Mutations in the Sec61p Channel Affecting Signal Sequence Recognition and Membrane Protein Topology*

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The orientation of most single-spanning membrane proteins obeys the “positive-inside rule”, i.e. the flanking region of the transmembrane segment that is more positively charged remains in the cytosol. These membrane proteins are integrated by the Sec61p/SecY translocon, but how their orientation is achieved is unknown. We have screened for mutations in yeast Sec61p that alter the orientation of single-spanning membrane proteins. We identified a class of mutants that are less efficient in retaining the positively charged flanking region in the cytosol. Surprisingly, these mutations are located at many different sites in the Sec61p/SecY molecule, and they do not only involve charged amino acid residues. All these mutants have a prl phenotype that so far have only been seen in bacteria; they allow proteins with defective signal sequences to be translocated, notype that so far have only been seen in bacteria; they allow proteins with very hydrophobic N-terminal signal-anchors and positive N-terminal flanking charge indicated that the signal initially enters the translocon to produce an Nexo/Ccyt orientation followed by inversion (16). Inversion appeared to be driven by charge interactions and to be slowed down by hydrophobic interactions of the signal core with the translocon or the membrane. At which point in the process, the plug domain moves out of its luminal binding cavity is not known.

Systematic mutation of conserved charged residues in Sec61p identified three residues that affected model protein topogenesis as predicted by the positive-inside rule (17): Glu382 and Arg67 and Arg74 in the plug domain, including its complete deletion, all prove inefficient in Sec61 complex assembly. To obtain more insight into the mechanism of topogenesis, we now used a screen to isolate mutations in Sec61p that influenced the orientation of diagnostic signal-anchor proteins. We identified 18 single-residue mutations that could be grouped into three classes with distinct effects on topology. One of them, also including all known plug mutations and the full plug deletion, showed suppression of signal sequence mutations, i.e. a prl phenotype, as previously observed only in prokaryotes (18–20). In Escherichia coli, prlA mutations in SecY were isolated as altering protein localization of proteins with mutated signal sequences. In view of the translocon structure and supported by

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The abbreviations used are: 5FOA, 5-fluoro-orotic acid; CPY, carboxypeptidase Y; DPAPB, dipeptidyl aminopeptidase B; ER, endoplasmic reticulum; GPD, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; HA, hemagglutinin; X-gal, (5-bromo-4-chloro-3-indolyl) -galactopyranoside.
Experimental data, _prlA_ effects are explained by destabilization of the closed state of the translocon and facilitated pore opening (4–6, 19, 20). The close correlation between a _prl_ phenotype and a specific effect on signal orientation thus sheds light on the role of translocon gating in the process of protein insertion.

**MATERIALS AND METHODS**

**Yeast Strains and Model Protein Constructs**—Yeast strain VGY61 (17) corresponds to RSY1293 (mato, ura3-1, leu2-3, -112, his3–11,15, trp1-1, ade2-1, can1–100, sec61::HIS3, [pDQ1]) (21) in which pDQ1 (i.e. YCplac111 (_LEU2 CEN_) containing _SEC61_ with codons 2–6 replaced by codons for _H_2_RS and with its own promoter) was exchanged for YCplac33 (_URA3 CEN_) with the same _SEC61_ gene. This made it possible to introduce mutant _sec61_ in YCplac111 (_LEU2 CEN_) by plasmid shuffling using 5-fluoro-orotic acid (5FOA). VGY61 with a disruption of _SSH1_ is described previously (17). _Sec61p_ mutant strains with the mutations _L63N_ and _L66N_, _L70N_, the triple mutation _LLNNN_, the deletions _ΔL70_, _Δtip_ (residues 67–72 replaced by a glycine), and _Δplug_ (residues 52–74 replaced by a glycine) have been reported previously (22).

Model constructs 40[Leu16] (shown schematically in Fig. 1) were described previously (17). _Sec61p_ mutant strains with the mutations _L63N_, _L66N_, _L70N_, the triple mutation _LLNNN_, the deletions _ΔL70_, _Δtip_ (residues 67–72 replaced by a glycine), and _Δplug_ (residues 52–74 replaced by a glycine) have been reported previously (22).

Model constructs 40[Leu16] (+5), 60[H1] (+1), and [Leu16] (−3) (shown schematically in Fig. 1) were described previously (17) and expressed in pRS426 (_URA3 2 μm_) with a glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter. Constructs 40[H1] (+5) and 60[H1] (+5) were generated in the same manner using the signal-anchor of _H1–4_ and _H1–3_ respectively. Construct 40[H1] (+5)_LacZ_ (Fig. 1) was made by inserting the sequence encoding residues 10–1024 of _E. coli_ β-galactosidase (_LacZ_) preceded by the linker GLINGACDP between the carboxypeptidase _Y_ (CPY) segment and the triple- _HA_ tag of 40[H1] (+5). This construct with a GPD promoter and phosphoglycerate kinase terminator was cloned into the _TRP1_ integration vector pRS404 and integrated into _trpl–1_ of VGY61 to produce the screening strain.

To analyze membrane insertion of dipeptidyl aminopeptidase _B_ (DPAPB), the CPY, invertease, and α-factor, the coding sequences of _DAP2, PRC1, SUC2_, and _MFα1_ were amplified by polymerase chain reaction (PCR), fused to a C-terminal triple- _HA_ epitope tag. Signal sequence truncations were produced by PCR using mutagenic primers. Codons 34–235 of _Atp2p_ (the β subunit of mitochondrial ATP synthase, deleting the leader peptide) and codons 1–200 of _Gal1p_ (cytosolic galactokinase) were fused to a sequence encoding a triple-HA epitope tag, cloned with the original promoter into VGY61, and grown on SD-Leu-Ura to maintain both wild-type _SEC61_ URA3 plasmid. Resistant colonies were replica-plated onto _YPD_ with adenine (YPDA), incubated for 1 day at 30 °C, overlaid with 0.5%-agarose in 0.5 M KHPO₄, pH 7, containing 0.1% SDS, and 400 μg/ml X-gal (Applichem) and incubated for 6 h at 30 °C and then at 4 °C for further color development. For each segment library, ~3000 colonies were screened. Colonies with darker or lighter color were streaked on YPDA plates, and the color assay was repeated. Plasmids were rescued by the procedure of Robzyk and Kassir (23), amplified in DH5α, transformed into VGY61, selected on SD-Leu, and replica-plated on plates containing 5FOA to eliminate the wild-type _SEC61_ plasmid. The plasmids encoding the model constructs were separately transformed into the resulting strains.

**Labeling and Immunoprecipitation**—Yeast cells were in vivo pulse-labeled for 5 min with 150 μCi/ml [35S]methionine (GE Healthcare), lysed with glass beads, heated at 95 °C for 5 min with 1% SDS, cleared by centrifugation, subjected to immunoprecipitation, and analyzed by SDS-gel electrophoresis and autoradiography as described previously (22). Signals were quantified by phosphorimagery.

**Competition between Wild-type and Mutant Sec61p**—To analyze the stability of _Sec61p_ mutants in the presence of wild-type _Sec61p_, the _sec61_ coding sequences were extended by a sequence encoding a _triple- _HA_ epitope tag, cloned with the original promoter into YCplac111 (_LEU2 CEN_), transformed into VGY61, and grown on SD-Leu-Ura to maintain both wild-type and _HA_ -tagged mutant copy of _Sec61p_. Cells were lysed in SDS-sample buffer with glass beads, boiled for 10 min, separated by SDS-gel electrophoresis, blotted onto nitrocellulose, and decorated with a rabbit antiserum against the C terminus of _Sec61p_, a gift by C. Stirling (University of Manchester, UK; 24), or with an anti-HA antibody. Antibody was detected using horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescence kit (Amersham Biosciences).
RESULTS

A Screen for Sec61p Mutations Affecting Protein Topology—To identify mutations in Sec61p that affect membrane protein topogenesis, we devised a screen based on the color reaction catalyzed by the model protein 40[H1](+5)LacZ (Fig. 1). It is a derivative of the asialoglycoprotein receptor H1, a type II single-spanning membrane protein. Because of inversion of the flanking charges of its signal-anchor sequence (resulting in a charge difference $\Delta(C-N)$ of +5 according to Hartmann et al. (11)), it inserts with mixed orientations into the ER membrane (25) and is thus a sensitive reporter of alterations affecting topology. The C-terminal sequence was replaced by a segment of yeast CPY and the coding sequence of bacterial -galactosidase (LacZ), which is active in the cytosol but inactive, when translocated. Mutations in Sec61p that affect the membrane orientation of 40[H1](+5)LacZ result in changes in enzyme activity detectable using the color substrate X-gal.

The coding sequence of Sec61p was mutagenized by error-prone PCR and transformed on a CEN plasmid with its own promoter into the yeast strain VGY61 with a chromosomally integrated 40[H1](+5)LacZ gene. After elimination of the wild-type copy of SEC61, colonies were analyzed for LacZ activity. Colonies deviating in color intensity from the background of colonies with wild-type Sec61p were picked, and the plasmids were isolated and transformed into VGY61 cells expressing 40[H1](+5)LacZ. Upon elimination of wild-type SEC61, the cells were labeled with $^{35}$S-methionine, and construct A was immunoprecipitated and analyzed by SDS-gel electrophoresis and autoradiography. In this manner, 29 Sec61p plasmids from a total of ~12,000 transformants were identified to produce an increase and 46 to produce a decrease in glycosylated, i.e. C-terminally translocated, forms of construct A. Upon sequencing, 18 single point mutations were identified to affect the topology of construct A.

Sec61p Mutations Display Distinct Topology Effects—The effects of these mutants as well as of R67E, R74E, and E382R analyzed previously (17) and I91T (one of four mutations in
Sec61-23; 21) on topology were tested for the five model proteins shown in Fig. 1. Construct B is identical to A except for the hydrophobic core of the signal-anchor that consists of a Leu$_{16}$ stretch instead of the natural transmembrane segment of H1. Upon expression in yeast cells, three forms were produced corresponding to the protein without glycans or with two or three glycans (17). Because the glycosylation sites are in the C-terminal domain, the glycosylated species represent polypeptides with a N$_{cyt}$/C$_{exo}$ orientation. In cells expressing wild-type Sec61p, 40% of construct A and 46% of construct B were glycosylated (22 and not shown). In cells expressing mutant Sec61p, this fraction was either increased or reduced as summarized in Fig. 2. The effects were essentially the same for constructs A and B, indicating that the hydrophobic cores of the two signals were handled identically. No singly glycosylated forms were detectable for any mutant (data not shown) indicating that glycosylation efficiency was not affected by the mutations.

The signal-anchors of constructs A and B have a positive charge difference ($\Delta$(C-N)$_{H1}$) typical of proteins with a N$_{exo}$/C$_{cyt}$ orientation. Based on studies in mammalian cells (12, 14, 25), they insert with mixed topologies because the 40-amino acid N-terminal domain of H1 hinders N-translocation. In construct C, this domain was in part replaced by the N-terminal translocated sequence of Ste2p, resulting in reduced C-translocation with wild-type Sec61p (12%). All Sec61p mutants that increased C-translocation of A and B, also did so for C (Fig. 2). Those that reduced C-translocation of A and B showed no significant effect, most likely because construct C was close to minimal C-translocation already with wild-type Sec61p. With a reduced charge difference ($\Delta$(C-N)$_{H1}$) of 1, i.e. with two positive flanking charges at the N- and three positive charges at the C-terminal end of the transmembrane domain, construct D was again at the center of the diagnostic range (60% C-translocation with wild-type Sec61p; 22), and all mutant translocons affected the ratio of orientations. Except for mutants W35R, T185K, and P200L, they did so in the same direction as with constructs A and B (Fig. 2).

With the Ste2p sequence at the N terminus of constructs C and D, an efficient N-glycosylation site was introduced so that polypeptides integrated into the ER membrane in either orientation were glycosylated: two or three times when the C terminus was translocated, and once when the N terminus was translocated. The appearance of unglycosylated products thus revealed polypeptides that were not integrated into the membrane. When expressed in cells with wild-type Sec61p, 5.3% (±1.4%, $n = 8$) of construct D were unglycosylated (17). Except for P292S, which generated <25% nonintegrated products, none of the mutants produced more than 10% of this form, indicating that they did not cause a significant defect in membrane integration.

Finally, model protein E differs from all others in that its signal-anchor...
chor is at the very N terminus (Fig. 1). Despite a negative charge difference of \( -3 \), this protein is inserted with \( \sim 30\% \) \( N_{\text{cyt}}/C_{\text{exo}} \) orientation by wild-type Sec61p, because of its highly hydrophobic signal-anchor (22). Many Sec61p mutants also affected the insertion behavior of this protein, mostly by reducing C-terminal translocation (Fig. 2).

In our analysis, as summarized in Fig. 2, the observed changes in model protein orientation versus wild-type Sec61p generally ranged from 5 to 20% age points and are therefore in the range that is also observed for single charge mutations flanking the signals of substrate proteins (17). Based on their effects on the different model proteins, the mutants could be grouped into three classes. Class 1 mutants consistently increased translocation of the more positively charged end of the signal-anchor of all substrates tested. This corresponds to the phenotype of a weakened positive-inside rule. The plug deletion and all other plug mutants previously prepared by directed mutagenesis also belong to this class (22). In contrast, class 3 mutants generally reduced C-translocation. Class 2 mutants only differed from class 3 in having the opposite effect on substrate D.

### TABLE 1

Summary of Sec61p mutant phenotypes

| Mutation in *Saccharomyces cerevisiae* Sec61p \(^a\) | Position in *M. jannaschii* SecY \(^b\) | Position in *E. coli* SecY \(^b\) | Localization | Class \(^c\) | Translocation defect | prl phenotype \(^d\) |
|--------------------------------------------------|--------------------------------------------|---------------------------------|--------------|----------------|----------------------|---------------|
| W235R                                           | W29                                       | F25                             | TM1 towards \( \beta \) and TM4 | 2            | \(-\) \(-\) \(\pm\) | \(-\)          |
| D61N                                            | A54                                       | T59                             | plug         | 1            | \(-\) \(-\) \(\pm\) | \(-\)          |
| R67E                                            | Q60                                       | N65                             | plug         | 1            | \(-\) \(-\) \(\pm\) | \(-\)          |
| R74E                                            | I67                                       | L72                             | plug         | 1            | \(-\) \(-\) \(\pm\) | \(-\)          |
| E79G                                            | T72                                       | A79                             | TM2 towards plug | 1    | \(-\) \(-\) \(\pm\) | \(-\)          |
| I86T                                            | V79                                       | I86                             | TM2 ring     | 1            | \(-\) \(-\) \(\pm\) | \(-\)          |
| I91T                                            | I84                                       | I91                             | TM2          | 3            | \(-\) \(-\) \(\pm\) | \(-\)          |
| Q93R                                            | Q86                                       | Q93                             | TM2 towards TM8 | 3   | \(-\) \(-\) \(\pm\) | \(-\)          |
| Q96R                                            | V89                                       | T96                             | TM2 towards TM8 | 3   | \(-\) \(-\) \(\pm\) | \(-\)          |
| L131P                                           | V124                                      | I133                            | TM3 end      | 1            | \(-\) \(-\) \(\pm\) | \(-\)          |
| S161T                                           | S151                                      | T68                             | TM4 towards inside | 1 | \(-\) \(-\) \(\pm\) | \(-\)          |
| D168A                                           | D158                                      | G175                            | TM4 towards inside | 3 | \(-\) \(-\) \(\pm\) | \(-\)          |
| T185K                                           | I174                                      | I191                            | TM5 ring     | 2            | \(-\) \(-\) \(\pm\) | \(-\)          |
| P200L                                           | P189                                      | L5 (not modeled)                | L5           | 2            | \(-\) \(-\) \(\pm\) | \(-\)          |
| Q261R                                           | E227                                      | E238                            | TM6/L6       | 3            | \(-\) \(-\) \(\pm\) | \(-\)          |
| K284E                                           | K250                                      | K268                            | L6/TM7; salt bridge to E460 | 1 | \(-\) \(-\) \(\pm\) | \(-\)          |
| P292S                                           | P258                                      | P276                            | TM7 start    | 1            | \(-\) \(-\) \(\pm\) | \(-\)          |
| T379I                                           | F333                                      | F331                            | TM8 end towards TM2 | 1  | \(-\) \(-\) \(\pm\) | \(-\)          |
| E382R                                           | E336                                      | A334                            | TM8 end towards TM2 | 1  | \(-\) \(-\) \(\pm\) | \(-\)          |
| M440K                                           | M354                                      | A351                            | Tip of L8    | 3            | \(-\) \(-\) \(\pm\) | \(-\)          |
| E460K                                           | E416                                      | E416                            | TM10 ring    | 3            | \(-\) \(-\) \(\pm\) | \(-\)          |
| L63N                                            | F56                                       | I61                             | plug         | 1            | \(-\) \(-\) \(\pm\) | \(-\)          |
| L66N                                            | W59                                       | F64                             | plug         | 1            | \(-\) \(-\) \(\pm\) | \(-\)          |
| L70N                                            | T63                                       | S68                             | plug         | 1            | \(-\) \(-\) \(\pm\) | \(-\)          |
| LLLNNN                                          | F56/W59/T63                               | I61/F64/S68                     | plug (L63N/L66N/L70N) | 1  | \(-\) \(-\) \(\pm\) | \(-\)          |
| ΔL70                                            | T63                                       | S68                             | plug         | 1            | \(-\) \(-\) \(\pm\) | \(-\)          |
| Δtip                                            | 60–65                                     | 65–70                           | plug (67–72 replaced by G) | 1 | \(-\) \(-\) \(\pm\) | \(-\)          |
| Δplug                                           | 46–67                                     | 42–72                           | plug deletion (52–74 replaced by G) | 1 | \(-\) \(-\) \(\pm\) | \(-\)          |

\(^a\) Single amino acid mutations in *S. cerevisiae* Sec61p isolated in the topology screen are listed in the upper portion of the table together with point mutations generated previously (in italics). In the lower portion, the plug mutations by Junne et al. (22) are summarized.

\(^b\) Corresponding position in the sequence of SecY from *M. jannaschii* or *E. coli* for comparison.

\(^c\) Classification according to the effect on substrate topology (Fig. 2 and 26). P292S was not classified because of its co-translational translocation defect.

\(^d\) prl phenotype based the translocation efficiency of CPYΔ3 in comparison to wild-type Sec61p (Fig. 6). \(-\), \(-\), and \(\pm\) indicates less and more than 2-fold the efficiency of wild-type Sec61p, respectively.
Translocation Efficiency and Stability of Mutant Translocons—

In addition to Sec61p, Sbh1p, and Sss1p, yeast expresses a second nonessential translocon complex consisting of Ssh1p (Sec61 homolog), Sbh2p, and Sss1p (26). In Δssh1 cells lacking the second translocon, topology changes by mutations D61N, W35R, and Q93R (as representatives of the three classes) were essentially the same4, as previously shown for R67E, R74E, and E382R (17). None of the Sec61p mutants displayed a growth defect at 30 °C (not shown) or at 39 °C even in the absence of Ssh1p (Fig. 3). Mutations I91T and P292S were cold sensitive and showed reduced growth in the presence of tunicamycin, which caused protein misfolding and ER stress (Fig. 3). I91T thus is responsible for the phenotype of Sec61–23, from which it is isolated. None of the other mutations showed sensitivity to cold or tunicamycin stress.

Efficiency of translocation was tested using CPY and DPAPB (Fig. 4A), established post- and co-translational substrates, respectively (27), for all mutants including the plug mutations prepared previously. Most mutants are as efficient in translocation as wild-type Sec61p. Exceptions are P292S and the full plug deletion Δplug that produces significant levels of unglycosylated CPY and DPAPB. A post-translational translocation defect was in addition observed for I91T and most severely, the partial plug deletion Δtip.

We previously observed that Δplug competes poorly with wild-type Sec61p for limiting factors in a heterozygous situation (22). When coexpressed with wild-type Sec61p, HA-tagged Δplug was hardly detectable in steady state by immunoblot analysis (Fig. 4B, lane 30). In contrast, all HA-tagged point mutants were present in similar amounts as HA-tagged wild type in the presence of untagged wild-type Sec61p (Fig. 4B, lanes 1–29). These results show that the full plug deletion has additional defects compared with the point mutations in the plug or elsewhere. These are most likely the result of joining the luminal ends of TM1 and TM2 by a very short connection and so immobilizing them in fixed relative position.

Class 1 Topology Effects Correlate with a prl Phenotype—The mutations affecting topology are listed in Table 1, and their locations are indicated in the structure model of the yeast Sec61 complex in Fig. 5. Most mutated residues are well conserved between species. The mutations are not limited to a single domain of the protein, but are found throughout the sequence. New mutations were found in the plug domain (D61N) and in TM2 facing the plug (E79G). Plugs destabilizing mutations as well as partial or complete deletion of the plug have previously been shown to affect topology with class 1 effects (22). In our screen, mutations were also isolated in three of the six residues forming the central constriction ring (I86T, T185K, and M450K). Several mutations are concentrated where TM2 contacts TM8 (I91T, Q93R, Q96R, T379I, and E382R) and two mutations (K284E and E460K) disrupt the ion-bridge connecting the cytosolic ends of TM7 and TM10.

4 T. Junne and M. Spiess, unpublished observations.
The distribution of the mutations in Sec61p is reminiscent of that of prlA mutations in E. coli SecY (19, 20). Several prlA mutations were found in the plug domain and in ring residues. Three mutations are exactly at corresponding positions: Asn\textsuperscript{65} in E. coli SecY (prlA91), Ile\textsuperscript{191} (prlA200), and Ile\textsuperscript{408} (prlA4–2), matching Arg\textsuperscript{67}, Thr\textsuperscript{185}, and Met\textsuperscript{450}, respectively, in Sec61p. In addition, it has very recently been shown that deletion of the plug domain of E. coli SecY has a prl phenotype (28, 29). We therefore set out to test our Sec61p mutations for a prl phenotype in yeast.

To inactivate the signal sequence of CPY, the first three residues of its hydrophobic core were deleted (CPYA\textsuperscript{3}; Fig. 6A). This reduced CPY translocation by wild-type Sec61p to \(~\)15%. Several of our Sec61p mutants significantly restored CPYA\textsuperscript{3} translocation, in some cases up to more than 70% (Fig. 6, B and C; top). When grouped according to their topogenic effects, most of the class 1 mutants (Fig. 6C, white bars), including all the plug mutants displayed the prl phenotype. Only one other mutant showed more than twice the translocation efficiency of the wild type: mutation of Glu\textsuperscript{460}, the ion-bridge partner of Lys\textsuperscript{284}, which in mutation K284E produced, class 1 and prl phenotypes. Deletion of the N-terminal sequence of DPAPB including half of its signal-anchor completely abolished translocation by wild-type Sec61p. This defect was again partially suppressed by a majority of the class 1 mutants (Fig. 6, B and C; bottom).

Further truncation or the complete deletion of the CPY signal abolished CPY translocation by the wild-type translocon, but was still rescued at a very low level by mutations L63N, Δplug, and L131P (Fig. 6D) that were tested as representatives of prl mutants identified with CPYA\textsuperscript{3}. In contrast, signal-less DPAPB was not translocated at all (Fig. 6D). The observed background of signal-independent CPY translocation by Sec61p mutants appears to be restricted to this protein and may be the result of a cryptic targeting signal within its mature portion. Deletion of the signal sequences of invertase and α-factor, additional co- and post-translationally translocated proteins, completely abolished export (Fig. 7, A–C). Translocation of the partial signal deletions was largely restored by the Sec61p point mutants tested, whereas Δplug at least recovered its relative translocation defect. No translocation was furthermore detected for polypeptides derived from a cytosolic (Gal1p) or mitochondrial (and thus intrinsically translocation competent) protein (Atp2p) equipped with a glycosylation site (Fig. 7D). There is thus no unspecific leakage of proteins through the mutant translocons.

As shown quite directly for the bacterial translocon (5, 6), prl mutations facilitate the opening of the translocation pore. The strong correlation between the prl phenotype and a distinct topology effect for a subset of our Sec61p mutations therefore suggests a role of timely pore opening in the proper orientation of signal sequences.

**DISCUSSION**

**Sec61p Mutations Affect Protein Orientation in Distinct Ways**

We have identified mutations in yeast Sec61p that affect the orientation of diagnostic signal-anchor proteins during insertion into the ER membrane. Point mutations that strongly interfere with transmembrane orientation (if they exist) are likely to have serious growth defects, precluding their isolation in our screen. This may explain why our mutations produce rather mild changes in topology, approximately in the range also caused by single charge mutations in signal flanking regions of the model substrates (17). This is generally not sufficient to perturb the topology of natural proteins. However, SecY mutations have recently been described that cause periplasmic stress and lactose permease misfolding in E. coli (30). Two of these mutations, secY238 and secY351, occurred at corresponding positions to our Q261R and M400K mutants in

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**FIGURE 7.** **prl Mutants do not allow translocation of signal-less proteins.** A–C, invertase (inv) and α-factor (αf) and the partial (invΔ4, αfΔ5) and complete (invΔ19, αfΔ18) signal deletion mutants were analyzed and quantified as in described in the legend to Fig. 6, B and C. D, as control proteins, the N-terminal portion of cytosolic galactokinase and of mature invertase and of mature Sec61p mutations are exactly at corresponding positions: Asn\textsuperscript{65} in E. coli SecY (prlA914), Ile\textsuperscript{191} (prlA200), and Ile\textsuperscript{408} (prlA4–2), matching Arg\textsuperscript{67}, Thr\textsuperscript{185}, and Met\textsuperscript{450}, respectively, in Sec61p. In addition, it has very recently been shown that deletion of the plug domain of E. coli SecY has a prl phenotype (28, 29). We therefore set out to test our Sec61p mutations for a prl phenotype in yeast.

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prl Mutations in Yeast Sec61p Affect Topogenesis

Sec61p, suggesting that mild topology defects in endogenous proteins were not unlikely.

Interestingly, the mutants, although distributed over the entire sequence, show distinct topology phenotypes on the model substrates tested (Fig. 2). The effects of class 1 mutants are consistent with a weakened positive-inside rule. Class 3 mutations, in contrast, appear to generally hinder translocation of the more sizable or less flexible portion of the model proteins and therefore to favor transfer of the smaller N-terminal sequence. Three mutants (class 2) deviate from this behavior for one substrate protein. The clearest insight into the mechanism of how some Sec61p mutations influence signal orientation is provided by the striking correlation between class 1 topology effects and a prl phenotype.

Sec61 Mutations Can Cause a prl Phenotype in Eukaryotes—In E. coli, prlA alleles of SecY are initially isolated as suppressors of signal sequence mutations in secretory proteins (18–20). This phenotype suggested a signal-dependent proofreading mechanism in SecY that prevents proteins with defective signals to be translocated by the wild-type translocon. Suppressor mutations are not allele-specific and suppress signal truncations as well as charge insertions. Interestingly, an effect on the topology of model membrane proteins is shown for two prlA alleles (31, 32). Secretory proteins completely lacking the signal, but not cytosolic proteins, are still exported by prlA mutants in a SecB-dependent manner (33). This is accounted for by the fact that SecB recognizes secretory proteins within their mature sequences (34, 35) and thus targets them to the translocon. Eukaryotic cells lack the SecB/SecA pathway of protein targeting, and no export-specific chaperones have been detected (36), which seems to explain why a prl phenotype has so far not been observed in eukaryotes. Yet, we found that a number of mutations in the Sec61 translocon of yeast also suppressed signal sequence mutations. However, unlike in the bacterial system, complete signal deletions were generally not suppressed, confirming the absence of signal-independent targeting mechanisms.

The closed state of the Sec61/SecY complex forms a compact structure stabilized from within by the bound plug domain. Many prlA mutations are altered in the plug domain of SecY in the pore ring or in residues contacting the plug (20), suggesting that they destabilize the closed conformation and facilitate plug movement and pore opening. This has been demonstrated experimentally by cysteine cross-linking of the plug with SecE outside the channel (5) and by observing spontaneous channel opening in conductance measurements (6). Accordingly, deletion of the plug domain is also found to have a prl phenotype (28, 29).

In agreement with this, also all plug mutations in yeast Sec61p as well as mutation of Asp61 forming part of the plug cavity and of the ring residue Ile86 produced a prl phenotype. In addition, elimination of the salt bridge between Lys284 and Glu460 that connected the cytosolic ends of TM7 and TM10 by mutation of either partner showed a prl phenotype, consistent with a stabilizing role of this ionic bond. For three other prl mutations, the mechanism of destabilization is not as obvious, but may well be a conformational change with that effect.

The Correlation between prl and Topology Phenotypes Suggests a Role for Regulated Pore Opening in Signal Orientation—The conspicuous correspondence between mutations causing a prl phenotype and a class 1 topology effect suggests a role for timely plug opening in the process of signal orientation. The mutants with facilitated plug movement and pore opening are apparently less efficient in rejecting the positive end of a signal sequence from entering the channel and translocation through. The central part of the plug domain contains two charged residues, Arg67 and Arg74, at least one of which is conserved in Sec61α sequence of eukaryotes and archaea. Plug movement out of the pore thus entails the removal of these positive charges from the channel (schematically illustrated in Fig. 8), thereby reducing repulsion of the more positively charged end of the signal from entering the pore. The class 1 phenotype thus corresponds to the effect of premature pore opening, as induced by prl mutations. Accordingly, timely plug movement contributes to the fidelity of membrane protein orientation in the ER. This model also suggests that signals orient themselves in the cytoplasmic pore cavity before full channel opening and insertion of the hydrophilic polypeptide. This is particularly plausible considering the narrow space within a monomeric translocation channel.

Glu382 is also identified to contribute to the positive-inside rule (17). Situated at the cytosolic end of TM8, it may attract the more positive end of signals. Its mutation to arginine
shows the corresponding class 1 effect on signal orientation. The absence of a prl phenotype for this mutation confirms that it does not exert its effect by promoting pore opening as for example the plug mutations. Other exceptions to the correlation between prl and class 1 phenotypes may be explained by the type of mutation at a particular position. K284E and E460K both destroy the stabilizing salt bridge and thereby cause the prl phenotype. The large lysine in place of Glu460, however, appears to produce an additional disturbance responsible for the different topology effects of the two mutations. In this light, it is also doubtful whether class 2 mutations represent a homogeneous group. The present list of mutations will serve as a starting point for further directed mutagenesis.

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