Genetic Complementation in Yeast Reveals Functional Similarities between the Catalytic Subunits of Mammalian Signal Peptidase Complex

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The abbreviations used are: SPC, signal peptidase complex; SP, signal peptidase; ER, endoplasmic reticulum; IMP, inner membrane peptidase; TM, transmembrane segment; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

SUMMARY

Type-I signal peptidases (SPs) comprise a family of structurally related enzymes that cleave signal peptides from precursor proteins following their transport out of the cytoplasmic space in eukaryotic and prokaryotic cells. One such enzyme, the mitochondrial inner membrane peptidase (IMP), has two catalytic subunits, which recognize distinct cleavage-site motifs in their
signal peptide substrates. The only other known type-I SP with two catalytic subunits is the signal peptidase complex (SPC) in the mammalian endoplasmic reticulum. To determine whether the SPC catalytic subunits exhibit nonoverlapping substrate specificity, we complemented yeast strains that lack endogenous SP with canine SPC18 and a truncation of canine SPC21 (SPC21ΔN), which lacks 24 N-terminal residues that prevent expression of SPC21 in yeast. By monitoring a variety of soluble and membrane-bound substrates, we find that, in contrast to the tested hypothesis, SPC catalytic subunits exhibit overlapping substrate specificity. SPC18 and SPC21ΔN do, however, cleave some substrates with different efficiencies, although no pattern for this behavior could be discerned. In light of the functional similarities between SPC proteins, we developed a membrane protein fragmentation assay to monitor the position of the catalytic sites relative to the surface of the endoplasmic reticulum membrane. Using this assay, our results suggest the active sites of SPC18 and SPC21ΔN are located 4-11 Å above the membrane surface. These data, thus, support a model that SPC18 and SPC21 are functionally and structurally similar to each other.

INTRODUCTION

Proteins transported out of living cells are usually synthesized as precursors with signal peptides that are cleaved following membrane translocation by a family of enzymes referred to as the type-I signal peptidases (SPs) (reviewed in ref 1). The signal peptides used to target precursors across the plasma membrane in prokaryotic cells and the endoplasmic reticulum (ER) membrane in eukaryotic cells contain three distinct amino acid stretches: a positively charged N-terminal region, a hydrophobic core, and a polar C-terminal region. A similar amino acid arrangement is present in a subset of signal peptides inside mitochondria and chloroplasts. The vast majority of signal peptides have a cleavage site motif consisting of a small, uncharged residue at the P₁ (also referred to as −1) position and a small, polar or hydrophobic residue at the P₃ (−3) position (2-5). The importance of this motif is illustrated by the fact that leader peptidase, the type-I SP in E. coli, has distinct binding pockets to accommodate the side chains of P₁ and P₃ amino acids in the soluble (extracytoplasmic) portion of this membrane-bound enzyme (6,7). Recognition of P₁ and P₃ amino acids places the signal peptide cleavage site near the catalytic site, which is also located in the extracytoplasmic portion of leader peptidase.
Whereas prokaryotic cells express monomeric type-I SPs, the ER and mitochondrion display multisubunit enzymes. The SP purified from canine microsomes [termed the signal peptidase complex (SPC)] has five distinct subunits (8). Two of these subunits, SPC18 and SPC21, are presumed to have catalytic activity based on their homology to characterized type-I SPs (9). The mitochondrial inner membrane peptidase (IMP) is a three-subunit complex also containing two catalytic subunits in the yeast *Saccharomyces cerevisiae* (10,11,12). A clue to this subunit complexity comes from an analysis of the IMP, whose catalytic subunits exhibit nonoverlapping substrate specificity (11). Notably, Imp1p recognizes cleavage-site motifs that can have structurally unrelated P<sub>1</sub> residues (13), and Imp1p has an unusual preference for acidic residues at the P′<sub>1</sub> position in the protein attached to the signal peptide (14). Imp2p, on the other hand, recognizes the canonical Ala-X-Ala motif in which Ala residues are located at the P<sub>1</sub> and P<sub>3</sub> positions (11,13,14).

Unlike the SPC in mammals, the SPC in the yeast *Saccharomyces cerevisiae* has only one catalytic subunit, Sec11p (15,16). Based on this difference and by analogy to the IMP catalytic subunits, a model has emerged in which mammalian cells evolved a second SPC catalytic subunit to cleave an unconventional signal peptide(s) not present in yeast (11). Here, we tested this model by expressing each catalytic subunit of mammalian SPC in yeast that lack endogenous Sec11p. Using this genetic complementation approach, we have identified soluble and membrane-bound substrates recognized by both SPC subunits.

**EXPERIMENTAL PROCEDURES**

*Yeast Strains, Media, and Antibodies.* Yeast strains used in this study are CVY1 (MATa ura3-52 his3-Δ200 lys2-80 trp-Δ901), CMY710 (MATα sec11Δ1::HIS3 ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 suc2-Δ9 ade2-101 lys2-80), CMY730 (MATα sec11Δ1::HIS3 ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 suc2-Δ9 ade2-101), and HLY1 (MATa leu2-3,112 ura3-52 trp1-Δ901 lys2-801 his4-619). Media for growing yeast have been described (17). A murine anti-FLAG M2 antibody conjugated to agarose (Sigma), anti-HA high affinity rat monoclonal antibody (Roche), and protein G-conjugated agarose beads (Roche) were used for immunoprecipitation. A rabbit anti-Kar2p antibody (18) was used for immunoblotting.
Construction of yeast expression vectors. A 1.5 kb BamHI fragment containing the ADH1 promoter (19) was inserted into plasmid pRS314 (CEN TRPI) (20). To eliminate the BamHI site upstream of the ADH1 promoter in the resulting construct, a unique NotI site adjacent to the BamHI site was cleaved and subjected to the Bal 31 exonuclease. The resulting plasmid was named pHF453. The ADH1 promoter was removed from pHF453 by digestion with SacI and BamHI and inserted into pRS424 and pRS426 (21), generating pHF454 (2 micron TRPI) and pHF455 (2 micron URA3), respectively. These vectors were used in constructions of plasmids encoding Sec11p, SPC18 and SPC21ΔN under control of the ADH1 promoter. The cDNA encoding SPC18 was amplified from canine spleen mRNA by reverse transcriptase PCR using primers P1 and P2 (Table 1). The cDNA encoding SPC21 was amplified using primers P3 and P4. P4 and P5 were used to amplify the DNA sequence encoding SPC21ΔN. The amplified DNA fragments were restricted with BamHI and EcoRI and inserted into pHF453 (CEN TRPI). The SEC11 gene was excised from pCM112 (16) and inserted into pHF453. FLAG-tagged Sec11p has been described before (16). To tag proteins at their N-termini with the FLAG epitope (22), primer P6 was used for SPC18; P7 was used for SPC21; and P8 was used for SPC21ΔN. To generate C-terminally FLAG tagged subunits, P9 was used in combination with P3 (for SPC21) and P5 (for SPC21ΔN). The amplified DNA fragments were restricted with BamHI and EcoRI and inserted into pHF454 (2 micron TRPI).

Construction of HA-tagged Preinvertase, Prepro-α-factor and Mnn2p. A DNA segment encoding three consecutive HA epitopes (23) followed by a stop codon (TAA) was inserted between the EcoRI and Kpn1 restriction sites of pHF454 (2 micron TRPI) and pHF455 (2 micron URA3). Mutations were introduced into the MNN2 gene (24) by two-step PCR. Two oligonucleotide primers, M5’ and M3’, were used in making the mnn2 mutations. M5’ and M3’ correspond to sequences at the 5’ and 3’ end of the MNN2 gene, respectively. Pairs of complementary internal primers were designed to introduce mutations. For example, to introduce mutant A (described in text), M5’ and Mut-A3’ (Table 1) were used to generate one PCR product. M3’ and Mut-A5’ were used to generate the second PCR product. We then obtained full-length mnn2 A mutant using M5’ and M3’, the products of the first two PCR reactions serving as template. Finally we inserted the mnn2 mutant genes between the BamHI and EcoRI sites in pHF454 and pHF455, and the mutations were confirmed by DNA sequencing. Mutation
of prepro-α-factor (P₁S) was introduced directly by a primer that was synthesized to contain the mutation. Primers for preinvertase, prepro-α-factor and prepro-α-factor (P₁S) were Inv5’, Inv3’, α5’ and α3’, α-ser5’ and α3’, respectively.

*In Vitro Coupled Transcription/Translation.* A BamHI - EcoRI DNA fragment that encodes SPC21-FLAG was inserted into the pCITE²⁻²c expression vector (Novagen). The resulting construct (pCV107) (1 µg) was used in an *in vitro* coupled transcription/translation reaction, employing the TNT² Coupled Reticulocyte Lysate System (Promega, Madison, WI). A 25-µl volume was used, and the reaction was allowed to proceed for 90 min. *In vitro* translated SPC21-FLAG was precipitated from a 10-µl reaction mix using agarose-conjugated anti-FLAG antibodies (20 µl).

*Biochemical Procedures.* Pulse-labeling, western blotting and immunoprecipitation procedures have been described before (18). Immunoprecipitations of proteins tagged with the FLAG epitope were performed as described (16). For Mnn2p fragmentation experiments, immunoprecipitates were subjected to endoglycosidase H treatment as described by the supplier (New England Biolabs).

**RESULTS**

*Complementation of Δsec11 with canine homologs of Sec11p.* The alignment depicted in Figure 1 reveals the high degree of sequence similarity between yeast Sec11p and its canine homologs, SPC18 and SPC21. Importantly, the alignment shows that SPC18 and SPC21 have conserved serine, histidine, and aspartic acid residues that are known to be essential for Sec11p catalytic activity (16). Based on these similarities, we asked whether SPC18 and SPC21 would function individually in yeast. The cDNAs encoding SPC18 and SPC21 were amplified from canine mRNA and inserted into yeast expression plasmid pHF453 under control of the *ADH1* promoter (19) as described in EXPERIMENTAL PROCEDURES. Plasmids pHF341 (*SPC18 TRP1*), pHF343 (*SPC21 TRP1*), and control plasmid pHF340 (*SEC11 TRP1*) were introduced into strain CMY710 (Δsec11) / pCM112 (*SEC11 URA3*). Cells were then placed on agar plates containing 5-fluoroorotic acid, which is used to select against the *URA3* gene (28). As expected, CMY710
(Δsec11) cells cured of pCM112 (SEC11 URA3) and containing no other plasmid were unable to grow, whereas cells that contained pHF340 (SEC11 TRP1) grew in the presence of 5-fluoroorotic acid (Figure 2). The pHF341 (SPC18 TRP1) plasmid also supported cell growth, but pHF343 (SPC21 TRP1) did not. To understand why SPC21 failed to complement the Δsec11 mutation, we noted the presence of a 24 residue, N-terminal extension in SPC21 that was not present in Sec11p (Figure 1). Although full-length SPC21 is present in the SPC purified from mammalian cells (25), we reasoned the extension could affect SPC21 function in yeast. We, therefore, prepared a new construct, pHF344 (TRP1), encoding SPC21ΔN without the N-terminal 24 residues (EXPERIMENTAL PROCEDURES). As shown in Figure 2, SPC21ΔN permitted growth of strain CMY710 (Δsec11) in the presence of 5-fluoroorotic acid (Figure 2). SPC18 also has an extended N-terminus relative to Sec11p, although this extension, 12 residues, is shorter than that present in SPC21 (Figure 1). Indeed, SPC18 lacking these 12 residues supported growth of Δsec11 yeast (data not shown). Furthermore, SPC21 lacking its N-terminal 12 residues and containing a T13M substitution for translation initiation complemented the Δsec11 mutation.

To determine whether SPC21’s inability to replace Sec11p was due to a protein expression problem, we C-terminally tagged SPC21 with the FLAG epitope (22) and expressed the protein in vitro and in yeast strain CVY1 (EXPERIMENTAL PROCEDURES). While we were able to detect in-vitro expressed SPC21-FLAG (Figure 3, lane 1), the protein was not present in yeast (lane 2). For control purposes, we attached the FLAG epitope to the C-terminus of SPC21ΔN (lacking the N-terminal 24 residues) and expressed the protein in strain CVY1. In this control analysis, SPC21ΔN-FLAG was present in yeast cell extracts (lane 4). Surprisingly, when we moved the FLAG-epitope to the N-terminus of intact SPC21, the protein was expressed in yeast (lane 3), and FLAG-SPC21 supported growth of the Δsec11 mutant (data not shown). We, therefore, conclude expression of SPC21 is affected by its N-terminus. Removing the first 12 or 24 residues from SPC21 or adding the FLAG epitope to the N-terminus of intact SPC21 permits expression of SPC21 catalytic activity inside yeast cells.

SPC18 and SPC21ΔN Cleave Conventional Signal Peptides. As SP activity is essential for yeast cell growth (26), the genetic data presented thus far are consistent with the notion that SPC18 and SPC21ΔN cleave signal peptides from yeast-expressed precursors. To generate corroborative
biochemical evidence, we analyzed three precursors, preKar2p, preinvertase and prepro-α-factor. We chose these precursors because their cleavage sites differ in amino acid composition at the critical P₁ and P₃ positions, even though each substrate has a conventional cleavage site motif (i.e. small, uncharged residues at the P₁ position and small, uncharged or hydrophobic residues at the P₃ position). The precursors also vary in signal peptide length and hydrophobicity, thus, presenting distinct features with which to analyze the substrate specificities of SPC18 and SPC21ΔN and to assess the tested hypothesis that these enzymes exhibit nonoverlapping substrate specificity.

PreKar2p is the precursor to ER-resident protein Kar2p, the yeast homologue of mammalian BiP (29). The signal peptide is unusually long (42 residues), is moderately hydrophobic (30), and has a cleavage-site motif consisting of (P₃) Val-X-Gly (P₁) (29). Strain CMY730 (∆sec11) bearing pCM112 (Sec11p), pHF341 (SPC18), or pHF344 (SPC21ΔN) was grown to early log phase, and cell extracts were immunoblotted with anti-Kar2p antibodies using methods described previously (18). As expected, Sec11p efficiently converted preKar2p to Kar2p (Figure 4A). SPC18 and SPC21ΔN also cleaved preKar2p although less efficiently than Sec11p. Thus, in contrast to the tested hypothesis, SPC18 and SPC21ΔN show overlapping substrate specificity with respect to preKar2p.

Preinvertase is the precursor to invertase, an enzyme secreted from yeast cells (31). The signal peptide is 19 residues in length, is strongly hydrophobic (32), and has the cleavage site motif, Ile-X-Ala (31). Invertase undergoes extensive core glycosylation in the ER, requiring use of a glycosylation inhibitor in order to see distinct protein bands on SDS-PAGE gels. Preinvertase presents an additional problem for monitoring signal peptide cleavage, namely the cleaved and uncleaved proteins cannot easily be resolved on protein gels. This problem necessitated our preparation and use of a truncated construct (residues 1-112) that encodes a protein with reduced molecular mass, termed preinvertase (112). Preinvertase (112) was C-terminally tagged with the HA epitope (23) and placed under control of the ADH1 promoter (EXPERIMENTAL PROCEDURES). This construct was introduced into strain CMY730 (∆sec11) harboring pCM112 (Sec11p), pHF341 (SPC18), or pHF344 (SPC21ΔN). Log-phase cells were treated with tunicamycin for 10-min to inhibit protein glycosylation (18), pulse-labeled with ³⁵S-methionine and cysteine for 10-min, and proteins were precipitated from cell extracts using anti-HA antibodies. As shown in Figure 4B, Sec11p, SPC18, and SPC21ΔN
converted similar amounts of preinvertase (112)-HA to invertase (112)-HA, a result consistent with the overlapping substrate specificity model.

Prepro-α-factor is the precursor to the α-factor pheromone (33). Its signal peptide has 19 residues, is less hydrophobic than signal peptides in the previously examined precursors (30), and has the cleavage-site motif, Ala-X-Ala (33). The cleaved and uncleaved forms of prepro-α-factor can be separated on SDS-PAGE gels, although the cleaved form migrates slower than the uncleaved form in this gel system (34). Prepro-α-factor (HA tagged) was expressed in the above-mentioned yeast strains, and cells were treated with tunicamycin. As shown by pulse labeling (Figure 5A), both mammalian enzymes were capable of cleaving prepro-α-factor-HA (Figure 5A), although SPC21ΔN exhibited slightly more robust activity with this substrate. This latter point is addressed in subsequent sections.

Among the precursors examined thus far, preinvertase and prepro-α-factor have alanine residues at the important P1 position, whereas preKar2p has a P1 glycine. Serine can also be found, albeit infrequently, at the P1 position in signal peptides (5). To assess the role of serine in the substrate specificities of SPC18 and SPC21ΔN, we asked whether these enzymes cleave prepro-α-factor when its P1 alanine is replaced by serine (EXPERIMENTAL PROCEDURES). As shown by pulse labeling (Figure 5B), both mammalian enzymes were able to recognize serine, although prepro-α-factor(P1S)-HA was cleaved slightly more efficiently by SPC21ΔN than by SPC18. As shown above, prepro-α-factor (P1A)-HA was also cleaved more efficiently by SPC21ΔN than by SPC18 (Figure 5A), arguing that prepro-α-factor is a slightly better substrate for SPC21ΔN irrespective of the P1 residue. Taken together, the data presented in Figure 4 and Figure 5 demonstrate that the SPC catalytic subunits recognize conventional cleavage site motifs in support of the overlapping substrate specificity model.

**SPC18 and SPC21ΔN Can Fragment a Membrane Protein.** In addition to cleaving signal peptides, ER SP can also fragment integral membrane proteins in mammals and yeast. Membrane-bound substrates include viral polyproteins and abnormal cellular proteins that contain cleavage site motifs similar to those present in signal peptides (17,35,36). To monitor membrane protein fragmentation in this study, we employed Mnn2p (α-1,2-mannosyltransferase), a type II membrane protein inserted into the ER and then functionally
expressed in the yeast Golgi (24). We chose this protein for the following reasons. (i) At least some type II membrane proteins can be fragmented by the SPC if they have appropriate cleavage site motifs (17,35). (ii) Mnn2p lacks a subunit partner (24), which was considered important because membrane protein complexes have been shown to be resistant to SPC fragmentation (35).

A systematic analysis of the “rules” governing membrane protein fragmentation has not been performed. However, the proximity of signal peptide cleavage sites to the hydrophobic regions in signal peptides led us to reason that a membrane protein cleavage site should be located near the transmembrane (TM) segment in Mnn2p. In addition, a signal peptide cleavage site is placed at the C-terminal end of the signal peptide, suggesting a membrane protein cleavage site should be positioned near the C-terminal end of the TM segment. By visual inspection, we did not find an appropriate SPC cleavage site in Mnn2p, later confirmed by pulse-labeling analysis (data not shown). We, therefore, prepared a series of constructs in which different numbers of amino acids were located between an introduced Ala-X-Ala motif and the TM segment of Mnn2p (HA tagged) (Figure 6, constructs A-H). In some of the constructs (D & E), an additional amino acid substitution was introduced to eliminate the possibility that the Ala-X-Ala motif would create an unintended cleavage site, which could be processed immediately following the introduced P3 Ala residue.

Before examining SPC18 and SPC21ΔN with this series of Mnn2 mutant proteins, we wished to establish our assay using Sec11p. Constructs A-H were placed under control of the ADH1 promotor and expressed in yeast strain CMY730 (∆sec11) bearing pCM112 (Sec11p). Cells were subjected to a 5-min pulse labeling, and proteins were precipitated from cell extracts using anti-HA antibodies. The immunoprecipitates were treated with endoglycosidase H (EXPERIMENTAL PROCEDURES) to remove core oligosaccharides before analysis by SDS-PAGE. As shown by pulse labeling (Figure 7A), Sec11p fragmented Mnn2-HA mutant proteins with different efficiencies. By plotting fragmentation efficiency against the number of amino acids between the cleavage site (P1 alanine) and the putative end of the TM segment, we observed a peak in activity when the P1 residue was located 1-2 amino acids from the TM segment (Figure 7B). The significance of this peak is discussed below. Next, we analyzed strain CMY730 (∆sec11) bearing pHF341 (SPC18) or pHF344 (SPC21ΔN). Cells were subjected to pulse labeling as described above, and radiolabeled Mnn2-HA was visualized by
autoradiography. As with Sec11p, SPC18 and SPC21DN fragmented the Mnn2 mutant proteins with different efficiencies (Figure 8A). When these data were quantitated, fragmentation efficiency peaked for both mammalian enzymes when the P1 residue was positioned 1-3 amino acids from the TM segment (Figure 8B and 8C). These exceedingly similar fragmentation patterns further support a model of overlapping substrate specificity for SPC18 and SPC21DN.

Despite these apparent similarities, SPC18 exhibited more robust activity than SPC21DN across the entire series of Mnn2 mutant proteins (Figure 8A). In contrast, SPC21DN cleaved prepro-α-factor more efficiently than SPC18 (Figure 5A), a pattern that held even when the P1 alanine of prepro-α-factor was changed to serine (Figure 5B). To determine whether a P1 serine had an effect on the fragmentation of Mnn2p, we took advantage of the fact that Mnn2p has a threonine residue conveniently located near the TM segment (Figure 6). Threonine is commonly found at the P3 position in signal peptide cleavage sites (5). As such, Mnn2p needed only an appropriate P1 residue, either alanine (construct J) or serine (construct K) (Figure 6). When constructs J and K were introduced into strains CMY730 / pHF341 (SPC18) and CMY730 / pHF344 (SPC21DN), Mnn2 (P1A) and Mnn2 (P1S) were fragmented better by SPC18 than by SPC21DN (Figure 9), although inclusion of the serine residue significantly reduced fragmentation efficiency by both SPC proteins. These data, thus, point, not to a role for serine in the substrate specificities, but to a model that Mnn2p is a slightly better substrate for SPC18 than SPC21DN.

**DISCUSSION**

The goal of this study was to test the hypothesis that type-I SPs in the mammalian ER, SPC18 and SPC21, exhibit nonoverlapping substrate specificity, such as that seen with the catalytic subunits of another type-I SP, the mitochondrial IMP. To this end, we attempted to complement yeast cells that lack endogenous SP with canine SPC18 and SPC21. We were successful using SPC18, but a 24 residue, N-terminal truncation (ΔN) was needed to achieve complementation with SPC21 (Figure 2). The truncation was required because intact SPC21 is not expressed in yeast (Figure 3). Using the complemented yeast strains, we demonstrate that SPC18 and SPC21DN cleave signal peptides from preKar2p, preinvertase, and prepro-α-factor (Figs 4 & 5), which have conventional cleavage-site motifs (ie small uncharged residues at the P1
and $P_3$ positions). Thus, in contrast to the original hypothesis, our data suggest mammalian SPC has two catalytic subunits with overlapping substrate specificity.

The functional analysis of leader peptidase, a type-I SP in *E. coli*, supports our use of SPC21ΔN to assess SPC21 catalytic specificity (7). As shown in that study, the isolated, catalytic domain of leader peptidase can cleave signal peptides accurately *in vitro*, even though the catalytic fragment lacks the entire cytoplasmic domain and transmembrane segments. On the other hand, SPC21ΔN lacks only the first 24 residues of the SPC21 cytosolic domain. Our inability to express intact SPC21 may be a problem unique to the system employed here, as full-length SPC21 and, for that matter, SPC18 are present in the SPC complex purified from canine microsomes (9,25). In this regard, the N-terminal extension may prevent assembly of SPC21 with one or more of the yeast SPC subunits. Sec11p, the yeast homolog of SPC18 and SPC21, binds to Spc3p, a noncatalytic subunit that is required to stabilize Sec11p (16,37,38). Thus, the N-terminus of SPC21 may interfere with Spc3p binding, leading to degradation of SPC21 in yeast.

Consistent with the model of overlapping substrate specificity, both SPC18 and SPC21ΔN exhibit membrane protein fragmentation activity. To monitor fragmentation, we introduced the “Ala-X-Ala” motif into Mnn2p at different positions relative to its TM segment (Figure 6). We, first, used this series of Mnn2 mutant proteins to examine the fragmentation activity of yeast Sec11p. In that analysis, we observed a peak in fragmentation activity when the cleavage (fragmentation) site was located 1-2 residues from the putative end of the TM segment (Fig 7B). To interpret the meaning of this peak, we considered the fact that Sec11p is an integral membrane protein whose catalytic site is anchored to the surface of the ER membrane (15,26,39). Therefore, a membrane protein substrate of Sec11p is likely to be fragmented when the distance separating its cleavage site from the ER membrane is similar to the distance separating the Sec11p catalytic site from the ER membrane. According to this interpretation, a distance encompassed by 1-2 amino acid residues is likely to correspond to the height of the Sec11p catalytic site. Approximately 3.5 Å is taken as the distance spanned by each residue in an extended protein (40), suggesting the Sec11p catalytic site is positioned 4-7 Å above the lumenal surface of the ER membrane. Accordingly, SPC18 / SPC21ΔN catalytic sites are located 4 – 11 Å (1-3 residues) above the ER membrane (Figs 8B & 8C). While we do not know the precise boundary between the TM segment and extracytoplasmic domain of Mnn2p, the protein has a
stretch of 19 consecutive residues exhibiting a hydrophobicity index greater than zero (27). In addition, the identified TM segment is flanked on both sides by multiple charged residues (Figure 6). This amino acid configuration is similar to TM segments in many integral membrane proteins, suggesting our calculated values are likely to be in error by, at most, only a few residues (angstroms). Furthermore, our estimates are in good agreement with a prior study (41) in which we suggested the SPC catalytic site is closer to the membrane surface than the oligosaccharyltransferase active site, positioned 30-40 Å above the lumenal surface of the ER membrane (40).

Obtaining our estimates for the height of the SPC catalytic sites would probably not be possible using signal peptide substrates because many are not firmly anchored to the ER membrane (H. Liang, N. Green and H. Fang, unpublished result). As a result, placing a cleavage site at different distances from the rather short hydrophobic region of a typical signal peptide would likely yield a broad range of efficiently processed sites, such as that seen previously (42). On the other hand, our use of an integral membrane protein substrate has identified a narrow range of efficiently processed cleavage sites, whose distance from the TM segment is likely to correspond directly to the disposition of the SPC catalytic site with respect to the ER membrane.

A major question remains unanswered. Why would mammalian SPC have two catalytic subunits with overlapping substrate specificity? The answer may come from our work showing differences in cleavage efficiency. Prepro-α-factor was cleaved more efficiently by SPC21ΔN than by SPC18 (Figure 5), whereas Mnn2p was fragmented more efficiently by SPC18 than by SPC21ΔN (Figure 9). We have not established a pattern for these differences, despite our use of substrates with different residues at the critical P$_1$ and P$_3$ positions, different degrees of hydrophobicity, and different numbers of residues between the cleavage site and hydrophobic segment. Instead, subtle differences in the conformation of the cleavage site may account for the observed differences in cleavage efficiencies. Such conformational effects could result from the absence of amino acid conservation outside the P$_1$ and P$_3$ positions. Considering the large number of soluble and membrane-bound proteins cleaved by mammalian SPC, a second catalytic subunit may be needed to ensure efficient cleavage of all encountered substrates.

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**FIGURE LEGENDS**

FIG. 1. **Alignment of SPC21, SPC18 and Sec11p sequences.** The sequences represent canine SPC21 and SPC18 (9,25) and yeast Sec11p (26). Open boxes enclose regions of amino acid sequence conservation. (*) Indicates residues essential for Sec11p catalytic activity (16). The transmembrane segment for Sec11p (underlined) was identified by Kyte-Doolittle analysis (27).

FIG. 2. **Genetic complementation of the sec11 growth defect.** Cells of yeast strain CMY710 (∆sec11) / pCM112 (SEC11 URA3) containing no additional plasmid or containing pHF340 (SEC11 TRP1), pHF341 (SPC18 TRP1), pHF343 (SPC21 TRP1), or pHF344 (SPC21ΔN TRP1) were grown to log phase. 10 μl of a 1:10 dilution of cells were placed on agar plates containing 5-fluoroorotic acid, and cells were allowed to grow 4 days at 30°C.
FIG. 3. Expression of different forms of SPC21 in yeast. DNA encoding SPC21-FLAG was expressed in a coupled in vitro transcription / translation reaction (EXPERIMENTAL PROCEDURES). The product was subjected to immunoprecipitation using agarose-conjugated anti-FLAG antibodies (lane 1). Cells of strain CVY1 bearing pCV105 (SPC21-FLAG) (lane 2), pCV104 (FLAG-SPC21) (lane 3), or pCV106 (SPC21ΔN-FLAG) (lane 4) were grown to log-phase. Cell extracts were subjected to immunoprecipitation followed by immunoblotting using anti-FLAG antibodies as described (16).

FIG. 4. Cleavage of preKar2p and truncated preinvertase. (A) Yeast strains bearing genes encoding Sec11p (lane 1), SPC18 (lane 2) and SPC21ΔN (lane 3) were grown to log phase, and cell extracts were immunoblotted with anti-Kar2p antibodies. (B) Yeast strains expressing a truncation of preinvertase, preinvertase (112)-HA, and bearing genes encoding Sec11p (lane 1), SPC18 (lane 2) and SPC21ΔN (lane 3) were pulse-labeled. Proteins were precipitated using anti-HA antibodies and subjected to SDS-PAGE.

FIG. 5. Cleavage of prepro-α-factor that contains P₁ alanine or P₁ serine. Yeast strains that express prepro-α-factor-HA (A) or prepro-α-factor(P₁S)-HA (B) and bear genes encoding Sec11p (lane 1), SPC18 (lane 2) or SPC21ΔN (lane 3) were pulse-labeled. Proteins were precipitated using anti-HA antibodies and subjected to SDS-PAGE.

FIG. 6. Position of fragmentation sites in Mnn2p. The relevant portion of the Mnn2p sequence is depicted, including the TM segment (underlined) and flanking sequences. Identity of the TM segment was derived from Kyte-Doolittle analysis (27), which identified 19 consecutive residues with hydrophobicity values greater than zero. The identities of amino acid substitutions in the sequence of Mnn2p are shown in boldface. Mutant constructs are labeled A-K.

FIG. 7. Fragmentation of Mnn2p by Sec11p. (A) Mnn2-HA fragmentation was monitored by pulse labeling as described in the text. (B) Protein-band intensities from panel A were quantitated, and the amount of fragmented Mnn2-HA was indicated as a percentage of total Mnn2-HA. Fragmentation efficiencies were plotted against the number of residues between a
fragmentation site and the end of the TM segment of Mnn2-HA. The fragmentation site refers to the second Ala residue in the Ala-X-Ala motif that was introduced into Mnn2-HA (see Figure 6).

FIG. 8. **Fragmentation of Mnn2p by SPC18 and SPC21ΔN.** (A) Yeast strains described in the text were subjected to a 5-min pulse, and Mnn2-HA (constructs A-H) (see Figure 6) was precipitated from cell extracts with anti-HA antibodies. The positions of Mnn2-HA and the corresponding protein fragments are indicated. Protein bands from (A) were quantitated to obtain fragmentation efficiencies for SPC18 (B) and SPC21ΔN (C). Fragmentation efficiencies were compared to the number of residues between the fragmentation site and the TM segment of Mnn2p as described in the legend to Figure 7.

FIG. 9. **Fragmentation of Mnn2p containing introduced alanine and serine residues by SPC18.** Yeast strains described in the text were subjected to a 5-min pulse followed by a 60-min chase, and Mnn2-HA [construct J (P1A) and construct K (P1S)] (see Figure 6) was precipitated from cell extracts with anti-HA antibodies.
Δsec11

Δsec11 / Sec11p

Δsec11 / SPC18

Δsec11 / SPC21

Δsec11 / SPC21ΔN

Fig.2
Fig. 3
Fig. 4
Fig. 5
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<th>TM Segment</th>
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Fig. 6
Fig. 7
Fig. 8
Fig. 9
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The sequences of mutations are in boldface.
Genetic complementation in yeast reveals functional similarities between the catalytic subunits of mammalian signal peptidase complex
Haobo Liang, Clint VanValkenburgh, Xuemin Chen, Chris Mullins, Luc Van Kaer, Neil Green and Hong Fang

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