Nearest Neighbor Analysis of the SecYEG Complex. 2. Identification of a SecY−SecE Cytosolic Interface†

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ABSTRACT: Although the importance of interactions involving both the cytosolic and transmembrane regions of SecY and SecE has been documented, no information has been available for the physical contact sites of these translocase subunits in their cytosolic domains. We now carried out site-specific cross-linking experiments to identify SecY and SecE regions that are physically close. Cysteines introduced into SecY residue 244 in the fourth cytosolic domain (C4) as well as into residues 354−356 and 362 in the C5 domain could be cross-linked with natural or engineered residues at positions 79 and 81 in the central part of the cytosolic loop of SecE. These cross-linkages were abolished by the Gly240 mutation in the SecY C4 region as well as by *prlG* alterations in SecE transmembrane segment 3, known to compromise SecY−SecE interaction. We suggest that the cytosolic and intramembrane interactions bring these two subunits together, forming a functionally crucial SecYE interface involving the SecY C5 region and the conserved cytosolic segment of SecE.

Among the membrane-embedded Sec translocase components, SecY and SecE are the principal factors that are thought to constitute a channel-like pathway in the membrane for transport of preproteins (*1*). In addition, SecG takes part in the SecYEG membrane-integrated complex. SecY has 10 transmembrane segments (TM1−TM10), six cytoplasmic regions (C1−C6), and five periplasmic regions (P1−P5) (*2*). It interacts independently with SecE and SecG (*3*). SecE has three transmembrane segments (TM1−TM3), two cytosolic regions (C1 N-terminal tail and C2 cytosolic loop), and two periplasmic regions (P1 loop and P2 periplasmic tail) (*4*). SecY−SecE interaction is important for the stable existence of SecY in the cell (*5*, *6*), and uncomplexed SecY subunit is rapidly degraded by the FtsH protease (*7*). Also, SecY and SecE provide a high-affinity binding site for SecA, the preprotein-translocating ATPase (*8*). By interacting with the SecYEG complex, SecA undergoes remarkable conformational changes that are termed the insertion−deinsertion cycles (*9*, *10*).

Genetic and biochemical studies indicate that C5 and C6 regions of SecY are important for the functional interaction with SecA (*11*−*13*). A C5 residue, Arg357, was identified as a residue essential for the SecY function (*14*). Genetic selections identified a special class of *secY* and *secE* mutations, called *prlA* and *prlG*, respectively, which circumvent the mutational defects in signal sequences on preproteins (*15*, *16*). Some of these mutations alleviate the proton-motive force requirement of the translocation reaction (*17*, *18*) and enhance the SecA-dependent *in vitro* translocation activity of the SecYEG channel (*17*−*21*). These mutations significantly weaken SecY−SecE interactions (*18*, *21*). Thus, proper SecY−SecE interaction may be important for the discriminating function of the translocation channel as well as for regulation of translocase activities.

The N-terminal region of SecE, up to the N-terminal end of the cytosolic loop, is dispensable (*22*, *23*), whereas the amino acid residues in the C-terminal side of the cytosolic loop are well-conserved among SecE homologues from different bacterial species (*24*). These "SecE signature" residues facing the cytosol (Figure 1) are important for cell viability and stabilization of SecY (*25*).

Recently, structural analyses of the Sec translocase have suggested that the SecYEG complex forms a ring-like structure with a hole at the center (*26*−*29*). It was estimated that two or more units of the SecYE(G) complex constitute a channel (*26*−*32*).

Despite the remarkable progress in the studies of SecY−SecE translocation components, there has been only limited information about how these proteins contact each other physically. Our studies using a dominant negative variant of SecY, which sequesters SecE, showed that the C4 domain of SecY is important for the SecY−SecE interaction. In particular, the importance of Gly240 has been documented (*33*, *34*). In addition to the cytoplasmic interaction, SecY and SecE interact via their transmembrane regions. Studies involving cysteine-scanning mutagenesis and intermolecular disulfide bond formation demonstrated that P1 of SecY and P2 of SecE (*35*), as well as TM2, TM7, and TM10 of SecY and TM3 of SecE, are close together (*30*, *36*, *37*). However, information about the cytosolic interface between SecY and SecE is still lacking. In this study, we carried out detailed cross-linking analysis of the SecYEG complex with a focus on the identification of the SecY−SecE contact regions on the cytosolic side of the membrane.

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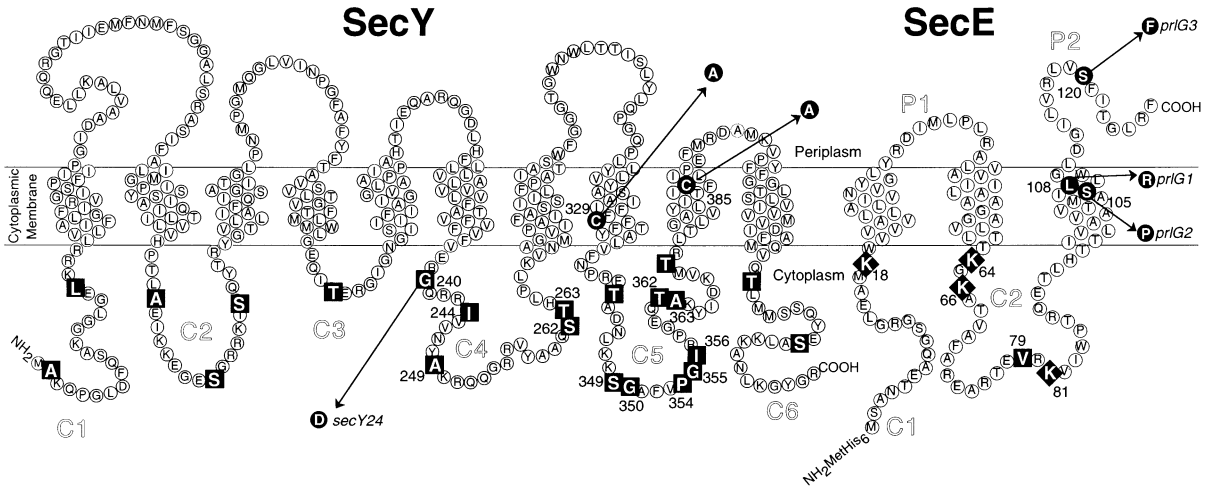

FIGURE 1: SecY and SecE variants used in this study. SecY and SecE amino acid sequences are shown along with their topology models (*2*, *4*). The two cysteine residues of SecY (shaded circles) were converted to alanine as indicated by the arrows. Single-cysteine mutations were introduced into residues depicted as shaded squares. SecE residues that were site-directedly converted to Cys are also depicted as shaded squares. Lys18, Lys64, Lys66, and Lys81 of SecE that were converted to arginine are highlighted as shaded diamonds. The *secY24* amino acid alteration and *prlG1*, *prlG2*, and *prlG3* are also shown.

MATERIALS AND METHODS

Escherichia coli Strains. Strains AD202 (an *ompT*::*kan* derivative of MC4100; *38*), GN31 (MC4100, *ompT*::*kan secY39*), and PR520 (MC4100, *secE501 argE*::Tn*10*) were described previously (refs *39*, *14*, and *40*, respectively).

Media. L medium contained 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 1.7 mmol of NaOH per liter. Ampicillin (50 μg/mL) and/or chloramphenicol (20 μg/mL) was included for growing plasmid-bearing strains, as required.

Plasmids. A pBR322-based plasmid pAJ57, a derivative of pCM4 (*34*), encoded a cysteine-less variant of SecY under the control of the *lac* promoter. A series of site-directed mutations were introduced further into pAJ57 to construct plasmids encoding single-cysteine SecY variants. pHMC5A encoded wild-type SecY under the *lac* promoter and was derived from pCM4. pHMC5A variants encoding the mutated SecYs were described by Mori and Ito (*14*). The plasmids encoding cysteine variants of SecY are summarized in Table 1.

A pACYC184-based plasmid pTYE100 encoded SecE and SecG under the control of the *tac* promoter (*14*). Site-directed mutations were introduced into its *secE* region to construct plasmids encoding mutant SecEs as summarized also in Table 1. Plasmid pKY250, a derivative of pACYC184, encoded SecE under the *lac* promoter (*6*). Plasmids pHM433, pHM434, and pHM435 were constructed by cloning the *Hin*dIII−*Xma*I fragment of pHM401, pHM402, and pHM403 (*21*), respectively, into pKY250.

Preparation of Membrane Fractions. Total membrane fractions were used for cross-linking experiments. Cells carrying an appropriate combination of SecY and SecEG plasmids were grown at 37 °C in L medium containing appropriate antibiotics and induced for the expression of SecYEG complexes. Cells were then harvested and converted to spheroplasts as described previously (*8*), which were finally resuspended in 10% (w/v) sucrose containing 3 mM EDTA (pH 7.0), 1 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride. Spheroplasts were then disrupted by sonication at 4 °C. After removal of debris materials, inner and outer membranes were recovered by ultracentrifugation and resuspended in 50 mM HEPES-KOH (pH 7.2) and 20% glycerol. The membrane preparation was divided into small portions, and they were stored at −80 °C.

Table 1: Plasmids Encoding Site-Directed SecY and SecE Variants

SecY plasmid	amino acid substitution in SecY	affected domain
pAJC7240[a]	Gly240Cys	C4
pAJC7244[a]	Ile244Cys	C4
pAJC7249[a]	Ala249Cys	C4
pAJC7262[a]	Ser262Cys	C4
pAJC7263[a]	Thr263Cys	C4
pS349C[b]	Ser349Cys	C5
pG350C[b]	Ser350Cys	C5
pP354C[b]	Pro354Cys	C5
pG355C[b]	Gly355Cys	C5
pI356C[b]	Ile356Cys	C5
pT362C[b]	Thr362Cys	C5
pA363C[b]	Ala363Cys	C5
pAJC7244-24[a]	Gly240Asp and Ile244Cys	C4
pP354C-24[b]	Gly240Asp and Pro354Cys	C4 and C5

SecE plasmid	amino acid substitution in SecE	affected domain
pSA32[c]	Lys18Arg	C1
pSA33[c]	Lys64Arg	C2
pSA34[c]	Lys66Arg	C2
pSA35[c]	Lys81Arg	C2
pSA41[c]	Val79Cys	C2
pHM433[d]	Leu108Arg	TM3
pHM434[d]	Ser105Pro	TM3
pHM435[d]	Ser120Phe	P2

[a] The *secY* mutations were introduced into pAJ57 which already included the Cys329Ala and Cys385Ala mutations. Therefore, these two alterations are present in all the constructs. [b] These *secY* mutations were introduced into pHMC5A encoding wild-type SecY. [c] These *secE* mutations were introduced into pTYE100 carrying *secE* and *secG*. [d] These *secE* mutations were introduced into pKY250 carrying *secE*.

Cross-Linking Experiments. The total membrane fraction in 50 mM HEPES-KOH (pH 7.2) and 20% glycerol was incubated with AMAS[1] or BMOE (purchased from Pierce), after addition of $^1/_{10}$ volume of a 10 mM stock solution in dimethyl sulfoxide (final concentration of 1 mM), at the specified temperature and for the specified time. The reaction

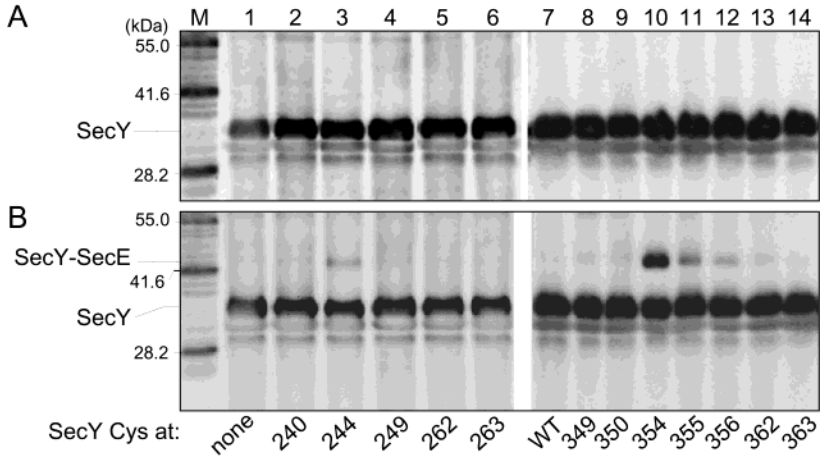

FIGURE 2: AMAS cross-linking of SecY and SecE. Strain AD202 carrying plasmid pTYE100 (encoding *secE* and *secG*) was transformed further with pAJ57 (Cys-less SecY), pHMC5A (wild-type SecY), or one of their derivatives encoding a variant of SecY having cysteine at the indicated residue numbers. The cloned proteins were induced, and total membrane fractions were prepared. Samples were analyzed by SDS−PAGE and anti-SecY immunoblotting before (A) or after (B) cross-linking with AMAS. The following SecY plasmids were introduced: lane 1, pAJ57 (Cys-less SecY); lane 2, pAJC7240 (Gly240Cys); lane 3, pAJC7244 (Ile244Cys); lane 4, pAJC7249 (Ala249Cys); lane 5, pAJC7262 (Ser262Cys); lane 6, pAJC7263 (Thr263Cys); lane 7, pHMC5A (wild-type SecY); lane 8, pS349C (Ser349Cys); lane 9, pG350C (Gly350Cys); lane 10, pP354C (Pro354Cys); lane 11, pG355C (Gly355Cys); lane 12, pI356C (Ile356Cys); lane 13, pT362C (Thr362Cys); lane 14, pA363C (Ala363Cys); and M, molecular mass markers.

was terminated by mixing the solution with a 2-fold concentrated SDS−PAGE sample buffer containing β-mercaptoethanol. Samples were subjected to SDS−PAGE. Subsequently, SecY and SecE, as well as their covalent complexes, were visualized with anti-SecY or His probe (purchased from Santa Cruz Biotechnology, Inc.) immunoblotting as described previously (*41*).

RESULTS

Identification of C4 and C5 Residues as Nearest Neighbors of SecE. The thiol−amine cross-linking experiments described in the preceding paper (*42*) revealed a cross-linked product for the Ile244Cys variant of SecY, which migrated faster than the SecY−SecG complex. This product, which proved to be a SecY−SecE complex (see below), was observed specifically for this C4 residue. We examined several other positions in the C4−C5 regions of SecY with respect to the formation of similar cross-linkage. Like the SecY single-cysteine variants studied previously, all the mutant constructs used below in this study are functional as they all exhibited positive complementation against the *secY39* mutant defect. For cross-linking experiments, each SecY variant was co-overexpressed with SecE and SecG. Total membrane preparations, in which the mutant SecY proteins accumulated normally (Figure 2A), were subjected to cross-linking with a thiol−amine reactive cross-linker, AMAS, having a spacer arm of 4.4 Å. Samples were separated by SDS−PAGE, and materials reacting with antibodies against each Sec component were detected by immunoblotting. Among the C4 variants that have been examined, a lower-mobility band was produced only for the Ile244Cys variant (Figure 2B, lane 3). We also examined SecY mutants having cysteine in the C5 domain, which had been constructed by Mori and Ito (*14*) in their random mutagenesis experiments targeted to a C5 segment. Among the seven variants, with cysteine placed at positions 349, 350, 354−356, 362, and 363, the one having cysteine at position 354 produced the cross-linked product most prominently (Figure 2B, lane 10), and positions 355 and 356 gave weaker but significant cross-linking (Figure 2B, lanes 11 and 12).

No cross-linked product of this size was detectable without AMAS (Figure 2A). Also, neither wild-type SecY nor the cysteine-less variant produced it (Figure 2B, lanes 1 and 7). Thus, the cross-linking was specifically generated through the introduced cysteine residues. The cross-linked products observed above reacted with antibodies against SecY (Figure 2B), but not with antibodies against SecG or SecA (data not shown). The SecE molecules supplied from the plasmid had a histidine tag in the amino terminus. Immunoblotting using antibodies against polyhistidine highlighted the His_6-tagged SecE as well as the cross-linked product (Figure 3, lanes 11 and 16). These results indicate that this cross-linking had occurred between SecY and SecE. Thus, C4 and C5 domains of SecY are adjacent to SecE.

Central Region of the SecE Cytoplasmic Loop Is Engaged in the SecY−SecE Cross-Linkage. It is known that the carboxy-terminal half of the cytoplasmic loop in SecE is important for the SecY-stabilizing and other functions of SecE (*24*, *25*) and that the N-terminal half of SecE is dispensable (*22*, *23*). Therefore, if the cytoplasmic SecY−SecE proximity observed above is functionally significant, the SecE region involved should be within the C2 loop of this protein. To identify a SecE residue responsible for the cross-linking, we mutated each of its four cytoplasmic lysine residues (at position 18 in C1 and at positions 64, 66, and 81 in C2) to arginine (Figure 1). All of these variants of SecE were functional as they retained the ability to complement a cold-sensitive *secE* mutant (data not shown). They were coexpressed with either the Ile244Cys or Pro354Cys variant of SecY as well as with SecG. Once AMAS cross-linking had occurred, the Lys81Arg form of SecE failed to produce the SecY−SecE complex (Figure 3, lanes 5, 10, 15, and 20), whereas the Lys18Arg, Lys64Arg, and Lys66Arg

[1] Abbreviations: AMAS, *N*-(α-maleimidoacetoxy)succinimide ester; BMOE, bis-maleimidoethane; PAGE, polyacrylamide gel electrophoresis.

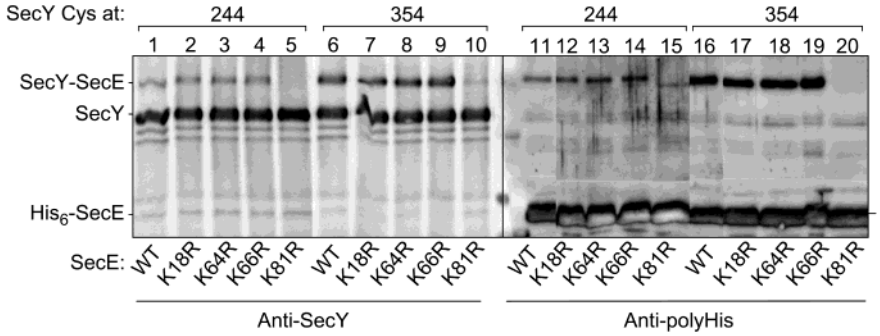

FIGURE 3: Lys81 of SecE in the cytoplasmic loop is the cross-linking partner. pTYE100 (SecE$^+$ and SecG$^+$; lanes 1, 6, 11, and 16), pSA32 [SecE(Lys18Arg) and SecG$^+$; lanes 2, 7, 12, and 17], pSA33 [SecE(Lys64Arg) and SecG$^+$; lanes 3, 8, 13, and 18], pSA34 [SecE(Lys66Arg) and SecG$^+$; lanes 4, 9, 14, and 19] or pSA35 [SecE(Lys81Arg) and SecG$^+$; lanes 5, 10, 15, and 20] were separately introduced into strain AD202. The resulting plasmid-bearing cells were transformed further with pAJC7244 (Ile244Cys; lanes 1−5 and 11−15) or pP354C (Pro354Cys; lanes 6−10 and 16−20). Total membrane fractions were subjected to the cross-linking reaction with AMAS, followed by visualization with anti-SecY (lanes 1−10) and the His probe (lanes 11−20), as described in the legend of Figure 2.

forms of SecE showed an unimpaired ability to cross-link (Figure 3, lanes 2−4, 7−9, 12−14, and 17−19). Thus, unlike the other lysines in SecE located in C1 or in the N-terminal region of C2, Lys81 in the central region of the cytoplasmic loop was responsible for the AMAS-mediated cross-linking with the cysteine residues in the C4 and C5 regions of SecY.

To substantiate the involvement of C2 in the SecY−SecE contact, we constructed SecE variants, in which either Thr68 (amino-terminal half of C2), Val79 (central part of C2), or Thr90 (carboxy-terminal half of C2) was converted to cysteine. They were also functional (complementation data not shown). Membranes containing one of the cysteine SecY variants and either SecE(Thr68Cys), SecE(Val79Cys), or SecE(Thr90Cys) were treated with an oxidant or with BMOE, a homobifunctional cross-linker having the reactivity against thiol groups and an 8.8 Å spacer arm. Although disulfide bond formation was not observed by simple oxidation of any SecY−SecE combination that was examined (data not shown), BMOE-mediated cross-linking was observed between cysteines at position 79 of SecE and at either position 244 or 362 of SecY (Figure 4, lanes 5, 6, 11, and 12). Cross-linking was not observed with SecE(Thr68Cys) or SecE(Thr90Cys) (data not shown). These results collectively showed that the central part of C2 in SecE is physically close to the C4 and C5 regions of SecY.

Reduction of the Level of SecY−SecE Cross-Linking by Mutations Known To Compromise SecY−SecE Interaction. A mutation, *secY24* with a Gly240Asp alteration in C4 of SecY [Figure 1 (*43*)], was reported to weaken SecY−SecE interaction (*3*, *33*, *34*). We examined whether the Gly240Asp alteration affected the cross-linking between the C4 and C5 residues of SecY and the C2 region of SecE. Thus, the Gly240Asp mutation was introduced into the SecY(Ile244Cys) and the SecY(Pro354Cys) variants of SecY. Upon treatment of the membranes with AMAS, neither of these SecY variants that included the double mutation formed a cross-linked product with SecE (Figure 5A, lanes 2 and 4). The extent of BMOE cross-linking between SecY Cys244 or Cys362 and SecE Cys79 was also decreased markedly by the introduction of the Gly240Asp mutation into SecY (data not shown). These results indicate that the SecY−SecE cross-linking depends on the integrity of the Gly240 region of SecY. Thus, not only the C4 (SecY)−C2 (SecE) proximity but also the C5 (SecY)−C2 (SecE) proximity requires the

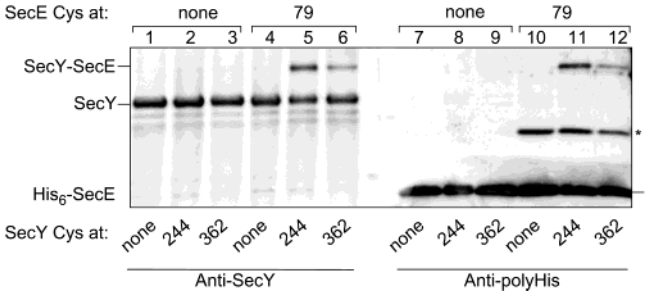

FIGURE 4: BMOE cross-linking of SecY and SecE. Combinations of two plasmids, one encoding SecG and a SecE single-cysteine variant and the other encoding a SecY with an engineered cysteine residue, were introduced into strain AD202. The following SecE−SecG plasmids were introduced: pTYE100 (SecE$^+$ and SecG$^+$; lanes 1−3 and 7−9) and pSA41 [SecE(Val79Cys) and SecG$^+$; lanes 4−6 and 10−12]. The SecY plasmids that were used are shown: lanes 1, 4, 7, and 10, pAJ57 (Cys-less SecY); lanes 2, 5, 8, and 11, pAJC7244 (Ile244Cys); and lanes 3, 6, 9, and 12, pT362C (Thr362Cys). These proteins were induced, and total membrane fractions were prepared. The samples were subjected to the cross-linking reaction with BMOE on ice for 1 h and analyzed by SDS−PAGE followed by anti-SecY (lanes 1−6) and His probe (lanes 7−12) immunoblotting. The band denoted with an asterisk is unidentified background protein.

normal interaction between these proteins, in which residue 240 plays an important role.

SecY−SecE association seems to require protein interactions between both their cytosolic regions and transmembrane regions. Indeed, another line of genetic evidence pointed to the importance of the latter interaction. This came from studies of some of the *prlA* and the *prlG* mutations (*15*, *16*), in *secY* and in *secE*, respectively, that were thought to compromise the interaction between SecY and SecE (*18*, *21*). We introduced each of the *prlG1*, *prlG2*, and *prlG3* mutations into the cloned *secE* gene. The AMAS-mediated SecY−SecE cross-linking was undetectable when SecE included the *prlG1* Leu108Arg alteration (Figure 5B, lanes 2 and 6). It was reduced markedly by the *prlG2* Ser105Pro alteration as well (Figure 5B, lanes 3 and 7), whereas the *prlG3* Ser120Phe alteration gave only a mild effect (Figure 5B, lanes 4 and 8). The PrlG1 and PrlG2 alterations are within TM3, whereas *prlG3* affects the P2 region. The results presented above indicate that both the cytosolic and intramembrane interactions contribute to the physical arrangement of SecY and SecE as detected experimentally by the site-specific cross-linking.

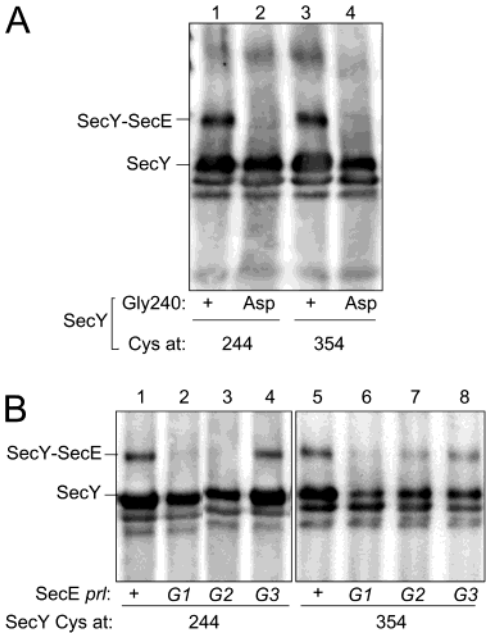

FIGURE 5: SecY−SecE cross-linking was abolished by *secY24* and some *prlG* mutations. (A) Effects of *secY24*. Strain AD202 was transformed with pKY250 ($SecE^+$) and a *secY* plasmid having the following mutations (Table 1): lane 1, Ile244Cys; lane 2, Gly240Asp and Ile244Cys; lanes 3, Pro354Cys; and lane 4, Gly240Asp and Pro354Cys. (B) Effects of *prlG* mutations. Strain AD202 carrying pAJC7244 [SecY(Ile244Cys); lanes 1−4] or pP354C [SecY-(Pro354Cys); lanes 5−8] was transformed further with pKY250 ($SecE^+$; lanes 1 and 5), pHM433 [SecE(Leu108Arg); lanes 2 and 6], pHM434 [SecE(Ser105Pro); lanes 3 and 7], or pHM435 [SecE-(Ser120Phe); lanes 4 and 8]. Cells were grown, and total membranes were prepared, which were subjected to cross-linking with AMAS. Samples were analyzed by SDS−PAGE and immunoblotting using antibodies against SecY.

DISCUSSION

Our earlier results indicated that the C4 region of SecY is important for the interaction between SecY and SecE and that this interaction is essential for the stable existence of the SecY molecule in the cell (*33*). In particular, the importance of a C4 residue, Gly240, was suggested (*34*). In the study presented here, we analyzed the cytosolic SecY−SecE arrangement at the amino acid level, using cross-linking approaches. It was found that cross-linking took place specifically via cysteine residues introduced into position 244, positions 354−356, and position 362. The partner residues in SecE were either Lys81 or an engineered cysteine at position 79, located in the central region of the SecE cytosolic loop. These results provide the first information about the physical proximity of the cytosolic regions of SecY and SecE. The C-terminal half of the SecE cytosolic loop contains several residues conserved among SecE homologues. The SecY-contacting residues identified here overlap this segment, which is crucial for the SecE functions, including its ability to stabilize SecY (*25*). The observed mutational effect of the *secY24* alteration indicates that the C4 domain of SecY and the C2 domain of SecE are not only physically close but also positively interacting with each other.

In contrast, the proximity of the C5 region of SecY to SecE may have more functional significance, without directly contributing to the molecular interaction itself. A number of mutations in the C5 region, including substantial deletions, exhibit the dominant-negative phenotype, which most probably is brought about by sequestration of SecE by the inactive SecY molecules (*14*, *44*). Thus, these C5 mutant proteins still interact with SecE. Introduction of one such loss-of-function mutations at residue 357, a residue identified as being particularly important for the SecY function (*14*), did not impair SecY−SecE cross-linking (data not shown). Thus, we believe that the C5 (SecY)−C2 (SecE) proximity is functionally crucial but that this proximity itself is passive, and the main binding force may be provided by the C4 (SecY)−C2 (SecE) interaction as well as by interaction between transmembrane segments of these proteins.

The C5 region of SecY is important for its function, activating the SecA translocation ATPase (*1*, *12*). The result that the C5 region is adjacent to SecE may suggest that the functionally important C5 segment, including the Arg357 residue, is positioned close to the SecE signature segment and this spatial arrangement is optimized for the productive interaction with SecA. The high-affinity binding site for SecA may be formed by the SecY−SecE subunit interface, to which both cytosolic (*8*) and transmembrane (*21*) SecY−SecE interactions contribute.

Recent studies show that the transmembrane segment-mediated SecY−SecE interaction can be modulated by a number of *secY* or *secE* mutations. Many of these mutations weaken the interaction. This was shown in this work with the *prlG* mutational effects that decreased the efficiency of SecY−SecE cross-linking. Alterations in this direction can enhance the translocation function under certain conditions, such as in the absence of the proton-motive force (*18*, *21*), in the presence of a defective mutation in the signal sequence (*15*, *16*), and in the presence of a translocation-compromising alteration in the SecYEG channel (*21*). In addition, these mutations seem to increase the affinity for SecA binding. These observations suggest that the SecY−SecE proximity must be subject to fine-tuning. At any rate, the information obtained from these cross-linking studies will be useful for our molecular understanding of the protein translocation channel, a major unsolved question in cell and structural biology.

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REFERENCES

1. Mori, H., and Ito, K. (2001) *Trends Microbiol. 9*, 494−500.
2. Akiyama, Y., and Ito, K. (1987) *EMBO J. 6*, 3465−3470.
3. Homma, T., Yoshihisa, T., and Ito, K. (1997) *FEBS Lett. 405*, 11−15.
4. Schatz, P. J., Riggs, P. D., Jacq, A., Fath, M. J., and Beckwith, J. (1989) *Genes Dev. 3*, 1035−1044.
5. Matsuyama, S., Akimaru, J., and Mizushima, S. (1990) *FEBS Lett. 269*, 96−100.
6. Taura, T., Baba, T., Akiyama, Y., and Ito, K. (1993) *J. Bacteriol. 175*, 7771−7775.
7. Kihara, A., Akiyama, Y., and Ito, K. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 4532−4536.
8. Matsuo, E., Mori, H., Shimoike, T., and Ito, K. (1998) *J. Biol. Chem. 273*, 18835−18840.
9. Economou, A., and Wickner, W. (1994) *Cell 78*, 835−843.
10. Matsumoto, G., Yoshihisa, T., and Ito, K. (1997) *EMBO J. 16*, 6384−6393.
11. Taura, T., Akiyama, Y., and Ito, K. (1993) *Mol. Gen. Genet. 243*, 261−269.

12. Taura, T., Yoshihisa, T., and Ito, K. (1997) *Biochimie 79*, 517−521.
13. Matsumoto, G., Yoshihisa, T., and Ito, K. (1997) *EMBO J. 16*, 6384−6393.
14. Mori, H., and Ito, K. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 5128−5133.
15. Osborne, R. S., and Silhavy, T. J. (1993) *EMBO J. 12*, 3391−3398.
16. Flower, A. M., Osborne, R. S., and Silhavy, T. J. (1995) *EMBO J. 14*, 884−893.
17. Nouwen, N., de Kruijff, B., and Tommassen, J. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 5953−5957.
18. Duong, F., and Wickner, W. (1999) *EMBO J. 18*, 3263−3270.
19. van der Wolk, J. P., Fekkes, P., Boorsma, A., Huie, J. L., Silhavy, T. J., and Driessen, A. J. (1998) *EMBO J. 17*, 3631−3639.
20. Mori, H., Shimizu, Y., and Ito, K. (2002) *J. Biol. Chem. 277*, 48550−48557.
21. Mori, H., Akiyama, Y., and Ito, K. (2003) *J. Bacteriol. 185*, 948−956.
22. Schatz, P. J., Bieker, K. L., Ottemann, K. M., Silhavy, T. J., and Beckwith, J. (1991) *EMBO J. 10*, 1749−1757.
23. Nishiyama, K., Mizushima, S., and Tokuda, H. (1992) *J. Biol. Chem. 267*, 7170−7176.
24. Murphy, C. K., and Beckwith, J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 2557−2561.
25. Pohlschroder, M., Murphy, C. K., and Beckwith, J. (1995) *J. Biol. Chem. 271*, 19908−19914.
26. Meyer, T. H., Menetret, J. F., Breitling, R., Miller, K. R., Akey, C. W., and Rapoport, T. A. (1999) *J. Mol. Biol. 285*, 1789−1800.
27. Manting, E. H., van Der Does, C., Remigy, H., Engel, A., and Driessen, A. J. (2000) *EMBO J. 19*, 852−861.
28. Collinson, I., Breyton, C., Duong, F., Tziatzios, C., Schubert, D., Or, E., Rapoport, T., and Kuhlbrandt, W. (2001) *EMBO J. 20*, 2462−2471.
29. Breyton, C., Haase, W., Rapoport, T. A., Kuhlbrandt, W., and Collinson, I. (2002) *Nature 418*, 662−665.
30. Kaufmann, A., Manting, E. H., Veenendaal, A. K. J., Driessen, A. J. M., and Does, C. (1999) *Biochemistry 38*, 9115−9125.
31. Nagamori, S., Nishiyama, K., and Tokuda, H. (2000) *J. Biochem. 124*, 122−129.
32. Mori, H., Tsukazaki, T., Masui, R., Kuramitsu, S., Yokoyama, S., Johnson, A. E., Kimura, Y., Akiyama, Y., and Ito, K. (2003) *J. Biol. Chem. 278*, 14257−14264.
33. Baba, T., Taura, T., Shimoike, T., Akiyama, Y., Yoshihisa, T., and Ito, K. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 4539−4543.
34. Matsuo, E., and Ito, K. (1998) *Mol. Gen. Genet. 258*, 240−249.
35. Harris, C. R., and Silhavy, T. J. (1999) *J. Bacteriol. 181*, 3438−3444.
36. Veenendaal, A. K., van der Does, C., and Driessen, A. J. (2001) *J. Biol. Chem. 276*, 32559−32566.
37. Veenendaal, A. K., van der Does, C., and Driessen, A. J. (2002) *J. Biol. Chem. 277*, 36640−36645.
38. Silhavy, T. J., Berman, M. L., and Enquist, L. W. (1984) *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory Press, Plainview, NY.
39. Akiyama, Y., and Ito, K. (1990) *Biochem. Biophys. Res. Commun. 167*, 711−715.
40. Riggs, P. D., Derman, A. I., and Beckwith, J. (1988) *Genetics 118*, 571−579.
41. Chiba, K., Mori, H., and Ito, K. (2002) *J. Bacteriol. 184*, 2243−2250.
42. Satoh, Y., Matsumoto, G., Mori, H., and Ito, K. (2003) *Biochemistry 42*, 7434−7441.
43. Shiba, K., Ito, K., Yura, T., and Cerretti, D. P. (1984) *EMBO J. 3*, 631−635.
44. Shimoike, T., Akiyama, Y., Baba, T., Taura, T., and Ito, K. (1992) *Mol. Microbiol. 6*, 1205−1210.

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