Structurally Related Spc1p and Spc2p of Yeast Signal Peptidase Complex Are Functionally Distinct*

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Two subunits of the mammalian signal peptidase complex, SPC12 and SPC25, share similar membrane topologies with the majority of each protein oriented toward the cytoplasm. Such similarities may suggest that these proteins perform redundant functions in signal peptidase activity. In the present study, we addressed this issue through analysis of the yeast homologs to SPC12 and SPC25, Spc1p and Spc2p. We show that both Spc1p and Spc2p are nonessential for signal peptidase activity and growth of yeast cells and that null mutations in the genes encoding Spc1p and Spc2p are synthetically lethal with a conditional mutation affecting Sec11p, an essential subunit of yeast signal peptidase. However, a high copy plasmid encoding Spc1p suppresses the conditional sec11 mutation, whereas the corresponding plasmid encoding Spc2p does not suppress sec11. Moreover, Spc2p, but not Spc1p, is important for signal peptidase activity and cell viability at high temperatures. These results indicate that although both Spc1p and Spc2p are noncatalytic, they are functionally distinct. Evidence is also presented that a double mutant lacking Spc1p and Spc2p grows well relative to wild type yeast cells, indicating that the signal peptidase complex missing at least two of its subunits is sufficient for signal peptidase activity in vivo.

Translocation across the membrane of the endoplasmic reticulum (ER) initiates protein transit through the secretory pathway. During or soon after translocation, ER membrane-bound signal peptidase catalyzes the proteolytic removal of NH₂-terminal signal peptides from newly synthesized precursor proteins (for review, see Ref. 1). Signal peptidase plays an additional role in the cell through participating in the degradation of some abnormal proteins in the ER membrane (2).

ER signal peptidase purified from a variety of eukaryotic cells does not exist as a monomeric enzyme as does leader peptidase, the enzyme that cleaves signal peptides in bacteria (3), but as a hetero-oligomeric protein complex (4). The signal peptidase complex (SPC) isolated from mammalian cells consists of five subunits, three of which (SPC18, SPC21, and SPC22/23) contain one transmembrane segment (5). This topology places a large COOH-terminal domain of each subunit in the lumen. The remaining subunits, SPC25 and SPC12, contain two transmembrane segments with relatively large hydrophilic NH₂- and COOH-terminal domains oriented toward the cytoplasm (6). The SPC of the budding yeast *Saccharomyces cerevisiae* contains at least four subunits (7). The genes encoding two of these subunits, Sec11p and Spc1p, have been cloned and characterized (2, 8, 9). Sec11p is essential for signal peptide cleavage, degradation of at least a subset of abnormal membrane proteins within the ER, and yeast cell growth. Based on the essential role of Sec11p in these activities and the fact that Sec11p contains distinct regions of homology to bacterial leader peptidase (10), this yeast subunit is presumed to contain the signal peptidase catalytic site. Spc1p, which is homologous to mammalian SPC12, is important for efficient enzyme activity (9). Spc1p is nonessential, however, for signal peptidase function and growth of yeast cells.

Here we have cloned the gene encoding the yeast homolog Spc2p to mammalian SPC25. As SPC12 and SPC25 of mammalian signal peptidase share similar membrane topologies, we questioned if the yeast homologs of these subunits perform redundant functions in signal peptidase activity in vivo. Results obtained through biochemical and genetic analyses indicate that Spc1p and Spc2p of the yeast SPC are functionally distinct.

EXPERIMENTAL PROCEDURES

Preparation of Antibodies—For the production of anti-Kar2p antiserum a 1.07-kb *Eco*RI DNA fragment derived from the *KAR2* coding sequence was cloned into pGEX-3X (13) to produce an in-frame fusion with glutathione S-transferase. The fusion protein was expressed in *Escherichia coli*, isolated as described previously (14), and antibodies were prepared from rabbits as described (15). Anti-Spc1p antibodies were made against an NH₂-terminal peptide MSEILQDVRQ. Anti-Spc2p antibodies were made against COOH-terminal peptides HNV-LDTKKNE and LVELVQKKGENSKKEL. Anti-Sec11p antibodies were made against an NH₂-terminal peptide MNLRFELQKL. The peptides were coupled to keyhole limpet hemocyanin that had been activated with sulfo-SMCC. The immunization of rabbits and the affinity purification of specific antibodies were performed as described (16).

Partial Purification of ER Signal Peptidase and NH₂-terminal Sequence Analysis—The isolation of the ER SPC from yeast membranes was performed as described previously (9) with the following modifications. Incubation of the digitonin extract with concanavalin A-Sepharose was done without adding extra amounts of CaCl₂ and MgCl₂ to avoid proteolytic degradation of Spc2p. Identification of the major bands of the peak fractions from the SP-Sepharose chromatography was confirmed by sequencing of either the NH₂ terminus or of fragments obtained after digestion with Lys-C.
Cleavage of Prepro-a-factor—The prepro-a-factor cleavage assay was performed as described (7). 5 μl (300 eq) of every fraction of the SP-Sepharose chromatography was examined. The samples were separated by a SDS-polyacrylamide gel. Cleavage products were visualized by autoradiography.

Cloning and Disruption of SPC2—The construct used for the disruption of SPC2 was produced as follows. Genomic SPC2 of strain NGY21B was amplified through the polymerase chain reaction (PCR) using the forward primer GTTGATCATCATCTTAGA and the reverse primer CGAGCGTCTGTAATAGA which flank the coding sequence. The reaction was carried out using a Gene Amp kit according to the manufacturer’s specifications (Perkin Elmer). The amplified fragment (1.8 kb) was restricted with BamHI and PstI sites. The resulting plasmid contained two HindIII sites located within the SPC2 coding sequence. A 1.2-kb HindIII fragment containing the URA3 gene was inserted into these HindIII sites resulting in the deletion of a 0.2-kb sequence of the SPC2 gene. This construct was linearized with SpeI and XhoI and then transformed into diploid strain SEY6210.5. Selection for the disruption mutation was performed on minimal agar plates supplemented appropriately but lacking uracil (15). Plasmid pHFS24 containing the GAL1/GAL10 divergent promoter fragment (17) followed by the URA3 gene was amplified by PCR using a forward oligonucleotide-bearing BamHI site upstream of the SPc2 stop codon and a reverse oligonucleotide bearing PstI site upstream of the SPC2 promoter (9). Peptide sequence analysis of the protein migrating with a molecular mass of 18 kDa was identified as Spc1p, whereas the 11-kDa protein was identified as Spc3p. A protein of 28 kDa (**) was seen from Fig. 2A, these fractions also showed the strongest reactions with anti-Spc1p and anti-Spc3p antibodies. Using anti-Spc2 antibodies a protein of approximately 20 kDa, which coeluted with fractions exhibiting both signal peptide cleavage activity and binding activity to antibodies directed against known yeast SPC subunits, was identified. To confirm the identity of the anti-Spc2 reactive protein, the fraction containing these SPC subunits was analyzed by peptide sequencing (Fig. 2B). Of the six major proteins detected three exhibited a molecular mass close to that predicted for Spc2p. The two protein bands of approximately 17 kDa both corresponded to Sec11p. The species exhibiting a slightly larger molecular mass may be a glycosylated form of Sec11p. The protein with apparent molecular mass of 18 kDa was identified as Spc2p, whereas the 11-kDa protein was identified as Spc1p (9). Peptide sequence analysis of the protein migrating with a molecular mass of 25 kDa, denoted by (*), revealed similarity to SPC2223 of the mammalian SPC (23).

RESULTS AND DISCUSSION

Identification of the Yeast Homolog to Mammalian SPC25—For the present study we employed a search of the database using Blastp (20) in an attempt to identify the gene encoding the yeast homolog to SPC25 of the mammalian SPC (21). This search identified a hypothetical protein (accession no. Z46729) with a predicted molecular mass of 19.6 kDa. Excluding the NH2-terminal 42 residues of the sequence, the yeast and mammalian proteins contain 15 glycine residues that are not present in Spc2p. Despite this difference, the isolectric points of Spc2p and SPC25 are similar (9.2 and 8.9, respectively). As shown in Fig. 1, Spc2p contains two hydrophobic stretches that may span the ER membrane. Corresponding regions in SPC25 have been shown to span the membrane and thereby orient the majority of SPC25 toward the cytoplasm (6). The yeast and canine proteins thus display sequence similarities, similar isolectric points, and very similar hydrophyt profiles, all suggesting that Spc2p is the yeast homolog to mammalian SPC25.

Spc2p Copurifies with Functional Signal Peptidase—The SPC from yeast was partially purified using a published procedure (9) with slight modifications (see “Experimental Procedures”). To identify the position of the SPC in the elution profile from the SP-Sepharose column, the fractions were assayed for their ability to convert prepro-a-factor (pp-a) to pro-a-factor (p-a) using a method described previously (7). In addition, these fractions were tested by Western blotting for the presence of known SPC subunits, Spc1p and Sec11p. The highest enzymatic activity was detected in fractions 13 and 14. As can be seen from Fig. 2A, these fractions also showed the strongest reactions with anti-Sec11p and anti-Spc1p antibodies. Using anti-Spc2 antibodies a protein of approximately 20 kDa, which coeluted with fractions exhibiting both signal peptide cleavage activity and binding activity to antibodies directed against known yeast SPC subunits, was identified. To confirm the identity of the anti-Spc2 reactive protein, the fraction containing these SPC subunits was analyzed by peptide sequencing (Fig. 2B). Of the six major proteins detected three exhibited a molecular mass close to that predicted for Spc2p. The two protein bands of approximately 17 kDa both corresponded to Sec11p. The species exhibiting a slightly larger molecular mass may be a glycosylated form of Sec11p. The protein with apparent molecular mass of 18 kDa was identified as Spc2p, whereas the 11-kDa protein was identified as Spc1p (9). Peptide sequence analysis of the protein migrating with a molecular mass of 25 kDa, denoted by (*), revealed similarity to SPC2223 of the mammalian SPC (23). This 25-kDa yeast protein has been named Spc3p. A protein of 28 kDa (***) was concanavalin A, a contaminant of the preparation.
produced viable haploid strains. The URA3 marker segregated with two spores from each tetrad. To confirm the disruption of SPC2, cell membranes were isolated from Ura+ and Ura− cells as described previously (15) and subjected to Western blotting using anti-Spc2p antibodies. This analysis revealed the presence of an immunoreactive protein of the correct apparent molecular mass for Spc2p in Ura+ (Fig. 3B, lane 1) but not in Ura− (lane 2) cells. Confirmation of the SPC2 deletion was also performed through PCR and Southern blotting according to published methods (9). Incubation of cells on agar plates revealed no apparent growth defects between wild type and Δspc2 strains at 18, 23, 30, or 37 °C. Furthermore, the Δspc2 mutant grew well on rich and minimal media and mated well. In combination, our data indicate that Spc2p is not required for cell viability or growth under tested conditions.

**Mutations Affecting Spc2p and Sec11p Genetically Interact**—Mutations in genes encoding previously identified subunits of the yeast SPC, Sec11p and Spc1p, display synthetic lethality (9). Synthetic lethality is a situation in which mutations in combination result in cell death under conditions that are permissive for either mutation alone. To determine whether a synthetic interaction exists between the temperature-sensitive (ts) sec11 mutation and the Δspc2 mutation, we crossed strains PBY408A (sec11) and HFY403 (Δspc2). Diploid cells were subjected to tetrad analysis at 23 °C. Following dissection of nineteen ascis, 13 were found to contain three viable spores (one Ura+ and ts, one Ura+ and non-ts, one Ura− and non-ts), four contained four viable spores (two Ura+ and ts, two Ura− and non-ts), and two contained two viable spores (both Ura− and non-ts). This 13:4:2 ratio is similar to the 4:1:1 ratio (parental ditype:nonparental ditype) expected for two independently segregating mutations. Our failure to recover any spores phenotypically both Ura− and ts out of 59 germinating indicated that combining the sec11 and Δspc2 mutations into the same cell results in cell death. Synthetic lethality was also observed for tetrads analyzed at 18 °C.

Given the synthetic lethality between the sec11 and Δspc2 mutations, we reasoned that the lack of Spc2p may increase the signal peptide processing defect seen in sec11 cells. To test this, a viable sec11 Δspc2 double mutant was produced by employing a plasmid (pHF324) expressing SPC2 under control of the regulatable GAL1 promoter (17). Isolation of the mutant on media containing galactose was accomplished as described previously (9). Resulting strain CMY60 (sec11 Δspc2)/pHF324 was grown to early log phase at which point cell aliquots were removed and suspended to A<sub>600</sub> = 0.6 in rich media containing glucose or galactose. Fig. 4A depicts relative growth curves of cells and demonstrates a clear growth defect for cells in glucose relative to those incubated in galactose, presumably due to depletion of Spc2p from the sec11 mutant in media containing glucose. At time points indicated in Fig. 4B, whole cell lysates were harvested from 1 A<sub>600</sub> eq of each culture and subjected to Western blot analysis using anti-Kar2p antibodies (see “Experimental Procedures”). Also termed yeast BiP, Kar2p is an ER resident protein that is targeted to the ER through an NH<sub>2</sub>-terminal signal peptide (25, 26). Cells incubated with glucose show an accumulation of preKar2p relative to those in galac-

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**Function of Signal Peptidase Subunits**

**Table I**

**Yeast strains used in this study**

| Strain  | Genotype                                      | Ref.  |  
|---------|-----------------------------------------------|-------|
| PBY408A | MATa sec11–7 ura3–52 his4–519 leu2–3,112 gal2–1 | 8     |
| RSY427  | MATa sec23–1 ura3–52 leu2–1 trp1–1 his4–419    | 2     |
| SEY6210.5 | MATa/MTu leu2–3,112 (leu2–3,112 ura3–52 ura3–52 his3–Δ200/his3–Δ200)  
trp1–Δ901/trp1–Δ901 suc2–Δ9/suc2–Δ9 ADE2/ade2–101 lys2–80 lys2–80 | 24    |
| NGY218  | MATa leu2–3 trp1–1 ura3–52 his4–619           |       |
| CMYD1   | MATa sec11–7 ura3–52 leu2–3 his4–619          | 9     |
| CMY10   | MATa sec23–1 sec11–7 trp1–1 ura3–52 his4–619  | 2     |
| CMY20   | MATa sec23–1 sec11–7/trp1–1 ura3–52 his4–619 |       |
| CMY30   | MATa sec23–1 sec2Δ1::URA3 trp1 lys2–80 his4  |       |
| CMY60   | MATa sec11–7 sec2Δ1::URA3 leu2 his4           |       |
| HFY402  | MATa ade2–101 ura3–52 leu2–3,112 trp1–Δ901 his3–Δ200 | 9     |
| HFY401  | MATa sec1Δ1::TRP1 ade2–101 lys2–80 ura3–52 leu2–3,112 trp1–Δ901 his3–Δ200 | 9     |
| HFY403  | MATa sec2Δ1::URA3 his3–Δ200 leu2–3,112 lys2–80 trp1–Δ901 |       |
| HFY404  | MATa sec1Δ1::TRP1 sec1Δ1::URA3 his3–Δ200 leu2–3,112 ade2–101 |       |
yeast strains containing Spc2p and therefore provide biochemical evidence of the synthetic interaction between the sec11 and Δspc2 mutations.

We have shown previously that Spc1p and Sec11p are functionally redundant, then it seemed plausible to ask whether a high copy plasmid encoding Spc2p suppressed the sec11 mutation. These results demonstrate that sec11 cells display an enhanced defect in preKar2p processing in the absence of Spc2p and therefore provide biochemical evidence of the synthetic interaction between the sec11 and Δspc2 mutations.

Yeast Cells Lacking Spc1p and Spc2p Are Viable—If Spc1p and Sec11p are functionally redundant, then it seemed plausible to ask whether a high copy plasmid encoding Spc2p suppressed the sec11 mutation. The sec11 mutant containing the high copy SPC2 expression plasmid was viable at 23 °C, a permissive temperature for the sec11 mutant, but inviable at 32 °C, the lowest nonpermissive temperature for the sec11 mutant. A high copy plasmid containing SPC2 thus did not suppress the sec11 mutation.

Preprotein processing in sec11 mutant cells depleted of Spc2p—We performed experiments to determine the importance of Spc2p to signal peptide cleavage in vivo. We subjected strains HFY402 (sec11 Δspc2)/pHF324 and HFY402 (wild type)/pHF324 to pulse-labeling analysis (2) at 30 °C. Conditional mutant CMYD1 (sec11) was similarly labeled following a preincubation at 30 °C, a temperature that induces a partial signal peptidase defect (2). As expected, mature Kar2p was precipitated from wild type strain HFY402 (Fig. 5A, lane 1), whereas preKar2p was present in the sec11 mutant (lane 2). The apparent absence of preKar2p in Δspc2 mutant cells (lane 4) indicated that the signal peptidase enzyme lacking Spc2p was functional. Furthermore, analysis of a strain containing the Δspc2 and Δspc1 mutations (lane 5) indicated that the absence of both Spc2p and Spc1p from yeast cells did not result in a detectable defect in preKar2p processing.

Similar analyses were performed for α-factor, a mating pheromone that is transported through the secretory pathway and exported from yeast cells (27). Here the sec23 mutation, which inhibits transport of proteins out of the ER (28), was employed to allow better resolution of precipitated preproteins and their signal peptide-processed forms. Strains RSY427 (sec23), CMY10 (sec11 sec23), and CMY30 (Δspc2 sec23) were subjected to pulse labeling following a 30 min incubation at 32 °C (a nonpermissive temperature for the sec11 and sec23 mutations). The presence of p-α-f in the control sec23 cells (Fig. 5B, lane 1) and pp-α-f in sec11 sec23 cells (lane 2) indicated functional and nonfunctional signal peptidase, respectively. The Δspc2 mutant exhibited no apparent defect in pp-α-f processing (lane 4).
migrates faster than p-

HFY403 (revealed an accumulation of unprocessed preKar2p in strains pulse-labeling analysis necessitated the use of Western blotting. Poor radiolabeling of cells at high temperatures required for phase cells or cells incubated in high salt (data not shown). No accumulation of preKar2p was apparent in stationary phase NaCl) for osmotic challenge, and incubated at high temperatures. As expected, yeast cells bearing the Δspc1 and sec23 mutations show no apparent defect in pp-α-f processing (lane 3). We have further shown that Spc2p is not important for signal peptidase-dependent protein degradation (data not shown), using chimeric membrane proteins described in our earlier report (2).

Precursor Proteins Accumulate in the Δspc2 Mutant at High Temperatures—In an attempt to find a circumstance under which Spc2p alone or in combination with Spc1p was critical for signal peptidase function we exposed cells to a variety of growth conditions. Strains HFY402 (wild type), HFY401 (Δspc1), HFY403 (Δspc2), and HFY404 (Δspc1 Δspc2) (all of which were derived from a single tetrad) were grown to stationary phase (A_{600} = 10–15), exposed to high salt (0.5–2 M NaCl) for osmotic challenge, and incubated at high temperatures. No accumulation of preKar2p was apparent in stationary phase cells or cells incubated in high salt (data not shown). Poor radiolabeling of cells at high temperatures required for pulse-labeling analysis necessitated the use of Western blotting as a means to study precursor processing at 42 °C. Western blot analysis of cells following a 3-h incubation at 42 °C revealed an accumulation of unprocessed preKar2p in strains HFY403 (Δspc2) (Fig. 6A, lane 3) and HFY404 (Δspc1 Δspc2) (lane 4). No preKar2p was visible in strain HFY402 (wild type) (lane 1) or HFY401 (Δspc1) (lane 2) at 42 °C or in any strains analyzed by Western blotting at 30 °C (data not shown). The absence of detectable levels of preKar2p in Δspc1 cells (lane 2) with no apparent increase in Δspc1 Δspc2 cells relative to Δspc2 cells (compare lanes 3 and 4) demonstrated that lack of Spc2p alone resulted in preKar2p accumulation at high temperatures.

We repeated the above analysis of Δspc2 cells to measure processing of carboxypeptidase Y (CPY), a vacuolar protein that is cleaved by signal peptidase prior to its transport to the Golgi apparatus in route to the vacuole (29). Upon incubation of Δspc2 cells at 42 °C, a form of CPY accumulated (Fig. 6B, lane 3) which was not visible in wild type cells incubated at 42 °C (lane 4) or in Δspc2 mutant cells incubated at 30 °C (data not shown). This form of CPY (denoted CPY*) migrated with mobility similar to that of the ER-glycosylated species seen to accumulate in sec11 (lane 1) and sec23 (lane 2) mutant cells following an incubation at the nonpermissive temperature for the sec11 and sec23 mutations. Based on the above results for Kar2p we suggest that accumulated CPY* represents glycosylated preproCPY that accumulates in the ER probably due to improper folding or retention of the signal peptide.

We next analyzed Δspc2 cells to distinguish between a possible defect in protein translocation versus a preprotein processing defect, either of which could account for the observed accumulation of preKar2p. For this we performed an in vitro protease sensitivity assay (2) on microsomal membranes derived from strain HFY403 (Δspc2) which had been incubated at 42 °C. Results demonstrated that the preKar2p seen in Δspc2 cells at high temperature was protected from proteases in the absence of detergent and cleaved producing a relatively stable...
the proteolytic fragment of Kar2p (~75 kDa) upon addition of detergent (data not shown), thus indicating that preKar2p did not accumulate in the cytoplasm. In combination these results indicate that \( \Delta \text{spc2} \) cells, when exposed to high temperature, exhibit a defect in cleavage of the preproteins examined and not in their translocation across the ER membrane.

In light of the observed defect in the \( \Delta \text{spc2} \) mutant at high temperatures, we next addressed the importance of Spc2p for cell viability. For this analysis, strains HFY402 (wild type), HFY401 (\( \Delta \text{spc1} \)), HFY403 (\( \Delta \text{spc2} \)), and HFY404 (\( \Delta \text{spc1} \Delta \text{spc2} \)) (closed circles) were grown (30°C) to early log phase (\( A_{600} = 1–3 \)). Cells were pelleted and resuspended in YEPD (rich) media to a concentration of 1 \( \times 10^6 \) cells/ml and placed at 42°C. Aliquots of each strain were removed, serially diluted, and placed on agar plates containing YEPD (rich) medium at indicated time points. Plates were incubated at 30°C for the appearance of colonies, which took approximately 2 days. Colony number was determined and used to calculate the number of viable cells/ml in cultures incubated at 42°C for different time intervals.

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**Fig. 7. Viability of \( \Delta \text{spc2} \) mutant cells at 42°C.** Strains HFY402 (wild type) (diamonds), HFY401 (\( \Delta \text{spc1} \)) (triangles), HFY403 (\( \Delta \text{spc2} \)) (open circles), and HFY404 (\( \Delta \text{spc1} \Delta \text{spc2} \)) (closed circles) were grown (30°C) to early log phase (\( A_{600} = 1–3 \)). Cells were pelleted and resuspended in YEPD (rich) media to a concentration of 1 \( \times 10^6 \) cells/ml and placed at 42°C. Aliquots of each strain were removed, serially diluted, and placed on agar plates containing YEPD (rich) medium at indicated time points. Plates were incubated at 30°C for the appearance of colonies, which took approximately 2 days. Colony number was determined and used to calculate the number of viable cells/ml in cultures incubated at 42°C for different time intervals.