

# An essential amino acid residue in the protein translocation channel revealed by targeted random mutagenesis of SecY

Hiroyuki Mori and Koreaki Ito\*

Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan

Edited by William T. Wickner, Dartmouth Medical School, Hanover, NH, and approved February 21, 2001 (received for review December 27, 2000)

The SecY/Sec61 $\alpha$  family of membrane proteins are the central subunits of the putative protein translocation channel. We introduced random mutations into a segment of *Escherichia coli* SecY within its cytoplasmic domain 5, which was shown previously to be important for the SecA-dependent translocation activity. Mutations were classified into those retaining function and those gaining a dominant-interfering ability caused by a loss of function. These analyses showed that Arg-357, Pro-358, Gly-359, and Thr-362 are functionally important; Arg-357, conserved in almost all organisms, was identified as an indispensable residue.

For proteins to make up a living cell, some of them must be translocated out of the cytosol. To allow transport of selected proteins, the cytoplasmic membrane in prokaryotic cells and the endoplasmic reticulum membrane in eukaryotic cells contain conserved heterotrimeric membrane protein complexes termed SecYEG and Sec61 $\alpha\beta\gamma$ , respectively (1). They are thought to constitute a protein-translocating channel (2). SecY is the central subunit of the SecYEG complex (3) and is in close proximity of the translocating polypeptide chain (4). It interacts specifically with SecA to activate the translocation ATPase activity (5–7). SecY has 10 transmembrane segments with both termini facing the cytoplasm (Fig. 1). Among its six cytoplasmic domains, the fourth domain (C4) is important for the interaction with SecE (8), whereas the fifth and the most C-terminal cytosolic domains (C5 and C6) are important for translocation facilitation (9, 10). Thus, a number of cold-sensitive and export-defective mutations including *secY39* (R357H) are known to affect the C5 domain (11). Consistent with the importance of C5, we isolated a dominant-negative mutation, *secY<sup>d1</sup>*, with an internal deletion of 3 amino acids at the C5-transmembrane segment 9 junction, which produces an inactive but SecE-sequestering product (9). *In vitro* assays showed that inverted membrane vesicles from the C5 and C6 cold-sensitive mutants are defective in supporting the SecA translocation ATPase activities (10). Also known are a cold-sensitive mutation in C6 that does not allow the productive mode of SecA insertion reaction and suppressor mutations in *secA* against the defects of the C5/C6 mutations (refs. 7 and 12; unpublished data). Thus, the C5 and C6 regions are important for functional SecY-SecA interaction.

The preferential occurrence of *sec* mutations in C5 prompted us to investigate the importance of this region more systematically at the amino acid residue level. We thus undertook extensive random mutagenesis of a C5 segment and found that Arg-357 is particularly important for SecY activity.

## Materials and Methods

**Escherichia coli Strains.** Strains AD202 (MC4100, *ompT::kan*), GN40 (AD202, *leu82::Tn10*) and KI438 [CSH26, *Δlac-pro secA-lacZ-f181* ( $\lambda$ PR9)] were described previously (7, 9, 13). MR1 (AD202, *secY39 leu82::Tn10*), NH178 (AD202, *secA173 leu82::Tn10*), and NH193 (AD202, *secA329 leu82::Tn10*) were constructed by appropriate P1 transductions. The *secA173* (H545L) and the *secA329* (I502S) mutations had been isolated as a suppressor against the *secY205* defect (12) and the *secY39* defect (unpublished data), respectively.

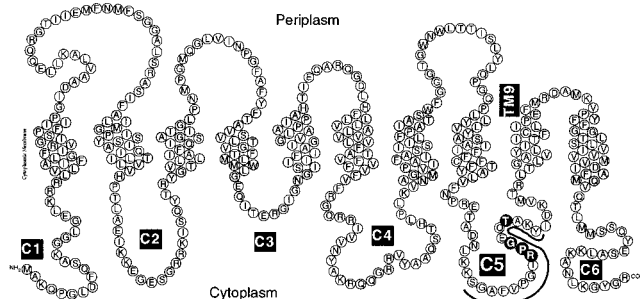


Fig. 1. Topology of SecY in the membrane and the target of mutagenesis. The cytoplasmic domains are indicated as C1–C6. The Ser-349 to Tyr-365 segment, shown by the wavy line, was subjected to the targeted random mutagenesis.

**Plasmid Libraries with Targeted Random Mutations for a Ser-349/Tyr-365 Segment of SecY C5 Domain.** A *secY* plasmid named pHMC5A was constructed by introducing a unique *Afl*III site at the position corresponding to Leu-346/Lys-347 without changing the coding (see Fig. 2) by site-directed mutagenesis. The parental plasmid had a *Nco*I–*Hind*III fragment (Fig. 2) from pCM10 (14) that was cloned into a variant of pTWV228 (11) having a *Nco*I site at the *lacZ* $\alpha$  initiation codon. Thus, pHMC5A carried unique *Afl*III, *Eco*RI, and *Cla*I sites in a segment of *secY* corresponding to Leu-346/Ile-366 (Fig. 2). To introduce directed random mutations, a series of degenerate oligonucleotides were synthesized chemically by using a mixture of four nucleotide precursors for the first and the second positions and a mixture of G and C precursors for the third position of each target codon of the Ser-349/Tyr-365 interval (Figs. 1 and 2). Two complementary chains were phosphorylated and annealed. They covered the *Afl*III–*Eco*RI, *Afl*III–*Cla*I, or *Eco*RI–*Cla*I segment, and they were used to substitute the corresponding portions of pHMC5A. The oligonucleotides additionally were designed to have their *Afl*III ends or the *Eco*RI ends (in the case of the *Eco*RI–*Cla*I fragments) ligatable but not cleavable after ligation with the vector (Fig. 2). This design enabled us to eliminate, by a restriction enzyme digestion, the original plasmid molecules that escaped the restriction or reincorporated the original fragment. We thus constructed a series of plasmid mutant libraries in which the *secY* gene had random mutations at each specified codon of Ser-349 to Tyr-365. Random sampling of transformants and sequencing of the plasmids indicated that almost all of the plasmids had a nucleotide alteration(s) at the target sites.

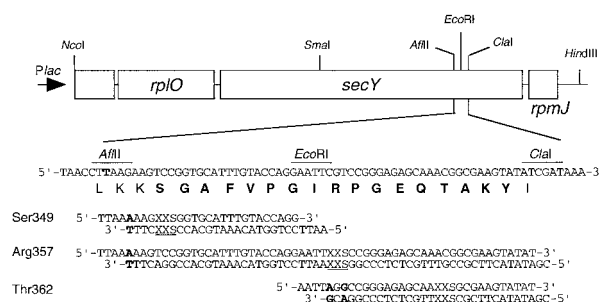
**Reconstitution of Translocase.** Wild-type SecA protein and SecA329 mutant protein were overproduced and purified as described (refs.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviation: MBP, maltose-binding protein.

\*To whom reprint requests should be addressed. E-mail: kito@virus.kyoto-u.ac.jp.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.



**Fig. 2.** Plasmid and oligonucleotides used for targeted random mutagenesis of a C5 segment of SecY. (Upper) The chromosomal segment cloned into pHMC5A, which contains an engineered *AflII* site created by a site-directed nucleotide change, is shown. (Lower) Either the *AflII*-*EcoRI*, *EcoRI*-*ClaI*, or the *AflII*-*ClaI* region was replaced by oligonucleotides that had mutations at a particular *secY* codon. Three representative oligonucleotides (mutation targets for Ser-349, Arg-357, and Thr-362, respectively) are shown. X, either A, C, G, or T; S, G or C. The *AflII* and *EcoRI* recognition sequences at the upstream end of the oligonucleotides had been modified as shown in bold face.

7 and 15; unpublished data). Plasmid pCM66 carried *lacI<sup>q</sup>* as well as a region encoding an N-terminally (His)<sub>6</sub>-tagged SecY, which was fused in-frame to the *lacZ $\alpha$*  initiation codon of a pUC-based *lac* promoter vector (E. Matsuo, personal communication). The R357E mutation was introduced into it to yield pCM66(R357E). One of these plasmids and a pACYC184-based pTYE100 (T. Yoshihisa, personal communication) encoding (His)<sub>6</sub>-tagged SecE and SecG (expression level of SecG was low for unknown reasons) were introduced into strain AD202 lacking OmpT (13). Overproduction of SecYE and SecY(R357E)E were achieved by induction with 1 mM isopropyl-thio- $\beta$ -D-thiogalactoside for 1 h. The SecYE complex was solubilized from a membrane fraction with 2.5%  $\beta$ -octylglucoside and purified by DEAE2 column chromatography. (His)<sub>6</sub>-tagged SecYE complexes were eluted earlier than the chromosomal product without the tag. The SecYE preparations were mixed with separately purified SecG and *E. coli* phospholipids for reconstitution of proteoliposomes essentially as described by van der Does *et al.* (16). The proteoliposomes were energized by valinomycin/K-acetate treatments (17) and subjected to proOmpA translocation reactions essentially as described (7).

## Results

**Functional Substitutions Identified Among SecY Mutant Libraries for the Ser-349/Tyr-365 Interval in Domain C5.** We constructed a series of plasmid mutant libraries in which *secY* had random mutations

at each specified codon of a Ser-349/Tyr-365 segment in domain C5. To assess functional importance of each residue, mutations that did not impair the functionality of SecY significantly were screened first; each mutant library was introduced into the *secY39*(Cs) mutant (18), and transformants (20–239 colonies) were examined for their ability to grow at 20°C (Table 1). The frequencies of Cs<sup>+</sup> transformants were 30% or more for most positions. Although a majority of the Cs<sup>+</sup> cells showed nearly full growth (Fig. 3A, dark gray), some of them, especially those with alterations at residues 358, 359, and 362, exhibited only slow growth (Fig. 3A, light gray). It was noticed that complementation-positive clones were less than 10% at three consecutive residues of Arg-357, Pro-358, and Gly-359. Functional mutations were infrequent also (23%) at the Thr-362 position. Most notably, no complementing mutation was obtained at the Arg-357 position. Nucleotide sequencing identified 132 amino acid changes in SecY, among which 123 single amino acid changes are summarized in Fig. 3A. Some changes were obtained repeatedly (Fig. 3A) among the initial 600 clones that had been sequenced.

Export of maltose-binding protein (MBP) was examined by pulse-labeling the *secY39* mutant cells carrying one of the mutant *secY* plasmids. Although only  $\approx$ 20% of labeled MBP in this mutant was in the mature form at 20°C (Fig. 4A, column 11 and broken line), the *secY* mutant plasmids that fully complemented the growth defect allowed  $\approx$ 80% processing (columns 1, 2, 3, and 9) as the wild-type *secY* plasmid did (column 12). Thus, the amino acid substitutions shown in dark gray in Fig. 3A have been assigned as compatible with the functioning of SecY. Partially complementing mutations (shown in light gray), which were common among mutations in residues 358, 359, and 362, gave lower processing efficiencies (40–70%; columns 4–8 and 10). The latter mutations were weakly dominant-negative when overexpressed in *secY*<sup>+</sup> cells (data not shown). Generally, good correlation was observed between protein export proficiency and growth of the complemented *secY39* cells.

**Nonfunctional and Dominant-Negative Substitutions.** A lack of function can result from any nonsense, deletion, and frame-shift mutations, which are not of our primary interests. Nonfunctionality caused by an extreme instability of the product is not informative either. We showed that loss-of-function mutations at the C-terminal region of SecY would be dominant-negative because they will still bind to SecE and sequester this partner molecule (8, 9, 11). To screen nonfunctional mutants, we introduced each plasmid mixture into a strain carrying a *secA-lacZ* fusion, which can report the

**Table 1. Targeted random mutagenesis of the Ser-349/Tyr-365 segment of SecY**

Target position	Screening for functional mutations			Screening for dominant-negative mutations		
	Number examined	Number of Cs <sup>+</sup>	Percentage of Cs <sup>+</sup>	Number examined	Number of Sec <sup>-</sup>	Percentage of Sec <sup>-</sup>
Ser-349	58	45	78	282	3	1
Gly-350	42	16	40	340	3	1
Ala-351	28	20	73	298	20	7
Phe-352	64	30	48	358	17	5
Val-353	50	19	38	266	7	3
Pro-354	36	16	44	288	0	0
Gly-355	20	19	95	863	42	5
Ile-356	26	23	88	494	35	7
Arg-357	22	0	0	612	415	68
Pro-358	64	3	5	836	152	18
Gly-359	34	2	6	1,018	111	11
Glu-360	92	57	62	811	5	1
Gln-361	80	24	30	791	7	1
Thr-362	138	31	22	1,171	221	19
Ala-363	239	174	73	355	17	5
Lys-364	33	31	94	802	9	1
Tyr-365	23	22	96	729	12	2

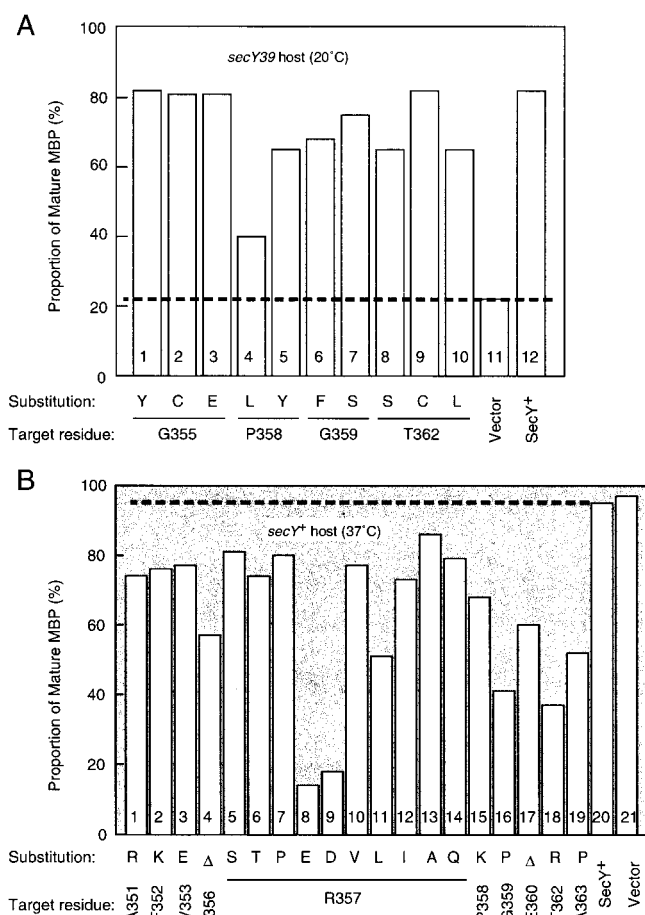
## A Functional substitutions

Asp	1										2				2		
Glu						1											
Lys					1		2					1		1		1	
Arg				1							1	3					
His	1						1			3				1	1		
Asn		1	2	2		1	1	1					2		2		
Gln		1	1	1	1					2	2				1		
Ser	1	2	1	7		2	1	1		5	4		3	7	3	4	
Thr			2	2								3	5	2		2	
Cys	1	1				2	1	1					1	2			
Gly		3	4				1				2		1		1	1	
Pro										3		4					
Tyr							1	1		4	1	1			2	1	3
Trp				1								3					
Phe	2	2	1	1		1					1	1			2		
Ala		1		2	1		1	2		1	1	2	1		5	1	2
Met			1	2	1					1		1					
Leu		1	4	3	2	2	2	2		1			7	1	1	4	
Ile	1		1		3	1	1			3		1	1		2		1
Val	1	4	1	9				1					2	3			2
	Ser	Gly	Ala	Phe	Val	Pro	Gly	Ile	Arg	Pro	Gly	Glu	Gln	Thr	Ala	Lys	Tyr
	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365

## B Dominant negative (non-functional) mutations

Asp				2				2									
Glu				4				3	2		*Δ1						
Lys			2						2				2			*	
Arg		4	1					*	2				3				
His									4								
Asn																	
Gln								2				*					
Ser	*							1	1								
Thr								1		2			*				
Cys																	
Gly		*					*			*			2				
Pro						*		1	*	6			5	10			
Tyr																	*
Trp																	
Phe			*														
Ala		*						1						*Δ1			
Met																	
Leu								1									
Ile							*Δ1	1									
Val				*				4									
	Ser	Gly	Ala	Phe	Val	Pro	Gly	Ile	Arg	Pro	Gly	Glu	Gln	Thr	Ala	Lys	Tyr
	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365

**Fig. 3.** Summary of the mutations obtained. (A) Functional substitutions. Shown at the bottom are the amino acid sequence of the target region (Ser-349 to Tyr-365) of SecY. Amino acid changes, specified at *Left*, are shown above each residue by dark gray boxes (which fully complemented secY39) and light gray boxes (which partially complemented secY39). Black boxes indicate the original amino acid. The numbers of the clones identified are shown in the boxes. (B) Dominant-negative (nonfunctional) mutations. Mutations that were obtained after screening for dominant-negative Sec phenotypes are summarized. Black boxes, substitutions; white boxes with thick frames and Δ, single residue deletions; \*, the wild-type residue. The numbers of the clones identified are shown in the boxes.



**Fig. 4.** Complementation and interfering phenotypes of the *secY* mutations. (A) Complementation of the *secY39* protein export defect. Strain MR1 (*secY39*) was transformed with a SecY mutant plasmid indicated at the bottom. Cells were grown with glycerol and maltose as carbon sources, first at 37°C and then at 20°C for 30 min, and pulse-labeled with [<sup>35</sup>S]methionine for 1 min (7). Labeled MBP was immunoprecipitated and proportions of radioactivities associated with the mature (presumably exported) form are quantitated by a PhosphorImager (7). The broken line shows the noncomplemented value. (B) Dominant interference. Strain AD202 (*secY*<sup>+</sup>) was transformed with two plasmids, pSTD343 (*lacZ*; Y. Akiyama, personal communication) and one of the dominant-negative SecY plasmids as specified at the bottom. Cells were grown at 37°C in the presence of 1 mM isopropyl-thio-β-D-thiogalactoside and 5 mM cAMP and pulse-labeled with [<sup>35</sup>S]methionine for 30 sec. The extents of MBP processing are shown as in A. The broken line shows the noninterfered value.

protein export status of the cell by the β-galactosidase activity (19). We saved transformant colonies that developed blue color because of increased β-galactosidase-mediated hydrolysis of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (9). Thus we were able to avoid simple knocking-out and destabilizing mutations.

Blue-colored colonies were obtained at frequencies that were correlated inversely with those of the functional mutations (Table 1; compare Fig. 2A and B). Thus, the dominant-negative mutations were frequent for the positions Arg-357, Pro-358, Gly-359, and Thr-362, at which functional mutations were rare. In particular, as many as 68% of the clones after mutagenesis of the Arg-357 position were of the nonfunctional/dominant-negative class. There were some variations in the extents of color development. We identified 27 single amino acid substitutions and three one-residue deletions (Fig. 2B) as well as 41 mutations (data not shown) with deletions, insertions, or substitutions of more than one amino acid residue in this region. These mutations may have been introduced by some aberrant products of oligonucleotides. None of the dom-

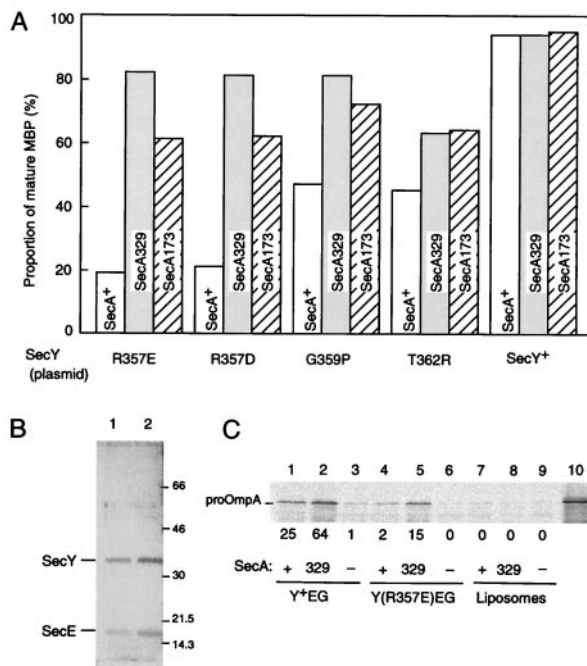
inant-negative class of mutations complemented the *secY39* mutant. Strikingly, 10 different dominant-negative substitutions were identified at the Arg-357 position, at which no functional substitution was obtained. Five and four different mutations were found at Pro-358 and Thr-362, respectively.

These mutant plasmids were expressed in a *secY*<sup>+</sup> strain, and export of MBP was examined at 37°C. Although MBP processing was ≈95% in the presence of the vector plasmid or a *secY*<sup>+</sup> plasmid (Fig. 4B, column 20 and broken line), it was retarded to below 80% in the presence of a mutant plasmid. Even this small reduction in the processing efficiency indicates a significant defect in the SecY mutant protein, because these assays were done in the presence of wild-type SecY molecules. The R357D and R357E substitutions conferred extremely strong translocation interference, giving only ≈20% processing of MBP (Fig. 4B, columns 8 and 9). These mutations had more adverse effects on cell growth than the other mutations, and they gave a deeper blue color in the colony assay. They seem to inactivate SecY almost completely, because their phenotypes were similar to those of deletions of as many as 12–15 residues in this region (data not shown). Mutations R357L, G359P, and T362R also inactivated SecY severely (Fig. 4B, columns 11, 16, and 18).

Although residue 357 was found to be particularly important, we did not obtain its substitution to a basic or aromatic amino acid. We thus constructed two substitutions, R357K and R357F, by site-directed mutagenesis. The former complemented weakly a *secY* defect and exhibited a weak dominant-negative character, whereas the latter was complementation-negative and strongly dominant-negative (data not shown). Thus, only a basic amino acid is allowed at this position, although Lys is still suboptimal.

**Arg-357 Is Crucial for the Translocase Activity of SecY.** In our characterization of the *secY205* (Cs) and the *secY39* (Cs) mutants, a number of extragenic suppressor mutations were isolated in *secA* (ref. 12; unpublished data). Large fractions of them proved to suppress a number of different *secY* alleles. It was found that the altered SecA proteins, called “superactive SecA,” possess highly elevated intrinsic ATPase activity (ref. 15; unpublished data). We combined a chromosomal suppressor mutation, *secA329* (I502S) or *secA173* (H545L), with a dominant-negative SecY (R357D, R357E, G359P, or T362R) plasmid. The dominant interference with the MBP export was alleviated significantly in the presence of these suppressors (Fig. 5A). The efficiencies of suppression varied combination-specifically to certain extents; *secA329* suppressed the severe SecY defects caused by R357E and R357D more effectively than the less severe T362R defect (Fig. 5A).

We overproduced (His)<sub>6</sub>-tagged versions of SecY and SecY(R357E) together with (His)<sub>6</sub>-tagged SecE and purified the (His)<sub>6</sub>-tagged SecYE complexes (Fig. 5B). By using these preparations and separately purified SecG, proteoliposomes containing purified (His)<sub>6</sub>-SecYEG or (His)<sub>6</sub>-SecY(R357E)EG were reconstituted. Their translocation activities were assayed by incubating them with radiolabeled proOmpA, SecA, and ATP. Under the conditions used, 25 and 2% of proOmpA was translocated into the SecY<sup>+</sup>- and SecY(R357E)-containing proteoliposomes, respectively (Fig. 5C, lanes 1 and 4). Thus, the SecYEG translocase activity is compromised markedly by the R357E substitution of SecY. When the purified SecA329 suppressor form of SecA was used to drive translocation, the translocation yield increased 2.5- and 7.5-fold, respectively, for the wild-type and mutant proteoliposomes (Fig. 5C, lanes 2 and 5). No significant translocation occurred into liposomes without added protein components (Fig. 5C, lane 8), showing that the superactive SecA only drives translocation in conjunction with SecYEG but not by itself or via some other pathway of translocation. The fact that the mutated SecY(R357E)EG responded *in vitro* to the superactive form of SecA to exhibit significant translocation activity indicates that it was not inactivated nonspecifically. Thus, the *in vivo* assessments used



**Fig. 5.** Translocase inactivation by dominant-negative *secY* mutations and reactivation by suppressor *secA* mutations. (A) Effects of the suppressor *secA* mutations on the dominant interference of protein export by some *SecY* alterations. Strains GN40 (*secA*<sup>+</sup> *secY*<sup>+</sup>; open columns), NH178 (*secA329 secY*<sup>+</sup>; shaded columns), and NH193 (*secA173 secY*<sup>+</sup>; striped columns) were transformed with two plasmids, pSTD343 (*lacI*<sup>R</sup>) and a mutant or a wild-type *SecY* plasmid, as specified at the bottom. The extents of MBP processing were assessed as described in Fig. 4B. (B) SDS/PAGE profiles of the purified preparations of (His)<sub>6</sub>-tagged *SecYE* (lane 1) and (His)<sub>6</sub>-tagged *SecY(R357E)E* (lane 2), each 1 μg of protein, as stained with Coomassie brilliant blue. (C) *In vitro* translocation activities of reconstituted proteoliposomes. Proteoliposomes were reconstituted with (His)<sub>6</sub>-tagged *SecYE* complex (0.2 μg, lanes 1–3) and *SecG* (0.2 μg), (His)<sub>6</sub>-tagged *SecY(R357E)E* complex (0.2 μg, lanes 4–6) and *SecG* (0.2 μg), or no added proteins (lanes 7–9). They were incubated at 37°C for 10 min with *in vitro*-translated and <sup>35</sup>S-labeled *proOmpA* in the presence of either *SecA*<sup>+</sup> (lanes 1, 4, and 7) or *SecA329* (lanes 2, 5, and 8) or in the absence of *SecA* (lanes 3, 6, and 9). Lane 10 shows input *proOmpA*. ATP (1 mM) was present in all assays. Samples were treated with proteinase K (0.1 mg/ml) on ice for 20 min and subjected to SDS/PAGE and PhosphorImager exposure. The values below the gels represent the proportions (percentage) of translocated *proOmpA*.

in our analyses faithfully represented the molecular functionality of *SecY* in facilitating protein translocation, in which Arg-357 plays a crucial role.

## Discussion

It is unlikely that a *SecYEG* channel is just a static structure in the membrane. It should be under precise gate control such that it is open only to allow transport of selected polypeptides without significant leakage of nonspecific solutes. Ribosomes, in the eukaryotic system, and *SecA*, in the prokaryotic system, may induce major subunit arrangements of the channel complex (20, 21). It is conceivable that there are specific regions in *SecY* that are crucial for the dynamic functionality of the channel. To identify such a region at the residue level, we worked out efficient strategies of mutagenesis and functional assignments of mutants. We focused the present analysis on a cytosolic domain of *SecY*, because cytoplasmic events such as interaction with a translocation substrate and with *SecA* for initiation of translocation will be fundamental for all the subsequent translocation reactions. We choose the Ser-349 to Tyr-365 interval as the target of mutagenesis on the basis of the locations of the previously isolated cold-sensitive *secY* mutations: *secY39* (R357H), *secY110* (R357C), *secY122* (G359R), and *secY40*

(or *secY115*, A363T). All these mutations impair the abilities of inverted membrane vesicles to activate the translocation ATPase of *SecA* (10).

In our screenings, simple knocking-out and destabilizing mutations must have been excluded, because such mutations will never complement a loss of function of the chromosomal *secY* gene or interfere with its normal functioning. In addition, mutations that cause extreme toxicity, if any, must have escaped the isolation. Partly because of the screening design and partly because of the nonexhaustive nature of our isolation, we did not obtain all of the possible changes of each residue. Nevertheless, the results were sufficiently informative to tell which residues are important for *SecY* functionality.

Newly synthesized *SecY* molecules are under mutual competition with respect to their binding with *SecE*, which determines stability of *SecY* (8, 22). Thus, those molecules that have failed in the binding are eliminated rapidly by the *FtsH* protease (23). We examined cellular accumulation of three weakly complementing and four dominant-negative *SecY* variants (including R357E) when they were overproduced together with *SecE*. They were all as stable as the wild-type *SecY* that was expressed similarly (data not shown). Thus, the different phenotypes of *SecY* variants cannot be explained primarily by stability difference.

Residues other than Arg-357, Pro-358, Gly-359, and Thr-362 can be mutated to a large variety of different amino acids without causing major defects (Fig. 3A), although basic amino acids are less favored at residues 351 and 352, and acidic amino acids are less favored at residue 353. Even the latter substitutions only mildly compromised the *SecY* function (Fig. 4B). In contrast, almost any changes at Arg-357 inactivated *SecY* and conferred dominant-negative properties (Figs. 3B and 4B), although dominant-negative substitutions were isolated also at Pro-358, Gly-359, Thr-362, and Ala-363. From these results, we surmise that Arg-357 is absolutely required, whereas the two residues C-terminally adjacent to it as well as Thr-362 and Ala-363 are important also.

We identified a total of 10 different substitutions of Arg-357 as dominant-negative mutations. Taken together with the two cold-sensitive mutations (*secY39* and *secY110*) as well as with the two site-directed mutations (R357F and R357K), a total of 14 substitutions have been characterized. Only the Lys substitution retained some weak complementation activity. In contrast, Asp and Glu substitutions caused extremely severe dysfunction of *SecY*. Thus this position should at least be basic, but Arg is required specifically for the full functionality. Our reconstitution experiments showed compellingly that the R357E mutated translocase was virtually inactive in translocation activity. However, this mutational defect, but not the defect caused by a deletion of an Ala-343/Arg-357 interval (data not shown), was still suppressible by the superactive forms of *SecA*. The suppression seems to require a preserved overall architecture of the *SecYE* complex and possibly represents a recovery of a productive *SecY*–*SecA* interaction (7, 12). The limited suppression specificity observed *in vivo* with respect to the *secY* alleles supports this notion. The R357E *SecY* alteration may impair an aspect of *SecA*–*SecY* interaction, in which *SecA* is activated to energize translocation.

The Arg-357, Pro-358, Gly-359, and Thr-362 residues, which were shown to be important in this study, are all well conserved among *SecY* homologues from prokaryotes as well as from chloroplasts, in which *SecA* acts as the primary “motor.” In addition, Pro-354 and Gly-355 are well conserved (Fig. 6, Left). This segment did not show highly significant homology with the eukaryotic *Sec61α* sequences (Fig. 6, Right). The *Sec61α* sequences themselves contain several absolutely conserved residues in this segment including Gly-404, Arg-406, Glu-413, and Leu-414 (numbering for *S. cerevisiae* *Sec61p*; Fig. 6, Right). These observations are consistent with a notion that this segment of *SecY/Sec61* is involved in some functions that are specific for prokaryotes and eukaryotes, respectively. The most

## Prokaryotes/Chloroplasts

## Eukaryotes

<i>Escherichia coli</i> (349)	SGAFVPGIRPGEQTAKY	<i>Saccharomyces cerevisiae</i> (398)	QGMVINGKRETSIYREL
<i>Vibrio cholerae</i> (347)	SGAFVPGIRPGEQTAKY	<i>Yarrowia lipolytica</i> (397)	QGIIVIAGRRETSAYREL
<i>Haemophilus influenzae</i> (345)	SGAFIPGIRPGEQTSRY	<i>Candida albicans</i> (397)	QGIIVIAGRHRTSAYREL
<i>Staphylococcus carnosus</i> (356)	QGSYVPGIRPGEQTAKY	<i>Schizosaccharomyces pombe</i> (397)	QQLVMAHGRECSMYREL
<i>Leptospira interrogans</i> (356)	YGFIPGIRPGSHTKKEY	<i>Saccharomyces cerevisiae Ssh1p</i> (403)	QGITLMLGRREONVAKEL
<i>Aquifex aeolicus</i> (343)	AGAFIPGVRPQDITVKY	<i>Homo sapiens</i> (397)	QQMVMRGHRETSVMHEL
<i>Streptomyces scabies</i> (348)	YGFIPGIRAGRPTEAY	<i>Canis familiaris</i> (397)	QQMVMRGHREKSMIHEL
<i>Streptomyces griseus</i> (347)	YGFIPGIRAGRPTEAY	<i>Mus musculus isoform 1</i> (397)	QQMVMRGHRETSVMHEL
<i>Rickettsia prowazekii</i> (340)	YGAVIPGKRPKNTSDY	<i>Mus musculus isoform 2</i> (397)	QQMVMRGHRDTSVMHEL
<i>Mycobacterium smegmatis</i> (353)	FGGFIPGIRPKPTADY	<i>Arabidopsis thaliana</i> (398)	QQMVMRGHRESNLQKEL
<i>Mycobacterium tuberculosis</i> (350)	FGGFIPGIRPKPTADY	<i>Triticum aestivum</i> (398)	QQMVMRGHRESNLQKEL
<i>Corynebacterium glutamicum</i> <sup>3</sup> (348)	YGFIPGIRPGRPTAEY	<i>Drosophila melanogaster</i> (344)	QHMVMRGHRENSMIHEL
<i>Thermotoga maritima</i> (336)	YGGYIPGIRPGRSTEQY	<i>Phaseolus vulgaris</i> (399)	QQMVMRGHRESNLQKEL
<i>Helicobacter pylori</i> (330)	NGGYIPGIRPGECTSSF	<i>Caenorhabditis elegans</i> (394)	QQMVMRGHREKSMIHEL
<i>Mycobacterium capricolum</i> (379)	SGTFIPGIRPKKDTIKY	<i>Halocynthia roretzi</i> (396)	QQMVMRGHREKSMIHEL
<i>Mycoplasma pneumoniae</i> (361)	SGRFIPGIRPKDTRKH	<i>Pyrenomonas salina</i> (402)	QQMVMKGRASSMCREL
<i>Mycoplasma gallisepticum</i> (356)	SHKFIPGVRPGLDTRKH	<i>Schizosaccharomyces pombe no.2</i> (262)	NQIVIAAGYREATMLKEL
<i>Bacillus sp.</i> (344)	QGGYIPGIRPKKATQTY	<i>Arabidopsis thaliana</i> (398).	QQMVMRGHRDSNLQKEL
<i>Bacillus subtilis</i> (335)	QGGYIPGVRPKMTQDR		
<i>Lactococcus lactis</i> (346)	QGSYIPGVRPKGTBKY		
<i>Synechocystis sp.</i> (353)	MGSIPGIRPKKTEQY		
<i>Chlamydia trachomatis</i> (358)	NGAFIPGIRPKKPTQTY		
<i>Antithamnion sp.</i> (319)	MGASIPNIRPGETIKY		
<i>Cyanophora paradoxa</i> (388)	MNALIPGVRPGETIKY		
<i>Synechococcus sp</i> (351)	MGSIPGVRPGRATSCY		
<i>Deinococcus radiodurans</i> (358)	AGGFIPGVRPGEPTADF		
<i>Pyrenomonas salina</i> (325)	MESSIPGVRPKKATDLY		
<i>Chlamydia pneumoniae</i> (358)	NNAFIPGIRPKKPTQHY		
<i>Odontella sinensis</i> (338)	MAVSIPGIRPGEVETIFY		
<i>Porphyra purpurea</i> (324)	MGASIPNIRPGEATDLY		
<i>Arabidopsis thaliana</i> (454)	QGASIPLVRPKKSTALF		
<i>Spinacia oleracea</i> (453)	QGASIPLVRPKKATAAY		
<i>Zea mays</i> (458)	QGASIPLVRPKKSTAAAY		

**Fig. 6.** Conservation of the Ser-349/Tyr-365 segment in different organisms. (Left) The amino acid sequence of the C5 domain was subjected to a homology search by using the BLAST program, and the Ser-349/Tyr-365 region is shown. The number in parenthesis indicates amino acid residue number of the residue that is equivalent to *E. coli* Ser-349 in each organism. Residues identical with those of *E. coli* SecY are shown in reverse font. (Right) *Saccharomyces cerevisiae* Sec61p sequence was aligned with *E. coli* SecY by using the CLUSTALW program. The region that corresponds to C5 of *E. coli* SecY was subjected to BLAST homology searches, and the Ser-349/Tyr-365 equivalent regions are shown. Residues identical with those of *S. cerevisiae* Sec61p are shown in reverse font.

likely function might be the SecA binding for the former and the ribosome binding for the latter.

Interestingly however, sequence alignment indicated that the Arg-406 residue of *S. cerevisiae* Sec61p may correspond to Arg-357 of *E. coli* SecY, making this Arg residue conserved in almost all organisms sequenced thus far (Fig. 6, \*). It is conceivable that this residue is involved in some fundamental characteristic of the protein translocation channel. One essential element common between prokaryotes and eukaryotes may be the signal sequence of presecretory proteins (24). Our characterization of the *secY39* (R357H) mutant suggests that the mutation impairs the initiation step of translocation that occurs after precursor protein binding and before the signal sequence cleavage (unpublished data). Thus,

Arg-357 may be involved in the initiation of translocation that occurs under the mutual interactions of SecYEG, SecA, and the signal sequence region of the precursor protein. The present results will guide further characterization of this interesting molecular machinery found in the membranes of all organisms.

We thank Ei-ichi Matsuo, Yoshinori Akiyama, Hitoshi Nakatogawa, Tohru Yoshihisa, and Gen Matsumoto for plasmids, *E. coli* strains, and stimulating discussion, and Yusuke Shimizu and Kiyoko Mochizuki for technical supports. This work was supported by grants from Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, and from the Ministry of Education, Science and Culture, Japan.

- Rapoport, T. A., Jungnickel, B. & Kutay, U. (1996) *Annu. Rev. Biochem.* **65**, 271–303.
- Johnson, A. E. & van Waas, M. A. (1999) *Annu. Rev. Cell Dev. Biol.* **15**, 799–842.
- Ito, K. (1996) *Genes Cells* **1**, 337–346.
- Joly, J. C. & Wickner, W. (1993) *EMBO J.* **12**, 255–263.
- Lill, R., Dowhan, W. & Wickner, W. (1990) *Cell* **60**, 271–280.
- Economou, A., Pogliano, J. A., Beckwith, J., Oliver, D. B. & Wickner, W. (1995) *Cell* **83**, 1171–1181.
- Matsumoto, G., Yoshihisa, T. & Ito, K. (1997) *EMBO J.* **16**, 6384–6393.
- Baba, T., Taura, T., Shimoike, T., Akiyama, Y., Yoshihisa, T. & Ito, K. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 4539–4543.
- Shimoike, T., Akiyama, Y., Baba, T., Taura, T. & Ito, K. (1992) *Mol. Microbiol.* **6**, 1205–1210.
- Taura, T., Yoshihisa, T. & Ito, K. (1997) *Biochimie* **79**, 512–517.
- Taura, T., Akiyama, Y. & Ito, K. (1994) *Mol. Gen. Genet.* **243**, 261–269.
- Matsumoto, G., Nakatogawa, H., Mori, H. & Ito, K. (2000) *Genes Cells* **5**, 991–1000.
- Akiyama, Y. & Ito, K. (1990) *Biochem. Biophys. Res. Commun.* **167**, 711–715.
- Matsuo, E. & Ito, K. (1998) *Mol. Gen. Genet.* **258**, 240–249.
- Nakatogawa, H., Mori, H. & Ito, K. (2000) *J. Biol. Chem.* **275**, 33209–33212.
- van der Does, C., Manting, E. H., Kaufmann, A., Lutz, M. & Driessen, A. J. (1998) *Biochemistry* **37**, 201–210.
- Driessen, A. J. & Wickner, W. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 201–210.
- Baba, T., Jacq, A., Brickman, E., Beckwith, J., Taura, T., Ueguchi, C., Akiyama, Y. & Ito, K. (1990) *J. Bacteriol.* **172**, 7005–7010.
- Riggs, P. D., Derman, A. I. & Beckwith, J. (1988) *Genetics* **118**, 571–579.
- Hanein, D., Matlack, K. E., Jungnickel, B., Plath, K., Kalies, K. U., Miller, K. R., Rapoport, T. A. & Akey, C. W. (1996) *Cell* **87**, 721–732.
- Manting, E. H., van Der Does, C., Remigy, H., Engel, A. & Driessen, A. J. (2000) *EMBO J.* **19**, 851–861.
- Taura, T., Baba, T., Akiyama, Y. & Ito, K. (1993) *J. Bacteriol.* **175**, 7771–7775.
- Kihara, A., Akiyama, Y. & Ito, K. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 4532–4536.
- Nielsen, H., Engelbrecht, J., Brunak, S. & von Heijne, G. (1997) *Protein Eng.* **10**, 1–6.