genes controlling protein translocation across the cytoplasmic membrane of *Escherichia coli* have been identified by means of genetic approaches [1–3]. They are the *secA* [4], *secB* [5], *secD* [3], *secE* [6], *secF* [3] and *secY* [7,8] genes. Of these genes, the *secD, secE, secF* and *secY* ones code for integral membrane proteins. Although extensive biochemical studies have been performed as to the functions of SecA [9] and SecB [10], both of which can be easily purified as soluble forms from overproducing cells, little is known about the functions of other Sec proteins. This is most likely due to that they are membrane proteins, and thus are hard to overproduce and purify.

The *secY* (or *prlA*) gene was identified as a temperature-sensitive mutations, which results in accumulation of presecretory proteins at a nonpermissive temperature [7,11]. The $secY^{ts}$ phenotype was also demonstrated *in vitro* [12]. This phenotype is suppressed by SecA in a cell-free *in vitro* translocation system, suggesting direct interaction between SecA and SecY [13]. A topological model of SecY in the cytoplasmic membrane was proposed [14]. Site-specific antibodies raised against synthetic peptides corresponding to the N- and C-terminal regions of SecY, which are exposed to the cytoplasmic surface of the membrane in the topological model, inhibited protein translocation into everted membrane vesicles [15]. Although these results suggest that SecY plays an important role in the protein translocation reaction, there is little direct biochemical evidence of it.

For biochemical studies, several groups recently proposed reconstitution systems for protein translocation [16–18]. Watanabe et al. [16] reported that presecretory proteins were efficiently translocated into SecY-deficient proteoliposomes, whereas two other groups [17,18] reported that the translocation into reconstituted proteoliposomes was markedly inhibited by anti-SecY IgG. More recently, Brundage et al. [19] isolated a fraction which is mainly composed of SecY, SecE and an uncharacterized protein. Although proteoliposomes reconstituted with this fraction exhibited translocation activity, it was unclear whether SecY directly par-

Abbreviations: SDS, sodium dodecyl sulfate; octyl glucoside, *n*-octyl β-D-glucopyranoside; sarcosyl, *N*-lauroylsarcosine sodium salt.

Correspondence: S. Mizushima, Institute of Applied Microbiology, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan.

ticipated in the translocation reaction or not. Thus, the essentiality of SecY for the translocation reaction is still a subject of controversy.

In the present work, using a reconstitution system, the involvement of SecY in the translocation of secretory protein was studied extensively. Anti-SecY antibodies, the synthetic SecY fragments used to raise these antibodies, proteinase OmpT, which specifically digests SecY, and partially purified SecY were effectively used in the reconstitution studies. The results obtained strongly support the view that SecY is an indispensable component of the translcoation machinery.

Materials and Methods

Bacterial strains and plasmids

Escherichia coli K003 (*lpp*$^-$, Δ*uncB-C*-Tn10) [20] was used as the source of everted membrane vesicles. *E. coli* RR1 harboring plasmid pMAN400 [21] which carries the high expression *secA* gene, was used as the source for SecA purification and reconstitution of proteoliposomes. *E. coli* W3110 and W3110 M25 (*ompT*) [22] were also used for reconstitution studies. *E. coli* W3110 M25 harboring pMAN809 and pMAN510 [23], which overproduces SecY and SecE, was also used. *E. coli* W3110 M25 harboring plasmid ptrp-GP, which carries the high expression *ompT* gene, was kindly supplied by K. Sugimura, Suntory Institute for Biomedical Research, and used for the preparation of OmpT proteinase. Plasmids pK127 [24] and pOAD26 [25] encode the genes for uncleavable OmpF-Lpp and proOmpA D26, respectively. These genes are under the control of the SP6 promoter.

Preparation of membrane vesicles, phospholipids and SecA

The everted membrane vesicles used for the *in vitro* translocation assay were prepared from *E. coli* K003 cells as described previously [26]. Membrane vesicles used for solubilization, fractionation and reconstitution of translocation components were prepared from cells of *E. coli* RR1 harboring pMAN400, W3110, W3110 M25 or W3110 M25 harboring pMAN809 and pMAN510, as described [18]. *E. coli* phospholipids were prepared as described [18]. SecA was purified from SecA-overproducing cells as described [27].

Preparation of site-specific antibodies against SecY

Five antibodies, which were denoted as antibodies SecY-1, SecY-2, SecY-3, SecY-4 and SecY-5, were raised against five synthetic peptides corresponding to the Met1–Arg22, Ala103–Arg121, Gly198–His216, Asn 338–Ala351 and Ser426–Arg443 regions of the SecY molecule [28], respectively, as described [18]. These peptides were designated as peptides 1 through 5, respectively. IgGs were isolated from these antisera on a protein A-agarose column as described [18]. Fabs were prepared, on a protein A-affinity column, from these IgGs after digestion with immobilized papain. The IgGs and Fabs, thus prepared, were dialysed against 50 mM potassium phosphate (pH 7.5), and then concentrated to 80 mg/ml by means of gel filtration.

Reconstitution of the protein translocation system

Solubilization of membrane vesicles with octyl glucoside and reconstitution of the protein translocation system were carried out as described previously [18]. The solubilization and reconstitution were also carried out in the presence of 150 mM NaCl in some experiments.

Assaying of protein translocation activity

Uncleavable OmpF-Lpp [24] and proOmpA D26 [25], both of which were synthesized *in vitro* in the presence of Tran^{35}S-label [20], were used. Uncleavable OmpF-Lpp is a model secretory protein, which is composed of proOmpF and the major lipoprotein (Lpp) with an uncleavable signal peptide and proOmpA D26 is a derivative of proOmpA lacking 250 amino acid residues at its C-terminus. The translocation into everted membrane vesicles was performed as described [26] with the following modifications. Everted membrane vesicles (2.5 μg in 5 μl) prepared from K003 cells were incubated on ice with 11 μl of an anti-SecY antibody. ATP and succinate were then added to final concentrations of 2 and 5 mM, respectively. After preincubation for 2 min at 37°C, a precursor protein (about 100000 cpm/assay) was added to initiate the reaction. The total volume of the reaction mixture was 25 μl. After 1 min, the reaction mixture was treated with 25 μg of proteinase K on ice for 20 min. The translocation into reconstituted proteoliposomes was carried out as described [18]. In both cases, the translocated proteins were detected as proteinase K-resistant radioactive bands on an SDS-polyacrylamide gel by means of fluorography as described [20]. Densitometric quantification of the bands was carried out with a Shimadzu CS-930 chromatoscanner.

SecY-depletion from the solubilized membrane

Membrane vesicles were solubilized with octyl glucoside as described [18]. An IgG was then added to the solubilized membrane (800 μg/200 μl) to a concentration of 45 μM (7.5 mg/ml). The mixture was incubated on ice for 20 min and subsequently mixed with 200 μl of protein A-agarose pretreated with 50 mM potassium phosphate (pH 7.5), 1.25% octyl glucoside. After 10-min shaking at 4°C, the mixture was centrifuged at 10000 × *g* for 5 min to remove the resin. The resultant supernatant was then used for the reconstitution of proteoliposomes.

For the preparation of a large amount of the SecY-depleted membrane fraction, 500 μl of the solubilized membrane was incubated with an IgG at the concentration of 60 μM (10 mg/ml) at room temperature for 30

min and then applied to a protein A-agarose column (1 ml) which had been equilibrated with 50 mM potassium phosphate (pH 7.5), 150 mM NaCl, 2.5 mg/ml *E. coli* phospholipids, 1.25% octyl glucoside. The column was then incubated for 30 min at room temperature and eluted with the buffer used for the column equilibration. The pass-through fraction (1.5 ml) was collected and glycerol was added to it to the final concentrations of 10% (v/v). The fraction was divided, frozen and then stored at −80°C until use.

Preparation of the 'peptide 1-released' fraction

IgG SecY-1 (2.3 mg) was added to the solubilized membrane (300 μl). After 20-min incubation on ice, the solution was mixed with 200 μl of protein A-agarose equilibrated with 50 mM potassium phosphate (pH 7.5), 1.25% octyl glucoside. The resin was then recovered by centrifugation at $10000 \times g$ for 5 min, washed three times with an equal volume of 50 mM potassium phosphate (pH 7.5), 1.25% octyl glucoside, and then treated with 60 μl of peptide 1 in 50 mM potassium phosphate (pH 7.5), 1.25% octyl glucoside. The final concentration of peptide 1 was 100 μM. The mixture was shaken for 30 min at 4°C and then the supernatant, designated as the peptide-1 released fraction, was obtained by centrifugation.

Purification of SecY

Cytoplasmic membrane vesicles were prepared from SecY/SecE-overproducing cells (W3110 M25/pMAN 809 and pMAN510), solubilized with octyl glucoside and then subjected to Mono S column chromatography. By means of immunoblot analysis, a SecY-rich and SecE-deficient fraction was detected and pooled. The procedure will be described in detail elsewhere. The buffer composition for the final preparation was 50 mM potassium phosphate, pH 7.5, 150 mM NaCl, 2.5% octyl glucoside, 10% glycerol. This preparation is called hereafter purified SecY.

Purification of OmpT proteinase

Everted membrane vesicles prepared from OmpT-overproducing cells were washed twice with 0.1% sarcosyl as described [29]. OmpT was then extracted from the washed membranes with 1.25% octyl glucoside in 50 mM Tris-HCl (pH 7.5), containing 5 mM ethylenediaminetetraacetic acid and 10% glycerol. The purity of the final preparation was 60%.

SDS-polyacrylamide gel electrophoresis and immunoblot analyses of SecY and SecE

A gel containing 12.2% acrylamide-0.33% *N,N'*-methylenebisacrylamide was used according to Laemmli [30]. All samples were applied to the gel without boiling. The method for immunoblot analysis was described previously [31]. A Protoblot system (Promega) was used for color development.

Materials

ATP, creatine kinase and succinate were obtained from Boehringer, Mannheim. Octyl glucoside was purchased from Dojindo Laboratories. Proteinase K was from Merck and SP6 RNA polymerase from Takara Shuzo Co., Ltd. Protein A-agarose and immobilized papain were from Pierce. Tran^{35}S-label, a mixture of 70% [^{35}S]methionine and 20% [^{35}S]cysteine, 1000 Ci/mmol, was obtained from ICN. Anti-SecE antiserum was raised against peptide corresponding to the Lys64–Lys81 of SecE [6].

Results

Effects of site-specific anti-SecY antibodies on protein translocation

We first examined the effects of anti-SecY antibodies raised against synthetic peptides corresponding to five regions of the SecY molecule on the protein translocation into everted membrane vesicles. These regions are thought to be exposed to the membrane surface [14]. IgGs SecY-1 and SecY-5 strongly inhibited the translocation of a model secretory protein, uncleavable OmpF-Lpp, whereas the other three IgGs did not (Fig. 1A). Essentially the same results were obtained when Fabs prepared from these antibodies were used (data not shown). The inhibition of translocation by antibodies which recognize the N- or C-terminus of the SecY molecule has been reported [15].

In the presence of peptides 1 and 5, the inhibition by IgGs SecY-1 and SecY-5, respectively, was abolished completely, the abolition being specific to antigenic peptides (Fig. 1B) Peptide 1 or 5 alone had no effect on the translocation. The abolition was observed irrespective of the order of the addition of the IgG and the peptide. These results indicate that the inhibition of the translocation was due to an antigen-antibody interaction.

Although antibodies SecY-2, -3 and -4 did not inhibit the translocation, immunoblot analysis revealed that these antibodies interacted with SDS-denatured SecY as extensively as antibodies SecY-1 and SecY-5 did (data not shown). The results suggest that the target SecY domains on everted membrane vesicles are inaccessible to these antibodies or that the interaction with these antibodies does not interfere with the function of SecY. The periplasmic localization of the SecY-3 site has been proposed [14]. Everted membrane vesicles were, therefore, solubilized with octyl glucoside, treated with IgGs and then reconstituted into proteoliposomes. No protein translocation activity was reconstituted in the presence of IgG SecY-1, whereas IgGs SecY-2, -3 and -4 did not inhibit the functional reconstitution of proteo-

liposomes (Fig. 1C). Essentially the same results were obtained when these IgGs were added to reconstituted proteoliposomes after freezing-thawing, which effectively destroys the membrane structure [32] (data not shown).

Depletion of SecY from the solubilized membrane results in failure of reconstitution of active proteoliposomes

The octyl glucoside-solubilized membrane was treated with IgG SecY-1 and subsequently treated batchwisely with protein A-agarose to remove the IgG. The extract thus treated with protein A-agarose contained little IgG, more than 99.99% of the added IgG being removed (data not shown).

Proteoliposomes were then reconstituted with the extract and subjected to the translocation assay (Fig. 2A). IgGs SecY-1 and SecY-5 effectively lowered the translocation activity. These results indicate that SecY or a component(s) coimmunoprecipitated with it is essential for the *in vitro* translocation reaction. IgGs SecY-2, -3 and -4 as well as non-immune IgG did not lower the translocation activity, suggesting that these IgGs were ineffective in binding to and hence removal of SecY.

Reconstitution of translocation activity from the solubilized and SecY-depleted membrane and the peptide 1-released fraction

The solubilized membrane preparation was treated with IgG SecY-1. The resulting immunoprecipitate was then treated with peptide 1, and the released fraction (peptide 1-released fraction) was mixed with the solubilized and SecY-depleted membrane. The mixture was then subjected to reconstitution. The reconstitution in the presence of the peptide 1-released fraction resulted in the appearance of translocation activity, which was comparable to that obtained with the whole solubilized membrane treated with non-immune IgG (Fig. 2B). Neither the SecY-depleted extract nor the peptide 1-released fraction alone resulted in functional reconstitution. This result further supports the view that SecY or a substance(s) coimmunoprecipitated with it participates in the translocation reaction. It also suggests that SecY is not the sole membrane component of the translocation machinery.

By means of immunoblotting, determination of SecY in the preparations used for these reconstitution studies was attempted. No quantitative results were obtained,

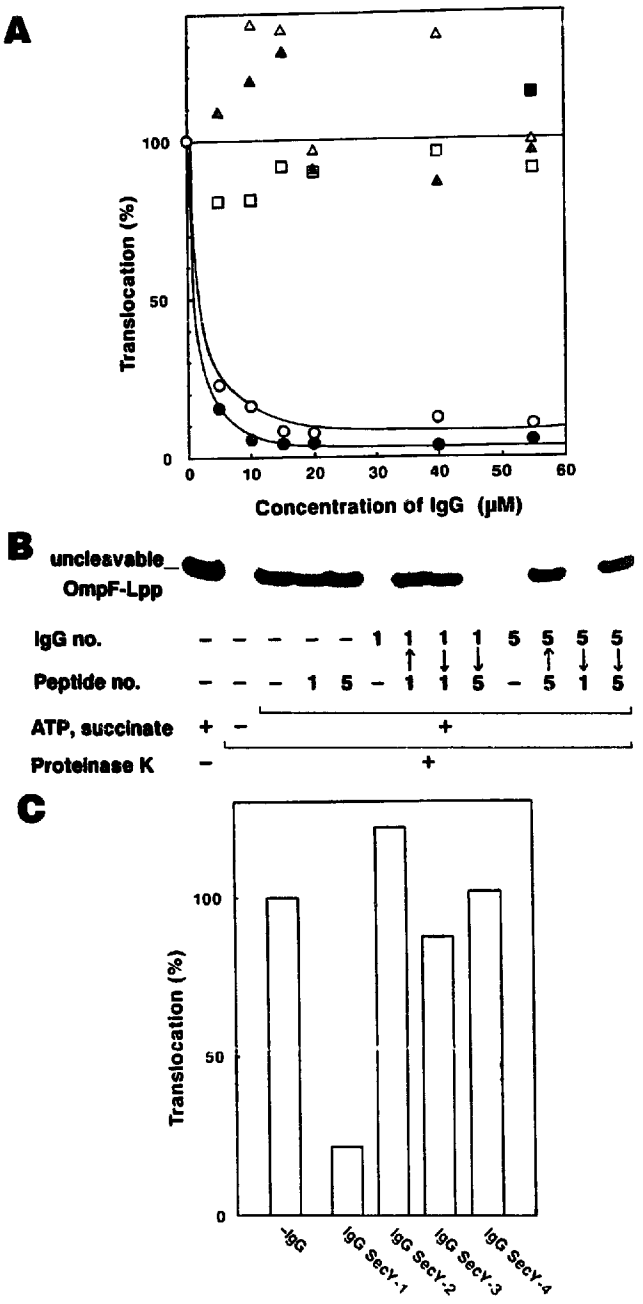

Fig. 1. Effects of five antibodies raised against distinct regions of SecY on the translocation of uncleavable OmpF-Lpp into everted membrane vesicles. (A) Everted membrane vesicles were incubated on ice for 30 min with an IgG at the indicated concentrations prior to the translocation assay. The amount of uncleavable OmpF-Lpp translocated in the absence of an antibody was taken as 100%. Symbols ●, □, △, ▲, ○ and ■ represent IgGs SecY-1, SecY-2, SecY-3, SecY-4, SecY-5 and non-immune IgG, respectively. (B) Synthetic peptide 1 or 5 was added to the final concentration of 100 μM to everted membrane vesicles which had been treated with the indicated species of IgG (12 μM) on ice for 30 min. After 30-min incubation on ice, they were then subjected to the translocation assay. A fluorogram of translocated uncleavable OmpF-Lpp is shown. The arrows indicate the order of the IgG and synthetic peptide treatments. (C) The effects of anti-SecY IgGs on the reconstitution of translocationally active proteoliposomes are shown. Solubilized membrane derived from SecA-overproducing cells was treated with 15 μM IgGs on ice for 30 min, followed by reconstitution. The resultant proteoliposomes were assayed for translocation activity at 37°C for 10 min using uncleavable OmpF-Lpp as a substrate. The activity of proteoliposomes which were not treated with an IgG was taken as 100%.

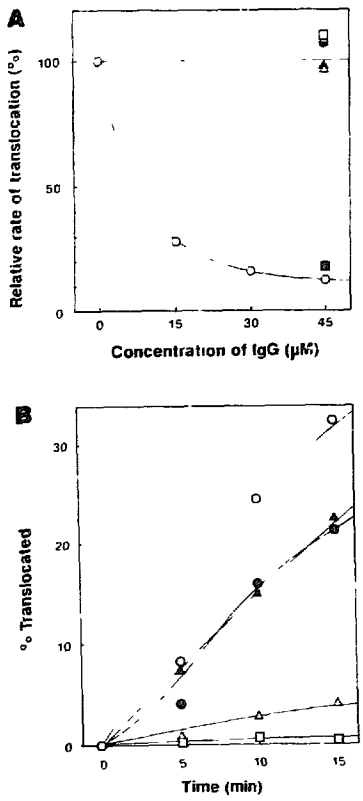

Fig. 2. SecY-depletion with anti-SecY IgGs resulted in the failure of the reconstitution of translocation activity, and the loss with IgG SecY-1 was restored by the addition of the peptide 1-released fraction. (A) Solubilized membrane prepared from SecA-overproducing cells was treated with the indicated concentrations of anti-SecY IgGs, followed by batchwise-treatment with protein A-agarose. The resultant supernatants were then subjected to reconstitution and translocation activity of the reconstituted proteoliposomes was assayed using uncleavable OmpF-Lpp as a substrate. The relative rates of translocation were then plotted against the concentrations of the IgGs. The rate of translocation, exhibited by proteoliposomes reconstituted in the absence of IgG, was taken as 100%. The IgGs used were SecY-1 (○), SecY-2 (△), SecY-3 (▲), SecY-4 (□), SecY-5 (■) and non-immune (●). (B) The solubilized membrane was treated with 45 μM IgG and protein A-agarose as in A. The IgGs used were none (○), non-immune IgG (●) and IgG SecY-1 (△). In another experiment (▲), the IgG SecY-1-treated sample (200 μl) was supplemented with 50 μl of the peptide 1-released fraction, which was prepared as described under Materials and Methods. The peptide 1-released fraction alone was also used (□). All samples were then subjected to reconstitution and the translocation activity reconstituted was assayed using uncleavable OmpF-Lpp as a substrate.

however. As will be discussed in the next section, this was most likely due to the rapid cleavage of the majority of SecY by the proteinase OmpT in the solubilized membrane.

Treatment of the solubilized membrane with OmpT results in degradation of SecY and failure of functional reconstitution

During the course of this work, it was reported that OmpT, an outer membrane proteinase [33,34], specifically cleaves SecY in the solubilized membrane into at least two fragments, 22 and 16 kDa ones [19,35]. We prepared everted membrane vesicles from both *ompT*$^+$ and *ompT*$^-$ cells and determined, by means of immunoblotting with anti-SecY-1 antiserum, the rate of breakdown of SecY in an octyl glucoside solution. Rapid degradation of SecY was observed with *ompT*$^+$ vesicles with a concomitant increase in the 22 kDa fragment representing the N-terminal half of SecY, whereas SecY was quite stable in the *ompT*$^-$ background, indicating that OmpT was responsible for the rapid degradation of SecY in the octyl glucoside solution (Fig. 3A). It should be noted that the overall protein profile of the *ompT*$^+$ membrane preparation was unchanged after 30-min incubation in the octyl glucoside solution (Fig. 3B), suggesting that the degradation by OmpT was highly specific to SecY.

The solubilized and incubated membrane was then reconstituted into proteoliposomes and examined for translocation activity (Fig. 3C). A clear difference in translocation activity between proteoliposomes derived from the *ompT*$^+$ membrane and those from the *ompT*$^-$ one was observed. Since the OmpT digestion was highly specific to SecY, this observation again supports the importance of SecY in the translocation reaction. It should be noted that the proteoliposomes reconstituted from the *ompT*$^+$ sample still exhibited significant translocation activity, even though the SecY content of the membrane thus treated was quite low. As will be discussed in the next paragraph, this is most likely due to the fact that the cleaved SecY fragment was still active.

In another set of experiments, the results of which are shown in Fig. 4, purified OmpT was used to examine the relationship between the SecY digestion and the failure of functional reconstitution. The membrane fraction prepared from *ompT*$^-$ cells overproducing SecY and SecE was solubilized with octyl glucoside and then treated with different amounts of purified OmpT. The cleavage of SecY into 22 and 16 kDa fragments was observed on immunoblot analysis with anti-SecY-1 and anti-SecY-5 antisera (Fig. 4A), whereas SecE (data not shown) and other membrane proteins (Fig. 4C) were highly resistant to OmpT under the conditions used. The 22 and 16 kDa fragments specifically reacted with anti-SecY-1 and anti-SecY-5 antisera, respectively, indicating that the 22 and 16 kDa fragments correspond to the N- and C-terminal halves of the SecY molecule, respectively (Fig. 4A) [19]. The OmpT-treated samples were then subjected to reconstitution, followed by assaying of translocation activity. Parallelism was observed between the extent of SecY degradation (Fig. 4B) and the loss of translocation activity (Fig. 4D). The more extensive degradation of SecY observed with the reconstituted samples (compare Fig. 4B with Fig. 4A) reflected further degradation during the reconstitution reaction.

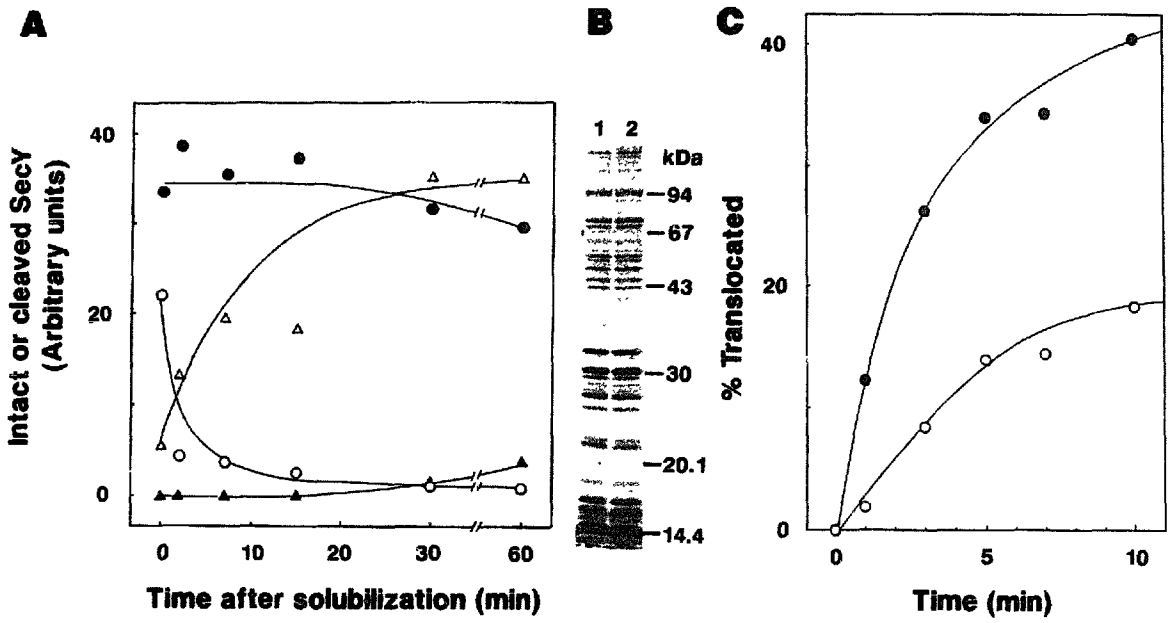

Fig. 3. SecY degradation by OmpT proteinase and its effect on reconstitution of translocation activity. (A) Membrane vesicles prepared from W3110 (*ompT*$^+$) (○, △) and W3110 M25 (*ompT*) (●, ▲) cells were solubilized on ice in the presence of 150 mM NaCl. At the indicated times after solubilization, an aliquot of each sample (15 μl; 75 μg of protein) was subjected to trichloroacetic acid (final concentration, 10%) precipitation. The acid-insoluble fraction was then subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblot analysis, and the density of the bands of the intact (○, ●) and cleaved (△, ▲) SecYs were quantified and plotted. (B) Solubilized membrane (W3110) before (lane 1) and after (lane 2) 30-min incubation on ice was subjected to SDS-polyacrylamide gel electrophoresis as described, followed by Coomassie brilliant blue staining. Each slot contained 50 μg of protein. (C) Solubilized membrane prepared from W3110 (○) and W3110 M25 (●) cells was incubated on ice for 30 min, subjected to reconstitution and then assayed for translocation activity using proOmpA D26 as a substrate. The amount of translocated proOmpA D26, densitometrically determined on a fluorogram, was expressed as a percentage of the input proOmpA D26.

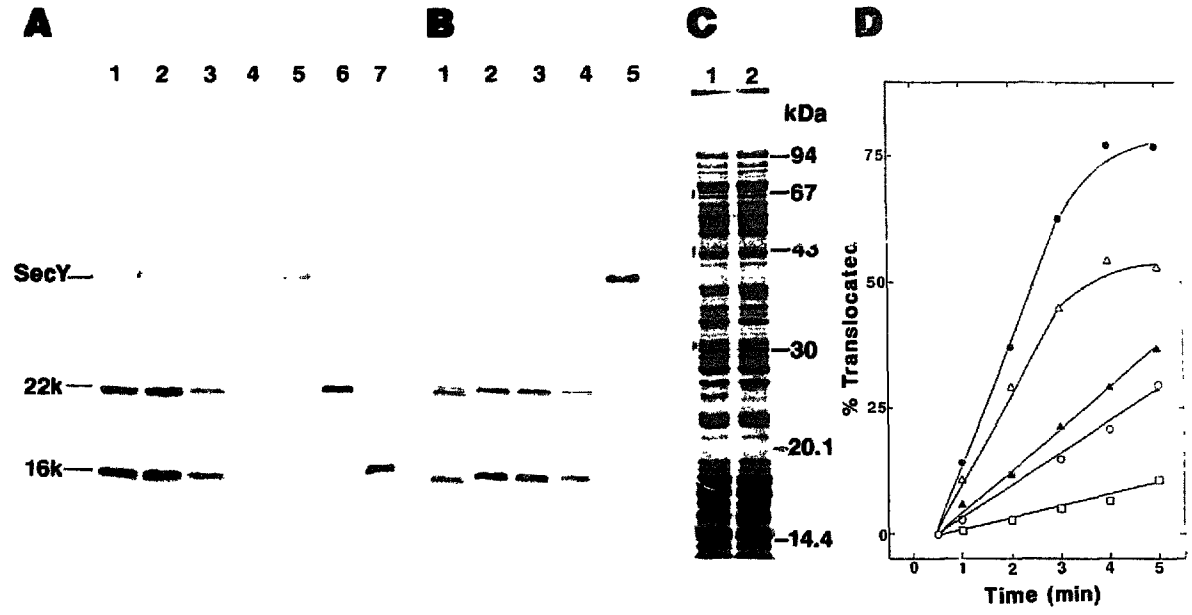

Fig. 4. Degradation of SecY by purified OmpT and its effect on reconstitution of translocation activity. (A) Solubilized membrane (45 μl, 225 μg of protein) prepared from cells of W3110 M25/pMAN809 and pMAN510, overproducing SecY and SecE, was treated with 5 μl of the OmpT preparation containing 10 (lane 1), 1 (lanes 2, 6 and 7), 0.1 (lane 3) and 0.01 μg (lane 4) of OmpT, or without OmpT (lane 5). The samples were then subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblot analysis with a mixture of antisera SecY-1 and SecY-5 (lanes 1 through 5), antiserum SecY-1 (lane 6) or antiserum SecY-5 (lane 7). The positions of intact SecY and 22 kDa and 16 kDa fragments of SecY are indicated. (B) The same samples were reconstituted into proteoliposomes and then aliquots (40 μl) were subjected to SDS-gel electrophoresis, followed by immunoblot analysis as in A. (C) The samples (25 μg protein) for lanes 2 and 5 in A were analysed by SDS-polyacrylamide gel electrophoresis, followed by Coomassie brilliant blue staining (lanes 1 and 2), respectively. (D) The reconstituted samples in B were assayed for translocation activity. The samples for □, ○, ▲, △ and ● correspond to those for lanes 1, 2, 3, 4 and 5 in B, respectively.

It was interesting that some reconstituted proteoliposomes (Fig. 4B, lanes 2 and 3) were appreciably active as to translocation although they did not contain a detectable amount of intact SecY, whereas further digestion into smaller fragments resulted in an almost complete loss of translocation activity (Fig. 4B, lane 1). These results suggest that the cleaved SecY fragments (22 kDa and 16 kDa ones) are active to some extent, unless they suffer further degradation. The cleavage of SecY by OmpT into at least two fragments and the possible participation of the latter in the translocation reaction have been discussed [19].

Purified SecY triggers reconstitution from the solubilized and SecY-depleted membrane

Everted membrane vesicles prepared from *ompT*$^-$ cells were solubilized with octyl glucoside, treated with IgG SecY-1 and then protein A-agarose, and then reconstituted into proteoliposomes. Immunoblot analysis clearly demonstrated the removal of SecY on the treat-

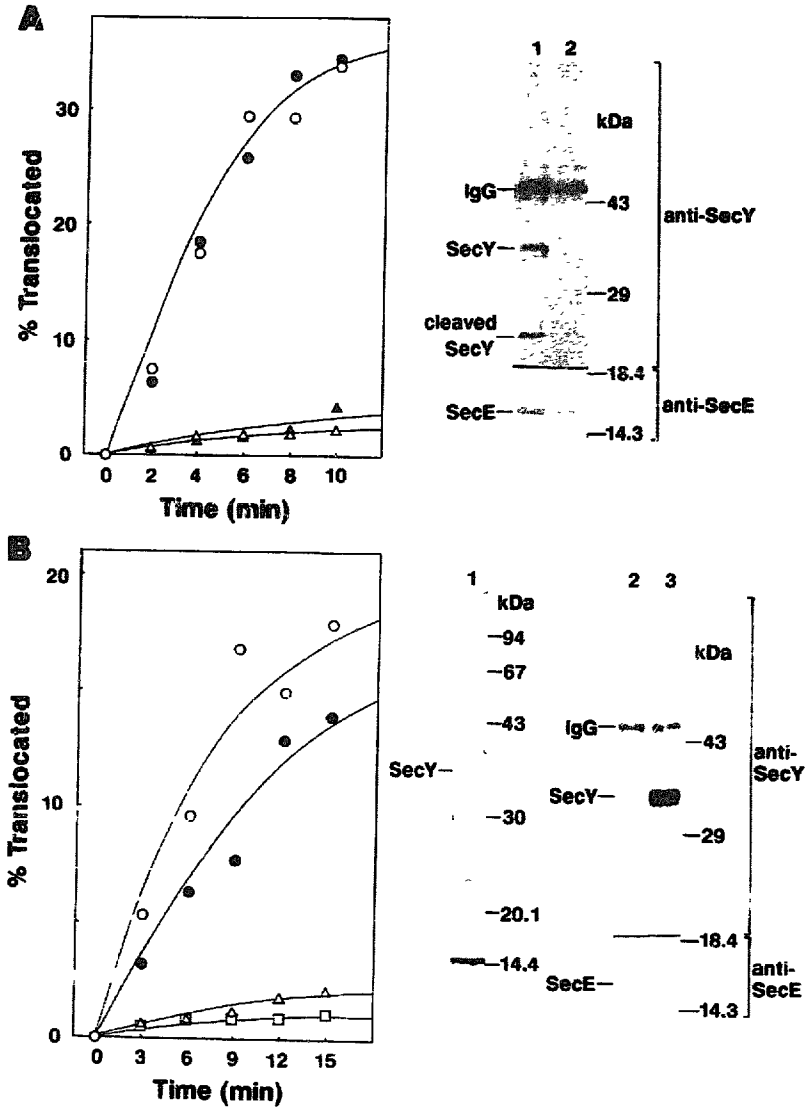

Fig. 5. Purified SecY triggered the reconstitution from the solubilized and SecY-depleted membrane. (A) Solubilized membrane prepared from W3110 M25 cells was treated with either non-immune IgG (○, ●) or IgG SecY-1 (△, ▲), applied on a protein A-agarose column and then subjected to reconstitution. The translocation of proOmpA D26 was then assayed in the absence (○, △) or presence (●, ▲) of 100 μM peptide 1. The amounts of protein translocated are expressed as percentages of the input proOmpA D26. Photographs: Reconstituted proteoliposomes (40 μg) were also subjected to immunoblot analysis after SDS-gel electrophoresis with anti-SecY-1 (upper half) or anti-SecE (lower half) antiserum: lane 1, non-immune IgG-treated; lane 2, IgG SecY-1-treated. The positions of SecY, the 22 kDa SecY fragment and SecE are indicated. (B) The SecY-depleted sample (80 μl) in A was mixed with 20 μl of purified SecY and 20 μl of the buffer, in which SecY was dissolved, (●) or 40 μl of the buffer (△), and then reconstituted into proteoliposomes. Solubilized membrane treated with non-immune IgG and then with protein A-agarose alone (○), and the purified SecY alone (□) was also reconstituted under the same conditions. All proteoliposomes were then assayed for translocation activity in the presence of 100 μM peptide 1. Photographs: Lane 1, Coomassie brilliant blue staining of the purified SecY preparation; lanes 2 and 3, immunoblot profiles with anti-SecY-1 (upper half) and anti-SecE (lower half) antisera of the samples (30 μg) reconstituted without (lane 2) and with (lane 3) purified SecY. The positions of SecY and SecE are shown.

ment with IgG SecY-1 and protein A-agarose (Fig. 5A). The treatment also resulted in the loss of translocation activity (Fig. 5A). The SecE content, on the other hand, remained unchanged after the treatment (Fig. 5A). The activity was not restored on the addition of peptide 1, indicating that the loss of activity was not due to contamination by IgG SecY-1 of the reconstituted proteoliposomes. The results shown in Fig. 5A also show that peptide 1 had no effect on the translocation.

Purification of SecY was carried out as described under Materials and Methods. The SecY preparation thus obtained did not contain detectable amounts of SecE and the uncharacterized protein, both of which have been reported to be copurified with SecY from the SecY-non-overproducing cells [19] (Fig. 5A). From the SDS-gel profile, the SecY content of the preparation was determined to be about 20% (Fig. 5B lane 1). Reconstitution experiments were then carried out starting from both the solubilized and SecY-depleted membrane and purified SecY. The proteoliposomes thus reconstituted in the presence of SecY contained an appreciable amount of SecY (Fig. 5B, lane 3) and exhibited appreciable translocation activity, which was comparable to that exhibited by the proteoliposomes reconstituted from the solubilized membrane treated with non-immune IgG (Fig. 5B). Neither the SecY-depleted preparation nor the purified SecY alone caused the reconstitution of active proteoliposomes. The results strongly suggest that SecY is an important component of the translocation machinery.

Discussion

Although genetic studies have clearly indicated the essentiality of SecY for protein secretion in living cells [7,11,36], the direct involvement of SecY in the translocation reaction has been a subject of controversy. In the present work, we intended to clarify biochemically, through the use of a reconstitution system for protein translocation, whether or not SecY is involved in the translocation reaction. All the results we obtained support the involvement of SecY. The results are discussed below:

(1) Inhibition of *in vitro* translocation activity by anti-SecY antibodies has been reported for everted membrane vesicles [15] and for reconstituted proteoliposomes [17,18]. We confirmed this in a more quantitative manner by using antibodies raised against synthetic SecY fragments. We further demonstrated that the synthetic SecY fragments specifically suppressed the inhibition of the translocation reaction by the antibodies. Moreover, a fraction which was immunoprecipitated with IgG SecY-1 and released from it on the addition of peptide 1 appreciably stimulated the reconstitution from the SecY-depleted membrane preparation. These results strongly support the involvement of SecY in the translocation reaction. However, the possibility that a component coimmunoprecipitated with SecY, but not SecY itself, is the factor which is directly involved in the translocation reaction could not be excluded. SecE and an uncharacterized protein were reported to be cofractionated through a purification process for SecY [19]. No coimmunoprecipitation of SecE with SecY was observed in the present work, however. No information was available as to the presence of the uncharacterized protein in the immunoprecipitated or the peptide 1-released fraction.

(2) OmpT, an outer membrane proteinase, digests SecY in a detergent-solubilized membrane into at least two fragments [19,35]. We found that the digestion was highly specific to SecY, the digestion of other membrane proteins, including SecE, being hardly detected under the same conditions. The cleavage was accompanied by a decrease in the reconstitution of translocation activity.

(3) When the SecY-depleted membrane preparation was subjected to reconstitution, translocation activity appeared only when the SecY preparation was added. Although the SecY preparation was contaminated by several proteins, no SecE was detected in it. Furthermore, none of the contaminating protein bands detected on an SDS-gel corresponded to the uncharacterized protein.

Thus, the involvement of SecY in the translocation reaction was supported by (a) the inhibition by anti-SecY antibodies, (b) suppression of the inhibition by epitopic SecY fragments, (c) active participation of the peptide 1-released fraction in the reconstituted translocation activity, (d) specific digestion of SecY by OmpT, which was accompanied by a failure of reconstitution, and (e) restoration by purified SecY of active reconstitution from the SecY-depleted sample. Although none of these observations alone is sufficient as proof for the direct involvement of SecY, they together strongly support the essentiality of SecY. Taken together with previous genetic and biochemical observations on *secY*^{ts} mutants [7,8,11–13,36], we conclude that SecY is an indispensable component of the secretory machinery.

Acknowledgements

We thank K. Sugimura for providing *E. coli* W3110 M25 and plasmid ptrp-GP, H. Yamada and K. Kanamaru for pOAD26, and I. Sugihara for excellent secretarial support. This work was supported by grants from the Ministry of Education, Science and Culture of Japan (Nos. 61060001, 02404013 and 02680153).

References

1 Benson, S.A., Hall, M.N. and Silhavy, T.J. (1985) Annu. Rev. Biochem. 54, 101–134.

2 Bieker, K.L., Phillips, G.J. and Silhavy, T.J. (1990) J. Bioenerg. Biomembr. 22, 291–310.
3 Gardel, C., Johnson, K., Jacq, A. and Beckwith, J. (1990) EMBO J. 9, 3209–3216.
4 Oliver, D.B. and Beckwith, J. (1981) Cell 25, 765–772.
5 Kumamoto, C. and Beckwith, J. (1984) J. Bacteriol. 154, 253–260.
6 Schatz, P.J., Riggs, P.D., Jacq, A., Fath, M.J. and Beckwith, J (1989) Genes Dev. 3, 1035–1044.
7 Ito, K., Wittekind, M., Nomura, M., Shiba, K., Yura, T., Miura, A. and Nashimoto, H. (1983) Cell 32, 789–797.
8 Akiyama, Y. and Ito, K. (1985) EMBO J. 4, 3351–3356.
9 Oliver, D.B., Cabelli, R.J. and Jarosik, G.P. (1990) J. Bioenerg. Biomembr. 22, 311–336.
10 Kumamoto, C.A. (1990) J. Bioenerg. Biomembr. 22, 337–351.
11 Emr, S.D., Hanley-Way, S. and Silhavy, T.J. (1981) Cell 23, 79–88.
12 Fandl, J.P. and Tai, P.C. (1987) Proc. Natl. Acad. Sci. USA 84, 7448–7452.
13 Fandl, J.P., Cabelli, R., Oliver, D. and Tai, P.C. (1988) Proc. Natl. Acad. Sci. USA 85, 8953–8957.
14 Akiyama, Y. and Ito, K. (1987) EMBO J. 6, 3465–3470.
15 Watanabe, M. and Blobel, G. (1989) Proc. Natl. Acad. Sci. USA 86, 1895–1899.
16 Watanabe, M., Nicchitta, C.V. and Blobel, G. (1990) Proc. Natl. Acad. Sci. USA 87, 1960–1964.
17 Driessen, A.J.M. and Wickner, W. (1990) Proc. Natl. Acad. Sci. USA 87, 3107–3111.
18 Tokuda, H., Shiozuka, K. and Mizushima, S. (1990) Eur. J. Biochem. 192, 583–589.
19 Brundage, L., Hendrick, J.P., Schiebel, E., Driessen, A.J.M. and Wickner, W. (1990) Cell 62, 649–657.
20 Yamane, K., Ichihara, S. and Mizushima, S. (1987) J. Biol. Chem. 262, 2358–2362.
21 Kawasaki, H., Matsuyama, S., Sasaki, S., Akita, M. and Mizushima, S. (1989) FEBS Lett. 242, 431–434.
22 Sugimura, K. (1988) Biochem. Biophys. Res. Commun. 153, 753–759.
23 Matsuyama, S., Akimaru, J. and Mizushima, S. (1990) FEBS Lett. 269, 96–100.
24 Yamane, K., Matsuyama, S. and Mizushima, S. (1988) J. Biol. Chem. 263, 5368–5372.
25 Tokuda, H., Akimaru, J., Matsuyama, S., Nishiyama, K., and Mizushima, S. (1991) FEBS Lett. 279, 233–236.
26 Yamada, H., Tokuda. H. and Mizushima, S. (1989) J. Biol. Chem. 264, 1723–1728.
27 Akita, M., Sasaki, S., Matsuyama, S. and Mizushima, S. (1990) J. Biol. Chem. 265, 8164–8169.
28 Cerretti, D.P., Dean, D., Daris, G.R., Bedwell, D.M. and Nomura, M. (1983) Nucleic Acids Res. 11, 2599–2619.
29 Sugimura, K. and Nishihara, T. (1988) J. Bacteriol. 170, 5625–5632.
30 Laemmli, U.K. (1970) Nature 227, 680–685.
31 Yamada, H., Matsuyama, S., Tokuda, H. and Mizushima, S. (1989) J. Biol Chem. 264, 18577–18581.
32 Pick, U. (1981) Arch. Biochem. Biophys. 212, 186–194.
33 Sugimura, K. and Higashi, N. (1988) J. Bacteriol. 170, 3650–3654.
34 Grodberg, J. and Dann, J. (1988) J. Bacteriol. 170, 1245–1253.
35 Akiyama, Y. and Ito, K. (1990) Biochem. Biophys. Res. Commun. 167, 711–715.
36 Bieker, K.L. and Silhavy, T.J. (1989) Proc. Natl. Acad. Sci. USA 86, 968–972.