Superactive SecY Variants That Fulfill the Essential Translocation Function with a Reduced Cellular Quantity*

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The fifth and the sixth cytoplasmic regions (C5 and C6) of SecY are important for the SecA-driven preprotein translocation reaction. A cold-sensitive mutation, secY205 (Tyr-429 Asp), in C6 impairs the ATP- and precursor-dependent SecA insertion into the membrane. We now identified second site mutations that suppressed the defect. Cis-placement of these mutations proved to suppress mutations at another essential residue (Arg-357) of SecY as well. Thus, they tolerate the otherwise defective SecY alterations in the same molecule. Two alterations (Ile-195 to Ser in TM5 region and Ile-408 to Leu in TM10 region) were found to make the translocation channel more active, because it enabled cells to survive with reduced content of the SecYE complex. These mutations only very weakly suppressed a signal sequence defect of the A receptor protein. The mutant SecYEG translocase exhibited higher than normal activity in vitro, being accompanied by striking independence of the proton motive force as well as by stabilization of a bound and active SecA species against urea treatment. These results have been interpreted in terms of balance shifts between channel closing and channel opening alterations in the SecYEG translocase.

In Escherichia coli, translocation of presecretory proteins across the cytoplasmic membrane is mediated by the Sec translocation machinery (translocase). SecA is the preprotein-driving ATPase, whereas SecY, SecE, and SecG form a membrane-embedded complex that presumably functions as a translocation channel. Although recent progress in the structural biology of the SecYE(G) complex indeed revealed a channel-like structure and provided some evidence that the active translocation channel may be composed of multiple (2–4) units of the SecYE(G) complex, the molecular nature of the translocation channel is largely unknown. In addition to ATP, the PMF contributes to the active translocation reactions to proceed (3, 4). Among the integral membrane Sec components, SecY and SecE are minimally required for a basal SecA-driven translocation activity (5). SecG is not essential in vivo or in vitro, but it greatly stimulates the SecA reaction cycles (6–8). SecY and SecE contain 10 and 3 transmembrane segments, respectively (9, 10). One important function of SecE is to stabilize SecY; SecY molecules that have failed in the complex formation are rapidly eliminated by the FtsH protease in vivo (11). Thus, a mutation, secE501, in the secE translation initiation region lowers cellular abundance of not only SecE itself but also SecY (12, 13).

The SecYE(G) complex also interacts with SecA, and specific interaction between these components is crucial for the SecA activation function (16). Specifically, a Tyr-429 to Asp substitution in the C6 domain by the secY205 mutation (see Fig. 1) impairs the SecA insertion reaction in response to ATP and a preprotein (16). We are particularly interested in this mutation because it may provide invaluable means to our structural and functional studies of the SecYEG channel. In this study, we carried out isolation and characterization of intragenic suppressor mutations against the secY205 deficiency. Although the mutations obtained turned out to suppress other defective alleles of secY as well, they proved useful for our understanding of how SecYEG channel is tuned in its activity to accept preproteins.

Materials and Methods

Media and Transduction—Minimal medium M9 was as described previously (20). L medium contained 10 g of bactotryptone, 5 g of yeast extract, and 5 g of NaCl per liter. P1 transduction was carried out by standard procedures (20), and unselected markers were scored as described previously (16).

E. coli Strains—E. coli strains used in this study are listed in Table 1. In order to introduce the zbd33/Tn10-linked secY mutations by P1 transduction, the Tet derivatives of the sec mutants, GN15 (secY205), TW155 (Δunc5BC) (16), and NH192 (secE501), were used. To construct strain NH192, prototrophic P1 transductants were selected to eliminate argE::Tn10 linked to secE501 (12).

Isolation of Revertants from the secY205 Mutant—Cells of the HM1001 (secY205 zbd33/Tn10) were UV-irradiated (14 J/m² for 10 s) and divided into several portions, which were cultured at 37 °C in L medium overnight and plated on L medium for selection at 20 °C. About 20–50 colonies (frequency, about 10⁻⁸) appeared after 3 days of incubation at 20 °C. Revertants from each independent source were pooled for preparation of a P1 vir phage lysate, which was used to infect GN15 (secY205 tet₅) to select Tet transductants at 20 °C. We only saved at most two transductants from an independent source. They were all confirmed to have a Cs⁺ determinant cotransducible with the transposon by individual transduction experiments into GN15 again. Thus we established 33 revertants from 19 independent pools. Their chromosomal secY regions were sequenced as described previously (21).

Introduction of the Mutations into the Cloned secY—Site-directed mutations were introduced into pHMC54 (Ref. 22; a pBR322-based plasmid carrying secY⁺) by the QuickChange method (Stratagene) us-
ing appropriate oligonucleotide primers. All the constructs were confirmed for nucleotide sequence changes as well as for the lack of any unwanted changes. Some segments were replaced by those from the original plasmid. 

Construction of secY743 and secY762 Single Mutant Strains—Strain SY92 (secY743) was constructed as follows. EM155 (polA1 secY24 zhd33::Tn10) was transformed with a plasmid pSY3 carrying the secY743 allele, which was introduced into AD202 by homologous recombination, and the plasmid-integrated strain was subjected to excision of the plasmid by P1 transduction to obtain strain HM1315. Strain HM1315 was used to construct strain SY92. Strain HM1315 (polA1 polA8) was transformed with a plasmid pHM448 by inserting the secY762 gene between the lamB60::Tn10 and MBP (a periplasmic protein) was examined by pulse-labeling cells for the indicated times with [35S]methionine and immunoprecipitation of these proteins. They were then separated by SDS-PAGE into the precursor and mature forms. Intracellular accumulation of SecY and SecE was examined by SDS-PAGE of whole cell proteins and their staining with respective antibodies. All the details of the immunoprecipitation and immunoblotting experiments were described previously (24). Visualization and quantification were by a Fuji BAS1800 PhosphorImager and a Fuji LAS1000 lumino-imager, respectively, for pulse-chase and immunoblotting experiments.

**In Vitro Translocation Assay—In vitro translocation reactions were carried out according to the published procedures (22, 25).** [35S]-Labeled proOmpA was prepared as described previously (25). Standard translocation reactions contained IMV (250 μg protein/ml), SecA (20 μg/ml), SecB (15 μg/ml), ATP (1 mM), phosphocreatine (60 mM), and creatine kinase (25 μg/ml) in buffer consisting of 50 mM Tris-HCl (pH 8.0), 5 mM MgSO4, and 500 μg/ml bovine serum albumin. After preincubation at 37°C for 20 s, [35S]-labeled proOmpA (1/20 the total volume) was added to start the reaction. To stop the reaction, samples were chilled on ice and treated with 0.1 mg/ml proteinase K on ice for 20 min followed by trichloroacetic acid precipitation and SDS-PAGE for quantification of translocated OmpA species. The PMF was generated by inclusion of 5 mM succinate and dissipated by 10 μM CCCP.

**Measurement of SecA ATPase Activity—A coupled spectrophotometric assay involving pyruvate kinase and lactate dehydrogenase was performed as described (26).** 

**Preparation of [35S]-Labeled SecA—A SecA overproducing strain, GN50 (16), was grown at 37°C in 100 ml of M9 medium supplemented with 18 amino acids (20 μg/ml), glucose (0.4%), and ampicillin (50 μg/ml) until a mid-log phase, and transcription of secA was induced with 1 mM isoproprop-β-D-thigalactoside and 5 mM cAMP for 12 min, followed by labeling with [35S]methionine for 30 min. Cells were resuspended in 1× SecA buffer (27) containing 1 mM dithiothreitol and 0.1% Pefabloc (Merck) and were disrupted by sonication. After removal of debris and membranes by ultracentrifugation, the supernatant was mixed with 350 μl (bed volume) of Blue-Sepharose (Amersham Bio-

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**TABLE I**

**Bacterial strains and plasmids**

| Strain   | Relevant genotype | Ref. |
|----------|------------------|------|
| MC4100   | F' araD139 ΔargF-lacU169 rpsL150 relA1 fbb5301 delC1 ptsF25 rbsR | 39   |
| CU141    | Δpro-lac thi ara'' polA1 Tn5 F' lacZ22ΔM15 Y' pro'' | 40   |
| AD292    | MC4100, amyT::kan | 41   |
| AD298    | AD202, secY92 zhd33::Tn10 rpsE | 42   |
| EM151    | CU141, secY24 zhd33::Tn10 rpsE | Laboratory stock |
| GN15     | AD202, secY206 Tet'* | 16   |
| GN31     | AD202, secY39 Tet'' | 16   |
| GN73     | MC4100, lamB60 | Laboratory stock |
| JE6631   | Hfr str thi polA1 | TIGER cell stock |
| HM1001   | K1438, secY205 lew::Tn10 ara'' | This study |
| HM1285   | JE6631, zhd33::Tn10 | This study |
| HM1307   | AD202, secY73 zhd33::Tn10 | This study |
| HM1308   | TW155, secY73 zhd33::Tn10 | This study |
| HM1311   | NI192, secY73 zhd33::Tn10 | This study |
| HM1312   | NH192, secY762 zhd33::Tn10 | This study |
| HM1314   | GN73, secY762 zhd33::Tn10 | This study |
| HM1315   | AD202, secY762 zhd33::Tn10 | This study |
| HM1316   | GN73, secY762 zhd33::Tn10 | This study |
| HM1320   | TW155, secY762 zhd33::Tn10 | This study |
| HM1585   | GN73, secY743 zhd33::Tn10 | This study |
| HM1589   | GN73, secY743 secY205 zhd33::Tn10 | This study |
| HM1592   | CN73, secY762 secY205 zhd33::Tn10 | This study |
| HM1607   | GN73, secY205 zhd33::Tn10 | This study |
| NI192    | AD202, secE501 | Laboratory stock |
| SY92     | AD202, secY743 zhd33::Tn10 | This study |
| SY95     | TW155, secY743 zhd33::Tn10 | This study |
| SY101    | NH192, secY743 zhd33::Tn10 | This study |
| TW155    | AD202, secY743 zhd33::Tn10 | This study |
| TYE077   | MC4100, lamB60 prlA3 | Laboratory stock |

**Plasmid**

| Plasmid | Cloned secY allele | Ref. |
|---------|--------------------|------|
| pHMC5A  | +                 | 22   |
| pHM440  | 205, 762           | This study |
| pHM448  | 762                | This study |
| pSY3    | 743                | This study |
| pSY4    | 205, 711           | This study |
| pSY5    | 205, 733           | This study |
| pSY6    | 205                | This study |
| pSY7    | 205, 743           | This study |
| pTW228  | Vector             | 18   |

**Superactive SecY Variants**
Superactive SecY Variants

RESULTS

Intragenic suppressors against the secY205 defect—The secY205 mutant that carried an insertion of zhd33::Tn10 was mutagenized with ultraviolet light, and revertants were selected at 20 °C. Nineteen independent revertant pools were used as the donor sources in P1 transduction into a Tet S mutant host. The double mutant plasmids allowed nearly wild-type growth for the suppression.

Suppressor Mutations Works against Mutations at Arg-357, an Essential SecY Residue—The intragenic suppressors secY711, secY733, secY743, and secY762 obtained against secY205 were also combined on plasmids, with the secY39 mutation affecting an essential SecY residue, Arg-357 (22). Each plasmid was introduced into the cold-sensitive strain carrying the chromosomal secY39 mutation. Each of the double mutant plasmids, unlike the nonsuppressed secY39 plasmid, showed positive complementation against this host strain. This was demonstrated with respect to cell growth (data not shown) and MBP export (Fig. 3 A and B, lanes 1–4). The SecY205 mutant IMV exhibited lower than normal translocation (28% depending on the suppressor alterations significantly restore the SecY functionality that had been compromised by the Arg-357 alterations.

In Vitro Effects of the Intragenic Suppressor Mutations—IMVs were prepared from the secY205 mutant as well as the same mutant that carried one of the seven suppressor mutations shown in Fig. 4. They were combined with SecA and assayed for activities to translocate proOmpA at 20 °C with or without imposition of the PMF. Even under the PMF-imposed conditions, the SecY205 mutant IMV exhibited lower than normal activity (13% translocation in 10 min as compared with the wild-type value of 50%). In contrast, IMVs from the suppressed strains gave much higher translocation (48–81% depending upon the suppressor alleles). Remarkably, translocation activities of the SecY762-SecY205 and the SecY752-SecY205 IMVs were even higher than that of the wild-type IMV, allowing 81 and 71% translocation, respectively.

At this reaction temperature (20 °C), wild-type IMV exhibited strong dependence on the PMF (Fig. 4, lane 18); the PMF independence value (translocation yield in the absence of PMF) that in the presence of PMF) was only 0.02. IMVs from some of the suppressed mutants showed strikingly higher values. For instance, PMF independence values for SecY762-SecY205 IMV, SecY743-SecY205 IMV, and SecY733-SecY205 IMV were 0.7,
0.49, and 0.3, respectively. The other double mutant IMVs examined also showed significantly increased PMF independence values (0.13–0.25). Assays of the SecA translocation ATPase revealed that the suppressed IMVs supported higher ATPase activities than the SecY205 single mutant IMV (data not shown).

We showed previously (16) that the SecY205 alteration impairs the SecA insertion reaction in the presence of ATP and proOmpA protein. This was based on experiments using 125I-labeled SecA, in which a 30-kDa C-terminal fragment was detected as a trypsin-protected “insertion segment” (14, 28). Eichler and Wickner (29) showed that [35S]methionine-labeled SecA enabled detection of another insertion segment of 65 kDa derived from the N-terminal region of SecA. We now used this latter reaction system. Additionally, NaN3 was added to stabilize the inserted state of SecA (15). Whereas reaction with the wild-type IMV evidently generated the protease-protected 65-kDa fragment when incubated with 35S-SecA, proOmpA, and ATP (Fig. 5, lane 13), the SecY205 IMV did not significantly support the reaction (Fig. 5, lane 10). The suppressed IMVs showed recovered abilities to support the generation of the 65-kDa trypsin-protected fragment (representative data for the SecY711, the SecY733, and the SecY743 effects are shown in lanes 1, 4, and 7). These results show that the secY205 mutation impairs the insertion of not only C-terminal but also N-terminal portions of SecA and that the suppressor mutations restored the SecA insertion defect caused by the secY205 mutation. They are consistent with the suppressor-dependent re-
exhibited normal ability of MBP export (Fig. 6). The phenotypes at different temperatures. The mutant cells showed slightly slower growth rate than the wild-type cells. Otherwise, these mutants had no obvious growth affect in vitro translocation assay at 20 °C for 10 min in the presence of 5 mM succinate (+PMF) or 10 μM CCCP (−PMF). Samples were treated with proteinase K and subjected to SDS-PAGE and PhosphorImager exposure. Precursor and mature bands of OmpA molecule are shown as p and m, respectively. The protease-resistant precursor and mature bands were quantified, and the translocation yields (%) are shown.

FIG. 5. Intragenic suppressors alleviate the SecA insertion defect of SecY205. SecA insertion assay was essentially according to the procedure described previously (15). The insertion reaction, using 35S-labeled SecA (7,000 cpm/assay) and urea-washed IMV (final 100 μg/ml), was allowed at 37 °C for 15 min in the presence or absence of ATP and/or proOmpA as indicated. Samples were then treated with 20 mM of NaN3 at 37 °C for 15 min. After trypsin treatment (final 1 mg/ml) on ice for 15 min, trypsin-resistant 65-kDa fragment was visualized by PhosphorImager exposure following SDS-PAGE. Relative amounts of the trypsin-resistant 65-kDa fragment of SecA are shown. 100% is defined as the amount of the 65-kDa fragment obtained with wild-type IMV in the presence of ATP and proOmpA.

cov ery of SecA translocation ATPase activity. However, the insertion-deinsertion equilibrium might have been shifted slightly toward the latter, because the suppression effect was less clear without NaN3 added to inhibit deinsertion (data not shown).

Phenotypes of a Single secY743 and secY762 Alleles—We constructed single mutant strains having the secY743 or the secY762 mutation on the chromosome as described under “Materials and Methods.” These two mutations most effectively alleviated the PMF dependence of in vitro translocation in the presence of the secY205 alteration (Fig. 4). The secY762 single mutant cells showed slightly slower growth rate than the wild-type cells. Otherwise, these mutants had no obvious growth phenotypes at different temperatures. The secY762 mutant exhibited normal ability of MBP export (Fig. 6A, lane 2).

We then introduced these secY alleles into the secE501 mutant strain that is cold-sensitive for growth (12). This secE mutation affects the translation initiation region of secE resulting in its reduced translation. Because unassembled SecY molecules are eliminated by rapid proteolysis, the mutant cells contain a reduced amount of SecY as well (13; see Fig. 6B, lane 3). Thus, the secE501 mutant cells contain an insufficient amount of the SecYE translocase complex. The secY762 and the secY743 mutations proved to suppress the cold sensitivity when combined with the secE501 mutation (data not shown) and to stimulate significantly the MBP export ability of this mutant (Fig. 6A, compare lanes 3 and 4; data not shown for the secY743,secE501 combination). Interestingly, this suppression was not accompanied by any increase in the cellular abundance of SecE or SecY, which was decreased by about 50% by the secE501 mutation (Fig. 6B, compare lanes 3 and 4; data not shown for the secY743,secE501 combination). Thus, the suppressor mutations enhance the SecYE translocase function such that the 50% reduction in its amount is tolerated. It is possible that a mutant translocation channel can handle an increased number of secretory proteins for their export per unit of time.

Superactive Translocation by the SecY Alterations—The mutated translocases were studied in vitro. IMVs were prepared from secY743,uncBC and secY762,uncBC strains and sub-
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In this study, we isolated intragenic suppressor mutations that suppressed the cold sensitivity of the secY205 mutant. These mutations significantly alleviated the in vivo protein export defect and the in vitro protein translocation defect of the secY205 mutation. Translocation mediated by the suppressor mutant translocase depended less on PMF than that mediated by the wild-type translocase. Suppression was also demonstrated (for some of the mutations examined) with respect to the ability to support the SecA translocation ATPase activity and to support the SecA insertion reaction. Some of the mutations were also shown to alleviate not only the original secY205 defect but also certain secY mutations that occurred at another residue (Arg-357) of SecY. The broad suppression specificity may suggest that the suppression is not through specific alteration in inter-domain interaction between the original Asp-429 mutation and the suppressor mutation. As a result, the suppressor mutation might affect the functional characteristics of the SecYEG channel in the opposite directions such that the impaired functionality is restored due to a balance shift.

Our previous proposal about the secY205 change was that the SecYEG translocation channel became more closed such that it cannot accept the preprotein-SecA complex for the productive insertion (16). Given this hypothesis, the intragenic suppressor might affect the channel conformation toward more “open” states. Our results indeed showed that the two suppressor mutations, secY743 and secY762, themselves hyperactivate the translocase. These mutations are able to suppress the cold-sensitive growth defect and protein export defect of the secE501 strain, in which quantity of the SecYE complex is reduced by ~50% due to the reduced translation of the SecE subunit and proteolytic elimination of uncomplexed SecY subunit. The possibility that they rendered the altered SecY protein unsusceptible to the FtsH protease could be ruled out because the abundance of the mutant SecY protein remained in the same lowered level. Thus, the mutations increase the “specific functionality” of the SecYE protein translocation channel such that its reduced quantity is counterbalanced.

The higher than normal translocation activity was reproduced in the in vitro reactions using IMVs. These IMVs were indeed able to mediate translocation of an increased number of proOmpA molecules. Moreover, they exhibited apparent resistance to urea treatment. Although treatment of wild-type IMV with 6 M urea rendered the translocation activity almost exclu-
sively dependent on exogenous SecA, as much as ~60% translocation activity, without added SecA, of the SecY762 IMV remained after the same treatment. Immunoblotting experiments showed that the urea treatment removed some 50–80% of SecA molecules that were bound to either the SecY7 or the SecY762 IMV, with no systematic difference between these IMV preparations. Thus, we speculate that a special “activat- ed” form of SecA molecules that had been bound to the translocation site was stabilized by the SecY alterations to resist the urea treatment. Perhaps this SecA species remains within the translocation channel, being ready to undergo productive insertion-deinsertion cycles upon encountering a preprotein. Thus, the altered channel may accept more readily the translocation initiation domain of the preprotein. Manting et al. (35) identified a mutant SecY, after Cys-scanning mutagenesis of the TM7 region, which exhibited higher SecA-binding affinity. Incidentally, this mutation also weakens the SecY-SecE association affinity.2

Enhancement of translocation activity reduced PMF dependence (34), and increased SecA affinity has been noted for some of the prlA mutations (35). Our examinations showed that of the two suppressor mutations examined, secY743 and secY762, exhibited a weak Prl phenotype. However, the extent of the suppression against a lamB signal sequence mutation was an order of magnitude lower than that of the prlA3 mutation. We examined whether the prlA3 mutation suppressed the secY205 defect by the complementation ability of a prlA3-secY205 double mutant plasmid. The results showed that the cold-sensitive growth defect was not complemented, although the export defects of MBP and OmpA were alleviated by this plasmid (data not shown). Thus, prlA3 is less effective than secY762 in the ability of intragenic suppression against secY205. These results suggest that suppression against the secY defective mutation includes some element distinct from suppression against a signal sequence mutation.

The weak Prl phenotypes of the suppressor mutations were canceled under the conditions of secY205 suppression. These observations are consistent with the balance shift model of the suppression. It should be noted that IMVs from the secY205-secY743 and the secY205-secY762 double mutants showed strikingly decreased PMF dependence in the translocation reaction, despite the fact that these double mutants were no longer Prl. Thus, Prl phenotype, PMF-independence, and sec suppression are related but distinct.

Duong and Wickner (34) proposed that some prl mutations weaken the SecY-SecE interaction and make the translocation channel more relaxed. We have shown that a secE105P alteration in a transmembrane segment of SecE suppresses a number of secY defects including those by the secY205 mutation.2 This “superactive” form of SecE might also shift the tight/relaxed balance of the translocation channel. Incidentally, this mutation also weakens the Sec-Y-SecE association affinity.2

In view of the recent observations (1, 2, 36–38) that more than one SecYEG complex forms an active translocase channel, it is conceivable that mutations like secY762 affect the intermolecular interaction of SecY in such a way that the channel is more easily activated. Indeed, Manting et al. (2) proposed that binding of SecA triggers the multimer formation, and such a notion is consistent with our implication that the superactive SecYE more effectively recruits a transloca-

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