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SecY, a Multispanning Integral Membrane Protein, Contains a Potential Leader Peptidase Cleavage Site

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SecY is an *Escherichia coli* integral membrane protein required for efficient translocation of other proteins across the cytoplasmic membrane; it is embedded in this membrane by the 10 transmembrane segments. Among several SecY-alkaline phosphatase (PhoA) fusion proteins that we constructed previously, SecY-PhoA fusion 3-3, in which PhoA is fused to the third periplasmic region of SecY just after the fifth transmembrane segment, was found to be subject to rapid proteolytic processing in vivo. Both the SecY and PhoA products of this cleavage have been identified immunologically. In contrast, cleavage of SecY-PhoA 3-3 was barely observed in a *lep* mutant with a temperature-sensitive leader peptidase. The full-length fusion protein accumulated in this mutant was cleaved in vitro by the purified leader peptidase. A sequence Ala-202–Ile–Ala located near the proposed interface between transmembrane segment 5 and periplasmic domain 3 of SecY was found to be responsible for the recognition and cleavage by the leader peptidase, since a mutated fusion protein with Phe-Ile-Phe at this position was no longer cleaved even in the wild-type cells. These results indicate that SecY contains a potential leader peptidase cleavage site that undergoes cleavage if the PhoA sequence is attached carboxy terminally. Thus, transmembrane segment 5 of SecY can fulfill both of the two important functions of the signal peptide, translocation and cleavage, although the latter function is cryptic in the normal SecY protein.

The signal (leader) sequence for protein translocation across the endoplasmic reticulum membrane or the bacterial cytoplasmic membrane is generally located at the amino terminus of a precursor secretory protein. It consists of a basic amino-terminal part, a hydrophobic central part, and a hydrophilic carboxy-terminal part (6). It is cleaved off after the cleavage site has reached the noncytoplasmic side of the membrane, where the active site of the enzyme responsible for the proteolytic processing (the signal or leader peptidase) resides. From comparison of a large number of sequences around the cleavage sites of signal peptides, some consensus features, but not precise conservation, emerged (36). The signal sequence (or its functional equivalent) is not unique to secreted proteins but also functions in the biogenesis of membrane proteins by causing translocation of its carboxy-terminally adjacent region. The signal sequences found in the class I membrane proteins are indistinguishable from those found in secreted proteins, whereas the class II membrane proteins contain uncleaved forms of signal peptide. Polytopic membrane proteins are also thought to contain internally located signal sequence equivalents (for a review, see reference 38).

The signal sequence-mediated protein translocation across the *Escherichia coli* cytoplasmic membrane is assisted by some cellular components. Genetic studies identified several *E. coli* genes participating in protein translocation; these include *secA* (29), *secB* (20, 21), *secD* (14), *secE* (30), and *secY/prlA* (12, 32). The *secY* gene product is an integral membrane protein that spans the cytoplasmic membrane 10 times (1, 2). The SecY function is required for protein translocation both in vivo (32) and in vitro (13). We have been particularly interested in SecY, since its multispanning structure in the membrane could entitle it to the translocator function. In our previous studies on the topological disposition of SecY, we constructed a series of SecY-alkaline phosphatase (PhoA) fusion proteins (2), using the Tn*phoA* approach (27). Our results show that SecY contains five periplasmic regions, the mature PhoA sequence attached to which is exported to the periplasm. Thus, transmembrane segments 1, 3, 5, 7, and 9 of SecY probably act like a signal sequence to translocate PhoA in the fusion proteins. Presumably, these segments also translocate the following periplasmic domain in the normal SecY protein. Taking advantage of the fact that the PhoA moiety shows different trypsin sensitivity when it is exported (trypsin resistant) or internalized (trypsin sensitive), we investigated the mode of the PhoA translocation promoted by the transmembrane segments of SecY (3). It was shown that this mode of PhoA export is dependent on the SecY function provided in *trans*, just like the PhoA export promoted by its own signal sequence (3). In the course of that study, we found that one of the SecY-PhoA fusions, named 3-3, was proteolytically and rapidly processed, generating a stable PhoA fragment. We pointed out that this cleavage might be carried out by the leader peptidase, since a sequence conforming to the signal sequence cleavage site was found in the SecY sequence just before the junction point. Here we report that the enzyme responsible for the in vivo cleavage of fusion 3-3 is in fact leader peptidase, which recognizes the SecY part of the fusion protein.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* K-12 strains used were as follows. AD90 was a Δ*phoA lon-100 tsx*::Tn*10* derivative of MC4100 (33) that has been described previously (3). IT41 [*lep-9*(Ts) *era*::Tn*10*] and its isogenic *lep*$^+$ strain IT42 were also described previously (16).

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Plasmids and site-directed mutagenesis. pKY3 (32) and pKY4 (2) had been constructed previously by cloning a *secY* fragment (the *Hae*II fragment containing *secY* that was linked with an appropriate linker) into the *Hin*dIII or *Eco*RI site, respectively, of the vector pNO1575. Expression of the *secY* gene on these plasmids is under the control of the *lac* promoter-operator.

The pKY4-derived plasmid carrying the *secY-phoA* 3-3 gene fusion (referred to here as pKY4-3-3) had been obtained by transposition of Tn*phoA* onto the *secY* gene of pKY4 (2). For site-directed mutagenesis of *secY-phoA* 3-3 or *secY*, the *Hin*dIII fragment of pKY4-3-3 or pKY3 which contained *secY-phoA* 3-3 or *secY*, respectively, was first cloned into the *Hin*dIII site of pUC118 (35) in the reverse orientation with respect to the direction of transcription. Oligonucleotide-directed mutagenesis was carried out by using these pUC118-derived plasmids according to the method of Kunkel et al., (22), using a kit (Mutan-K) purchased from Takara. Deoxyuracil-containing single-stranded DNA was prepared from these plasmids and annealed with a mutagenic primer, an oligonucleotide (32-mer) designed so as to replace both GGC (alanine) codons at positions 202 and 205 of *secY* by TTC (phenylalanine). After polymerization, ligation, and transfection, mutant bacteriophages were screened by sequencing the relevant region, using a primer (a 17-mer corresponding to residues 4786 to 4802 [numbering as in reference 8] of the *spc* operon) provided by T. Sako. The *Hin*dIII fragments of the mutated plasmids were isolated and recloned into the *Hin*dIII site of pNO1575. The resulting plasmids pKY4-3-3F (fusion 3-3 mutant, designated *secY-phoA* 3-3F) and pKY159 (*secY* mutant) were confirmed by sequencing.

Pulse-chase, immunoprecipitation, and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Cells were grown in M9 medium (28) supplemented with amino acids (20 μg/ml each other than methionine and cysteine), thiamine (1 μg/ml), glucose (0.4%), and ampicillin (50 μg/ml) to early log phase (reading of about 20 on a Klett-Summerson colorimeter with a no. 54 filter) at 30°C and then at 42°C for 1 h unless otherwise indicated. After induction of *lac*-controlled genes on the plasmids with 5 mM cyclic AMP (for AD90) or 5 mM cyclic AMP plus 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (for IT41 and IT42), cells were labeled with 15 to 100 μCi of [^{35}S]methionine per ml (1,000 Ci/mmol; American Radiolabeled Chemicals) for the period specified in each experiment. Chase with unlabeled methionine was initiated by addition of 200 μg of L-methionine per ml. At each chase point, a portion (0.1 ml) was removed and either chilled with crushed ice (for analysis of intact SecY) or mixed with an equal volume of 10% trichloroacetic acid (for analysis of the SecY-PhoA fusion proteins). In the former case, labeled cells were collected by centrifugation, subjected to lysozyme/freezing-thawing as described previously (1), and solubilized with 1% SDS by incubation at 37°C for 5 min. The trichloroacetic acid-treated samples were placed at 0°C for 20 min or longer, and protein precipitates were collected by centrifugation, washed with acetone, and finally dissolved in 60 μl of 1% SDS–50 mM Tris hydrochloride (pH 8.1)–1 mM EDTA at 37°C. Immunoprecipitation of SDS-solubilized samples was carried out after dilution of the samples with a buffer containing a nonionic detergent as described previously (17) except that Lubrol buffer containing 0.1% Lubrol (type PX), 50 mM Tris hydrochloride (pH 8.1), 0.1 mM EDTA, and 0.15 M NaCl was used instead of Triton buffer. This replacement reduced the background proteins. Proteins in the immunoprecipitates were solubilized in SDS sample buffer (23) at 37°C for 5 min, separated in 10% PAGE (23) or 15% polyacrylamide–0.12% *N,N'*-methylene bisacrylamide PAGE (17), and visualized by fluorography with sodium salicylate (9). ^{14}C-labeled molecular weight standards were as described previously (17).

Conformation assay of SecY-PhoA 3-3. Trypsin sensitivity of the PhoA domain of SecY-PhoA fusion 3-3 was assayed as described previously (3). Briefly, spheroplasts were lysed with 1% Triton X-100 and mixed with trypsin at a final concentration of 50 μg/ml. After incubation at 0°C for 30 min, the samples were mixed with the inhibitors of trypsin and subjected to trichloroacetic acid treatment and immunoprecipitation.

In vitro cleavage of SecY-PhoA 3-3 by leader peptidase. IT41 cells carrying pKY4-3-3 were labeled with [^{35}S]methionine, rapidly chilled with crushed ice, and mixed with NaN_3 (0.02%) and chloramphenicol (100 μg/ml). They were collected, washed with 10 mM Tris hydrochloride (pH 8.1)–0.02% NaN_3–100 μg of chloramphenicol per ml, and suspended in 85 μl of the sample buffer. The cells were lysed by incubating at 0°C with 10 μl of lysozyme (1 mg/ml in 0.1 M EDTA [pH 7.5]) and 5 μl of 20% Triton X-100 for 30 min. The lysate was divided into two 50-μl portions and incubated at 37°C for 60 min with or without 1 μl (17 ng) of purified leader peptidase provided by W. Wickner. The reaction was stopped by addition of an equal volume of 10% trichloroacetic acid, and samples were subjected to immunoprecipitation as described above.

RESULTS

Identification of the cleavage products of SecY-PhoA fusion protein 3-3. The SecY-PhoA fusion protein 3-3 (2) contains the amino-terminal part of SecY up to residue 208, which is followed by the PhoA sequence of Tn*phoA*. The junction is located within the third periplasmic region of SecY, just after the fifth transmembrane segment (see Fig. 4). As described previously, the fusion protein 3-3 is rapidly cleaved in vivo, generating a 48-kilodalton (kDa) fragment reactive with antibodies against PhoA (PhoA** in Fig. 1, lanes 1 to 3) (3). This fragment represents the PhoA moiety of the fusion protein, which has been translocated to the periplasmic space; it can be recovered from the periplasmic fraction, and it is in a protease-resistant conformation characteristically observed with the exported PhoA protein (3). We now identified the SecY portion of the cleavage products by immunoprecipitation, using antibodies against the amino-terminal SecY peptides (24). A cross-reacting fragment of about 19 kDa was observed after a chase for 1.5 min or longer (SecY* in Fig. 1, lanes 5 and 6). The calculated mass of 22 kDa for the SecY part agrees with the observed electrophoretic mobility (19 kDa) of the SecY fragment (note that SecY migrates in SDS-PAGE faster than expected; 17). These results indicate that fusion protein 3-3 is cleaved only at one site around the junction between SecY and PhoA.

In vivo evidence for the cleavage of SecY-PhoA fusion protein 3-3 by leader peptidase. Fusion 3-3 contains the sequence Ala-202–Ile–Ala in the SecY region just before the junction. The region containing this sequence was found to conform to the leader peptidase cleavage site (36, 37), raising a possibility that the cleavage is catalyzed by leader peptidase. We used a mutant strain IT41 (*lep-9*) with temperature-sensitive leader peptidase (16) to examine this possibility. In this strain, unprocessed precursor forms of exported proteins accumulate at the nonpermissive temperature (42°C) as

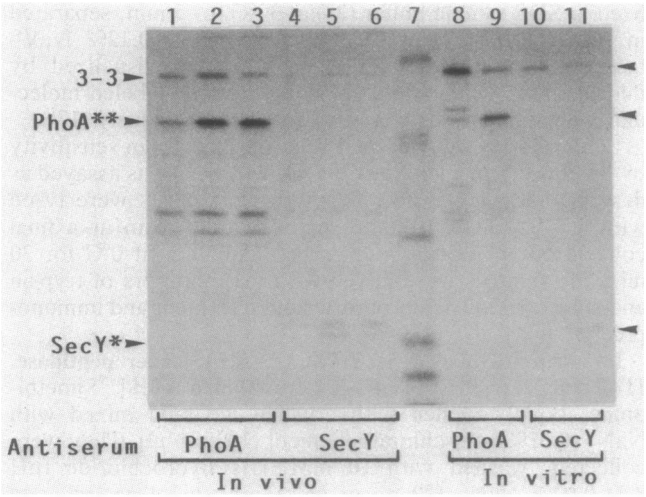

FIG. 1. Leader peptidase-catalyzed cleavage of SecY-PhoA 3-3 in vivo and in vitro. For in vivo assays, AD90(pKY4-3-3) cells were grown at 37°C and induced with 1 mM IPTG and 5mM cyclic AMP. After 6 min, cells were pulse-labeled for 30 s (lanes 1 and 4) with [^{35}S]methionine, followed by a chase for 1.5 (lanes 2 and 5) and 3 (lanes 3 and 6) min. For in vitro assays, IT41 (*lep-9*)(pKY4-3-3) cells were pulse-labeled for 1 min at 42°C, lysed by lysozyme-detergent treatment, and incubated at 37°C for 60 min with (lanes 9 and 11) or without (lanes 8 and 10) leader peptidase. Both the in vivo and in vitro samples were subjected to trichloroacetic acid treatment and immunoprecipitation with antiserum against PhoA (lanes 1 to 3, 8, and 9) or SecY (lanes 4 to 6, 10, and 11), followed by 10% SDS-PAGE and fluorography. PhoA** and SecY* indicate, respectively, the PhoA and the SecY fragments of SecY-PhoA 3-3 after cleavage by leader peptidase. Lane 7 was loaded with the molecular size standards (69, 46, 30, 18, and 12 kDa from top to bottom).

a result of inactivation of leader peptidase. The precursor OmpA protein accumulated under such conditions was susceptible to externally added protease in spheroplasts (data not shown), in agreement with the currently accepted notion that the leader peptidase function is not required for the translocation reaction per se (11). When 3-3 was expressed in IT41 (*lep-9*) and IT42 (*lep*$^+$) cells at 42°C, its cleavage in the former strain was found to be markedly retarded (Fig. 2; compare panels A and B). For some unknown reason, the kinetics of the 3-3 cleavage in the IT42 cells was somewhat slower than in AD90 cells previously studied (3), but the

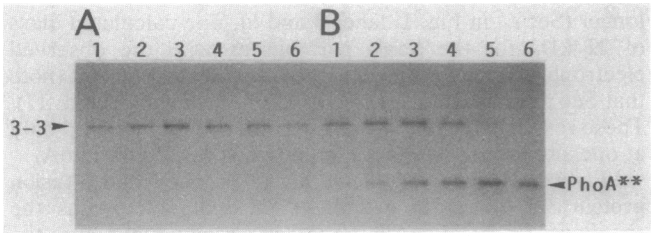

FIG. 2. Effect of the *lep* mutation on the in vivo cleavage of SecY-PhoA 3-3. IT41 (*lep-9*)(pKY4-3-3) (A) and IT42 (*lep*$^+$)(pKY4-3-3) (B) were grown first at 30°C and then at 42°C for 1 h. Cells were pulse-labeled for 1 min with [^{35}S]methionine at 6 min postinduction and chased for 0 (lanes 1), 1.5 (lanes 2), 3 (lanes 3), 6 (lanes 4), 12 (lanes 5), or 24 (lanes 6) min with unlabeled methionine. Proteins were immunoprecipitated with antiserum against PhoA and analyzed by 10% SDS-PAGE and fluorography.

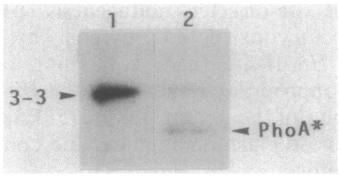

FIG. 3. Export of the PhoA domain of the SecY-PhoA fusion 3-3 in the *lep* mutant. IT41 (*lep-9*)(pKY4-3-3) cells were induced, pulse-labeled at 42°C, and converted to spheroplasts, which were then incubated at 0°C for 30 min with (lane 2) or without (lane 1) a combination of 1% Triton X-100 and 50 μg of trypsin per ml. The reaction was terminated by protease inhibitors and trichloroacetic acid precipitation. Immunoprecipitates with antiserum against PhoA were analyzed by 10% SDS-PAGE. The PhoA fragment generated by trypsin is indicated by PhoA*.

distinction between IT41 (*lep-9*) and IT42 was very clear; the full-length fusion protein was the major labeled species in IT41 even after a 24-min chase (Fig. 2A, lane 6).

Although the uncleaved fusion protein also accumulated under the translocation-impaired conditions in the *secY24* mutant (3), the full-length product observed in the *lep-9* mutant was distinct from the one observed in the *secY24* mutant in that its PhoA portion had been translocated to the periplasm. Thus, upon trypsin treatment of the Triton X-100-solubilized cell lysates, the fusion protein in IT41 (*lep-9*) generated the undigestible PhoA fragment (PhoA* in Fig. 3), whereas the fusion protein in the *secY24* mutant was completely digested (3). Such trypsin resistance is indicative of translocation for the PhoA sequence that assumes an enzymatically active (5) and trypsin-resistant (3) conformation only after localization to the periplasmic space.

In vitro evidence for the cleavage of fusion protein 3-3 by leader peptidase. The cleavage of the fusion protein by leader peptidase was confirmed in vitro, using a purified preparation of the enzyme (Fig. 1, lanes 8 to 11). When a detergent extract of IT41 containing the full-length fusion protein 3-3 was incubated with leader peptidase, produced were both the SecY fragment (Fig. 1, lane 11) and the PhoA fragment (lane 9) of the same sizes as the respective in vivo fragments (Fig. 1; compare lanes 2 and 9 for PhoA and lanes 5 and 11 for SecY). We conclude from these results that leader peptidase is the enzyme that is responsible for the cleavage of the SecY-PhoA fusion protein 3-3.

Leader peptidase recognition sequence is located within SecY. To determine whether leader peptidase indeed recognizes the Ala-202–Ile–Ala sequence and cleaves the Ala-204–His bond, we constructed a mutant form of fusion 3-3 with amino acid substitutions in this region. An oligonucleotide was designed to introduce site-directed alterations to the plasmid DNA such that the fusion gene now encodes phenylalanine instead of alanine at both residues 202 and 204 (Fig. 4). We chose the phenylalanine substitutions because the positions −1 and −3 of signal peptides generally contain small and uncharged amino acid such as alanine or serine. Such amino acid residues in the M13 coat protein leader peptide have indeed been shown to be critical for the recognition by leader peptidase and to be nonreplaceable by phenylalanine with a bulky side chain (19). Pulse-chase experiments revealed that the mutant form of the fusion (named 3-3F) was not cleaved in the *lep*$^+$ *sec*$^+$ cells (Fig. 5); no PhoA fragment could be detected after the chase time when the parental fusion was completely processed (compare lanes 1 and 6). Thus, the Ala-202–Ile–Ala sequence is

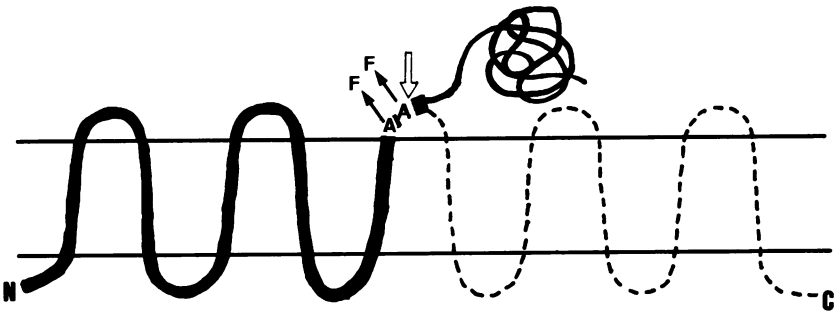

FIG. 4. Schematic representation of the leader peptidase recognition site in SecY-PhoA fusion 3-3. The thick line represents the SecY part, the thin line represents the PhoA part, and the dotted line represents the carboxy-terminal region of SecY which is missing in this fusion protein. Filled arrows indicate the two alanine residues that have been changed to phenylalanine in the SecY-PhoA 3-3F mutant; the open arrow indicates the putative site of the cleavage. It is not known whether the amino-terminal region of SecY in the fusion protein assumes the topology shown.

indeed critical for the cleavage by leader peptidase, which probably recognizes this sequence and hydrolyzes a peptide bond, presumably between Ala-204 and His-205.

DISCUSSION

The SecY-PhoA fusion protein 3-3 is peculiar among the several SecY-PhoA fusion proteins characterized previously in that it is subject to rapid cleavage as soon as its PhoA part is translocated to the periplasmic side. This cleavage occurs as rapidly as the cleavage of the ordinary signal sequences in protein secretion. The results presented above demonstrated that fusion 3-3 is cleaved by leader peptidase. We have identified both the amino-terminal (SecY part) and the carboxy-terminal (PhoA part) products of this endoproteolysis in vivo. The purified leader peptidase was found to convert 3-3 into these fragments in vitro. The in vivo cleavage was retarded in the temperature-sensitive *lep* mutant. Finally, the mutation that changed Ala-202–Ile–Ala of SecY to Phe-Ile-Phe rendered the fusion protein uncleavable in vivo. Thus, SecY contains a site at around amino acid residue 204 that can be recognized and cleaved by leader peptidase at least in an artificial construction of the hybrid protein.

Previously, the *degP* and *ompT* proteolytic systems were known to be involved in degradation of some fusion proteins in the periplasm (4, 7, 34). The results presented here show that leader peptidase can also participate in cleaving some special class of fusion proteins in vivo. Whether this observation is fortuitous or has some general significance depends on how frequently membrane proteins contain a potential leader peptidase cleavage site at the periplasmic side of their transmembrane segments. There have indeed been some precedents in which a transmembrane sequence is followed by a potential signal peptidase-processing site and in which some structural manipulations make such a cleavage site actually get cleaved (25, 26, 31). However, these studies relied on the eucaryotic cell-free translocation systems, and in none of these cases has it been examined whether such cleavage also occurs in vivo. Also, the enzyme responsible has not unequivocally been identified, although the signal peptidase has been assumed to be responsible. Such proteins include human invariant chain of class II histocompatibility antigen-chloramphenicol acetyltransferase fusion proteins (25, 26) and asialoglycoprotein receptor (31), both of which consist of a positively charged and amino-terminally located cytoplasmic domain, a single transmembrane segment, and a large carboxy-terminal exocytoplasmic domain. They became proteolytically processed during in vitro membrane translocation when the cytoplasmic domain or the carboxy-terminal end of the transmembrane segment was deleted. It was thought that the deletions induced a structural or steric change to the transmembrane region such that the cryptic cleavage site became accessible to the signal peptidase.

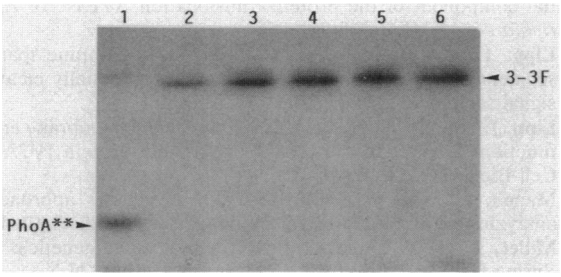

FIG. 5. Pulse-chase of SecY-PhoA 3-3F. AD90(pKY4-3-3) (lane 1) and AD90(pKY4-3-3F) (lanes 2 to 6) were induced, pulse-labeled for 30 s, and chased for 0 (lane 2), 1 (lane 3), 2 (lane 4), 4 (lane 5), or 6 (lanes 1 and 6) min at 37°C. Samples were analyzed as described in the legend to Fig. 2.

In contrast to the above-mentioned examples, the SecY-PhoA 3-3 fusion protein contains the element that unmasks the cleavage site carboxy terminally; the PhoA sequence that follows the SecY part activates the cleavage site. The PhoA sequence might cause some structural change to the preceding SecY region and make it more susceptible to the cleavage by leader peptidase. A calculation by the method of Chou and Fasman (10) showed that the β-turn probability of the sequence around the proposed cleavage site is higher in the context of fusion protein 3-3 than in the intact SecY. Such β-turn structure may favor the cleavage by leader peptidase (6). Alternatively, the force generated by the folding reaction of the PhoA moiety may "pull out" the cleavage site, which may otherwise be buried in the membrane.

We showed previously that SecY contains multiple regions, namely, the alternate transmembrane segments (starting from the most amino-terminal one) that can promote translocation of the following PhoA mature sequence in a *secY*$^+$-dependent manner. These transmembrane sequences thus possess one of the features of signal sequence, that is, to promote translocation of adjacent carboxy-terminal part. The fifth transmembrane segment has now been shown to

have another feature of signal sequence as well: to be recognized and cleaved by leader peptidase.

It is intriguing to ask why transmembrane segment 5 of SecY is followed by a leader peptidase cleavage site that could be activated, albeit artificially. As already pointed out, the existence of such a site could be fortuitous, but the possibility cannot be excluded that it has some evolutionary or functional significance. For instance, the signal peptides and the transmembrane sequences may have evolved from a common origin, or one of them may have diverged from the other.

It is possible that under certain circumstances, the cleavage site actually undergoes cleavage even in SecY itself. We have pursued this possibility but so far failed to obtain evidence for this assumption. However, one observation suggested a possible link between the leader peptidase function and the biogenesis of SecY. We found that the apparent synthesis level of plasmid (pKY4)-encoded SecY was severalfold higher in the *lep* mutant (IT41) than in the isogenic *lep*$^+$ strain, and the cells of IT41 harboring pKY4 were very unstable (unpublished results). This observation could be interpreted to mean that the overproduced SecY protein was cotranslationally cleaved by leader peptidase. However, the phenylalanine substitution mutation that prevented the cleavage of the fusion protein did not cancel the effect of the *lep* mutation; even the mutated SecY protein was synthesized at an apparently higher rate in *lep-9* cells than in wild-type cells. Thus, if leader peptidase indeed has a hitherto unrecognized role in triggering the scavenging of excess SecY protein, some unidentified cleavage site in addition to Ala-202–Ile–Ala should be involved. It is important to examine more systematically how frequently integral membrane proteins contain potential leader peptidase cleavage sites.

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