

The Hydrophobic Core of the Sec61 Translocon Defines the Hydrophobicity Threshold for Membrane Integration

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The Sec61 translocon mediates the translocation of proteins across the endoplasmic reticulum membrane and the lateral integration of transmembrane segments into the lipid bilayer. The structure of the idle translocon is closed by a luminal plug domain and a hydrophobic constriction ring. To test the function of the apolar constriction, we have mutated all six ring residues of yeast Sec61p to more hydrophilic, bulky, or even charged amino acids (alanines, glycines, serines, tryptophans, lysines, or aspartates). The translocon was found to be surprisingly tolerant even to the charge mutations in the constriction ring, because growth and translocation efficiency were not drastically affected. Most interestingly, ring mutants were found to affect the integration of hydrophobic sequences into the lipid bilayer, indicating that the translocon does not simply catalyze the partitioning of potential transmembrane segments between an aqueous environment and the lipid bilayer but that it also plays an active role in setting the hydrophobicity threshold for membrane integration.

INTRODUCTION

Protein translocation across the endoplasmic reticulum (ER) membrane is initiated by a hydrophobic signal sequence that, mediated by signal recognition particle (SRP) and SRP receptor, is targeted to the Sec61 translocon (Osborne *et al.*, 2005). Here, the signal is oriented to transfer one end across the membrane and to integrate itself into the membrane. The translocon provides a pore for hydrophilic polypeptide segments to pass through, while simultaneously facilitating the integration of apolar segments into the lipid bilayer.

Mutagenesis of substrate proteins showed that charged residues flanking the hydrophobic core of a signal or signal-anchor sequence are important to define its final orientation according to the “positive-inside rule” (generally positioning the more positive end on the cytoplasmic side; von Heijne, 1986; Hartmann *et al.*, 1989; Beltzer *et al.*, 1991). The hydrophobicity of the signal influences the orientation process (Goder and Spiess, 2003) and drives integration into the membrane and insertion of the adjacent hydrophilic segment into the pore (Kida *et al.*, 2009). Furthermore, subsequent apolar segments integrate into the membrane depending on their hydrophobicity. Cross-linking studies led to the proposal that membrane integration is a multistep process involving intermediate binding sites (Do *et al.*, 1996; Ismail *et al.*, 2006). Systematic analysis of potential transmembrane segments (TM) in mammalian *in vitro* and *in vivo* systems, in bacteria, and in yeast (Hessa *et al.*, 2005, 2007, 2009; Xie *et al.*, 2007; Lundin *et al.*, 2008) yielded “biological hydropho-

bicity scales” and suggested that membrane insertion is fundamentally a thermodynamic partitioning process. Based on this interpretation, it was proposed that the function of the Sec61p channel is to provide a site in the membrane through which TMs can equilibrate between the lipid and aqueous phases (Heinrich *et al.*, 2000; von Heijne, 2006).

Crystal structures of the archaeal SecYE β translocon (van den Berg *et al.*, 2004) provided a first basis to understand these processes mechanistically. SecY/Sec61 α is a compact 10-helix bundle of two halves that may open a lateral gate toward the lipid membrane between TM helices 2/3 and 7/8 (illustrated in Figure 1 for the model of the yeast Sec61 complex; Junne *et al.*, 2006). In the idle translocon, the central pore is obstructed by a luminal plug domain (highlighted in Figure 1A), but in addition by a central constriction (Figure 1, B and C). The latter is generated by six, almost invariably hydrophobic side chains provided by TMs 2, 5, 7, and 10. This constriction ring might be responsible for the good viability of yeast cells and the short-term survival of bacteria with full deletion of the plug domain in Sec61p and SecY, respectively (Junné *et al.*, 2006; Maillard *et al.*, 2007).

Mutations in the plug, in the constriction ring, as well as in the helices forming the lateral gate were found to destabilize the closed state of the translocon, resulting in a *protein localization (prl)* phenotype that suppresses inactivating mutations in signal sequences, both in bacteria (Emr *et al.*, 1981; Veenendaal *et al.*, 2004; Li *et al.*, 2007) and in yeast (Junné *et al.*, 2007). Such mutations in bacterial SecY produced transient channel openings in planar membrane permeability measurements (Saparov *et al.*, 2007). In addition, *prl* mutants were shown to affect signal-anchor topology by premature opening of the translocation pore, before the orientation of the signal is completed (Junné *et al.*, 2007).

To specifically analyze the importance and function of the hydrophobic constriction ring, we have here mutated all of its contributing residues to more hydrophilic or even charged amino acids, alone or in combination with a point mutation in the plug or a full plug deletion, and then we analyzed the resulting phenotypes with respect to viability,

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Abbreviations used: CPY, carboxypeptidase Y; DPAPB, dipeptidyl aminopeptidase B; ER, endoplasmic reticulum; HA, hemagglutinin; PCR, polymerase chain reaction; TM, transmembrane.

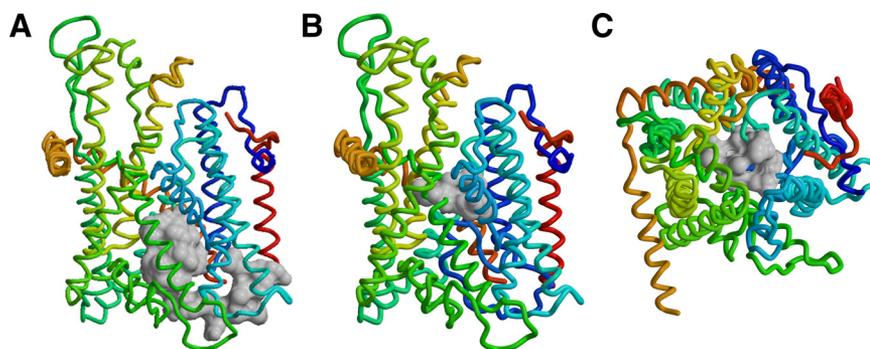


Figure 1. Plug domain and constriction ring of Sec61p. The model of yeast Sec61 complex is shown as the polypeptide backbone (Sec61p in blue to yellow, Sbh1p in red, and Sss1p in orange) with the plug domain (residues 52-74; A) or the residues of the constriction ring (V82, I86, I181, T185, M294, and M450; B and C) in space-filling representation in gray. Views from within the membrane (A and B) or from the cytosol (C) are shown with the lateral exit gate to the front or bottom, respectively.

translocon assembly and stability, translocation defects, and TM integration. The translocon was found to be surprisingly tolerant to even drastic mutations in the constriction ring. Most interestingly, ring mutants were found to affect the integration of hydrophobic sequences into the lipid bilayer, indicating that the translocon does not simply catalyze the partitioning of potential TM segments between an aqueous environment and the lipid bilayer but also that it plays an active part in setting the threshold for lipid integration.

MATERIALS AND METHODS

Yeast Strains

Yeast strain VGY61 (Goder *et al.*, 2004) corresponds to RSY1293 (*mata*, *ura3-1*, *leu2-3-112*, *his3-11,15*, *trp1-1*, *ade2-1*, *can1-100*, *sec61::HIS3*, [pDQ1]) (Pilon *et al.*, 1997) in which pDQ1 (i.e., YCplac111 (*LEU2 CEN*) containing *SEC61* with codons 2–6 replaced by codons for H₆RS 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. The absence of wild-type *SEC61* was confirmed by polymerase chain reaction (PCR) and restriction enzyme digestion of the products. VGY61 with a disruption of *SSH1* was described previously (Goder *et al.*, 2004).

Mutagenesis of Sec61p

Sec61p mutant strains with the mutations L63N and Δ plug (residues 52-74 replaced by a glycine) have been described previously (Junne *et al.*, 2006). Ring mutations were introduced sequentially in each of four quarters of ~350 base pairs of the coding sequence delimited by unique restriction sites XbaI, SacI (created by a silent mutation), StuI, AccI, and EcoRI (464 base pairs after the stop codon) at nucleotide positions 27, 343, 710, 1099, and 1916 from the initiation codon, respectively. V82/I86 and I181/T185 were mutagenized simultaneously, whereas mutations of M294 and M450 were generated separately. In proximity of these four loci new silent restriction sites, Asp718, BamHI, PstI, and again BamHI were created at positions 232, 541, 892, and 1336, respectively. Mutagenesis was performed by PCR using appropriate mutagenic primers and Vent polymerase (New England Biolabs, Ipswich, MA). Ring and plug mutations were combined via Asp718. All constructs were verified by sequencing.

Growth Analysis and Sec61p Levels

For serial dilution experiments, yeast strains were grown in YPDA medium at 30°C to mid-log phase and diluted to 0.1 OD₆₀₀. Aliquots of 6.6-fold serial dilutions were transferred onto YPDA plates and incubated at 15, 30, or 37°C.

To determine steady-state levels of Sec61p by immunoblot analysis, 10 OD₆₀₀ equivalents of yeast cells were lysed in SDS-sample buffer with glass beads and boiled for 10 min. Aliquots of equal total protein were separated by SDS-gel electrophoresis, blotted onto nitrocellulose, and decorated with a rabbit antiserum against the C terminus of Sec61p. Antibody was detected using horseradish peroxidase-conjugated anti-mouse secondary antibody and the enhanced chemoluminescence kit (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Equal protein loading was approximated based on Coomassie Blue staining of a separate gel.

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-hemagglutinin (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 HA-tagged mutant copy of Sec61p. Translocons were analyzed by immunoblotting as described above using antibodies directed against the C terminus of Sec61p and against the HA epitope, respectively.

Model Proteins

The substrate proteins dipeptidyl aminopeptidase B (DPAPB), carboxypeptidase Y (CPY), and CPY Δ 3 were described previously (Junne *et al.*, 2007). In CPY Δ C, the C-terminal 209 amino acids of CPY were deleted by PCR mutagenesis and fused to a triple-HA tag. To determine the effect of Sec61p mutations on membrane integration, the potential TM segments developed by Hessa *et al.* (2005) and shown in Table 1 were inserted into the translocated domain of DPAPB replacing codons 170–378, by PCR mutagenesis. The resulting model proteins thus consisted of an N-terminal cytoplasmic domain; a signal-anchor; a spacer sequence; the potential TM segment; and a C-terminal sequence of 29, 16, 124, 27, and 470 residues, respectively. Spacer and C-terminal sequence contain four and three potential glycosylation sites, respectively. They were expressed in pRS426 (*URA3 2 μ*) with a glyceraldehyde-3-phosphate dehydrogenase promoter and a C-terminal triple-HA tag.

Labeling and Immunoprecipitation

Yeast cells were in vivo pulse labeled for 5 min with 150 μ Ci/ml [³⁵S]methionine/cysteine (PerkinElmer Life and Analytical Sciences, Boston, MA), and, if indicated, they were chased with 30 μ g/ml each of unlabeled methionine and cysteine and 3 mM ammonium sulfate. Cells were 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 (Junne *et al.*, 2006). Signals were quantified by phosphorimager.

RESULTS

Sec61p Mutants with Hydrophilic or Even-charged Constriction Residues Retain Functionality

To test the importance of the hydrophobic constriction ring in Sec61p, the six amino acids constituting it, V82, I86, I181,

Table 1. Potential TM segments to test membrane integration behavior of Sec61p mutants

DPAPB-H nX/(19-n)A	Potential TM sequence ^a
0L/19A	GGPG AAAAAAAAAAAAAAAAAAAAAA GPGG
1L/18A	GGPG AAAAAAAAAALAAAAAAAAAAAA GPGG
2L/17A	GGPG AAAALAAAAAAAAAAAAAAAAAAA GPGG
3L/16A	GGPG AAAALAAAAALAAAAAAAAAAAA GPGG
4L/15A	GGPG AAAALALAAALAAAAAAAAAAAA GPGG
5L/14A	GGPG AAAALALAAALAAALALAAAA GPGG
6L/13A	GGPG AAAALALALALALALAAAA GPGG
1S/18A	GGPG AAAAAAAAAASAAAAAAAAAAAA GPGG
2S/17A	GGPG AAAASAAAAASAAAAAAAAAAAA GPGG
3S/16A	GGPG AAAASAAAAASAAAAASAAAA GPGG

^a Essentially the same model sequences were used as had been described previously by Hessa *et al.* (2005, 2007, 2009), including flanking glycine/proline tetrapeptides to “insulate” the central 19-residue stretch from the surrounding sequence. For simplicity of construction, 2S, 2L, and 4L guest residues were left asymmetric, because position dependence had been shown to be negligible (Hessa *et al.*, 2007). Guest residues are shown in bold.

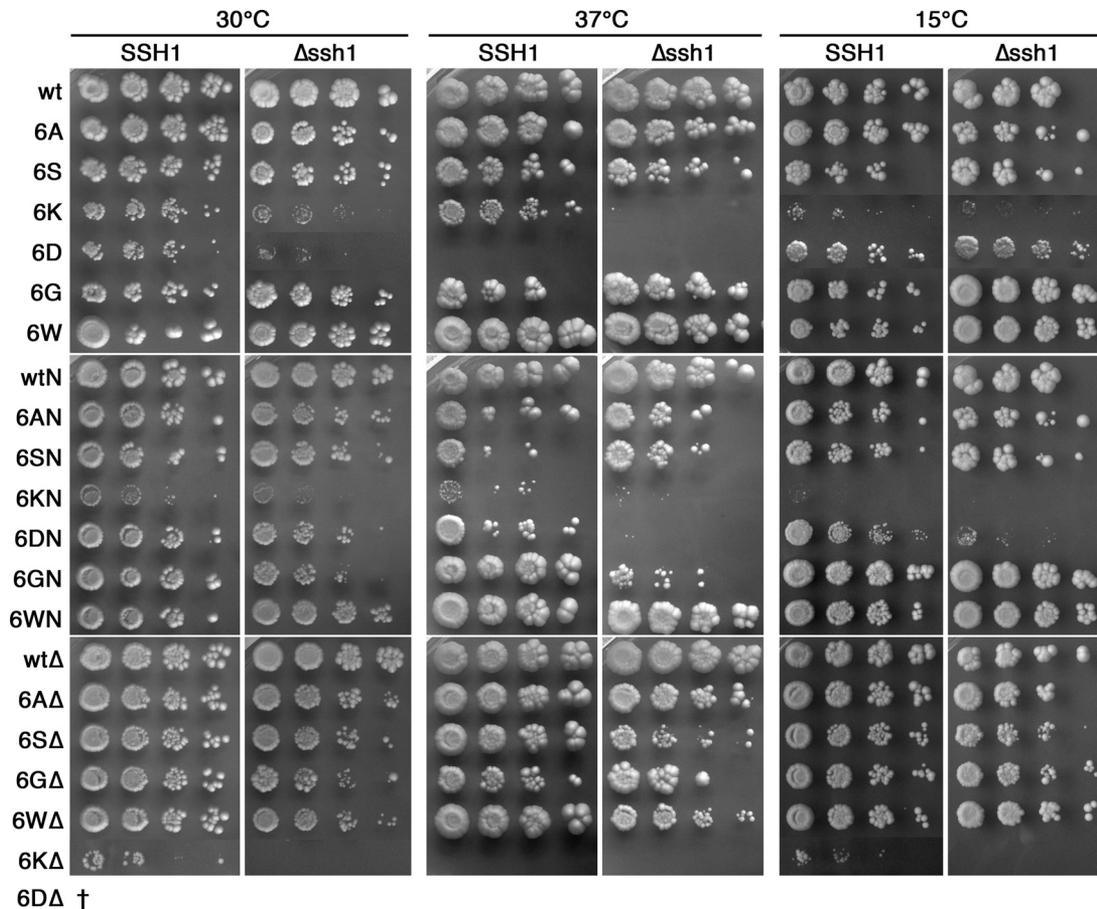


Figure 2. Growth of yeast cells with wild-type or mutant Sec61p in the presence or absence of Ssh1p. *SSH1* or $\Delta ssh1$ cells expressing the indicated Sec61p mutants were plated at serial dilutions onto YPDA plates and incubated for 3 d at 30°C, 5 d at 37°C, or 11 d at 15°C.

T185, M294, and M450, were first mutated to either alanines (6A) or serines (6S). Because these mutants produced no striking growth defects, we also generated mutants in which the ring residues were replaced by glycines (6G), lacking any side chain. In addition, an opposite mutant with six tryptophans, the most bulky amino acid, was produced (6W). Finally, all six ring residues also were mutated to the charged amino acids aspartate (6D) or lysine (6K). We expected these latter mutants to be nonfunctional, because they were likely to interfere with the proper insertion of the Sec61p TM segments or because charge repulsion might prevent the formation of the helix bundle. To our surprise, however, all mutants supported growth at 30°C, even in the absence of the second, nonessential Sec61p homologue Ssh1p (Figure 2).

We further constructed Sec61p mutants in which the ring mutations were combined with the L63N point mutation in the plug domain (Junne *et al.*, 2006) named 6XN (X standing for A, S, G, W, D, or K), or with the full plug deletion (replacement of residues 52–74 by a glycine; Junne *et al.*, 2006) named 6X Δ . As is shown in Figure 2, yeast cells with any of these mutants in place of wild-type Sec61p and in the absence of the nonessential *SEC61* homologue *SSH1* were viable except for cells containing 6D Δ , which could not lose the wild-type copy of *SEC61*. In addition, cells with 6K Δ grew so poorly that they were not yet visible after 3 d. *SSH1* rescued growth of cells with 6K Δ but not with 6D Δ . Not unexpectedly, it was the charge mutants that showed the severest growth defects: 6K, 6D, 6KN, and 6K Δ had the lowest growth rates, and 6K, 6D, 6KN, 6DN, and 6K Δ

showed heat and/or cold sensitivity, in some cases rescued by expression of *SSH1*. Ssh1p is functional only in cotranslational translocation, because it does not assemble with the Sec62–Sec63 complex essential for posttranslational translocation (Finke *et al.*, 1996), but it is found associated with translating ribosomes (Prinz *et al.*, 2000) and cotranslational substrate proteins (Wittke *et al.*, 2002). Rescue of growth in the presence of Ssh1p thus suggests that cotranslational translocation was limiting. The growth behavior of the other mutants showed little, if any, deviation from wild type.

Ring Mutations Affect Translocation Efficiency

To test the functionality of mutant translocons with respect to co- and posttranslational translocation, the translocation efficiency was tested for DPAPB and CPY (Figure 3, A–C), established co- and posttranslational substrates, respectively (Ng *et al.*, 1996). Rather modest defects were detected for cotranslational translocation of DPAPB with <30% nonintegration even for the charge mutants. However, ~50% of CPY precursor failed to be translocated by 6K, 6D, and 6KN translocons during the 5-min labeling period, and even more by 6K Δ , whereas the other mutants showed only mild defects in comparison with the respective control (wt, wtN, or wt Δ ; Figure 3, A and C).

Although unglycosylated full-length products of an obligatory cotranslational substrate directly reflect the defect in translocation, unglycosylated products of a posttranslational substrate primarily indicate a reduced rate of translocation resulting in an increased pool of cytosolic precursor. To test whether the CPY precursors not translocated after the label-

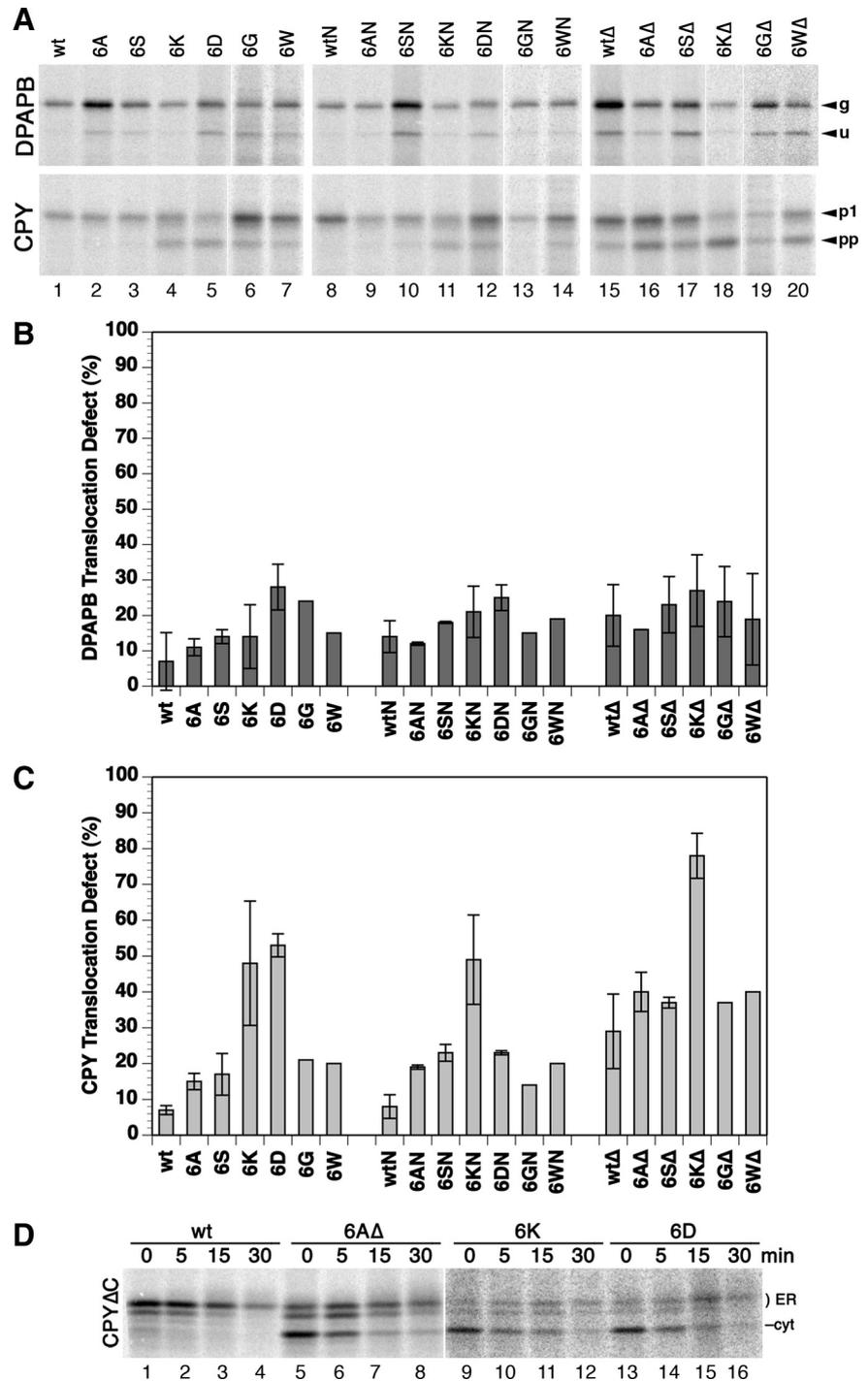


Figure 3. Translocation efficiency of wild-type and mutant Sec61p. Integration of DPAPB as a cotranslational and of CPY as a posttranslational substrate of the Sec61 translocon was analyzed in a $\Delta ssh1$ background by pulse labeling for 5 min with [35 S]methionine, immunoprecipitation, gel electrophoresis, and autoradiography (A). The products correspond to glycosylated (g) and unglycosylated (u) forms of DPAPB and to the glycosylated first proform (p1) and the unglycosylated preproform (pp) of CPY. Results were quantified by phosphorimaging, and the fraction of untranslocated DPAPB (B) and CPY (C) was plotted (mean and SD of three determinations; single measurements for 6G Δ and 6W Δ in C). In D, C-terminally truncated CPY Δ C was expressed in cells with the indicated wild-type and mutant translocons, pulse labeled for 5 min, and chased with unlabeled methionine for up to 30 min before immunoprecipitation, gel electrophoresis, and autoradiography to separate the translocated, two- and threefold glycosylated ER forms (ER) from cytosolic precursor (cyt).

ing period can still be translocated later on, we performed pulse-chase experiments. With CPY, this is complicated by the fact that mature CPY, or after deglycosylation the ER and Golgi forms, comigrate with the unglycosylated precursor. For this reason, we analyzed CPY Δ C, a C-terminally truncated version of CPY that cannot fold and is retained in the ER. Expressed with wild-type Sec61p, CPY Δ C was almost completely glycosylated and thus translocated within the pulse period (Figure 3D, lane 1). During the chase, the signal was gradually reduced to ~25% within 30 min by degradation (lanes 2–4). In cells with mutant translocons 6A Δ , 6K, or 6D (lanes 5–16), the signal of the glycosylated ER forms

initially increased during the chase and then decreased more slowly, indicating that cytosolic precursors continued to be translocated in this period. The posttranslational defects observed by pulse-labeling in Figure 3 (A and C) reflect reduced translocation rates and not the final loss of translocated protein, which is defined by competition between the rates of translocation and cytosolic degradation.

The Hydrophobic Constriction Ring Stabilizes the Closed State of the Translocon

Single point mutations in the constriction ring have been shown previously to produce a *pri* phenotype, i.e., the sup-

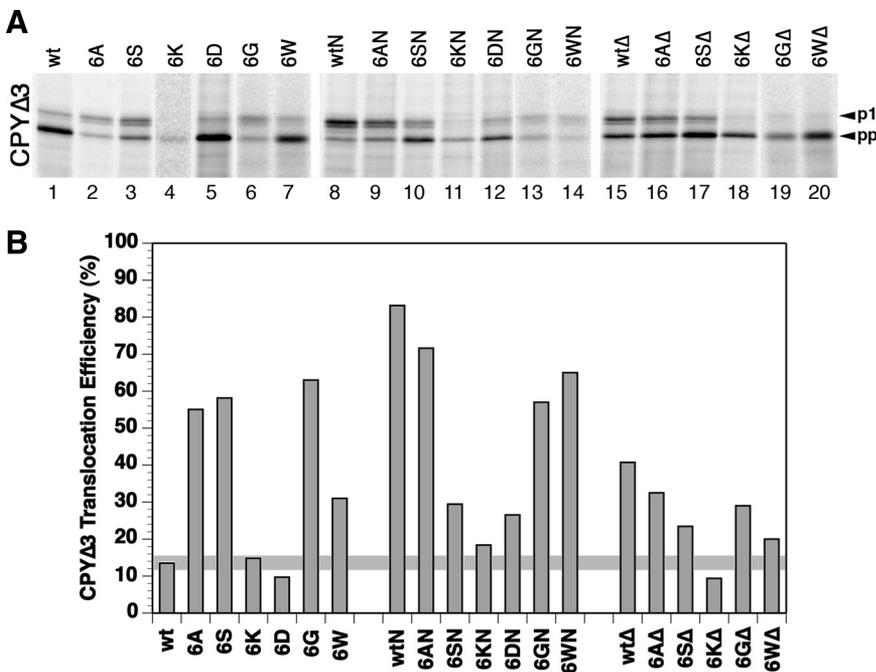


Figure 4. *prl* phenotype of mutant Sec61p. (A) CPYΔ3 (CPY with a signal sequence lacking 3 apolar residues) was expressed in Δ *ssi1* cells with wild-type (wt) or the indicated mutant Sec61p, labeled, and analyzed as described in Figure 3A. (B) Translocation efficiency was quantified by phosphorimager. The average of one to three determinations is shown. The horizontal line indicates the wild-type levels.

pression of inactivating mutations in signal sequences, both in the bacterial system (Smith *et al.*, 2005) and in yeast (Junne *et al.*, 2007). In general, *prl* mutations are interpreted to destabilize specifically the closed state of the translocon by disturbing the structure of the plug, its binding site in the luminal cavity, or lateral gate closure, and thus facilitate pore opening. As a result, even marginally hydrophobic signal sequences obtain access to the membrane that are rejected by the wild-type translocon. To test for a *prl* phenotype, translocation of CPYΔ3 was tested, a mutant CPY in which the signal was inactivated by deletion of three apolar residues to yield <15% translocation with wild-type Sec61p. Replacement of all six hydrophobic constriction residues by alanines, serines, or glycines showed a clear *prl* effect, because >50% of CPYΔ3 was translocated (Figure 4). 6W showed a smaller effect, whereas 6D and 6K did not suppress the signal mutation, even when taking into account their general translocation defect. The effect of the charge mutations and in part of 6W thus seems not to specifically destabilize the closed state in favor of the open one, but to disturb the structure in a more general manner.

Ring mutations and the L63N plug mutation or the full plug deletion, which both generate a *prl* phenotype on their own, were not additive in suppression of the signal defect (Figure 4). The *prl* phenotype was always reduced, only mildly by alanines and most significantly by the charge mutations. This suggests that the effects of the combined mutations are not limited to facilitate pore opening.

To test the stability of Sec61p mutants, their steady-state levels in cells lacking wild-type *SEC61* were analyzed by immunoblot analysis. Surprisingly, charged residues replacing the hydrophobic constriction residues did not significantly reduce protein levels in comparison with the respective wild-type translocons (wt, wtN, and wtΔ; Figure 5A). In contrast, in a heterozygous situation, when coexpressed with a wild-type copy of *SEC61*, several of the mutant translocons (tagged with an HA-epitope for independent detection) were observed at strongly reduced levels (Figure 5B). This phenomenon was previously observed for ΔTM2 (deletion of codons 77–107; Wilkinson *et al.*, 2000) and Δplug

(Junne *et al.*, 2006; Figure 5B, lane 15). It indicates competition of wild-type and mutant Sec61p for limiting interaction partners that are required for stability. In support of this notion, overexpression of the β and γ subunits Sbh1p and Sss1p at least partially rescued Δplug and ΔTM2 (Junne *et al.*, 2007). Reduced levels in a heterozygous situation therefore suggest an altered protein surface with reduced binding affinity to partner molecules. Most affected were the mutations to lysines, aspartates, and glycines, whereas mutations to serines and tryptophans caused minor effects. Only the mutations to alanines showed no reduction of protein levels compared with the corresponding wild-type version of the translocon.

Properties of the Constriction Ring Regulate Membrane Insertion

In addition to offering a passage for polypeptides through the membrane, the translocon also provides a lateral gate for the insertion of TM segments into the lipid bilayer. To analyze the effect of ring mutations on integration of TM sequences into the lipid bilayer, we tested the integration efficiency of moderately hydrophobic H-segments previously used by von Heijne and colleagues to characterize this process in mammalian *in vitro* and *in vivo* systems and in yeast (Hessa *et al.*, 2005, 2009). They consisted of a 19-alanine host segment in which an increasing number of residues were replaced by leucines (Table 1), thus creating a series of increasing hydrophobicity. These sequences were inserted into the exoplasmic domain of DPAPB, generating a protein (DPAPB-H) with an uncleaved signal-anchor sequence for cotranslational ER targeting and translocation of its C terminus, and a potential stop-transfer sequence (as illustrated in Figure 6A). Depending upon whether this sequence is integrated into the membrane or is translocated, the protein is glycosylated only at sites between the signal-anchor and the H-sequence or also at downstream sites, respectively. The fraction of translocated to integrated H-segments can thus be determined after pulse-labeling, immunoprecipitation, gel electrophoresis, and autoradiography from the intensities of the fully and partially glycosylated forms corresponding to

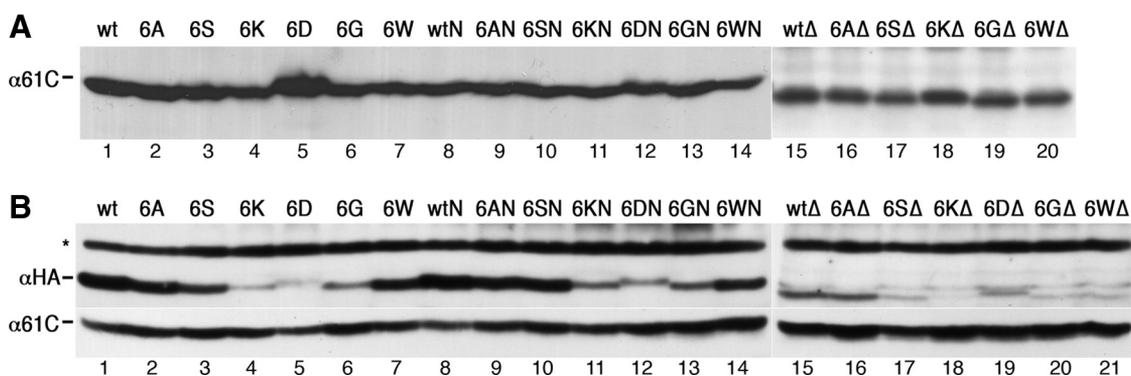


Figure 5. Levels of wild-type or mutant translocons in the absence (A) or presence (B) of a second wild-type copy of Sec61p. (A) Steady-state amounts of wild-type and mutant Sec61p were determined in an *SSH1* background by immunoblot analysis of total cell lysate. Equal loading was approximated based on protein determination and Coomassie staining of SDS-gels. (B) Yeast cells expressing equal amounts of wild-type Sec61p and the indicated HA-tagged mutants were analyzed by immunoblot analysis using an antiserum against the C terminus of Sec61p ($\alpha 61C$) and an anti-HA antibody (αHA) recognizing wild-type and mutant Sec61p, respectively. Mutation of constriction residues to aspartates consistently resulted in slightly reduced electrophoretic mobility. The asterisk indicates a background band recognized by the anti-HA antibody that also serves as a loading control. Data for *SSH1* cells are shown; the same result was obtained with $\Delta ssh1$ cells (unpublished data).

the translocated (T) and integrated H-segments (I), respectively. Unglycosylated products (U) generated by some of the partially defective mutant translocons were clearly separated and ignored as irrelevant to the process of membrane integration of the H-segments.

Oligo-alanine H-segments with no or one leucine were fully translocated by the wild-type translocon, whereas increasing membrane integration was observed with additional leucines (Figure 6B, and quantified as the integrated fraction in Figure 7A). Fifty percent integration was obtained with approximately four leucines. Cells expressing the Δ plug translocon dealt with the DPAPB-H constructs identically, indicating that the plug domain does not affect the outcome of the integration process. Similarly, the Q96R translocon, a Sec61p mutant altered at the cytoplasmic end of TM2 and affecting signal-anchor orientation (Junne *et al.*, 2007), did not affect membrane integration. Also, the replacement of the constriction ring by six tryptophans, which are quite hydrophobic, had no effect (Figures 6B and 7B).

In contrast, exchange of the ring residues to hydrophilic amino acids, 6G, 6S, 6K, and 6D, clearly affected the insertion of H-segments (Figures 6B and 7, B and C). The required number of leucines to allow 50% membrane insertion was reduced to approximately two with 6S, 6G, and 6K, and the 6D translocon already mediated >60% integration for a pure 19-alanine sequence. Insertion of one to three serines into the oligo-alanine H-segment (Table 1) was necessary to prevent integration completely (Figure 6B and 7, B and C). In addition, the transition from predominantly translocated to mostly integrated H-segments occurred over a wide range of hydrophobicity for 6K and 6D, rather than within three leucines as for the other translocons. Surprisingly, mutation of the constriction residues to 6A had the opposite effects on H-domain insertion: it required five leucines for 50% insertion and the transition was completed in a range of only two additional leucines.

If the results are interpreted as an equilibration between a membrane inserted and a free state (according to Hessa *et al.*, 2005), the ratio of integrated to translocated fractions, $K_{app} = f_i/f_t$, i.e., the apparent equilibrium constant, can be used to calculate apparent free energies of membrane insertion as $\Delta G_{app} = -RT \ln K_{app}$, where R is the gas constant and T the absolute temperature of 303 K. The resulting plots (Figure 7, A'-C') reveal a good linearity, consistent with the equilibrium assumption. The number of leucines in the oligo-alanine host sequence necessary for 50% membrane insertion

with each mutant translocon was interpolated from these plots and listed in Table 2. These values and the apparent free energies of insertion ΔG_{app} are generally reduced with mutant translocons containing hydrophilic or charged ring residues. The amino acids lining the core of the translocation pore thus clearly influence the hydrophobicity threshold for membrane insertion, most likely by defining the polarity of the environment of the substrate sequence within the pore, i.e., of one of the two compartments between which the H-segment is partitioning. Increased polarity in the more polar compartment is expected to favor membrane integration of a moderately hydrophobic sequence. This is what we observe for the 6G, 6S, 6K, and 6D mutant translocons. The behavior of the 6A mutant, however, does not simply correlate with the increase in polarity in the ring residues and requires a different explanation.

DISCUSSION

Constriction Ring Mutants Retain Translocon Functionality

The crystal structure of the idle translocon shows a pore that is closed by the luminal plug domain and a central constriction formed by six hydrophobic residues. A likely function of this apolar constriction is thus to prevent or reduce ion permeability when the plug is out and during protein translocation. This is supported by the viability of yeast cells expressing plugless mutant translocons (Junne *et al.*, 2006) and the fact that in *Escherichia coli* expression of translocons with their plug locked open, although lethal, did not lead to an immediate membrane depolarization (Harris and Silhavy, 1999). Indeed, it has recently been shown that *prl* mutants of SecY with single hydrophobic-to-asparagine mutations in the constriction ring or with a deletion in the plug domain caused ion conductance across inner membrane vesicles, but with strong selectivity for anions, particularly chloride (Dalal and Duong, 2009). This selectivity, which preserves the seal for protons, is also functional during protein translocation through the wild-type translocon.

Here, we have tested the effects of replacing all six constriction residues to various less hydrophobic, polar, and even charged amino acids. Only minor defects were detected for mutations to alanines, serines, tryptophans, or glycines, and significant functionality was retained even with charged residues (aspartates or lysines). Mutation to

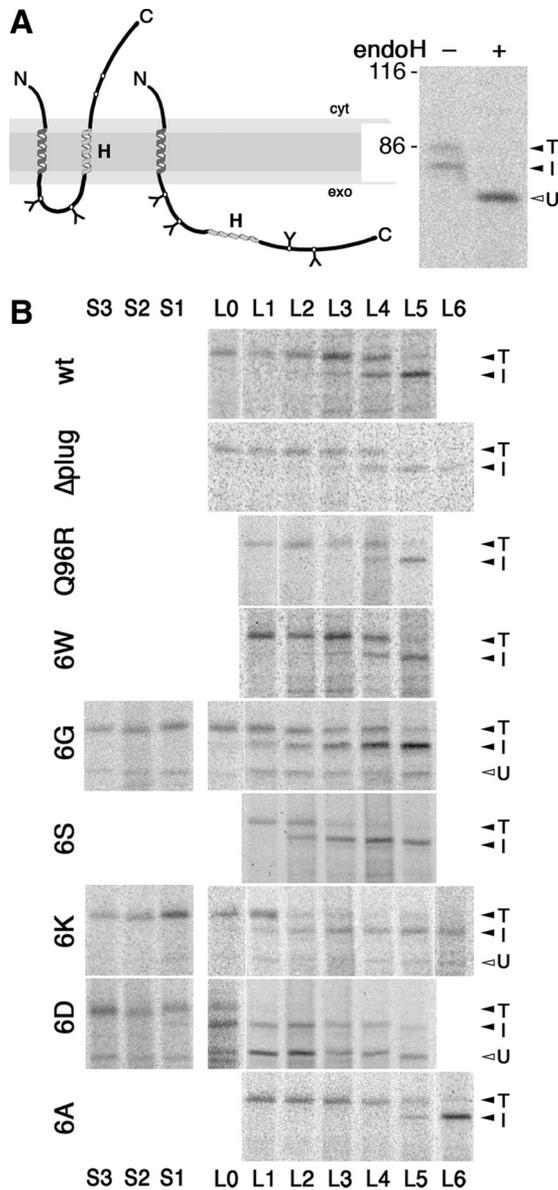


Figure 6. Membrane insertion of H-segments of various hydrophobicities mediated by wild-type and mutant Sec61p. (A) Schematic representation of the DPAPB-H model proteins (left) and products of the DPAPB-H substrate with four leucines expressed in cells with wild-type Sec61p after [^{35}S]methionine labeling, immunoprecipitation, incubation with (+) or without (-) endoglycosidase H (endoH), and gel electrophoresis (right). Integration of the H-segment results in a partially glycosylated double-spanning membrane protein (I), whereas its translocation yields a fully glycosylated type II protein (T). U, unglycosylated form, cyt, cytoplasmic; exo, exoplasmic. The position of molecular weight markers (in kilodaltons) is indicated. (B) *SSH1* cells expressing wild-type or mutant translocons (as indicated on the left) as well as a DPAPB-H substrate (with the number of leucine or serine residues indicated above and below) were pulse labeled with [^{35}S]methionine, and the substrate products were immunoprecipitated, separated by gel electrophoresis, and visualized by autoradiography.

the uncharged amino acids, 6A, 6S, 6G, and 6W, produced a *prl* phenotype, indicating specific destabilization of the closed state and facilitated translocon opening. *prl* mutations specifically disturb interactions that must be overcome for preprotein insertion. Therefore, not every ring mutation

causes a *prl* phenotype (in Sec61p I86T does, but not T185K or M450K; Junne *et al.*, 2007), and multiple ring mutations do not necessarily have a stronger phenotype than single mutations (6S showed less suppression than I86T). The charge mutations in 6D and 6K, but also the single mutations T185K or M450K (Junne *et al.*, 2007), caused less specific perturbations and thus no *prl* effect. Similarly, by cumulation of *prl* mutations upon combining mutations in the ring residues and in the plug domain may generally destabilize the structure and thus not enhance or even reduce suppression of signal defects.

Because the constriction ring forms part of the plug binding site, ring mutations are expected to simultaneously disturb plug insertion. This could explain that an additional point mutation or even deletion of the plug did not strongly aggravate the phenotypes. Translocon stability was not significantly compromised by the ring mutations, not even by charged residues. Only in competition with a wild-type copy of Sec61p were the levels of mutant translocons clearly reduced in the order $A < S \approx W < G < D \approx K$, suggesting altered or less stable surface binding sites for limiting interaction partners. Again, this is a surprisingly mild effect for considerable alterations in the center of the protein. The ring mutations 6A, 6S, 6G, and 6W also did not significantly affect the orientation of sensitive diagnostic constructs (as used previously in Junne *et al.*, 2007), and 6D and 6K did not have strong and opposite effects that could be correlated with their charges (unpublished data). It suggests that the positive-inside rule is not dominated by charged residues in the core of the translocon.

The Translocon Core Regulates Membrane Integration

It is long known that hydrophobicity is the essential property of a sequence for membrane integration (Davis and Model, 1985). Cross-linking experiments with reconstituted proteoliposomes demonstrated that the translocon allows a TM domain to bypass the barrier posed by the polar head groups of the lipid bilayer and to come into contact with the hydrophobic interior of the membrane (Heinrich *et al.*, 2000). It was proposed that Sec61 provides a site through which a TM domain can dynamically equilibrate between the lipid and aqueous phases, depending on its hydrophobicity. The systematic analyses by von Heijne and colleagues (Hessa *et al.*, 2005, 2009) yielded a biological hydrophobicity scale for membrane integration consistent with a thermodynamic partitioning process. The observed position dependence of residues such as tryptophan or tyrosine in the H-segment reflects the symmetry of the lipid bilayer (Hessa *et al.*, 2007) and is also consistent with equilibration. Based on this interpretation, an apparent free energy contribution (ΔG^{app}) for membrane integration could be calculated for each amino acid. Interestingly, the values, although generally similar, were different for different systems (mammalian ER *in vitro* and *in vivo*, yeast, bacteria, and biophysical measurements). From our measurements in yeast, we obtained $\Delta G^{\text{Leu,app}} = -0.51$ kcal/mol, $\Delta G^{\text{Ala,app}} = 0.12$ kcal/mol and an interpolated 3.6 leucines in a 19-residues oligo-alanine sequence for 50% integration (compared with previous measurements in yeast of -0.21 kcal/mol, 0.06 kcal/mol, and 4.4 leucines, respectively (Hessa *et al.*, 2009).

An alternative model to partitioning is a kinetically controlled mechanism in which H-segments trigger the opening of the lateral gate for (irreversible) exit into the lipid phase. Membrane integration would reflect the probability of gate opening, as supported by the molecular modeling study by Zhang and Miller (2010). *In vitro* crosslinking studies with arrested nascent chains showed extended association of TM segments with translocon proteins in defined positions (e.g. McCormick *et al.*,

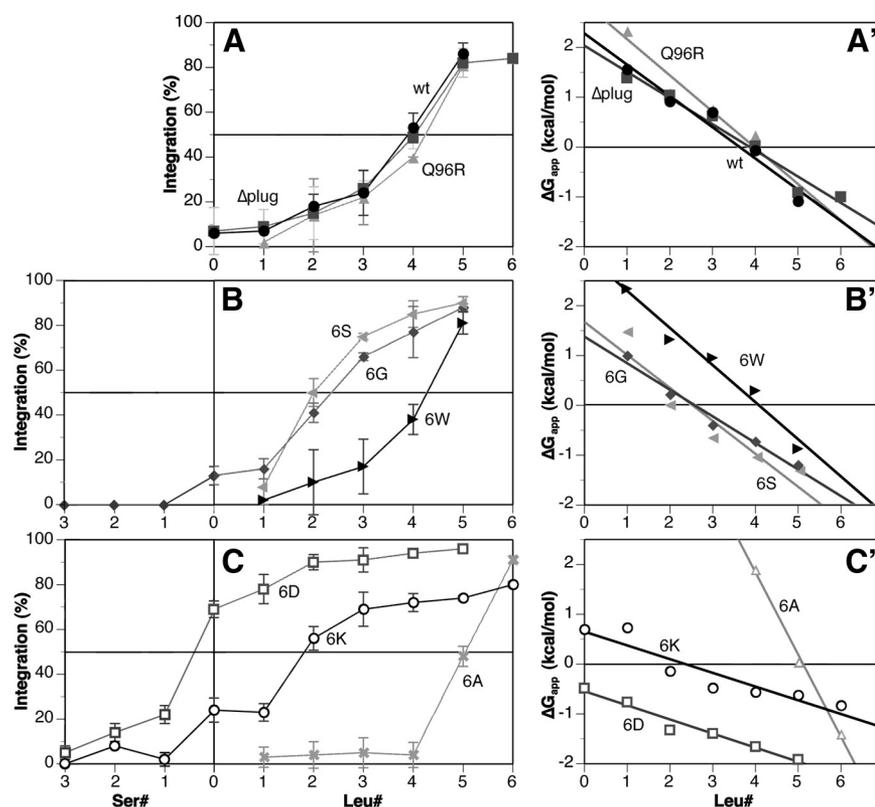


Figure 7. Efficiency of H-segment integration by wild-type and mutant Sec61p. (A–C) The membrane-inserted fraction of two to five experiments like those shown in Figure 6 was quantified and plotted (with SDs) versus the number of leucines or serines in the H-segment. (A'–C') The data for the H-segments containing zero to six leucines also were plotted as apparent free energies of membrane insertion, ΔG_{app} with straight lines determined by linear regression.

2003; Pitonzo et al., 2009), arguing that the H-segments might not immediately be free in the lipid phase. If these mechanisms depend on the hydrophobicity of the H-segment, the result might be difficult to distinguish from that of a free equilibration mechanism. Here, we found that mutations in the translocon affect TM integration, seemingly in support of a kinetic model, because a pure catalyst of partitioning should not affect the equilibrium. However, the translocon not only mediates the transition between two environments but also defines the properties of one of them. The hydrophobic constriction ring provides an apolar core that has also been observed experimentally at the center of the SecYEG translocon of *E. coli* (Bol et al., 2007). In addition, the narrowness of the pore (particularly

in the presence of a substrate) partially excludes water to create conditions less polar than in bulk solution. The choice for an H-segment is therefore not the lipid environment versus the aqueous solution, but versus a less polar pore environment. Constriction ring mutations change the conditions inside the pore and thus also the outcome of a partitioning process with the membrane. An increase in polarity inside the pore by replacing the hydrophobic constriction residues with polar amino acids reduces the hydrophobicity required for 50% membrane integration (Table 2). Replacement with serines or glycines reduced it by at least one leucine, replacement with lysines by even more. The effect was most dramatic for aspartates that resulted in predominant membrane integration even of the Ala₁₉ H-segment. The reason is probably that the charges on the short side chains of aspartates are more concentrated whereas those on the long lysine side chains are more delocalized. Tryptophans, as relatively hydrophobic amino acids and excluding water by their size, did not alter membrane integration significantly.

Interestingly, mutation of the ring residues to alanines had a clear effect of raising the hydrophobicity threshold for membrane integration to approximately five leucines. This is contrary to the expectation for introducing less hydrophobic ring residues. Of course, the effect of the mutations on the conformation of the translocon is not known. It is conceivable that the small side chains allow the pore to contract and thus to further exclude water, resulting in conditions corresponding to increased hydrophobicity. Glycines may not have this effect, because they introduce increased conformational flexibility.

It has been shown by cysteine cross-linking that the plug can move out of its binding cavity to reach SecE and that plug movement is triggered by polypeptide translocation (Tam et al., 2005). Molecular dynamics simulations, however, suggested that the plug is not necessarily fully displaced by a translocation polypeptide, but remains posi-

Table 2. Summary of membrane integration parameters of wild-type and mutant translocons

Sec61	n for 50% membrane integration of nL/(19–n) ^a
Wt	3.6
Δ plug	3.8
Q96R	4.0
6W	4.1
6G	2.6
6S	2.5
6K	2.3
6D	–2.0
6A	5.1

^a The number of leucines in the oligo-alanine host sequences (shown in Table 1) for $\Delta G_{app} = 0$ was interpolated from the linear regressions shown in Figure 7 (A'–C').

tioned either toward the lateral gate for a hydrophilic substrate or toward the inner side of the pore for a hydrophobic substrate (Zhang and Miller, 2010). In our experiments, full deletion of the plug did not affect H-segment integration, suggesting that the plug does not play an active role in regulating membrane integration.

Yet, our results clearly show that the properties of the residues forming the central constriction in the Sec61 translocon adjust the hydrophobicity threshold at which translocating sequences prefer the apolar environment of the lipid bilayer and thus stop further transfer to be anchored as TM domains.

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