SecY alterations that impair membrane protein folding and generate a membrane stress

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We report on a class of Escherichia coli SecY mutants that impair membrane protein folding. The mutants also up-regulate the Cpx/σE stress response pathways. Similar stress induction was also observed in response to a YidC defect in membrane protein biogenesis but not in response to the signal recognition particle–targeting defect or in response to a simple reduction in the abundance of the translocon. Together with the previous contention that the Cpx system senses a protein abnormality not only at periplasmic and outer membrane locations but also at the plasma membrane, abnormal states of membrane proteins are postulated to be generated in these secY mutants. In support of this notion, in vitro translation, membrane integration, and folding of LacY reveal that mutant membrane vesicles allow the insertion of LacY but not subsequent folding into a normal conformation recognizable by conformation-specific antibodies. The results demonstrate that normal SecY function is required for the folding of membrane proteins after their insertion into the translocon.

Introduction

Newly synthesized membrane proteins in Escherichia coli are believed to be delivered by the signal recognition particle (SRP) to the SecYEG translocon cotranslationally (Lee and Bernstein, 2001; for review see Luirink and Sinning, 2004). Thus, the SecYEG translocon is used both for the transport of secretory proteins from the cytosol to the periplasm (Mori and Ito, 2001b) and for the integration of membrane proteins (for reviews see Dalbey and Chen, 2004; Rapoport et al., 2004). However, the role of SecYEG in the latter is only poorly understood. It is assumed that a signal-anchor sequence or a stop-transfer hydrophobic segment of a membrane protein is inserted into the translocon first and then moves laterally and is integrated stably into the lipid bilayer. In the processes of membrane protein integration/assembly, an integral membrane protein, YidC, also plays crucial roles (Dalbey and Kuhn, 2004). Although integration of a class of membrane proteins is mediated directly by YidC, other membrane proteins are believed to be inserted first into the SecYEG channel and are transferred to YidC, which will facilitate final anchoring into the lipid phase (Urbanus et al., 2001) or folding into physiologic conformations (Nagamori et al., 2004). The SecDF–YajC complex has large periplasmic domains and interacts with both SecYEG and YidC (Nouwen and Driessen, 2002; Xie et al., 2006). It is thought to mediate the interplay between SecYEG and YidC.

According to the crystal structure of the SecYEβ translocon from an archaea (van den Berg et al., 2004), an hourglass-shaped polypeptide-conducting channel is formed within the SecY subunit of the heterotrimer in which two halves of SecY are arranged in a pseudosymmetrical manner. It was proposed that the front side of the complex can be opened laterally for the exit of hydrophobic segments of newly synthesized proteins from the translocon to enter the hydrophobic environment. To maintain the impermeability of the membrane to ions, the conductance of the translocon is proposed to be blocked by a plug that covers the pore from the periplasmic side in the resting state and by a ring of hydrophobic residues in the pore that forms a seal around the translocating peptide chain. Despite this remarkable progress, several questions remain about translocon function in membrane protein integration. (1) What are the pathways from the translocon into either the periplasm or the membrane for exported versus membrane proteins, respectively? (2) What are the mechanisms by which movement of a membrane protein in the vertical direction is redirected to

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Abbreviations used in this paper: IMV, inverted membrane vesicle; PSBT, 1.3 S subunit of Propionibacterium shermanii biotin transcarboxylase; SRP, signal recognition particle; TM, transmembrane.

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lateral transfer toward the lipid phase? (3) Is the translocon sufficiently flexible to allow the reorientation of hydrophobic helices (Goder and Spiess, 2003; Goder et al., 2004)? (4) How and when do lipid interactions occur to generate the final topology (Hessa et al., 2005)?

Whatever the mechanisms involved, it is likely that membrane protein assembly involves thermodynamic partitioning and is catalyzed by proteinaceous machinery (for review see Rapoport et al., 2004; Hessa et al., 2005). Given the requirement for macromolecules, the system may be sensitive to environmental stresses and substrate overloading. Although cells have a regulatory mechanism to cope with defects in biogenesis, little is known about the system that deals with abnormalities in membrane proteins. E. coli possesses two extracytoplasmic stress response pathways called Cpx and ςE, which up-regulate the expression of a class of proteins involved in folding and proteolysis (Akiyama et al., 2004; Alba and Gross, 2004; Duguay and Silhavy, 2004; Ehrmann and Clausen, 2004). Both systems were shown to sense abnormal proteins in either the outer membrane or the periplasmic space (Duguay and Silhavy, 2004). Additionally, we have shown that the Cpx/ςE systems sense the accumulation of certain abnormal plasma membrane proteins. For example, the lack of FtsH, a membrane-bound protease, results in up-regulation of the Cpx stress response pathway, which is exaggerated by the overproduction of a membrane protein substrate of FtsH (Shimohata et al., 2002). We reasoned that the stress response mechanism can sense some abnormal states of plasma membrane proteins. To further explore this notion, we examined whether extracytoplasmic stress responses are up-regulated when a cellular factor for membrane protein assembly/folding is functionally impaired. For this purpose, we used the arabinose-controlled expression system of YidC (Samuelson et al., 2000). When arabinose was removed from the medium, YidC abundance in the engineered strain decreased with time (Fig. 1 A, lanes 5–8). In parallel to this decrease, Cpx pathway gene expression elevated up to about fourfold (Fig. 1 B, closed circles). Another assay using a reporter of the ςE pathway showed that YidC-deficient conditions also up-regulate this stress response pathway (Fig. 1 C, closed circles).

A previous study indicates that YidC depletion results in the defective folding of a multipath membrane protein, LacY (Nagamori et al., 2004). It is also known that YidC is associated with the SecYEG translocon via the SecDF–YajC complex (Nouwen and Driessen, 2002; Xie et al., 2006). We found that the secDJ mutation (Gardel et al., 1987), which decreases the expression level of the secD–secF operon (Chiba, K., personal communication), also induces the Cpx stress response markedly (Fig. 2 B, third bar). In parallel, we observed that this mutation considerably impaired the insertion of a model membrane protein, MalF–PSBT(J) (1.3 S subunit of Propionibacterium shermanii biotin transcarboxylase (J); Fig. 2 A, lane 3; see the next section and Materials and methods for the significance of this indication of insertion defect). From these results, we surmise that the YidC and SecD deficiencies result in the generation of some aberrant forms of membrane proteins, which, in turn, activate the extracytoplasmic stress response mechanisms.

The roles of YidC and SecD in membrane protein biogenesis are probably at stages after the initial targeting of newly synthesized membrane proteins. Defects at the targeting steps caused by an SRP mutation is known to induce the ς17-dependent

**Results**

**Defective assembly of plasma membrane proteins up-regulates the Cpx and ςE stress response pathways**

We showed previously that the accumulation of certain classes of membrane proteins under the conditions of compromised activities of quality control proteases results in up-regulation of the Cpx extracytoplasmic stress response (Shimohata et al., 2002). We reasoned that the stress response mechanism can sense some abnormal states of plasma membrane proteins. To further explore this notion, we examined whether extracytoplasmic stress response is induced when a cellular factor for membrane protein assembly/folding is functionally impaired. For this purpose, we used the arabinose-controlled expression system of YidC (Samuelson et al., 2000). When arabinose was removed from the medium, YidC abundance in the engineered strain decreased with time (Fig. 1 A, lanes 5–8). In parallel to this decrease, Cpx pathway gene expression elevated up to about fourfold (Fig. 1 B, closed circles). Another assay using a reporter of the ςE pathway showed that YidC-deficient conditions also up-regulate this stress response pathway (Fig. 1 C, closed circles).

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The roles of YidC and SecD in membrane protein biogenesis are probably at stages after the initial targeting of newly synthesized membrane proteins. Defects at the targeting steps caused by an SRP mutation is known to induce the ς17-dependent
cytosolic stress response, presumably by producing uninserted membrane proteins in the cytosolic part of the cell (Bernstein and Hyndman, 2001; Park et al., 2002). To examine the effects of a defect in the SRP-targeting factor on the envelope stress response, we used the \textit{ffh10}\(\text{(Ts)}\) mutation (Park et al., 2002). It was found that these mutant cells contained lower than normal levels of the Cpx-controlled LacZ activity (Fig. 2 D). Thus, this mutation in the targeting factor does not up-regulate the Cpx extracytoplasmic stress response. We also examined the consequence of a simple reduction of the cellular abundance of the translocon. The \textit{rplO215}\(\text{(amber)}\) mutation (Ito et al., 1984), which decreases the expression level of SecY by \(\sim 70\%\) (Fig. 2 C, lane 4), did not activate the Cpx pathway at all (Fig. 2 B, fourth bar), although a model membrane protein, MalF-PSBT(J), exhibited a considerable insertion defect in this mutant (Fig. 2 A, lane 4). Also, the \textit{secE501} mutation, which decreases \textit{secE} expression (Schatz et al., 1991) and consequently leads to rapid degradation of the uncomplexed fraction of SecY (Fig. 2 C, lane 2; Taura et al., 1993), impaired insertion of the model membrane protein (Fig. 2 A, lane 2) but activated the stress response only insignificantly (Fig. 2 B, second column). These results indicate that the lack of insertion process itself does not generate a membrane stress. Collectively, we suggest that membrane protein folding/assembly defects that occur within the plasma membrane after the targeting event lead to the up-regulated envelope stress responses.

**Strategy to classify defects in membrane protein biogenesis**

Membrane protein biogenesis is accomplished by a series of sequential steps: translation, targeting, translocation via the translocon, lateral exit from the translocon, folding, assembly, and stable integration into the membrane. Although previous genetic and biochemical studies revealed cellular mechanisms that mediate these steps (for reviews see Dalbey and Chen, 2004; Luirink and Sinning, 2004), these studies primarily addressed whether a particular model membrane protein was inserted into the membrane with the proper topology of the extracytoplasmic domain. In most cases, defects in the pathway were detected only by the accumulation of uninserted molecules. In this study, we use the term type 1 defect to describe this phenomenon (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200611121/DC1). A defect in subsequent steps can occlude the translocon or interfere with earlier steps in the pathway (i.e., by backup). As the biogenesis pathway is constantly loaded with newly synthesized proteins, the accumulation of uninserted molecules can result not only when the primary defect is at the targeting step but also when the defect is at a later stage (Fig. S1). In other words, a defect in any step of the pathway can appear to be type 1.

From the results presented in the preceding section, we suggest that the induction of envelope stress responses can be

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**Figure 2.** Stress response induction does not always accompany defects in membrane protein integration. (A) Effects of different sec mutations on MalF-PSBT insertion. Plasmids pSTD343 (\textit{lacI}) and pGJ78J (MalF-PSBT(J)) were introduced into SH463 \(\text{[sec]}\), NH192 \(\text{[secE501]}\), SH625 \(\text{[secD1]}\), and KI200 \(\text{[rplO215]}\). The plasmid-bearing cells were grown at 30°C, induced with IPTG for 1 h, and analyzed for biotinylation (top) and accumulation (bottom) of the fusion protein. (B) Effects on Cpx pathway activation. Plasmid pSH10 (\textit{cpxP-lacZ}+) was introduced into the \textit{sec}+, \textit{secE501}, \textit{secD1}, and \textit{rplO215} strains for \(\beta\)-galactosidase measurements at 30°C. (C) Effects on the SecY contents. Portions of cultures used in B were examined by SDS-PAGE and anti-SecY immunoblotting. The graph shows the cellular contents of SecY relative to the wild-type abundance. (D) The \textit{ffh10}\(\text{(Ts)}\) mutation does not activate the Cpx pathway. Plasmid pSH10 [\textit{cpxP-lacZ}+] was introduced into SKP1101 \(\text{[ffh10(Ts)]}\) and SKP1102 \(\text{[ffh1]}\). The plasmid-bearing cells were grown first at 30°C and then were shifted to 42°C for 1 h for the measurement of \(\beta\)-galactosidase activities. Error bars represent SD.
used as a specific indicator of postinsertion defects such as folding and assembly. We use the term type 2 defect for this phenomenon. Although a membrane stress response may suggest a postinsertion defect, a more direct demonstration of the folding/assembly failure is required. To demonstrate this point, we use the system of Nagamori et al. (2003, 2004), in which in vitro translation of LacY is coupled to insertion into the membrane and folding into the tertiary structure, which is recognized by conformation-specific mAbs. We use the term type 3 defect to describe a failure that is detectable by this assay (Fig. S1). It should be noted that the in vitro system would suffer minimally from the backup phenomena because it uses very minute amounts of radiolabeled membrane protein. In the following analyses of secY mutations, we used the MalF-PSBT(J) fusion protein (see Materials and methods; Jander et al., 1996) and the SecY-PhoA C6 fusion protein (Fig. S2; Akiyama et al., 1990) to reveal the type 1 defect, induction of the Cpx/σ^E-stress response to identify the type 2 defect, and acquisition of conformational epitopes in LacY to identify the type 3 defect.

### Mutations in secY that impair the integration function do not correlate with those impairing export function

We examined a series of secY mutants that were described previously (Taura et al., 1994) as well as additional secY mutants isolated in this study (see Materials and methods and Table I) with respect to their defects in protein export and in membrane protein insertion (type 1 defect). These mutants were originally isolated as those up-regulating either a secretion monitor reporter (Taura et al., 1994) or a membrane stress reporter (see Materials and methods) at 37 or 30°C. Whereas many of them are either cold sensitive or temperature sensitive for growth, we assessed their export/integration phenotypes at the growth-allowing temperature (37 or 30°C), at which their lesion had been recognized by the original screening procedures. The export of OmpA, as examined by pulse labeling and immunoprecipitation, was evidently retarded in the secY39, secY205, secY125, and secY124 mutants (Fig. 3 A, lanes 2–5) but was much less pronouncedly retarded in the secY40, secY129, secY351, secY238, secY929, secY351, and secY403 mutants (Fig. 3 A, lanes 6, 7, and 9–12). The export of maltose-binding protein showed secY dependence similar to that of OmpA (unpublished data).

We examined membrane protein insertion phenotypes of the mutants using MalF-PSBT(J) and SecY-PhoA(3-3). These two model membrane proteins gave consistent results, and results with the latter are presented in Fig. S2 (A and B; available at http://www.jcb.org/cgi/content/full/jcb.200611121/DC1). All of the mutant strains were normal in biotinylating a control fusion protein (MalF-PSBT(K)) that had the PSBT domain on the cytosolic side (unpublished data). In contrast, MalF-PSBT(J), having PSBT on the periplasmic side, was not appreciably biotinylated in the secY^+_, secY39, or secY205 cells (Fig. 3 B, top; first to third lanes), in which the fusion protein itself, as visualized by anti-MalF immunoblotting, accumulated normally (Fig. 3 B, bottom). Remarkably, the integration process appeared to have proceeded normally in the two strongly export-defective mutants, secY39 and secY205. The remaining secY mutants examined all proved to be considerably defective in the integration process, as indicated by appreciable biotinylation of the fusion protein (Fig. 3 B, fourth to seventh lanes and ninth to twelfth lanes).

The aforementioned results allow classification of the secY mutations into three groups. First, the secY39 and secY205 mutations are severely defective in export but are nearly normal in integration (see Fig. S2 B for the normal kinetics of SecY-PhoA processing in secY39). Second, secY40, secY129, secY238, secY929, secY351, and secY403 are nearly normal in export but are considerably defective in integration. Finally, secY125 and secY124 are defective in both processes. We also examined OmpA export in the presence of simultaneously expressed MalF-PSBT. In this case, export was considerably retarded even in the secY40 and secY129 mutants (Fig. 3 C, bottom; lanes 6

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### Table I. SecY alterations characterized in this study

<table>
<thead>
<tr>
<th>Allele name</th>
<th>Amino acid change</th>
<th>Domain affected</th>
<th>OmpA export</th>
<th>MalF-PSBT insertion</th>
<th>Cpx/σ^E stress</th>
<th>LacY folding</th>
<th>LacY stability</th>
</tr>
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<tbody>
<tr>
<td>secY^+_</td>
<td>None</td>
<td>None</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>secY125^a</td>
<td>S76F</td>
<td>P1 [TM2a]</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>secY124, secY84^b</td>
<td>P84L</td>
<td>TM2</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>secY238^b</td>
<td>E238K</td>
<td>TM6/C4</td>
<td>[+]</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>secY299^c</td>
<td>G299D</td>
<td>P4</td>
<td>(+)</td>
<td>–</td>
<td>[–]</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>secY351^b,c</td>
<td>A351T</td>
<td>C5</td>
<td>(+)</td>
<td>–</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>secY39</td>
<td>R357H</td>
<td>C5</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>secY40</td>
<td>A363T</td>
<td>C5</td>
<td>(+)</td>
<td>–</td>
<td>[–]</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>secY129^a</td>
<td>C3859P</td>
<td>TM9</td>
<td>(+)</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>secY403^c</td>
<td>G403R</td>
<td>TM10</td>
<td>(+)</td>
<td>–</td>
<td>[–]</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>secY205</td>
<td>Y429D</td>
<td>C6</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>rplO215</td>
<td>None (low abundance)</td>
<td>None (low abundance)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

In the third column, SecY domains are indicated by TM, C, and P for transmembrane, cytoplasmic, and periplasmic domains, respectively, numbered from the N terminus in this order. In the fourth and fifth columns, – indicates a considerable defect, + indicates no defect, and [+] indicates a weak defect. In the sixth column, + indicates considerable induction, – indicates no induction, and [+] indicates weak induction. In the seventh column, + and – indicate normal and defective folding, respectively. In the last column, + and – indicate normal and decreased stability, respectively. NT, not tested.

^aStress response inducers, which are referred to as membrane protein folding-defective mutants in this paper.
^bMutations newly identified in this study by the stress response-based screening described in Materials and methods. The rplO215(amber) mutation reduces the expression level of SecY.

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and 7) but not in the wild-type cells (Fig. 3 C, lane 1). Overproduced MalF-PSBT seems to occlude even the relatively normal export pathway of the translocons having these alterations. As already discussed in the previous section, the accumulation of untranslated MalF-PSBT observed in some of the secY mutants studied in Fig. 3 B may have been produced secondarily by the primary defect that lies in a later step of the biogenesis pathway.

Membrane stresses are generated in some secY mutants

We then proceeded to screen the secY mutants by monitoring the type 2 defect. Thus, we combined each of the aforementioned secY mutations with a LacZ reporter, cpxP’-lacZ, of the Cpx stress response pathway (Danese et al., 1995). LacZ (β-galactosidase) levels were found to be elevated appreciably in the secY125, secY124, secY238, and secY351J mutants (Fig. 4). The extent of this induction was approximately threefold for secY125 and secY238, which was comparable with the extent of induction observed with the cpxA24 constitutive mutant (Shimohata et al., 2002). In contrast, the Cpx pathway was not strongly up-regulated in the secY40, secY299, or secY403 mutant (not depicted for secY403) despite their integration defects. Stress response was not at all induced in the export-integration mutant secY39 or secY205 (Fig. 4 C). The σ^54 pathway was also up-regulated in secY125 but not in secY205 (unpublished data). Thus, a class of integration-impairing secY mutations activates the stress response mechanisms (Table I).

The LacY folding assay establishes membrane protein folding-impairing secY mutations

To study the type 3 defect, we used LacY, a polytopic membrane protein of known 3D structure (Abramson et al., 2003). Inverted membrane vesicles (IMVs) were prepared from cells in which the stress response induction was evidently observed (secY129, secY238, and secY40) as well as from cells in which the stress response induction was evident (secY129, secY238, and secY125). They were combined with the reaction system for in vitro transcription/translation of LacY to examine its insertion and folding. Initially, we observed that several secY mutant IMVs were far less active than wild-type IMVs (Fig. S3, lanes 2–4; available at http://www.jcb.org/cgi/content/full/jcb.200611121/DC1) than wild-type IMVs (Fig. S3, lane 1). These IMVs were prepared without a urea wash. We then washed the IMVs with 4 M urea (see Materials and methods) before the assay. Remarkably, the urea wash activated the mutant IMVs, all of which exhibited LacY insertion activities associated with mutant IMVs even after a 5-M urea wash (Fig. 3). The urea wash cleared the mutated translocons by removing abnormal polypeptides so that the translocons were then capable of accepting LacY, making it possible to observe the initial integration of LacY into the mutant IMVs.

To characterize LacY inserted in vitro, immunoprecipitation was performed with mAb 4B1, which binds specifically to a conformational epitope in periplasmic loop VII/VIII of LacY (Sun et al., 1996). LacY synthesized in vitro was inserted into
findings, we examined the stability of newly synthesized and membrane-integrated LacY-His10 by pulse-chase experiments, or mutant does not efficiently fold into a normal conformation.

Another mAb, 4B11, recognizes a discontinuous epitope that contains determinants from both loops VIII/IX and X/XI on the cytoplasmic surface of LacY (Sun et al., 1997; Nagamori et al., 2004). As shown in Fig. 5 B (lanes 4 and 8), mAb 4B11 binds very weakly to LacY synthesized in vitro with secY129 and secY238 IMVs as compared with the mAb binding to LacY inserted into wild-type IMVs (Fig. 5 A, lanes 5, 11, and 16). In contrast, the mAb binds much less effectively to LacY inserted into secY129, secY238, or secY238 IMVs (Fig. 5 A, lanes 7, 8, and 18). These results strongly indicate that LacY inserted into the membrane of the secY10, secY238, or secY125 mutant does not efficiently fold into a normal conformation.

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Discussion

The results presented in this paper extend our previous conclusion that abnormal plasma membrane proteins can generate a stress that is sensed by the Cpx signal transduction mechanism (Shimohata et al., 2002). Failure in membrane protein biogenesis subject to affinity isolation. Thus, LacY-His10 was expressed in secY10, secY39, secY205, secY40, secY129, secY238, and secY125 cells, which were labeled briefly with [35S]methionine and chased with unlabeled methionine for 1, 2, and 4 min. As reported previously (Nagamori et al., 2004), LacY was stably maintained in membranes of wild-type cells (Fig. 6, top). Its stability was not affected adversely in the secY39, secY205, or secY40 mutant (Fig. 6). In contrast, LacY was markedly destabilized in the secY125 mutant such that the radioactive band decreased to an almost undetectable level after a 4-min chase (Fig. 6). LacY synthesized in the secY129 and secY238 mutants was also unstable, although less so than in the secY125 mutant. These results are consistent with the notion that LacY molecules that were membrane integrated in the secY125, secY129, and secY238 mutants are degraded by a proteolytic mechanism of the cell, presumably as a result of a failure in folding. They agree well with the results obtained in the in vitro experiments. Collectively, we conclude that LacY, a paradigm for polytopic membrane proteins, is misfolded when membrane insertion is mediated by a translocon with the SecY129, SecY238, or SecY125 alteration.
can generate a stress that is sensed by the Cpx and the σE regulatory mechanisms that have generally been believed to sense protein abnormalities at the outer membrane–periplasmic regions of the cell. The Cpx pathway induction in *E. coli* mutants lacking phosphatidylethanolamine (Mileykovskaya and Dowhan, 1997) may also be ascribed to the generation of some unfolded membrane proteins. The Cpx and σE regulatory systems contain both plasma membrane–integrated and periplasmic components (Duguay and Silhavy, 2004; Grigorova et al., 2004), and some of them could participate in this sensing. Molecular mechanisms of membrane stress recognition are emerging new subjects.

In contrast to the YidC and SecDF defects, the *ffh* SRP defects that occur at the targeting step do not elicit the envelope stress responses; instead, the σE-mediated cytosolic stress response is up-regulated under Ffh-compromised conditions (Bernstein and Hyndman, 2001; Park et al., 2002). It is noteworthy that the secY40, secY299, and secY403 mutants are defective in membrane protein insertion but are only weak or insignificant inducers of envelope stress responses. It is possible that some of these mutations primarily affect the targeting step of membrane protein assembly. Indeed, synthetic phenotypes with SRP alterations have been described previously for the secY40 mutation (Newitt and Bernstein, 1998; Angelini et al., 2005) affecting a cytosolically exposed part of SecY (van den Berg et al., 2004).

Importantly, we have identified several amino acid substitutions in SecY that not only impair membrane protein biogenesis but also induce the extracytoplasmic stress responses. A formal possibility that the altered SecY protein itself is sensed as malformed by the stress response mechanisms is unlikely for the following reasons. First, stress response induction is abolished by the introduction of wild-type secY in trans (unpublished data), indicating that a loss of function is responsible. Second, the secY238, secY299, secY351, and secY129 mutants are nearly normal in the protein export ability, making it unlikely that the mutated SecY proteins have been considerably denatured. We propose that the secY mutations lead to membrane protein biogenesis failures, generating abnormally folded proteins within the plasma membrane. This stress response seems to be mechanistically distinct from the phage shock protein induction associated with some *yidC* and sec defects (Jones et al., 2003), as we confirmed the lack of PspA induction in some of the SecY alterations (unpublished data).

Conclusive evidence that a class of secY alterations lead to inserted but abnormally folded membrane proteins came from our analyses of in vitro translation and integration of LacY. In vitro–synthesized LacY inserted competently into urea-washed membrane vesicles from the secY mutants in manners that were resistant to further treatment with urea, which removes LacY molecules only peripherally attached to the membrane (Roepke and Kaback, 1989; Nagamori et al., 2003). Strikingly, however, there is a clear distinction between LacY molecules synthesized in the presence of the wild type, SecY39, SecY40, or SecY205 IMVs and those synthesized in the presence of the SecY125, SecY129, or SecY238 IMVs. Whereas the products of the former reactions react with the conformation-specific mAbs against LacY, the products of the latter reaction do so only weakly. These antibodies have been used to probe folding states of this integral membrane protein under a variety of conditions, including the presence or absence of YidC (Bogdanov and Dowhan, 1998; Nagamori et al., 2004).

Thus, we conclude that a class of secY mutations alters the translocon in such a way that its function to effectively support the folding of membrane proteins, at least that of LacY, is compromised. Consistent with this notion, LacY is destabilized in these mutant cells. Unstable LacY is also produced in YidC-depleted cells (Nagamori et al., 2004). Our results showing that such SecY defects are accompanied by the induction of membrane stresses suggest that the folding-assisting role of SecY is not limited toward LacY but is exerted toward several membrane proteins. Previously, Prinz et al. (1998) showed that the *prlA* class of secY mutations affects localization of the PhoA domain attached to a membrane protein. However, the significance of this observation with respect to the normal function of SecY in membrane protein assembly is obscure; for instance, this mode of PhoA translocation depends on SecB, which is considered to be unrelated to membrane protein integration. Goder et al. (2004) described that in yeast, some *secl6* mutations affect the topological preference of a mutated transmembrane (TM) segment that naturally assumes dual orientations. Our present observations with SecY are distinct from the aforementioned precedents in that the mutations impair folding of a native, unaltered membrane protein, LacY.
Different amino acid substitutions of SecY affected OmpA export and Malf-PsBT/SecY-PhoA insertion differentially (Table I). Because the secY39 and secY205 alterations, which impair export but not integration, are suggested to affect aspects of SecY–SecA interactions (Matsumoto et al., 1997; Mori and Ito, 2001a, 2003), it is conceivable that the aforementioned differentiation comes from the differential utilization of SecA by exported versus membrane proteins (Koch and Müller, 2000). However, it should be noted that insertion of both Malf-PsBT (Jander et al., 1996) and SecY-PhoA (unpublished data) depends on SecA. Intriguingly, we showed that export of a periplasmic protein, DsbA, exhibits a similar spectrum of dependence on different secY mutations as the membrane protein integration studied here (Shimohata et al., 2005). As Schierle et al. (2003) argue, higher hydrophobicity of the signal sequence of DsbA might direct it to the SRP-dependent pathway of translocation. Thus, membrane proteins and DsbA may use a targeting route different from that used by OmpA and maltose-binding protein, and the mode of targeting may determine the mode of translocon utilization. Recently, Mitra et al. (2005) speculated on the basis of their electron microscopic data of E. coli SecYEG, which is in complex with the ribosome bearing a nascent membrane protein, that a working form of SecYEG is its front-to-front dimer. Furthermore, they proposed that the two SecYEG units are nonidentical such that one of them has a more hydrophobic channel than the other. Although this model should be examined critically by higher resolution structural studies, it offers a nice explanation for our finding of differential translocon utilization by different classes of exported or membrane proteins.

We mapped positions affected by the five membrane protein folding–defective SecY alterations on the 3D structure of E. coli SecY (Mori et al., 2004) modeled on the basis of the published coordinates for the Methanococcus jannaschii SecYEβ complex (van den Berg et al., 2004). Although the mutations are located in diverse positions of SecY (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200611121/DC1), some comments are possible. First, Ala351 (the site of secY351 mutation) is located within the cytosolic loop 5 (C5), which is of implicated importance in the interaction with SecA or the ribosome. Second, Glu238 (secY238) and Cys385 (secY129) are located near the cytosolic end of TM6 (before C4) and TM9 (after C5), respectively, which face each other. Finally, secY125 and secY124, impairing both export and integration, are mapped within regions of implicated importance in vertical and/or lateral gating of the translocon (van den Berg et al., 2004). Ser76 (the site of secY125 alteration) is located near the C-terminal end of the short plug helix called TM2a by van den Berg et al. (2004). The bulky Phe residue here may compromise the proposed movement of this plug toward the periplasm to open the gate, which is in agreement with the late step translocation defect by secY125 (Matsumoto et al., 2000a). Our results seem to suggest that this plug movement is also important for the effective clearance of the pore by the lateral release of a membrane protein. Pro84 (secY124) is located in the middle of TM2. The importance of Pro84 in membrane protein integration has been suggested from our reisolation of the same Pro84-Leu substitution by the new mutant screening designed for membrane stress induction (Table I). The TM2, TM7, and TM3 regions of SecY were proposed to comprise the lateral gate, through which substrate signal peptide or a region destined to a TM configuration moves in or out (van den Berg et al., 2004). Pro84 in TM2 could contribute to the gate-opening function possibly by perturbing the helix structure of this TM segment.

How does SecY contribute to the correct folding of membrane proteins? One obvious function is to recognize a TM sequence and assist its movement out of the translocon. Additionally, it may have a role of reorienting a hydrophobic segment to generate its correct TM topology (Goder and Spiess, 2003; Goder et al., 2004). Finally, SecY may interact with SecDF and/or YidC to create a productive assembly line for a membrane protein to follow. Each of the membrane protein folding–defective mutations identified in this study may affect one of the aforementioned functions or still other functions of the SecY translocon, and their further analysis will be useful for our full understanding of the mechanisms of membrane protein integration and folding that occur in living cells.

Materials and methods

Media

Minimal medium M9 (Miller, 1972) and complete nutrient medium L (containing 10 g bacto-tryptone, 5 g of yeast extract, and 5 g NaCl per liter; pH was adjusted to 7.2 by NaOH) were used. For growing plasmid-carrying strains, 50 μg/ml ampicillin, 20 μg/ml chloramphenicol, and/or 25 μg/ml tetracycline were added to the medium.

Bacterial strains and plasmids

The following E. coli K-12 strains were used in this study. MC1400 (araD139 Δ[argF-lac]U169 rpsL150 relA1 Fllb5301 ΔdeoC1 pft253 β5; Casadaban, 1976) was used as a wildtype strain. K1200 (MC1410, pRlOam215 Tn10 [Δtap023supSPM]), SP56 [MC1400, ΔrpsB8 [degP-lacZ]], and NH192 (MC1400, secE5O1[C]) were previously described by Ito et al. (1984). Shimohata et al. (2002), and Matsumoto et al. (2000b; note that NH192 was erroneously named as NH195 in this reference), respectively. AD202, an ompT::kan derivative of MC4100 (Akaiyama and Ito, 1990), was the parental strain for the following strains: TY0 (rpsE secY129 zhd33::Tn10), TY18 (rpsE secY125 zhd33::Tn10), TY24 (rpsE secY129 zhd33::Tn10), and AD208 (secY39 zhd33::Tn10; Baba et al., 1990; Taura et al., 1994; for TY strains), SKP1101 (MC1400, ara- rffh1::kan/pSKP10) and SKP1102 (MC1400, ara- rffh1::kan/pSKP11) carried rffh10(Ts) and the wild-type allele of rffh on the plasmids, respectively (obtained from G. Phillips, Iowa State University, Ames, IA; Park et al., 2002). The YidC conditional expression strain JS7131 (MC1060, ΔyidC attB::R6Kori Par μö-yidC [Spec]), having the chromosomal yidC disruption and arabinose promoter-controlled copy of yidC, and its isogenic yidC strain JS71 were described previously (Samuelson et al., 2000).

MC4100 mutants having secY alleles, SH470 [secY39], SH464 [secY205], SH465 [secY125], SH466 [secY124], SH467 [secY40], and SH468 [secY129], were constructed by cotransduction of the respective secY mutations in the original mutant alleles (Ito and Akaiyama, 1991; Taura et al., 1994) with zhd33::Tn10. Note that the secY115 mutation (Taura et al., 1994) is identical to secY40 (Baba et al., 1990), SH625 [secD1] was a transductant of MC1400 using THE521 (Kihara and Ito, 1994) is identical to MC1060, ΔyidC attB::R6Kori Par μö-yidC [Spec], having the chromosomal yidC disruption and arabinose promoter-controlled copy of yidC, and its isogenic yidC strain JS71 were described previously (Samuelson et al., 2000).

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Plasmid pSH10 carried the cpxP-lacZ transcriptional fusion controlled by the Cpx regulatory pathway (Shimohata et al., 2002). Plasmid pG78J carried a PSBT domain fused to the second periplasmic domain of MalF under the lac promoter (Jander et al., 1996).

Identification of new secY alleles by screening of localized chromosomal mutations for those up-regulating the extraplasmic stress responses. Localized mutagenesis of the chromosomal region around zhd233-30 to 31 and screening for SecYaffected mutants were performed as described previously (Taura et al., 1994) except that SP556 (degP-lacZ) was used as a recipient in P1 transduction (Miller, 1972). Blue-colored transductants were looked for on agar plates containing 25 μg/ml tetracycline, 40 μg/ml Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and 0.5 mM phenylmethylsulfonyl fluoride at 30 or 37°C. They were purified and checked for growth phenotypes at 20, 30, 37, and 42°C. Plasmid pHMCSA (see†; Mori and Ito, 2001a) was then introduced to examine whether the growth defect (if any) and color development on the X-gal plates were corrected. Those complemented were further tested for cotransduction of the phenotypes with zhd332.30 to 31 when they were used as a donor in backcross P1 transduction into the original recipient. We established each mutant by saving a transductant with the mutant phenotypes at this stage, which was further subjected to sequence determination for the secY ORF. Mutants thus established are summarized below in a format of allele name given, nucleotide changes with a deduced amino acid change in parentheses, and growth phenotypes (Ts, Cs, and Gs/Cs indicate poor growth at 42°C, 37°C, and at both of these temperatures, respectively): secY84, C251T (Pro244Leu) and C3901T (silent); Cs; secY238, G712A (Glu238Lys), G986 (Gly299Glu), Ts; secY351, G1051A (Ala351Thr), Ts/Cs; and secY403, G1207A (Gly403Arg). The amino acid substitution by secY84 is identical to that by secY124; thus, data for secY84 were omitted. Established strains of the SP556 (degP-lacZ) background were named SH237 (secY238), SH245 (secY299, SH247 (secY351)), and SH253 (secY403). We also used these mutants of the MC4100 background named SH469 (secY238), SH472 (secY299), and SH471 (secY351) as well as AD2397 (AD202; secY124, secY238, secY299, secY351, secY403); thus, data for secY124 were also omitted.

Pulse-chase assay of LacY stability. Cells transformed with both a LacYHis10 expression plasmid (Guan et al., 2006) and a lacY plasmid (pSTD343) were precultured in LB-ampicillin-chloramphenicol (100 μg/ml and 34 μg/ml, respectively) medium at 37°C overnight, centrifuged down, washed with M9 minimal medium, and inoculated into 50 ml M9 medium supplemented with amino acids (except Met and Cys) and thiamine for further growth at 37°C. Cells were then induced for the lac translocation with 1 mM IPTG for 5 min at OD600 = 0.6–0.8 and were pulse labeled with [35S]Tran (MP Biomedicals) for 1 min followed by chase with excess concentrations of unlabeled Met and Cys for indicated times. Samples were placed on ice, and cells were harvested by centrifugation. Membranes were prepared by sonification and subjected to a 5-M urea wash as described previously (Weinglass and Kaback, 2000; Nagamori et al., 2004). Radioactive LacYHis10, was isolated by metal affinity chromatography and visualized as described previously (Nagamori et al., 2004).

Online supplemental material

Fig. S1 provides graphical explanations of different types of membrane protein biogenesis defects. Fig. S2 shows the results of integration assays using the SecYPhoA(3-3) fusion protein. Fig. S3 shows LacY integration activities of IMVs prepared from secY mutants as well as their activation by urea washings. Fig. S4 shows locations of the membrane protein folding–impairing amino acid substitutions on the 3D structure of SecY. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200611121/DC1.

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References


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