The homologue of mammalian SPC12 is important for efficient signal peptidase activity in Saccharomyces cerevisiae*

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The multisubunit signal peptidase catalyzes the cleavage of signal peptides and the degradation of some membrane proteins within the endoplasmic reticulum (ER). The only subunit of this enzyme functionally examined to date, yeast Sec11p, is related to signal peptidase I from bacteria. Since bacterial signal peptidase is capable of processing both prokaryotic and eukaryotic signal sequences as a monomer, it is unclear why the analogues enzyme in the ER contains proteins unrelated to signal peptidase I. To address this issue, the gene encoding Spc1p, the yeast homologue to mammalian SPC12, is isolated from the yeast Saccharomyces cerevisiae. Spc1p co-purifies and genetically interacts with Sec11p, but unlike Sec11p, Spc1p is not required for cell growth or the proteolytic processing of tested proteins in yeast. This indicates that only a subset of the ER signal peptidase subunits is required for signal peptidase and protein degradation activities in vivo. Through both genetic and biochemical criteria, Spc1p appears, however, to be important for efficient signal peptidase activity.

In eukaryotic and prokaryotic cells, secretory signal sequences (signal peptides) function in the transport of polypeptide chains through membranes separating the extracytoplasmic space from the cytosolic milieu (reviewed in Ref. 1). While not all signal peptides are proteolytically processed upon transport across the membrane, cleavable signals consist of a stretch of hydrophobic amino acids usually preceded by one or more positively charged amino acids and followed by the signal peptide cleavage site. Signals exhibiting this motif are ubiquitous in nature as demonstrated by their presence in polypeptide chains targeted to and across membranes of bacteria and mitochondria of the endoplasmic reticulum (ER), mitochondria, and chloroplasts of eukaryotes. Furthermore, signal sequences of precursor proteins in bacteria and eukaryotic cells may be exchanged and then recognized and cleaved correctly by the signal peptidase of the other cell type both in vitro and in vivo.

One of the earliest studied enzymes capable of precisely cleaving signal peptides off of newly synthesized precursors, signal peptidase I, resides within the inner cell membrane of the bacterium Escherichia coli (2). Also referred to as leader peptidase I, this enzyme consists of a single polypeptide chain and therefore contains all of the amino acids essential for catalyzing the signal peptide cleavage reaction. This enzyme contains three distinct regions, including an essential serine residue, common to members of the signal peptidase I family (3, 4). Additional members of this family include Sec11p of the ER signal peptidase complex in the yeast Saccharomyces cerevisiae, and two distinct subunits in both the ER signal peptidase from mammals and the mitochondrial inner membrane protease from yeast. The requirement for two signal peptidase I homologues in the mammalian ER signal peptidase is unclear; however, it has been shown that different sets of precursor proteins are cleaved by different subunits of the mitochondrial inner membrane protease (5). A similar theme is demonstrated by E. coli which contains signal peptidase II cleaving a different set of precursors from that recognized by signal peptidase I (6, 7).

Signal peptidase isolated from the ER membrane is more complicated than other proteins in the signal peptidase I family in that it contains several polypeptide chains that are unrelated to signal peptidase I. There is some controversy, however, as to the number of polypeptide chains present in ER signal peptidase. The enzyme purified from canine pancreas contains five membrane-bound proteins with the following molecular masses: 25, 22/23 (a glycoprotein), 21, 18, and 12 kDa (8). The analogous complex in yeast seems to consist of four proteins with molecular masses ranging from 13 to 25 kDa (9). Interestingly, a two-subunit complex isolated from hen oviduct is sufficient to perform the signal peptide cleavage reaction in vitro (10). Despite these differences, canine SPC21 (mammalian signal peptidase complex protein with molecular mass of 21 kDa) and SPC18 are highly similar to Sec11p of the yeast signal peptidase complex (11, 12, 13). Strong similarity also exists between Sec11p and one of the proteins in the two-subunit avian signal peptidase (14). The remaining protein of this two-subunit complex is homologous to mammalian SPC22/23 (15). SPC22/23, SPC25 and SPC12 are dissimilar to each other and to proteins of the signal peptidase I family (16, 17, 18).

These five canine subunits fall into two topologically distinct groups: single-spanning proteins exhibiting a type II orientation (the C terminus localizes to the luminal side of the membrane), and proteins containing two membrane-spanning segments oriented with their N and C termini facing the cytoplasmic side of the membrane. Proteins in the first group, represented by SPC18, SPC21, and SPC22/23, possess relatively large C-terminal domains (19). SPC12 and SPC25, which fall into the second topological group, contain large cytoplasmic domains and very short luminal stretches of amino acids (17). The fact that the catalytic site of signal peptidase is probably...
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**RESULTS**

Isolation of a SPC1 Homologue—We used a high copy suppressor approach (32, 33) to search for genes encoding proteins of the ER signal peptidase complex (see “Experimental Procedures”). This search yielded the high copy (2µ) plasmid pSPC1 that contained a suppressor, termed SPC1, of the sec11 mutation. The growth pattern of sec11 mutant strain CMYD1 bearing and not bearing pSPC1 is shown in Fig. 1 (genotypes of relevant strains are listed under “Experimental Procedures”). Through subcloning of DNA fragments derived from pSPC1 into a high copy (2µ) plasmid, a 500-base pair SpeI restriction fragment was found to confer growth to sec11 mutant strain CMYD1 at 35 °C (plasmid constructions are described under “Experimental Procedures”). A low copy (CEN) plasmid containing the SpeI fragment did not correct the growth defect of strain CMYD1 at 35 °C, indicating the suppressor’s effect on the sec11 mutant was dependent on its overexpression in cells.

DNA sequencing of this 500-base pair SpeI fragment revealed the presence of an open reading frame containing 94 codons and no other open reading frame containing AUG followed by a stop codon. The predicted polypeptide encoded by the above-described open reading frame is 31 kDa and a 0.9-kb XbaI fragment from pSPC1 was inserted into vector pUC19 (26). The resulting plasmid was digested with EcoRI and BamHI restriction enzymes, and the 0.4-kb fragment was subcloned into vector pUC19 (26). The DNA sequence of this XbpI fragment corresponded to 31 kDa. **Fig. 1.** Effect of SPC1 overexpression on growth of the sec11 mutant. Strains FC2-128 (wild-type), CMYD1 (sec11), and CMYD1/pSPC1 were plated on agar plates containing YEPED (rich) medium (22) and then incubated at 23 and 35 °C for 3–5 days. Diagram at left depicts the arrangement of the strains under study.

To construct a frameshift mutation in SPC1, plasmid pRS426 carrying a 0.5-kb SpeI fragment bearing SPC1 was digested with NdeI, which degrades within the SPC1 gene. The protruding 5′ ends were then filled in by using the Klenow fragment of E. coli DNA polymerase I. The blunt-ended DNA was religated, thus creating a frameshift.

Partial Purification of ER Signal Peptidase and N-terminal Sequence Analysis—Membranes from strain RSYS21 were prepared and extracted as described elsewhere (31) with the modification that saponin was deleted from the first membrane extraction. Dignonin extracts corresponding to 100 eq were supplemented with CaCl2 and MgCl2 (1 mM each) and was incubated overnight with 7 ml of Concanavalin A-Sepharose (Pharmacia Biotech Inc.) at 4 °C. After washing with D-buffer containing 420 mM potassium acetate, the salt concentration was diminished by a wash with D-buffer alone. Bound proteins were eluted at room temperature with D-buffer containing 1 M-methylmannoside for at least 4 h. The glycoproteins were then applied to 5 ml of Q-Sepharose Fast Flow (Pharmacia) and signal peptidase was recovered from the flow-through of the column. This fraction was subjected to further purification using a 1-ml cartridge of SP-Sepharose HiLoad (Pharmacia). Elution was carried out at 4 °C with a 42-ml linear salt gradient ranging from 0 to 500 mM potassium acetate. Signal peptidase was eluted with about 200 mM salt. The fractions of the SP-Sepharose chromatography were analyzed by SDS-PAGE and Coomassie staining. Proteins corresponding to 8 eq of yeast membranes were applied per lane. Partial amino acid sequences of the two lowest protein bands were obtained by sequencing of either the N terminus (Sec11p) or fragments obtained after digestion with Lys-C (Spc1p).
The nucleotide sequence of Spc1p has been submitted to GenBank\(^2\), accession no. U26257, and, independently, the nucleotide sequence of the region of chromosome 10 containing SPC1 has been entered into the data base through the effort of the yeast genome sequencing project (accession nos. Z49511, Z49510, and X87611).

FIG. 2. Comparison of yeast Spc1p to canine SPC12. The amino acid sequences of Spc1p (94 residues) and SPC12 (102 residues) are aligned. Aligned amino acid sequences that are identical are indicated by (i); aligned amino acids that are similar are indicated by (.). Underlined amino acids correspond to sequenced peptides. The boxes enclose stretches of amino acids that putatively span the ER membrane as determined by hydropathy analysis (34).

The calculated molecular mass of high copy suppressor protein Spc1p was 10.8 kDa, a size that was similar to the SPC12 subunit of the mammalian signal peptidase complex. Using the analysis of Kyte and Doolittle (34), Spc1p and SPC12 contained two closely spaced stretches of hydrophobic amino acids, both of which were of sufficient length to span the membrane (amino acids contained within putative transmembrane segments are enclosed by a box in Fig. 2). The predicted isoelectric point of Spc1p was 9.3, in good agreement with SPC12 (pl = 9.4). Sequence comparisons were performed by aligning the putative transmembrane segments of Spc1p and SPC12, with the remainder of the amino acids aligned accordingly. From this comparison, the sequence identity was 17%, and the similarity was 50% (Fig. 2). Alternatively, an analysis that introduced gaps in the sequences of Spc1p and SPC12 (35) gave an identity value of 24%, with the overall similarity equal to 63%. Taken together, these data indicated that Spc1p was a homologue of mammalian SPC12.

Signal peptidase was partially purified from yeast cells to directly assess whether Spc1p was a signal peptidase subunit. A complex containing four prominent bands with apparent molecular masses of 12, 17, 18, and 25 kDa was identified (Fig. 3). These molecular masses were similar to sizes reported previously for subunits of partially purified signal peptidase from yeast (9). As expected, the 17-kDa band corresponded to Sec11p as demonstrated by N-terminal sequencing which gave the sequence MNLRFE (11). Internal peptide sequencing of the 12-kDa protein revealed distinct sequences that matched the predicted sequence of Spc1p (matched sequences are underlined in Fig. 2). These data thus indicate Spc1p is the lower of four prominent proteins in the ER signal peptidase isolated from yeast cells.

Overexpression of SPC1 Facilitates Signal Peptide Cleavage in the sec11 Mutant—Because Spc1p is a high copy growth suppressor (Fig. 1), we reasoned its overexpression may correct the sec11 defect as measured biochemically. To test this, strains CMYD1 (sec11) and CMYD1/pSPC1 were grown to log phase at 23°C, shifted to 35°C for 1 h; then signal peptidase activity was examined by pulse-chase (20) using anti-Kar2p antibodies (36). The data show that preKar2p, the precursor to ER resident protein Kar2p (36, 37), was present in the sec11 mutant after the pulse (Fig. 4, lane 1), with a small amount of preKar2p conversion to mature Kar2p occurring during the chase (lane 2). This result is consistent with our previous observation that preKar2p is cleaved in the conditional sec11 mutant in a temperature-dependent manner (20). Examination of the signal peptide cleavage reaction in sec11 mutant cells containing the SPC1 high copy expression plasmid (strain CMYD1 (sec11)/pSPC1) revealed no apparent processing of preKar2p during the pulse at 35°C (lane 3). However, a greater amount of preKar2p was converted to Kar2p during the chase in strain CMYD1 (sec11)/pSPC1 (lane 4) than in sec11 cells lacking pSPC1 (lane 2). Overexpression of SPC1 therefore increased the amount of preKar2p cleavage occurring in the sec11 mutant during a 30-min chase analysis.

Construction and Phenotypic Analysis of a Δspc1 Mutant—A plasmid construct was prepared containing a replacement of the entire SPC1 open reading frame with the TRP1 gene (Fig. 5A). This construct was linearized and introduced into diploid strain SEY6210.5 (Δtrp1). Transformed cells (Trp⁺) were sporulated and tetrads were dissected. This dissection revealed two Trp⁺ spores in each of ten tetrads. PCR and Southern blot analyses confirmed that the haploid spores phenotypically Trp⁺ contained a disruption of SPC1 (Fig. 5, B and C). Furthermore, Southern blot analysis indicated SPC1 was present in only one copy in cells (Fig. 5C). From observing strain HFY401 (Δspc1) incubated on agar plates at various temperatures, it was apparent that the Δspc1 mutant grew normally at
Spc1p is nonessential for cell viability.

An in vivo pulse-labeling analysis (22) of preKar2p processing was employed to examine signal peptidase activity in the Δspc1 mutant. This analysis depicted in Fig. 6 shows that functional signal peptidase was present in wild-type strain HFY402 as measured by the presence of Kar2p after a 5-min pulse (lane 1). Control strain CMYD1 containing the temperature-sensitive sec11 mutation displayed preKar2p after a 5-min pulse at its nonpermissive temperature (37 °C) (lane 2), while in Δspc1 mutant strain HFY401 preKar2p was converted to Kar2p in a manner similar to that observed in wild-type cells (compare lanes 1 and 3). These results thus show no measured defect in the processing of the preKar2p signal peptide in the Δspc1 mutant.

We repeated the pulse-labeling analysis examining α-factor (αf), a secreted pheromone (38), and carboxypeptidase Y (CPY), a vacuolar protease (39). A 5-min pulse-labeling experiment (22) revealed that, while the control sec11 mutation inhibited the cleavage of the αf and CPY precursors, the Δspc1 mutation did not affect their processing (not shown). Moreover, since the active site of signal peptidase localizes to the ER lumen, the presence of cleaved forms of Kar2p, CPY, and αf demonstrate that the Δspc1 mutation does not strongly affect translocation pathways operating at the ER membrane in yeast cells (31, 40–42). Although these data do not exclude the possibility that very small defects were present in the Δspc1 mutant or that signal peptides of proteins not examined were uncleaved, we conclude that Spc1p does not perform an essential role in the processing of tested precursor proteins, and, based on the absence of a measured growth defect, we further conclude that a sufficient level of processing of the precursors to essential proteins occurs in Δspc1 mutant cells.

Depletion of Spc1p from the sec11 Mutant Exacerbates the Signal Peptide Cleavage Defect—A diploid strain heterozygous for the Δspc1 and sec11 mutations was produced through a cross between strains CMYD1 (sec11) and HFY401 (Δspc1::TRP1). The heterozygote was allowed to sporulate, and tetrads were dissected. Interestingly, not all tetrads yielded four colonies at temperatures permissive for cells containing the sec11 mutation (23 and 18 °C). Indeed, the tetratype (3 viable spores/1 Trp' ts, 1 Trp', 1 Trp'):parental ditype (4 viable spores/2 Trp', 2 Trp-ts):nonparental ditype (2 viable spores/2 Trp') ratio of 7:5:8 was detected with tetrads incubated at 23 °C. This pattern of viable and inviable progeny indicated the sec11 and Δspc1 mutations were synthetically lethal (i.e., the double mutant was unable to grow).

In order to produce a viable sec11 Δspc1 double mutant for analysis of signal peptidase activity, we prepared a construct, phF314, expressing SPC1 under control of the regulatable GAL1 promoter (see "Experimental Procedures"). phF314 was introduced into the above-described diploid cells heterozygous for the Δspc1 and sec11 mutations. Transformants were sporulated, and random spores were placed on agar plates containing galactose. Out of 15 analyzed spores, 4 were identified that did not grow after shifting to medium containing glucose. These glucose-sensitive cells were temperature-sensitive for growth, demonstrating the presence of the sec11 mutation. PCR analysis, similar to that shown in Fig. 5B, confirmed that the glucose-sensitive cells contained a disruption of SPC1.

A typical growth curve of the sec11 Δspc1 double mutant containing the regulatable SPC1 expression plasmid is shown in Fig. 7A. Strain CMY50 (sec11 Δspc1)/pHF314 was grown to early log-phase (A600 = 1.5); cells were then divided with one half of the cells suspended in a medium containing glucose and the other half remaining in the medium containing galactose. Through monitoring the optical densities of cultures in the respective growth media, it was apparent that, approximately 10 h after shifting to glucose, cell growth had begun to be inhibited. At this time point, an aliquot representing 3 A600 cell equivalents was removed from each flask, diluted to A600 = 1, then subjected to a pulse-chase analysis at 30 °C, a condition that only partially inactivates signal peptidase in the sec11 mutant (20). Data shown in Fig. 7B (lanes 1–3) demonstrate that cells utilizing galactose for growth (and thus phenotypically Spc1p−) displayed partial processing of preKar2p. In contrast, cells grown in the presence of glucose to inactive expression of plasmid-borne SPC1 displayed strong inhibition in the processing of preKar2p (lanes 4–6). This revealed a dependence for Spc1p in the sec11 mutant.

PreKar2p Accumulates in the Δspc1 Mutant Expressing Abnormal Membrane Protein AHDK2—The data presented thus
Spreading far show that Spc1p is important for efficient signal peptidase activity as measured in **sec11** mutant cells. If Spc1p does indeed function in facilitating enzyme activity, then it should be possible to demonstrate a role for Spc1p in cells wild-type for **sec11**. To this end, we asked whether the signal peptidase enzyme lacking Spc1p could function efficiently in cells expressing precursor proteins and abnormal membrane protein AHDK2. A chimera containing a fragment of histidinol dehydrogenase tethered to the ER membrane through its attachment to a luminal domain of membrane protein arginine peptidase (20). AHDK2 has been shown to be degraded with a luminal domain of membrane protein arginine peptidase tethered to the ER membrane through its attachment. The 2 distinct proteolytic fragments produced in a Sec11p-dependent manner. The **Δspc1** mutation even though AHDK2 was expressed from a high copy expression plasmid. The proteolysis of preKar2p in cells expressing high levels of AHDK2 was examined using a pulse-labeling analysis (22). Fig. 8A shows that the AHDK2 protein was converted to Kar2p in control strain HFY402/pAHDK2 (lane 1), indicating overexpression of AHDK2 did not strongly affect signal peptide cleavage by wild-type signal peptidase. In contrast, Kar2p and preKar2p were detected in strain HFY401 (Δspc1)/pAHDK2 (lane 2). We conclude from the presence of preKar2p in the Δspc1 mutant expressing AHDK2 (Fig. 8, lane 2) and from the absence of preKar2p in the Δspc1 mutant lacking AHDK2 (Fig. 8, lane 3) that Spc1p has a role in facilitating efficient signal peptidase activity in cells expressing abnormal membrane protein AHDK2. This degree of precursor accumulation in strain HFY401 (Δspc1)/pAHDK2 was insufficient, however, to produce an apparent growth defect.

**DISCUSSION**

This study describes the first in vivo characterization of a signal peptidase subunit other than subunits from the bacterial signal peptidase I protein family. Spc1p, the yeast homologue of mammalian SPC12, co-purifies with Sec11p, an essential subunit of the yeast signal peptidase complex, and exhibits the following two genetic interactions with Sec11p. Overexpression of Spc1p suppresses the temperature-sensitive **sec11** mutation, and the **spc1** and **sec11** mutations are synthetically lethal. Despite these genetic and physical interactions with an essential component of the signal peptidase complex, Spc1p is non-essential for the proteolytic cleavage of tested precursor proteins and the degradation of abnormal membrane protein AHDK2. Our results are therefore in good agreement with in vitro data obtained from analysis of enzymatically active avian signal peptidase which contains a Sec11p homologue but lacks a Spc1p homologue (10). Furthermore, our results agree with topology studies (17), suggesting that mammalian SPC12 does not contribute amino acids to the catalytic site of signal peptidase.

While Spc1p is probably not itself catalytic, we show that Spc1p is important for increasing the efficiency of signal peptidase action. Specifically, overexpression of Spc1p promotes cleavage of the preKar2p signal peptide in the temperature-
sensitive sec11 mutant, and depletion of Spc1p exacerbates the sec11 defect. In addition, preKar2p accumulates in Δspc1 mutant cells expressing abnormal membrane protein AHDK2. The latter result argues that competition exists between preKar2p and AHDK2 for the active site of signal peptidase and supports the idea that abnormal membrane protein AHDK2 is degraded through a signal peptidase-dependent pathway.

Since enzymatically active signal peptidase I purified from E. coli contains only one polypeptide, it was expected that Spc1p, which is unrelated to signal peptidase I, would be important for activities other than signal peptide cleavages. Instead, our data suggest that Spc1p is important for efficient signal peptidase activity. We thus conclude that either bacterial cells contain a homologue of Spc1p that does not bind tightly to signal peptidase I or eukaryotic cells have a particular requirement for noncatalytic subunits such as Spc1p. This requirement could derive from the fact that, in contrast to bacterial signal peptidase, ER signal peptidase must recognize signal peptides of proteins targeted to various compartments of the secretory pathway. Since signal peptides vary in length and primary sequence, the diversity of signal peptides recognized by the ER enzyme is probably greater than that encountered in bacteria. In addition to the demand placed on ER signal peptidase to efficiently process a variety of precursor proteins, the multisubunit signal peptidase complex is also involved in protein degradation. The observed accumulation of preKar2p in the Δspc1 mutant expressing abnormal membrane protein AHDK2 suggests that Spc1p may function to ensure efficient signal peptide cleavages in situations where the protein degradation activity within the ER is high.

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