Many type I signal peptidases from eubacterial cells appear to contain a serine/lysine catalytic dyad. In contrast, our data show that the signal peptidase complex from the endoplasmic reticulum lacks an apparent catalytic lysine. Instead, a serine, histidine, and two aspartic acids are important for signal peptidase activity by the Sec11p subunit of the yeast signal peptidase complex. Amino acids critical to the eubacterial signal peptidases and Sec11p are, however, positioned similarly along their primary sequences, suggesting the presence of a common structural element(s) near the catalytic sites of these enzymes.

Cleavable signal sequences, which are usually located at the N termini of precursor proteins, function in the delivery of their protein cargo to specific destinations within both eukaryotic and eubacterial cells. A large number of signal sequences possess a common motif consisting of an n-region that is often positively charged and a hydrophobic core (the h-region) followed by a polar c-region containing the cleavage site (1). Small uncharged amino acids are usually present at the −1 and −3 positions from the cleavage site. Signals exhibiting this motif are recognized and cleaved by type I signal peptidases. Type I signal peptidases are found within the endoplasmic reticulum (ER) membrane, the mitochondrial inner membrane, and the cytoplasmic membrane of eubacterial cells (reviewed in Ref. 2). There is a strong functional conservation of the signal sequence cleavage reaction, revealed by the fact that signal sequences of proteins targeted normally to the ER can be cleaved by eubacterial signal peptidase (3), and eubacterial signal sequences can be cleaved by ER signal peptidase (4).

Site-directed mutagenesis studies suggest that many eubacterial signal peptidases contain a serine/lysine dyad with which to catalyze the cleavage reaction (5–9). A similar catalytic dyad is thought to be present in the LexA and UmuD proteins of Escherichia coli (10, 11) and in both catalytic subunits of mitochondrial signal peptidase (12). Recent x-ray crystallographic analysis confirms the role of serine as a nucleophile and is consistent with a lysine acting as a general base in catalysis by E. coli leader peptidase (13).

At least one eubacterial signal peptidase, SipW of Bacillus subtilis, may exhibit a catalytic site more like that of Sec11p of the ER signal peptidase. B. subtilis contains five distinct chromosomally encoded signal peptidases, SipW being the only one like Sec11p (14, 15). As shown in Fig. 1, Sec11p contains a serine residue that aligns to the catalytic serine of leader peptidase; however, within the limited regions of homology that exist between Sec11p and leader peptidase, Sec11p contains a histidine that has been aligned to the catalytic lysine of leader peptidase (16, 17). From this, the type I signal peptidase family may contain a subgroup, represented by Sec11p and SipW, that uses a distinct catalytic mechanism (14). It has been noted previously, however, that an alignment of Sec11p to the E. coli leader peptidase sequence is of low statistical significance (5). In addition, two subunits, Sec11p and Spc3p, of the ER signal peptidase are essential in the yeast Saccharomyces cerevisiae (18–22), thus raising the possibility that Spc3p may contribute amino acids to the catalytic site.

To identify the probable catalytic site residues of ER signal peptidase and thus explore the evolutionary relationship of the type I signal peptidase family, all serine, lysine, histidine, and aspartic acid residues that are conserved among Sec11p- and Spc3p-type subunits have been changed using site-directed mutagenesis. We have found that Ser-44, His-83, Asp-103, and Asp-109 are essential for signal peptidase activity by Sec11p. Because all conserved and partially conserved lysines were nonessential, this work strengthens the notion that the type I signal peptidase family is comprised of two groups, one containing a serine/lysine dyad and one that appears to lack a catalytic lysine.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, Reagents, and Antibodies—Yeast strains constructed for this study include CVY1 (MATa ura3–52 his3–Δ200 lys2–80 trp1–Δ901), CMY710 (MATa sec11Δ::HIS3 ura3–52 leu2–3, 112 his3–Δ200 trp1–Δ901 sucl2–3 ade2–101 lys2–80), and HYF409 (MATa sec11–7 leu2–3, 112 ura3–52 trp1–Δ901 lys2–801 his4–619). Strain HYF405 (MATa sec11Δ::LEU2 ura3–52 leu2–3, 112 his3–Δ200 trp1–Δ901 sucl2–3) has been described previously (19). To create a chromosomal replacement of the SEC11 gene with the his3 gene, pCM111(23) was digested with SmaI, thereby removing the entire SEC11 open reading frame, and the fragment was replaced by a 1.5-kilobase HIS3 fragment (24). This construct was introduced into the yeast chromosome by homologous recombination using methods published previously (20). Media for growing yeast have been described (25, 26). A murine anti-FLAG M2 antibody and anti-FLAG M2 antibody conjugated to agarose (Sigma), a rat anti-HA antibody (clone 3F10) (Roche Molecular Biochemicals), and a rabbit anti-Kar2p antibody (from Mark Rose, Princeton) were used in this study. Purified digitonin was a gift from Kent Matlack and Tom Rapoport (Harvard Medical School).
rylated following the directions from the Sculptor® in vitro mutagenesis kit (Amersham Pharmacia Biotech) except that T4 DNA ligase buffer was added instead of T4 polynucleotide kinase buffer, and the reaction time was extended to 30 min. After production of plasmids, the steps for single-stranded DNA synthesis outlined in the Sequenase™ Version 2.0 kit (U. S. Biochemical Corp.) were followed. Oligonucleotides used for mutagenesis of SEC11 (Table I) and SPC3 (Table II) are listed.

Enrichment of ER Membranes—Preparation of ER membranes was based on work done previously (27, 28). Two thousand A₅₅₀ yeast cell equivalents were resuspended in distilled water (50 ml), pelleted, re-suspended in 100 ml of zymolase buffer (1.4 M sorbitol, 50 mM potassium acetate by adding 750 mM KOH-HEPES (pH 7.5), 10% -mercaptoethanol, and 50 μg/ml protease inhibitor mixture consisting of 1 μg/ml each of pepstatin A, chymostatin, aprotinin, leupeptin, and antipain). Homogenization was performed in the cold room using a Potter homogenizer (10 strokes). The homogenized spheroplasts were subjected to centrifugation at 10,000 × g for 10 min. The supernatant was saved, and the pellet was subjected to a second homogenization and centrifugation using 3 ml of HS buffer. The supernatants from the two homogenizations were combined and subjected to centrifugation at 10,000 × g for 10 min. The final supernatant was overlaid on a discontinuous sucrose gradient containing 1 ml of 70% sucrose, 2 ml of 48% sucrose, 2 ml of 39% sucrose, and 1 ml of 30% sucrose (pH 7.5), 40 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 25 mM KCl. The gradient was subjected to centrifugation at 3000 rpm for 45 min in a Beckman Ti50 rotor. Membranes at the 48/39 interface were collected, mixed with HS (2 ml), subjected to centrifugation at 40,000 rpm for 45 min in a Beckman Ti50 rotor. The agarose was sedimented and washed as described (26). Proteins were analyzed by Western blotting using a 1/6000 dilution of anti-HA antibodies (Roche Molecular Biochemicals).

RESULTS

One Conserved Serine, One Conserved Histidine, and Two Conserved Aspartic Acids Are Essential in Sec11p—To identify amino acids important for ER signal peptidase activity, we employed a site-directed mutagenesis approach. We reasoned that catalytic amino acids are probably conserved among various Sec11p- and Spc3p-type subunits. We therefore aligned the sequence of Sec11p to the sequences of the two related Spc3p-type subunits (33) and to the sequence of Sec11p to the sequences of the two related Spc3p-type subunits (33). One Conserved Serine, One Conserved Histidine, and Two Conserved Aspartic Acids Are Essential in Sec11p—To identify amino acids important for ER signal peptidase activity, we employed a site-directed mutagenesis approach. We reasoned that catalytic amino acids are probably conserved among various Sec11p- and Spc3p-type subunits. We therefore aligned the sequence of Sec11p to the sequences of the two related Spc3p-type subunits

[Image: Sequence homology between leader peptidase and Sec31p. Box I, Box II, and Box III represent sequences that are homologous in E. coli leader peptidase and the Sec11p subunit of the yeast ER signal peptidase. The positions of the catalytic serine and lysine of leader peptidase and the corresponding serine and basic amino acids important in Sec11p and their corresponding amino acids in leader peptidase are indicated (*). Gaps in these sequences are indicated by dashes. Identical residues are indicated by stacked dots, and similar residues are indicated by single dots.]

**Table I**

| Mutation | Oligonucleotide |
|----------|----------------|
| S33A     | 5'-CCATTGTCCTAATCCGCTTCTCCAGCT-3' |
| S35A     | 5'-CTACCTAAGCCTGCTGCATGTTGGA-3' |
| S42A     | 5'-CGTGTTGGTCTGCTGGAACCTTGAAGAC-3' |
| S42T     | 5'-CTGGTGGTTCTGACTAACCTGATTACGAC-3' |
| S44A     | 5'-GTGCTTGGCAAGGCCATGAAACCAGC-3' |
| S44C     | 5'-GTGCTTGGCAAGGCCATGAAACCAGC-3' |
| H83A     | 5'-CAAAATCCCCATGTTGCTAGTGGTGGAAGGC-3' |
| H83F     | 5'-CAAAATCCCCATGTTGCTAGTGGTGGAAGGC-3' |
| H83K     | 5'-CAAAATCCCCATGTTGCTAGTGGHGATGGC-3' |
| D53N     | 5'-CCACGTTTCCAAAGGGGTGATACCTTTTCTATTGGC-3' |
| D53N     | 5'-CCACGTTTCCAAAGGGGTGATACCTTTTCTATTGGC-3' |
| D103E    | 5'-CCTCCTCGACAGAGTGAAGAAACCGCAGAATG-3' |
| D103N    | 5'-CCTCCTCGACAGAGTGAAGAAACCGCAGAATG-3' |
| D109E    | 5'-CAATTGCCTCGACAGAGTGAAGAAACCGCAGAATG-3' |
| D109N    | 5'-CAATTGCCTCGACAGAGTGAAGAAACCGCAGAATG-3' |
| K101R    | 5'-CAATTGCCTCGACAGAGTGAAGAAACCGCAGAATG-3' |
| K112S    | 5'-GAAATTTCTTCGAACTTGAACCTGAGGTAGTGAG-3' |
| K112S    | 5'-GAAATTTCTTCGAACTTGAACCTGAGGTAGTGAG-3' |
| K112S    | 5'-GAAATTTCTTCGAACTTGAACCTGAGGTAGTGAG-3' |

**FIG. 1.** Sequence homology between leader peptidase and Sec31p. Box I, Box II, and Box III represent sequences that are homologous in E. coli leader peptidase and the Sec11p subunit of the yeast ER signal peptidase. The positions of the catalytic serine and lysine of leader peptidase and the corresponding serine and basic amino acids important in Sec11p and their corresponding amino acids in leader peptidase are indicated (*). Gaps in these sequences are indicated by dashes. Identical residues are indicated by stacked dots, and similar residues are indicated by single dots.
aspartic acids were conserved upon alignment of yeast Spc3p to its canine, chicken, Caenorhabditis elegans, and Schizosaccharomyces pombe counterparts (Fig. 2B) (21).

Mutations that alter amino acids critical for signal peptide activity are lethal to yeast cells (18, 19). A cell growth assay, based on the plasmid shuffle technique (35), was therefore employed to determine which of the amino acids was important for cell viability. In this assay, yeast cells bearing a chromosomal disruption of an essential signal peptide gene and a plasmid bearing both a wild-type signal peptide gene and the \textit{URA3} gene were used. The \textit{URA3} gene is toxic to yeast cells incubated in the presence of 5-fluoroorotic acid (36); however, by introducing a plasmid bearing a mutated form of the signal peptide gene and a marker other than \textit{URA3}, cell growth in the presence of 5-fluoroorotic acid indicates that the \textit{URA3}-based plasmid has been cured and that the encoded protein is functional.

Mutations altering the conserved amino acids in Sec11p were constructed by site-directed mutagenesis and introduced into a \textit{TRP1}-based plasmid. This series of plasmids and the control vector lacking the \textit{SEC11} gene were transformed into yeast strain CMY710 (\textit{sec11}pCM112 (\textit{SEC11} \textit{URA3}). Cells were then subjected to plasmid shuffling. Mutations that alter amino acids Ser-44, His-83, Asp-103, and Asp-109 were found to be lethal (Table III), whereas mutations altering the conserved amino acids Ser-42 and Lys-101 were nonlethal. As expected, the vector control that lacked \textit{SEC11} was unable to support growth of strain CMY710 (\textit{sec11}). To determine whether the amino acids conserved in Spc3p were essential, a series of \textit{TRP1}-based plasmids carrying the appropriate \textit{spc3} mutations and the vector control were transformed into strain HFT405 (\textit{spc3}pHF331 (\textit{URA3} \textit{SPC3}), and transformed cells were subjected to plasmid shuffling. Results from monitoring growth of cells bearing each of these mutations indicated that the conserved amino acids Lys-91, Asp-115, and Asp-109 in Sec11p were nonessential (Table IV).

The fact that the conserved lysines in Sec11p and Spc3p were nonessential (Tables III and IV) suggests that a fundamental difference may exist in the catalytic mechanism of ER signal peptide catalytic dyad. We therefore constructed additional mutations in the \textit{SEC11} and \textit{SPC3} genes, paying particular attention to lysine residues that were conserved in only a subset of the sequences presented in Fig. 2. A and B. In the \textit{SEC11} gene, mutations altering Lys-122, Lys-124, Lys-148, Asp-53, Ser-33, and Ser-35 were found to be nonlethal (Table III). In the \textit{SPC3} gene, mutations affecting Lys-106, Lys-116, Lys-134, Lys-144, Lys-148, Lys-181, Lys-183, and Ser-103 were nonlethal (Table IV). Most of the nonlethal mutations listed in Tables III and IV produced no detectable growth defect. The only exception was the mutations altering Ser-42 of Sec11p. The S42T mutation inhibited cell growth ~10-fold, whereas the S42A mutation inhibited the growth rate of mutant cells ~20-fold as judged by colony size on agar plates at 30 °C. None of the mutants tested exhibited a temperature-sensitive or cold-sensitive phenotypic at 37 and 18 °C.

**Conserved Serine, Histidine, and Aspartic Acid Residues Are Essential for Signal Peptidase Activity by Sec11p**—We next monitored signal peptide activity in mutant cells using an in vivo assay. Because the temperature-sensitive \textit{sec11} mutant inhibits signal peptidase activity almost completely at the nonpermissive temperature (18, 23), the \textit{sec11} mutant serves as a suitable host to assess enzyme function in the presence of the mutations constructed by site-directed mutagenesis. Before using this mutant, we chose to sequence the \textit{sec11}–7 mutation. This mutation was found to produce a single amino acid change, Gly to Asp, at position 67 of Sec11p (Table III).

To monitor signal peptide activity, a series of plasmids bearing the above-described lethal mutations (see Table III) was introduced into strain HFY409 (\textit{sec11}–7). Cells were grown to early log phase at the permissive temperature, incubated at the nonpermissive temperature (37 °C) for 75 min, and pulse-labeled for 10 min. Proteins were precipitated from cells extracts using anti-Kar2p antibodies. Kar2p, an ER lumenal heat shock protein, provided a simple assay for signal peptidase activity because its precursor, preKar2p, was distinguishable from Kar2p on sizing gels (37). As can be seen in Fig. 3, little or no mature Kar2p was present in the \textit{sec11}–7 mutant control at the nonpermissive temperature, whereas \textit{sec11} cells carrying the wild-type \textit{SEC11} gene exhibited processing of preKar2p. In contrast, \textit{sec11} mutations S44A, H83K, H83F, D103E, D103N, K106S, and D109N inhibited the conversion of preKar2p to Kar2p.

**The Stability of Sec11p Is Affected by Some Amino Acid Substitutions at the His-83, Asp-103, and Asp-109 Positions**—Having identified a number of new \textit{sec11} mutations that abolish signal peptidase activity, we next asked whether these mutations affect the stability of Sec11p, as a mutation may alter an amino acid important for folding of the enzyme (and stability in yeast cells) but not its catalytic site. Sec11p was epitope-tagged immediately after its N-terminal methionine using the FLAG epitope and shown to function in a \textit{sec11} mutant. However, our initial attempts to detect this protein above background proteins using Western blotting were unsuccessful, even though enriched ER membranes were used. We therefore employed an approach based on the immunoprecipitation of FLAG-tagged Sec11p followed by Western blotting with anti-FLAG antibodies (“Experimental Procedures”). Strain CMY710 (\textit{sec11}pCM112 (\textit{SEC11})) was transformed with plasmids bearing wild-type \textit{SEC11} or the above-described mutations that were lethal to yeast cells (see Table III). The plasmids used allow for overproduction of FLAG-tagged Sec11p because gene expression is under control of a strong promoter (\textit{ADH1}) (31), and each plasmid contains a 2-μm (high copy) origin of replication. We did not, however, determine the extent of overexpression, and we relied on endogenous levels of Spc3p.
Fig. 2. Alignment of Sec11p and Spc3p to their homologs. Alignment of Sec11p (S. cerevisiae), CFSPC21 (canine), CFSPC18 (canine), and BsSipW (B. subtilis) (A) and Spc3p (S. cerevisiae), GGSPC22/23 (avian), CESPC22/23 (C. elegans), and SPSPC22/23 (S. pombe) (B) were performed using the algorithm contained in the MacVector software from IBI (New Haven, CT) (19). Conserved residues are indicated using bold letters. Arrows point to amino acids that were changed as listed in Tables III and IV.
for complexing to Sec11p, not overexpressed levels.

ER membranes from 1000 A_{go} units of cell was used for each lane of an SDS-PAGE gel, and because the same anti-FLAG antibody was used for the immunoprecipitation and Western blot analyses, the light chain of the anti-FLAG antibody preparation was present in this analysis (Fig. 4). In cells bearing FLAG-tagged Sec11p (see lane denoted wild-type), two forms of the protein were found, one exhibiting an apparent molecular mass of 20 kDa (labeled as Sec11p) and another of 23 kDa (labeled with *). The 20- and 23-kDa species were absent from cells lacking FLAG-tagged Sec11p (see Fig. 4; lane denoted control). The 20-kDa species had the size expected of FLAG-tagged Sec11p. The 23-kDa form was probably ER-glycosylated Sec11p, as its sequence contains one Asn-linked glycosylation site (18). Indeed, two forms of Sec11p were seen previously in ER signal peptidase purified from yeast cells (22).

We observed the absence of Sec11p in yeast cells carrying the H83A, H83K, D103E, and D109N mutations (Fig. 4). In contrast, mutations S44A, H83F, D103N, and D109E did not have a dramatic effect on the steady state levels of Sec11p. One interpretation of the fact that the H83A, H83K, D103E, and D109N mutations caused a loss of detectable Sec11p is that His-83, Asp-103, and/or Asp-109 are important structurally but not catalytically, and therefore specific alterations of these amino acids inhibit the normal folding of Sec11p (causing enzyme inactivity as indicated in Fig. 3) without affecting Sec11p stability or (ii) His-83, Asp-103, and/or Asp-109 are important catalytically; however, specific alterations of these residues can lead to protein instability.

**Binding of Inactive Sec11p Mutant Proteins to Spc3p**—Because Sec11p and Spc3p associate with each other in a complex, a more direct assay of Sec11p structural integrity was to determine whether Sec11p communoprecipitated with Spc3p. To this end, we devised a method to monitor the association of HA-tagged Spc3p with FLAG-tagged Sec11p. Sec3p was tagged immediately after its N-terminal methionine, and the tagged protein was shown to function in a Δspx3 mutant. For determinations of binding of FLAG-tagged Sec11p to HA-tagged Spc3p, plasmids pCV101 (SEC11-FLAG TRP1) and pCV102 (SPC3-FLAG TRP1) were introduced into cells of strain CVY1 (wild-type). The SEC11 and SPC3 genes carried by these high copy (2 μm) plasmids were under control of a strong (ADH1) promoter and, thus, overexpressed in yeast cells. ER mem-

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Catalytic Difference among Signal Peptidases

In this study, we have taken a site-directed mutagenesis approach aimed at identifying amino acids that may serve catalytic roles within the two essential subunits, Sec11p and Spc3p, of ER signal peptidase from the yeast *S. cerevisiae*. The following conclusions are presented. (i) Among the conserved serines, histidines, lysines, and aspartic acids in Sec11p and Spc3p, Sec11p contains one serine, one histidine, and two aspartic acids necessary for signal peptidase activity, and (ii) none of the lysines changed in Sec11p and Spc3p are essential for enzyme function. The absence of an apparent catalytic lysine suggests that, although related functionally, the mechanism of proteolysis by ER signal peptidase differs fundamentally from that of *E. coli* leader peptidase, which contains a serine/lysine catalytic dyad (13). Our data suggest that Ser-44, His-83, Asp-103, and Asp-109 of Sec11p are candidate catalytic residues, because certain amino acid substitutions at these positions abolish signal peptidase activity without inhibiting the overall structure of Sec11p as judged by the stability of Sec11p and the binding of Sec11p to Spc3p. A subset of changes at the His-83, Asp-103, and Asp-109 positions interfere, however, with the stability of Sec11p (Fig. 4). This finding is unexpected, as conservative changes of catalytic site amino acids do not typically result in protein instability (7, 9). The instability detected here suggests that the folding and/or assembly of Sec11p may be unusually sensitive to alterations of at least a subset of catalytic site residues. As with any site-directed mutagenesis approach, however, we cannot eliminate the possibility that the structure of Sec11p has been changed locally by the subset of mutations that inhibit Sec11p function without affecting its stability or its binding to Spc3p. Further analyses will be necessary to establish the identity of the catalytic site amino acids in ER signal peptidase, especially because it is unlikely that both Asp-103 and Asp-109 serve catalytic roles.

Ser-44 and His-83 of Sec11p have been aligned previously to serine/lysine dyad of leader peptidase (16). These similarities suggest a commonality in the positioning of catalytically important amino acids and may portend a structural similarity in Sec11p and leader peptidase at or near their catalytic sites. The two essential aspartic acids of Sec11p (Asp-103 and Asp-109) align to Asp-273 and Asp-280, respectively, which are positioned near the catalytic site of leader peptidase (13). Indeed, Asp-273 and Asp-280 seem to play important structural roles in leader peptidase (7, 13). Interestingly, sequence alignments suggest that this arginine residue has been replaced by Ser-111 in Sec11p (Fig. 1), which thus leaves open the possibility that Asp-109 may serve a catalytic role.

**DISCUSSION**

The sequence of the luminal domain of Sec3p (amino acids 36–184) was aligned to the sequence of leader peptidase that resides between Box II and Box III (amino acids 154–271) (see Fig. 1 for a description of Boxes II & III) using the computer-based analysis described previously (19). Gaps in these sequences are indicated by dashes. Identical residues are indicated by stacked dots, and similar residues are indicated by single dots.

**FIG. 5. Coimmunoprecipitation of Sec11p and Spc3p.** Strains CVY1/pCV102 (SPC3) (control), CVY1/pCV102/pCV101 (SEC11-FLAG) (wild type), and CVY1 harboring pCV102 and a derivative of pCV101 containing each of the sec11 mutations shown (S44A, H83F, D103N, and D109E) were grown to log phase. Enriched ER membranes from 2000 equivalents of cells was represented on a single lane of an SDS-PAGE gel.

**FIG. 6. Alignment of a portion of leader peptidase to the luminal domain of Spc3p.** The sequence of the luminal domain of Sec3p (amino acids 36–184) was aligned to the sequence of leader peptidase that resides between Box II and Box III (amino acids 154–271) (see Fig. 1 for a description of Boxes II & III) using the computer-based analysis described previously (19). Gaps in these sequences are indicated by dashes. Identical residues are indicated by stacked dots, and similar residues are indicated by single dots.

| Leader peptidase | Sec3p |
|------------------|-------|
| Ser-44 and His-83 | Ser-111 |
| Asp-103 and Asp-109 | Asp-273 and Asp-280 |

**FIG. 5. Coimmunoprecipitation of Sec11p and Spc3p.** Strains CVY1/pCV102 (SPC3) (control), CVY1/pCV102/pCV101 (SEC11-FLAG) (wild type), and CVY1 harboring pCV102 and a derivative of pCV101 containing each of the sec11 mutations shown (S44A, H83F, D103N, and D109E) were grown to log phase. Enriched ER membranes from 2000 equivalents of cells was represented on a single lane of an SDS-PAGE gel.

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| Asp-103 and Asp-109 | Asp-273 and Asp-280 |

**FIG. 5. Coimmunoprecipitation of Sec11p and Spc3p.** Strains CVY1/pCV102 (SPC3) (control), CVY1/pCV102/pCV101 (SEC11-FLAG) (wild type), and CVY1 harboring pCV102 and a derivative of pCV101 containing each of the sec11 mutations shown (S44A, H83F, D103N, and D109E) were grown to log phase. Enriched ER membranes from 2000 equivalents of cells was represented on a single lane of an SDS-PAGE gel. The use of a rat-derived anti-HA antibody was needed to eliminate the mouse-derived anti-FLAG light chain band, which was found to obscure Spc3p on the Western blot. As a control, strain CVY1/pCV102 (SPC3-FLAG) that contains Spc3p-FLAG but lacks Sec11p-FLAG was treated identically. Derivatives of pCV101 bearing the S44A, H83F, D103N, and D109E mutations, which did not dramatically affect the steady-state levels of Sec11p (Fig. 4), were also introduced into cells of strain CVY1/pCV102 (SPC3-FLAG). All of the material from 2000 equivalents of cells was represented on a single lane of an SDS-PAGE gel.

In the Western blot probed with anti-HA antibodies, two major forms of Spc3p co-immunoprecipitated with FLAG-tagged Sec11p (Fig. 5, see lane denoted wild type). The 27-kDa form was the size expected for HA-tagged Spc3p. The 35-kDa form (denoted by *) probably represents Spc3p that had been transported out of the ER (because of its overproduction) and hyper-glycosylated by Golgi-derived enzymes. In the lane denoted control, the 27- and 35-kDa species were absent, indicating that, as expected, these proteins were not precipitated by anti-FLAG antibodies from cells lacking FLAG-tagged Sec11p yet containing HA-tagged Spc3p. In contrast, Sec11p containing the S44A, H83F, D103N, or D109E mutation was found to coimmunoprecipitate with both the 27- and 35-kDa species, although variable amounts of the 35-kDa form were detected in cells containing the D103N mutation. The data thus reveal that the S44A, H83F, D103N, and D109E mutations do not alter the overall structure of Sec11p, determined through the binding of mutant Sec11p to wild-type Spc3p.
Catalytic Difference among Signal Peptidases

We have observed here that some changes at the Asp-103 and Asp-109 positions abolish Sec11p function but not its binding to Spc3p. This suggests that one of these aspartic acids (along with Ser-44 and His-83) may be important catalytically, a scenario that is consistent with the more typical serine proteases that contain a catalytic triad consisting of serine, histidine, and aspartic acid (38). That one of these aspartic acid residues may be catalytic suggests that changing the other essential aspartic acid residue alters only a local structure of Sec11p. Alternatively, both of these aspartic acid residues may be important structurally, leading to the idea that Sec11p uses a serine/histidine dyad for catalysis, such as that found in an esterase from Streptomyces scabies (39). Because a subset of amino acid changes at the His-83 position results in instability of Sec11p, we cannot rule out the possibility that His-83 is important not for catalysis but for the structural integrity of the protein. However, the fact that His-83 has been aligned previously to the catalytic lysine of leader peptidase (16, 17) and that substitution of His-83 with a phenylalanine residue leads to a Sec11p that is stable in yeast cells, binds to Spc3p, and lacks detectable enzymatic activity (Figs. 3, 4 and 5) argues for the importance of histidine in catalysis.

The recently solved crystal structure of leader peptidase (13) reveals that this enzyme contains two distinct domains, one possessing both amino acids of the serine/lysine dyad, and a second domain, which we refer to here as the noncatalytic domain. From sequence alignments, we suggested previously that Sec11p and Spc3p may correspond to distinct regions of leader peptidase (19). The strongest homology between the sequences of Sec11p and leader peptidase resides within Boxes I, II, and III (Fig. 1). However, a notable difference exists in the number of amino acids located between Box II and Box III. In Sec11p, this intervening stretch consists of 10 amino acids, whereas the corresponding region of leader peptidase contains 118 amino acids. Most of this 118-amino acid stretch is placed within the noncatalytic domain of leader peptidase (13). Moreover, a portion of these 118 amino acids exhibits detectable homology to the luminal domain of Spc3p (Fig. 6) (refer also to Ref. 19). These similarities and the finding of probable catalytic amino acids in Sec11p thus raise the possibility that the luminal portions of Sec11p and Spc3p correspond to the catalytic and noncatalytic domains, respectively, of leader peptidase. Considering the apparent catalytic differences between Sec11p and leader peptidase, this model suggests an unusual evolutionary history among members of the type I signal peptidase family.

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REFERENCES

1. von Heijne, G. (1983) Eur. J. Biochem. 133, 17–21
2. Dalley, R. E., Lively, M. O., Bron, S., and van Dijl, J. M. (1997) Protein Sci. 6, 1129–1138
3. Talmodge, K., Kaufman, J., and Gilbert, W. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3988–3992
4. Lingappa, V. R., Chaidiez, J., Yost, C. S., and Heddgath, J. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 456–460
5. Black, M. T. (1993) J. Bacterial. 175, 4957–4961
6. Paetzel, M., Rynsbad, N. C. J., Tschampa, W. R., Casareno, R., Bullinger, P. R., and Dalley, R. E. (1997) J. Biol. Chem. 272, 9994–10005
7. Sung, M., and Dalley, R. E. (1992) J. Biol. Chem. 267, 13154–13159
8. Tschampa, W. R., Sung, M., Delgado-Partin, V. M., and Dalley, R. E. (1993) J. Biol. Chem. 268, 27349–27354
9. van Dijl, J. M., de Jong, A., Venema, G., and Bron, S. (1995) J. Biol. Chem. 270, 3611–3616
10. Peat, T. S., Frank, E. G., McDonald, J. P., Levine, A. S., Woodgate, R., and Veid, M. T. (1993) Proc. Natl. Acad. Sci. U. S. A. 84, 3987–3991
11. Nunnari, J., Fox, T. D., and Walter, P. (1993) Science 262, 1997–2004
12. Paetzel, M., Dalley, R. E., and Rynsbad, N. C. J. (1998) Nature 396, 186–190
13. Tjalsma, H., Bolhuis, A., van Roosmalen, M. L., Wiegert, T., Schumann, W., Broekhuizen, C. P., Quan, W. X., Venema, G., Bron, S., and van Dijl, J. M. (1998) Genesis 12, 2318–2331
14. Tjalsma, H., Noback, M. A., Bron, S., Venema, G., Yamane, K., and van Dijl, J. M. (1997) J. Biol. Chem. 272, 25883–25992
15. Dalley, R. E., and von Heijne, G. (1992) Trends Biochem. Sci. 17, 474–478
16. van Dijl, J. M., de Jong, A., Veelmaapera, J., Venema, G., and Bron, S. (1992) EMBO J. 11, 2819–2828
17. Bolhuis, A., Deshaies, R. J., and Schekman, R. W. (1988) J. Cell Biol. 106, 1035–1042
18. Fang, H., Mullins, C., and Green, N. (1997) J. Biol. Chem. 272, 13152–13158
19. Fang, H., Panzer, S., Mullins, C., Hartmann, E., and Green, N. (1996) J. Biol. Chem. 271, 16460–16465
20. Meyer, H. A., and Hartmann, E. (1997) J. Biol. Chem. 272, 13159–13164
21. Mullins, C., Meyer, H. A., Hartmann, E., Green, N., and Fang, H. (1996) J. Biol. Chem. 271, 29078–29084
22. Mullins, C., Ly, C., Campbell, A., Fang, H., and Green, N. (1995) J. Biol. Chem. 270, 71319–71347
23. Boeke, J. D., Deshaies, R. J., and Schekman, R. W. (1987) in Current Protocols in Molecular Biology, Vol. 2, pp. 13.0.1–13.14.17, John Wiley & Sons, Inc., New York
24. Green, N., and Walter, P. (1992) Mol. Cell. Biol. 12, 276–282
25. Hansen, W., Garcia, P. D., and Walter, P. (1986) Cell 45, 397–406
26. Sandersson, C. M., and Meyer, D. J. (1991) J. Biol. Chem. 266, 13423–13430
27. Wilson, I. A., Niman, H. L., Houghten, R. A., Cheresson, A. R., Connolly, M. L., and Lerner, R. A. (1984) Cell 37, 767–778
28. Sikorski, R. S., and Hieter, P. (1989) Genetics 122, 19–27
29. Bennett, J. L., and Hall, B. D. (1982) J. Biol. Chem. 257, 3018–3025
30. Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H., and Hieter, P. (1992) Gene (Amst.) 110, 119–122
31. Hopk., T. P., Pritchett, K. S., Price, V. L., Libby, R. T., March, C. J., Cerretti, D. P., Urdal, D. L., and Conlon, P. J. (1988) Biotechnology 6, 1204–1210
32. Shelnos, G. S., and Blobel, G. (1990) J. Biol. Chem. 265, 9512–9519
33. Sikorski, R. S., and Boeke, J. D. (1991) Methods Enzymol. 194, 302–318
34. Boeke, J. D., Trueheart, J., Natsoulis, G., and Fink, G. R. (1987) Methods Enzymol. 154, 164–175
35. Rose, M. D., Miura, L. M., and Vogel, J. P. (1989) Cell 57, 1211–1221
36. Carter, P., and Wells, J. A. (1988) Nature 332, 564–568
37. Wei, Y., Schottel, J. L., Derewenda, U., Swenson, L., Patkar, S., and Derewenda, Z. S. (1995) Nat. Struct. Biol. 2, 218–223