Determination of the Transmembrane Topology of Yeast Sec61p, an Essential Component of the Endoplasmic Reticulum Translocation Complex*

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Sec61p is a highly conserved integral membrane protein that plays a role in the formation of a protein-conducting channel required for the translocation of polypeptides into, and across, the membrane of the endoplasmic reticulum. As a major step toward elucidating the structure of the endoplasmic reticulum translocation apparatus, we have determined the transmembrane topology of Sec61p using a combination of C-terminal reporter-domain fusions and the in situ digestion of specifically inserted factor Xa protease cleavage sites. Our data indicate the presence of 10 transmembrane domains, including several with surprisingly limited hydrophobicity. Furthermore, we provide evidence for complex intramolecular interactions in which these weakly hydrophobic domains require C-terminal sequences for their correct topogenesis. The incorporation of sequences with limited hydrophobicity into the bilayer may play a vital role in the formation of an aqueous membrane channel required for the translocation of hydrophilic polypeptide chains.

Protein translocation across the membrane of the endoplasmic reticulum (ER)1 is a decisive step in the biosynthesis of many classes of proteins in eukaryotes. The process of signal sequence recognition and targeting of proteins to the ER has been substantially elucidated (1), but the mechanism by which they subsequently cross the membrane remains less well understood. Increasingly, genetic, biochemical, and biophysical approaches have been applied in probing the translocation process.

The suggestion that the passage of nascent polypeptides across the membrane occurs through a proteinaceous, aqueous channel (2) is supported by a growing body of evidence. Electrophysiological studies indicate the presence of large aqueous channels in rough ER membranes (3), and fluorophores incorporated into a translocating polypeptide report an aqueous environment when trapped within the membrane (4, 5). Considerable support for the proposal has been provided by the identification of proteins located in proximity to translocating nascent polypeptides by use of cross-linking techniques (6).

Genetic studies in Saccharomyces cerevisiae have led to the identification of a number of genes involved in the translocation process (7–13). These include SEC61 that is an essential gene encoding the 53-kDa integral membrane protein, Sec61p (13). Sec61p appears to exist as part of a stable complex together with Sss1p and Sbh1p (14, 15). Like Sec1p, Sss1p is essential for viability and for protein translocation in vivo (9). In contrast, Sbh1p is encoded by a nonessential gene, and its role in translocation is uncertain (16). Both the Sec61 complex and a second membrane protein complex (comprising Sec62p, Sec63p, Sec71p, and Sec72p) are required for the efficient posttranslational translocation of prepro-a-factor into reconstituted proteoliposomes (15). Cross-linking studies indicate that Sec61p is in close proximity to prepro-a-factor at different stages of its translocation through the bilayer (17, 18), suggesting that Sec61p may play a direct role in the formation of a protein-conducting channel in the ER membrane. A mammalian homologue of Sec61p, Sec61α (19), also contacts nascent polypeptides during their membrane transfer (19–21) and exists in a homologous Sec61 complex together with Sec61β (Sbh1p) and Sec61γ (Sss1p) (22).

The determination of transmembrane topology is essential for any understanding of the structure-function relationship of Sec61p in the translocation process. Hydrodynamic analysis of the Sec61p sequence reveals a number of regions comprising predominantly hydrophobic residues, which may correspond to transmembrane domains. The establishment of transmembrane topology at the ER membrane is thought to depend upon topogenic sequences directing sequential translocation and membrane integration events (23, 24). Support for this comes largely from the generation of predicted topologies by expression of protein chimeras encoding signal, signal-anchor, and stop-transfer sequences (25–27).

In this report we have analyzed the orientation of the yeast Sec61p in the ER membrane. We used a C-terminal reporter technique whereby N-terminal portions of Sec61p were fused to a topological reporter protein. This provided evidence for seven transmembrane domains. As a result of anomalies arising from this approach, the topology was probed using insertion fusions and expression of Sec61p as complementary polypeptide fragments. This provided evidence for a further three transmembrane domains, resulting in a model in which Sec61p spans the ER bilayer 10 times. Several of the transmembrane domains are relatively weakly hydrophobic and may be crucial in the formation of an aqueous protein-conducting channel. This combined approach to topology determination also strongly suggests that some transmembrane domains are dependent on C-terminal sequences for their correct topogenesis.

EXPERIMENTAL PROCEDURES

Materials and DNA Manipulations—DNA restriction and modification enzymes, Endo H, and in vitro mutagenesis reagents were pur-
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chased from Boehringer Mannheim. Sequenase™ version 2.0 and sequenase from U. S. Biochemical Corporation. The negative factor Xα, and proteinase K were from ICN, Promega, and Sigma, respectively. All other chemicals and reagents were purchased from Boehringer Mannheim, Sigma, and British Drug House at analytical grade. Routine DNA manipulations and sequencing were carried out according to standard protocols (28, 29).

Strains, Growth Media, and Conditions — The following yeast strains were used in this study: CSY142 (MATα, leu2, pep4::URA3); YT555 (30); MATα, ura3, ade2, Δsec2; CSY110 (13); MATα/MATα, sec61::HIS3/SEC61, his3/hib3, leu2/leu2, ura3/ura3, ade2/ade2, trp1/trp1, can1/can1); and BW474 (this study); MATα, leu2, ade2, ura3, his3, trp1, can1::SEC61 (pBW26). See below for BW474 construction. Microbiological culture media were obtained from Difco. Yeasts were grown in YP (complete) medium supplemented with 2% glucose (YPD) or 2% galactose supplemented with appropriate nutrients. Solid media were Bacto-peptone (33) supplemented with 2% glucose (YPD) or 2% galactose supplemented with appropriate nutrients. Solid media were supplemented with 2% Bacto-agar (Difco). Yeast transformations were carried out by the one-step procedure (31).

Construction of a Yeast Strain with Conditional Expression of SEC61—In order to generate a yeast strain with conditional expression of Sec61p, SEC61 was placed under the control of the GAL1 promoter. A 2.4-kbp HindIII fragment containing SEC61 from pCS43 (13) was cloned into pRS316 (32) to provide multicopy, -selectable expression. Construction of Site-specific Sec1p-Suc2p Fusion Proteins—Eight of the pBW11-BamHI plasmids were used to make Suc2p fusions. Digestion with XhoI, subsequent end-filling, followed by digestion with BamHI removed the C-terminal portion of the SEC61 gene. A 2.2-kbp HindIII/PvuII fragment from pCS29 (see above) was then ligated to make an in-frame fusion. Each fusion was confirmed by DNA sequence analysis. pCS21-BamHI plasmids were digested jointly with BamHI and Smal to remove the C-terminal region of SEC61, and a 2.2-kbp BamHI/PvuII fragment from pCS29 was cloned in its place. BamHI sites at nucleotides 349, 637, 826, 1000, 1336, and 1423 were used to make Sec61p fusions to amino acid residues Lys 70, Gly445, and Gly475, respectively. In each case the Sec61p was fused to the third residue of the mature portion of Suc2p.

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RESULTS

Hydropathic Profile of the Sec61p Sequence—The polypeptide chain of Sec61p is 480 amino acids in length, and its hydropathic profile is shown in Fig. 1A. The 10 most hydrophobic segments (HS) have been indicated. Several of these segments exhibit the extreme mean hydropathies typical of transmembrane domains (e.g. HS1, -3, -4, and -6) (13, 41). However, others are much less hydrophobic, and their membrane disposition is uncertain. The shortest hydrophobic segment being HS5 representing a stretch of 12 residues in which only 11 are nonpolar (see Fig. 1A).

Construction of Sec61p-Suc2p Fusion Proteins—We have analyzed the transmembrane topology of yeast Sec61p using a C-terminal reporter approach originally developed in Escherichia coli for the study of cytoplasmic membrane proteins (42) and adapted in yeast for the study of ER and plasma membrane protein topology (43–45). Suc2p was chosen as a topological reporter, because it does not possess any membrane topogenic preferences and becomes rapidly modified by asparagine-linked glycosylation at multiple sites upon translocation to the lumen of the ER, resulting in a 20–26-kDa increase in molecular mass (46). Suc2p fusions have also been successfully applied in determining the topology of both Sec62p and Sec63p (30, 47).

A total of 22 Sec61p-Suc2p fusions were constructed either randomly by exonuclease III deletion or by the use of specific BamHI restriction sites introduced at various points throughout the SEC61 coding sequence (see “Experimental Procedures”). In all cases the resulting fusion proteins were expressed from the native SEC61 initiation codon under the control of its own promoter. The precise positions of the fusions are shown in Fig. 1B.

Analysis of Fusion Protein Topology—Plasmids encoding the Sec61p-Suc2p fusion proteins were transformed into suitable yeast strains, and whole cell extracts were prepared from transformants grown under selective conditions. Extracts were incubated with Endo H to remove asparagine-linked oligosaccharide. Control samples were mock-digested in the absence of Endo H. Reaction products were resolved by SDS-PAGE and then immunoblotted with antisera raised against either Suc2p or Sec61p (Fig. 2).

In the case of a fusion in which Suc2p is fused to Sec61p after the glutamine residue at position 261 (Gln261), a single immunoreactive species was observed in mock-digested extracts with a relative molecular mass of 96 kDa (Fig. 2A, lane 9). This band is absent in control extracts prepared from cells carrying a vector plasmid (Fig. 2D, lanes 1 and 2). Upon Endo H digestion the immunoreactive band exhibited a higher gel mobility with a mass of 75 kDa (Fig. 2A, lane 10). This gel mobility shift is indicative of the removal of 21 kDa of asparagine-linked oligosaccharides. From these results we concluded that the Gln261-Suc2p fusion is a glycoprotein, from which we infer that the Suc2p domain is oriented within the ER lumen. In contrast, the gel mobility of a Suc2p fusion at residue arginine 412 (Arg412) is unaffected by Endo H treatment (Fig. 2A, lanes 11 and 12), indicating that it is unglycosylated. This result is consistent with the Suc2p domain in this case being oriented toward the cytoplasmic compartment. A more complex result is seen for fusion Gln156 (Fig. 2A, lanes 1 and 2) where two forms of the fusion protein are observed in mock extracts, the larger of which shifts upon Endo H treatment, suggesting that this fusion exists as a mixture of glycosylated and unglycosylated forms at steady state. We interpret this result as indicating that fusion Gln156 exists as a mixture of topological forms.

The data for all other fusion constructs shown in Fig. 2 were interpreted by the same criteria, taking into consideration the following notable points. First that extracts prepared from CSY142 transformants contain an immunoreactive band of 60 kDa also seen in a vector control extract (Fig. 2C, lanes 1 and 2) corresponding to the cytosolic form of Suc2p. Second, the observed relative molecular masses for the deglycosylated/unglycosylated forms of the Sec61p-Suc2p fusions fell in the range of 65 kDa (for fusion Leu70), to 96 kDa (for fusion Gly475). These compare with the predicted molecular masses of 68 to 112 kDa for Leu70 to Gly475. The apparent anomaly for fusions carrying larger portions of Sec61p sequence would be expected given that Sec61p itself migrates aberrantly in SDS-PAGE (13). In all cases where an Endo H-induced shift in gel mobility was observed this shift was of the order of 20–24 kDa consistent with a fully glycosylated Suc2p domain (46). The most N-terminal fusion for which we have data is Leu70 which is extensively glycosylated (Fig. 2C, lanes 3 and 4). The simplest interpretation of this result is that the first 70 amino acids of Sec61p contain sufficient information to translocate the Suc2p domain to the ER lumen. The hydropathy analysis indicates that the first major hydrophobic segment (HS1) is formed by residues 33–55 (Fig. 1A). This result indicates that the C-terminal end of HS1 is located toward the ER lumen. The Leu70 fusion can be detected with an antiserum raised against...
residues 12–26 of Sec61p (not shown), from which we conclude that HS1 is not cleaved during biogenesis. It therefore follows that HS1 spans the bilayer with its N-terminal end oriented toward the cytoplasm.

The next Sec61p-Suc2p fusions created at Sec61p residues Pro\textsuperscript{166} and Ile\textsuperscript{116} are both downstream of HS2. If HS2 spans the bilayer then one might have expected the C terminus of each fusion to be located in the cytosol and therefore be unglycosylated. However, both Pro\textsuperscript{166} and Ile\textsuperscript{116} were found to be expressed exclusively as glycoproteins (Fig. 2D, lanes 5 and 6; Fig. 2B, lanes 1 and 2) indicating that HS2 does not span the bilayer in the context of these fusion proteins. Yet more surprising was the finding that fusion Ala\textsuperscript{141} is also expressed exclusively as a glycoprotein (Fig. 2D, lanes 7 and 8). The simplest interpretation of these results would be to conclude that neither HS2 nor HS3 is capable of spanning the membrane. However, this would seem unlikely given the extremely hydrophobic nature of HS3 (Fig. 1A). Residue Ala\textsuperscript{141} is in a very short hydrophilic region between HS3 and HS4 (Fig. 1B), and this proximity to the end of HS3 may interfere with its membrane insertion. An alternative explanation for this anomaly would arise were HS2 to require the presence of HS3 in order to assemble into the bilayer. Under these circumstances HS2 would be excluded from the membrane in fusions Pro\textsuperscript{166} and Ile\textsuperscript{116}, but both HS2 and HS3 would span the bilayer in fusion Ala\textsuperscript{141}. Evidence for such an interaction will be presented in a later section.

The glycosylation of Ala\textsuperscript{141} is consistent with the hydrophilic loop between HS3 and HS4 being located on the luminal face of the ER membrane. A fusion at Ser\textsuperscript{179} is then unglycosylated (Fig. 2D, lanes 9 and 10) consistent with HS4 spanning the membrane with its C terminus toward the cytoplasmic compartment. A fusion within HS4, at Gln\textsuperscript{219}, is expressed as a mixture of glycosylated and unglycosylated forms (Fig. 2A, lanes 1 and 2) suggesting that the truncated HS4 domain can function, albeit inefficiently, as a stop-transfer domain during fusion protein biogenesis.

Fusions Gln\textsuperscript{192} and Gly\textsuperscript{213} occur at, or near, the beginning of HS5 and, like Ser\textsuperscript{179}, are unglycosylated (Fig. 2A, lanes 3 and 4; Fig. 2B, lanes 3 and 4). However, fusion Lys\textsuperscript{229} that lies clearly within a hydrophilic sequence between HS5 and HS6 (Fig. 1B) is also unglycosylated (Fig. 2D, lanes 11 and 12), suggesting that the relatively short HS5 does not span the bilayer. Fusions Phe\textsuperscript{256} and Gln\textsuperscript{261} are exclusively glycosylated (Fig. 2A, lanes 7–10) consistent with HS6 being the next transmembrane sequence. This simple interpretation is made more complex by the finding that, while Gln\textsuperscript{261} is glycosylated, the vast majority of Tyr\textsuperscript{264} (Fig. 2C, lanes 5 and 6) and all detectable Gly\textsuperscript{276} (Fig. 2B, lanes 5 and 6) are found to be unglycosylated. These results suggest the existence of two transmembrane spanning domains between residues Lys\textsuperscript{229} and Gly\textsuperscript{276} and appear to exclude HS5 as a candidate for a transmembrane domain. However, data provided in a following section indicate that the interpretation of the Sec61p-Suc2p fusion data in this region is complicated by an interaction between HS5 and downstream sequences.

The remaining fusion data appear to be consistent with HS7, -8, -9, and -10 having the ability to span the ER membrane. Fusion Gly\textsuperscript{276} places the sequence between HS6 and HS7 within the cytoplasm. Fusions Gln\textsuperscript{314} and Ser\textsuperscript{315} were both very substantially glycosylated (Fig. 2B, lanes 7 and 8; Fig. 2C, lanes 7 and 8) consistent with HS7 acting as a transmembrane domain. The subsequent absence of glycosylation in fusions Gln\textsuperscript{314} (Fig. 2D, lanes 3 and 4) and Arg\textsuperscript{312} (Fig. 2A, lanes 11 and 12) is consistent with HS8 spanning the bilayer with its C terminus on the cytosolic face of the membrane. A fusion immediately following HS9 at residue Ser\textsuperscript{336} is not glycosylated (Fig. 2C, lanes 9 and 10), whereas a small proportion of fusions Gly\textsuperscript{445} and Gly\textsuperscript{460} is glycosylated (Fig. 2B, lanes 9 and 10; Fig. 2A, lanes 13 and 14). Finally fusion Gly\textsuperscript{475} is again unglycosylated.
ated (Fig. 2B, lanes 11 and 12). These data suggest the presence of two transmembrane sequences in this region. We therefore interpret these data to suggest that HS9 is capable of spanning the bilayer with an end point between Ser436 and Gly445 and that HS10 also spans the membrane with an end point between Glu460 and Gly475. Both HS9 and HS10 are only moderately hydrophobic and are linked by a very short hydrophilic loop. It is therefore possible that HS9 and HS10 may interact with one another during normal topogenesis. This might explain the very limited glycosylation of fusions Gly445 and Glu460. The Sec61p-Suc2p fusion data are summarized in Fig. 1B.

Hydrophobic Segment 2 Requires C-terminal Sequences in Order to Adopt a Transmembrane Topology—The C-terminal reporter data described above suggests the absence of functional transmembrane domains between residues Leu70 → Ala141, despite the fact that this region contains two strikingly hydrophobic segments, HS2 and HS3 (Fig. 1A). The C-terminal fusion approach might be expected to be problematic should any given domain be dependent upon downstream sequences for its correct membrane assembly. Such a consideration led us to develop an alternative strategy that was not dependent upon radical C-terminal deletions. We have employed an insertion approach similar to reported methods (48–50) using the recognition motif for a highly specific protease, factor Xa, with the intention of using in situ cleavage of these Sec61p derivatives in microsomes as an assay for the topology of the cleavage site. This protease recognizes the tetrapeptide motif IEGR, cleaving C-terminal to the arginine residue (51). We inserted this recognition motif into hydrophobic segments 1/2 (at Leu70) and 2/3 (at Pro105) in order to examine the potential of hydrophobic stretches HS2 and HS3 to span the bilayer in the context of the intact Sec61p molecule. An insertion containing two tandem fXa sites at Leu70 (HS1x2) and a single site at Pro105 (HS2x3) were subsequently used (see “Experimental Procedures”).

Any insertion into a multi-spanning integral membrane protein might interfere with either the targeting or assembly of the protein, thus invalidating any topological assessments. In order to guard against this possibility, we have examined the ability of these different Sec61p-fXa derivatives to functionally complement the normally lethal sec61::HIS3 null mutation. Single copy plasmids encoding Sec61p-fXa fusions HS1x2 or HS2x3 were capable of complementing the sec61::HIS3 mutation resulting in cells with normal growth rates in both cases (Fig. 3). The ability of these fusions to provide the essential function of Sec61p represents the best possible evidence that these insertion mutants attain their native conformation and are correctly assembled in the ER membrane. All subsequent experiments with these fusions were performed with strains after passage on 5-FOA medium to remove the GAL-SEC61 plasmid, thus shuffling the fusions into the null mutant background (see “Experimental Procedures”).

Initially, the ability of fXa to cleave these various Sec61p-fXa derivatives was tested in detergent-solubilized membrane preparations. Immunoblot analysis of cleavage products revealed that both HS1x2 and HS2x3 proteins could be efficiently cleaved after membrane solubilization with Nonidet P-40 (Fig. 4A). Before proceeding to cleave these derivatives in situ, we first tested the membrane orientation and integrity of our microsomal preparations by examining the protease sensitivity of the ER membrane protein, Sec63p (10, 30), and the ER luminal protein, Kar2p (53).

Yeast microsomes were exposed to proteinase K in the presence or absence of 0.2% Nonidet P-40 (sufficient to permeabilize membranes), prepared for SDS-PAGE, and analyzed by immunoblotting with either Sec63p or Kar2p antiserum. Using membranes derived from cells expressing the HS1x2 derivative, we found that the 73-kDa protein Sec63p was completely susceptible to proteinase K resulting in a major 68-kDa protected form (Fig. 4B) consistent with reported data (30). Crucially only trace quantities of a 43-kDa species produced in the presence of detergent (30) were observed in the absence of detergent, indicating the presence of a very low level of broken membranes (Fig. 4B). The integrity and stability of these membranes was further confirmed by the complete protection of the ER luminal protein Kar2p from proteinase K digestion unless membranes were first permeabilized with 0.2% Nonidet P-40 (Fig. 4C). These results demonstrate that our microsomal membranes were predominantly comprised of sealed membrane vesicles oriented with their cytoplasmic side facing out.

Having established suitable conditions for topological cleavage, in situ cleavage of Sec61p-fXa proteins was examined. Microsomes prepared from strains expressing either the HS1x2 or HS2x3 fusion proteins were incubated with fXa in the presence or absence of 0.2% Nonidet P-40. The reaction products were analyzed by SDS-PAGE and immunoblotted with antisera directed against the C terminus of Sec61p (Fig. 4D). Native Sec61p does not contain any fXa recognition sites and was not cleaved under the conditions described (not shown). An fXa cleavage product was generated from HS1x2 membranes only under conditions of permeabilization (Fig. 4D, lane 3) strongly suggesting that the fXa site and hence segment 1/2 are located in the ER lumen, consistent with the Suc2p fusion data. With HS2x3 microsomes, an fXa cleavage product was generated in the absence of permeabilization (Fig. 4D, lane 5), and the appearance of this product was not affected by 0.2% Nonidet P-40 (Fig. 4D, lane 6). These data are only consistent with a cytosolic location of the fXa site and hence hydrophilic segment 2/3, from which it follows that HS2 must span the bilayer in the intact protein. This result conflicts with the observation that Suc2p fusions within loop 2/3 (at residues P105 and I116) report a luminal topology for Suc2p indicating that HS2 does...
not span the bilayer in the context of these fusion proteins.

Analysis of the C-terminal Membrane Location by \textit{fXa} Insertion—Factor Xa recognition site insertion was also used to examine the membrane location of the Sec61p C terminus. A \textit{Bam}III site created at the codons representing residues Gly\textsuperscript{477} and Phe\textsuperscript{488} was used as the site of insertion for two tandem \textit{fXa} motifs to generate Sec61p mutant HS10xC. A low copy plasmid encoding this fusion was able to complement the \textit{sec61::HIS3} null mutation (Fig. 3) confirming that the insertion of \textit{fXa} motifs into the C terminus does not significantly perturb the structure and function of Sec61p. Microsomes were prepared from cells expressing HS10xC and tested for factor Xa cleavage both \textit{in situ} and after membrane solubilization. The solubilized fusion protein proved to be an efficient substrate for \textit{fXa} protease yielding a fragment detected by immunoblotting with antisera raised against the N terminus of Sec61p migrating slightly faster than the intact fusion protein (Fig. 4A, lane 6). Cleavage \textit{in situ} was detected in the absence and presence of detergent (Fig. 4D, lanes 8 and 9). These data are consistent with the location of the C terminus on the cytosolic face of the membrane as determined with the Suc2p fusion made to residue Gly\textsuperscript{475}.

Hydrophobic Segment 5 Can Span the Bilayer in the Presence of C-terminal Sequences That Are Capable of Acting in Trans—We sought to investigate the inconsistencies obtained with the Suc2p fusion analysis around hydrophobic regions HS5 and HS6. The topology of the HS5 and HS6 region was investigated by \textit{fXa} site insertion at residues Ser\textsuperscript{179} and Lys\textsuperscript{229}, but despite retaining function the Sec61p-\textit{fXa} proteins could not be cleaved \textit{in situ} (not shown). Interestingly, while neither a 243-residue polypeptide encoding HS1–5 (N\textsubscript{5}) nor a 264-residue polypeptide encoding HS6–10 (C\textsubscript{5}) could complement \textit{sec61::HIS3}, the co-expression of these two fragments provided functional complementation of \textit{sec61::HIS3}. This result clearly indicates that N\textsubscript{5} must assemble into its correct, functional topology at least in the presence of C\textsubscript{5}. We therefore re-examined the topology of fusion Lys\textsuperscript{229} (N\textsubscript{5}, Suc2p) in the presence of the C\textsubscript{5} domain.

A low copy \textit{LEU2} plasmid encoding the Lys\textsuperscript{229} fusion was co-transformed together with a multicopy \textit{TRP1} plasmid encoding the C\textsubscript{5} fragment into the \textit{sec61::HIS3} strain, BWY47. Remarkably, transformants grew upon gal shut-off, although with a severe growth defect (not shown), indicating that at least some of the Lys\textsuperscript{229}–Suc2p/C\textsubscript{5} form of Sec61p was analyzed by immunoblotting using antisera directed against Suc2p and the C terminus of Sec61p. The C\textsubscript{5} fragment and three higher molecular weight species were detected (Fig. 5, lane 1). Of the three fusion protein bands, two migrated as a doublet similar in molecular weight to the Unglycosylated form of the fusion protein detected previously (Fig. 2D, lane 11). Endo H digestion revealed that the third, and largest, fusion protein species was glycosylated (Fig. 5, lane 2) indicating that HS5 can become transmembrane in the presence of its cognate C\textsubscript{5} fragment. These data suggest that HS5, a weakly hydrophobic membrane anchor, requires downstream sequences for its stable topogenesis and that these sequences can be provided in trans. It is not known if the effect of this co-expression is to promote translocation of HS5 and the Suc2p moiety in the Lys\textsuperscript{229} fusion or the stabilization of the glycoform that may be particularly unstable when individually expressed and hence not detected previously (Fig. 2D, lane 11).

\footnote{B. M. Wilkinson, Y. Esnault, F. Kepes, and C. J. Stirling, manuscript in preparation.}

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\caption{Factor Xa cleavage of Sec61p-\textit{fXa} fusion proteins. A, cleavage of solubilized Sec61p-\textit{fXa} proteins. Microsomes prepared from strains expressing the HS1x2, HS2x3, and HS10xC Sec61p fusions were solubilized in 2% Nonidet P-40 at 4 °C with rotation for 15 min, diluted 10-fold into \textit{fXa} buffer (100 mM NaCl, 25 mM Tris-HCl, pH 7.4, 2 mM MgOAc) containing 1.1 \textmu{}g of \textit{fXa}, 0.1 A\textsubscript{280} microsome equivalent units, and digested for 18 h at 4 °C. Controls samples were mock-digested. The reactions were terminated by addition of Laemmli sample buffer (52), and digested for 18 h at 4 °C. Membranes were prepared from cell expressing HS10xC and tested for factor Xa cleavage \textit{in situ} and after membrane solubilization. The solubilized fusion protein proved to be an efficient substrate for \textit{fXa} protease yielding a fragment detected by immunoblotting with antisera raised against the N terminus of Sec61p migrating slightly faster than the intact fusion protein (Fig. 4A, lane 6). Cleavage \textit{in situ} was detected in the absence and presence of detergent (Fig. 4D, lanes 8 and 9). These data are consistent with the location of the C terminus on the cytosolic face of the membrane as determined with the Suc2p fusion made to residue Gly\textsuperscript{475}.}
\end{figure}
DISCUSSION

We have used a combination of approaches to determine the topology of yeast Sec61p in the ER membrane. Our data suggest the presence of 10 transmembrane domains as shown in diagrammatic form in Fig. 6. Given the high level of amino acid sequence identity and the similarity of hydropathy profiles, it would seem likely that Sec61α (19) also has a similar structure. The 10 transmembrane structures and orientation of membrane anchors of Sec61p are similar to the topological model proposed for the SecY subunit of the bacterial cytoplasmic membrane translocase (54). SecY has been widely implicated in protein translocation across the cytoplasmic membrane of E. coli (55) and appears to be distantly related to Sec61p (56). The hydrophobic segments in Sec61p vary enormously in their length and relative mean hydropathies (Fig. 1A). Our data indicate that several domains of quite limited hydrophobicity are incorporated into the ER bilayer where they might play an important role in the formation of an aqueous protein-conducting channel.

Our data indicate that HS1 spans the bilayer with its N terminus oriented toward the cytoplasmic compartment. While, by inference, this would place the N terminus of Sec61p in the cytoplasm, we cannot exclude the possibility that sequences N-terminal to HS1 might be buried within or might even span the bilayer. In particular, residues 3–21 have the potential to form a highly amphipathic α-helical structure. Intriguingly, the introduction of an fXa tetrapeptide motif (IEGR) within this region resulted in the loss of Sec61p function, whereas insertion of an inverted motif (STLD) retained function. Significantly, the fXa motif is predicted to disrupt the amphipathic helix, whereas the inverted motif could conform to an amphipathic structure. These findings would be consistent with the predicted amphipathic helix playing an important role in Sec61p biogenesis and/or function. A structure such as this may be partially embedded in the plane of the bilayer (as shown in Fig. 6) or might actually span the bilayer with its hydrophilic surface lining the interior of the translocation pore. Such a domain might play an important role in the gating of the translocation pore, either during the initiation of translocation or perhaps to permit the lateral diffusion of hydrophobic domains into the lipid bilayer during the assembly of integral membrane proteins. The potential importance of this putative amphipathic structure is underlined by its conservation both in Sec61α (19) and in a Schizosaccharomyces pombe homologue. 4

Our data indicate that both HS2 and HS5 require C-terminal sequences for their topogenesis. In the case of HS2, the C-terminal fusion data clearly report that neither HS2 nor HS3 span the bilayer. However, in the intact Sec61p (HS2x3) the presence of downstream sequences facilitates the stable membrane insertion of HS2 resulting in the observed cytoplasmic orientation of the HS2x3 loop. If the “stabilizing” sequence were itself a transmembrane domain then one would predict that the inclusion of this domain should lead to the coordinated assembly of HS2. This would create an apparent anomaly in our Suc2p-fusion data in which the addition of one hydrophobic segment would lead to the formation of two transmembrane domains, such that the topology of the Suc2p reporter would be unaltered. Exactly such an anomaly appears to occur in fusion Ala141, where the addition of the extremely hydrophobic HS3 domain has no apparent effect on the topology of the fusion protein. While our data present no direct evidence that HS3 actually spans the ER membrane, the simplest interpretation of our data would be to conclude that HS3 functions as a signal-anchor sequence and that it serves to promote, or stabilize, the membrane insertion of HS2. Our data do not indicate whether the proposed interaction between HS2 and HS3 occurs after their sequential insertion in the bilayer or whether it is required to mediate their pair-wise insertion in a manner similar to that described for domains of human P-glycoprotein (57, 58), a plasma membrane ATPase (59), and lactose permease (50). The nature of the interaction between HS2 and HS3 is unclear. HS2 contains a conserved glutamate residue, Glu79, which may reduce the ability of this domain to act as a stop transfer sequence in the context of fusions Pro105 and Ile116. However, HS3 does not contain a cognate positively charged residue that would allow the formation of a salt bridge, as has been suggested in the stabilization of the M9-M10 domains of lactose permease (60).

Our results also indicate that HS5 requires downstream sequences in order to function as a signal anchor sequence. In this case the Suc2p moiety in fusion Lys229 could be translocated to the ER lumen only when the C-terminal portion of

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3 B. M. Wilkinson and C. J. Stirling, unpublished observations.

4 J. Broughton and C. J. Stirling, manuscript in preparation.
Sec61p was present in trans. This effect is clearly specific to the cognate C-terminal fragment since intact Sec61p does not promote the glycosylation of Lys229 when present in trans (in strain CSY142; see Fig. 2). This, coupled to the observed complementation of a sec61 null mutant, leads us to conclude that the Lys229 fusion interacts directly with the C-terminal fragment of Sec61p to form a functional ER translocase. Our current data do not indicate whether the assembly of the glycosylated form of Lys229 occurs co- or post-translationally. Should it prove to be post-translational, then this particular phenomenon may be uniquely dependent upon the role of Sec61p as a protein translocase. In other words, the insertion of Lys229 with a cytoplasmically oriented C terminus may occur in, or around, HS6. Fusion Lys229 is unglycosylated in contrast to Sec61p, where the assembly of a functional translocation channel might facilitate the "auto-catalytic" translocation of the Sec62p domain into the ER lumen giving rise to a glycosylated form. This hypothesis will require further study.

Clearly, sequences C-terminal to HS5 are required for its topogenesis. Our Sec2p fusion data around this region are complex but appear to indicate that the stabilizing sequences occur in, or around, HS6. Fusion Lys229 is unglycosylated indicating a failure of HS5 to form. Fusions Phe256 and Gln361 are then completely glycosylated, whereas Tyr425 is again fully glycosylated (Fig. 1B). These data might indicate that transmembrane domain 5 extends significantly beyond residue Lys229 despite the fact that residues 225–238 are predominately charged. There are several alternative explanations for these findings, but given the partial glycosylation of Val447 and the assembly of Lys229 in the presence of the C5 fragment, we believe that the most likely explanation to be as follows. First, that sequences between Lys229 and Phe256 serve to stabilize the formation of transmembrane domain 5. Second, that the C-terminal boundary of transmembrane domain 6 extends beyond Gln361 such that the additional residues present in fusion Tyr425 are required for it to span the bilayer. HS5 may contain at least one charged residue dependent upon its exact position, but the lack of any charged residues in HS6 again appears to rule out the formation of a salt bridge between these domains. It is worth noting that HS5 corresponds to a stretch of 12 residues of which 11 are nonpolar (residues Gly231–Val242), flanked on both sides by several polar residues. Our data do not indicate the precise end points of any given transmembrane domain, only that particular hydrophobic segments have membrane spanning potential. For example, HS5 may span the membrane in α-helical conformation thus incorporating numerous polar residues in the bilayer. Alternatively, HS5 would be sufficiently long to span the bilayer in β-sheet conformation. Either scenario would be consistent with the observation that HS5 requires some stabilizing interaction(s) within the bilayer.

The proposed topology of Sec61p (Fig. 6) provides a basis for the functional dissection of this essential protein. Deletion of individual domains would determine whether the essential function of Sec61p can be retained upon loss of significant portions of the protein, and a systematic isolation and characterization of point mutations defective in Sec61p function would identify the critical residues and domains. Using the deduced topology the position of mutations with respect to the ER membrane would be known, thus aiding the detailed molecular description of the function(s) of Sec61p in ER translocation.