The Yeast SPC22/23 Homolog Spc3p Is Essential for Signal Peptidase Activity*  

(Received for publication, January 13, 1997, and in revised form, March 17, 1997)  

Hellmuth-A. Meyer and Enno Hartmann‡  
From the Max-Delbrück-Center for Molecular Medicine, Robert-Rössele-Strasse 10, 13125 Berlin-Buch, Germany

Protein translocation into the endoplasmic reticulum (ER) is triggered by signal sequences (1) that direct precursor proteins to the translocation sites on the ER membrane (for review, see Ref 2). The trimeric Sec61 complex, the central constituent of these translocation sites (3), is involved in various functions directly linked to the transport of the nascent chain. Its α-subunit is a major part of the protein conducting channel, and during the co-translational mode of translocation, the majority of ribosome binding sites are provided by the Sec61 complex (4). In addition, the Sec61 complex is reportedly involved in a second signal sequence recognition event that takes place in the membrane during the protein translocation (6). Moreover, recent electronmicroscopic data demonstrate that the Sec61 complex forms pore-like structures in the ER membrane (7).

In addition to trimeric Sec61 complexes, yeast contains a heptameric Sec complex consisting of the subunits of the Sec61p complex (Sec61p, Shb1p, and Sss1p) as well as of the subunits of the Sec62/63 subcomplex (Sec62p, Sec63p, Sec71p, and Sec72p) (8, 9). This Sec complex is sufficient to translocate proteins post-translationally into reconstituted proteoliposomes in the presence of the luminal chaperone Kar72p (9).

Two other protein complexes that are part of the translocation site modify the nascent chain while being translocated, the oligosaccharyl transferase complex and the signal peptidase complex (SPC). The SPC cleaves the signal peptides of most secretory and many membrane proteins as soon as the luminal domain of the translocating polypeptide is large enough to expose its cleavage site to the enzyme (10). Mammalian signal peptidase has been purified from dog pancreas microsomes as a complex of five different polypeptide chains (11). The cDNAs of all five subunits have been cloned and sequenced (12–15). Remarkable differences exist between the topologies of these proteins within the ER membrane. Three of them, SPC18, SPC21, and the glycoprotein SPC22/23 are single-spanning membrane proteins with their amino termini facing the cytosol. The major part of these proteins is located within the lumen of the ER, and they all contain a second only moderately hydrophobic sequence close to their carboxyl termini (11, 16). SPC18 and SPC21 show high homology to each other (12). Moreover, their sequences are related to leader peptidases (17), the enzyme responsible for signal sequence cleavage during translocation of proteins across the plasma membrane of bacteria (18). Therefore, these polypeptides may function as catalytic subunits. The functions of the SPC22/23, however, remains unclear.

In contrast, SPC12 and SPC25 contain two membrane spanning segments with their amino and carboxyl termini facing the cytosol and almost no residues in the lumen of the ER. Therefore, it has been speculated, that SPC12 and SPC25 are involved in processes other than substrate binding or the actual enzymatic reaction. This assumption was supported by the fact that an active signal peptidase that comprises only an SPC18 homolog and an SPC22/23 homolog could be purified from hen oviduct (19, 20).

In yeast, the SPC contains four subunits (21, 22). Three of them have been characterized to date. The Sec11p is homologous to SPC18 and SPC21 (23) as well as to leader peptidases of bacteria (17, 24). SEC11 is essential for cell viability. The essential nature of Sec11p is seen when haploid cells containing a temperature-sensitive allele of SEC11 are shifted to non-permissive temperature. These cells rapidly accumulate precursors of secretory proteins and stop growing (25). Spc1p and Spc2p, the other two subunits that were analyzed until now, are homologs of the canine SPC12 and SPC25, respectively. Both yeast proteins are nonessential for cell viability and signal peptide cleavage under normal growth conditions (22, 26). However, they modulate the activity of yeast SPC as overproduction of Spc1p suppresses the sec11 temperature-sensitive phenotype and depletion of Spc2p results in a defect in signal transduction of Spc1p. The Sec12p is homologous to SPC12 and SPC25 (27, 28).

In eukaryotic cells signal sequences of secretory and membrane proteins are cleaved by the signal peptidase complex during their transport into the lumen of the endoplasmic reticulum. The signal peptidase complex in yeast consists of four subunits. To date, three of these subunits have been functionally characterized. One of them, the Sec11p, is essential for viability of yeast cells. It shows significant homology to the mammalian SPC18 and SPC21 as well as to bacterial leader peptidases. Two other subunits, Spc1p and Spc2p, have been shown to be homologous to mammalian SPC12 and SPC25, respectively, and are not essential for protein translocation or signal peptide cleavage.

We have purified and analyzed the fourth subunit of yeast signal peptidase, Spc3p. The protein is essential for viability of yeast cells. Depletion of SPC3 leads to accumulation of precursors of secretory proteins in vivo and to the loss of the signal peptidase activity in vitro. Therefore, in contrast to the bacterial leader peptidases, yeast signal peptidase requires a second subunit for its function.

Protein translocation into the endoplasmic reticulum (ER) is triggered by signal sequences (1) that direct precursor proteins to the translocation sites on the ER membrane (for review, see Ref 2). The trimeric Sec61 complex, the central constituent of these translocation sites (3), is involved in various functions directly linked to the transport of the nascent chain. Its α-subunit is a major part of the protein conducting channel, and during the co-translational mode of translocation, the majority of ribosome binding sites are provided by the Sec61 complex (4, 5). In addition, the Sec61 complex is reportedly involved in a second signal sequence recognition event that takes place in the membrane during the protein translocation (6). Moreover, recent electronmicroscopic data demonstrate that the Sec61 complex forms pore-like structures in the ER membrane (7).

In addition to trimeric Sec61 complexes, yeast contains a heptameric Sec complex consisting of the subunits of the Sec61 complex (Sec61p, Shb1p, and Sss1p) as well as of the subunits of the Sec62/63 subcomplex (Sec62p, Sec63p, Sec71p, and Sec72p) (8, 9). This Sec complex is sufficient to translocate proteins post-translationally into reconstituted proteoliposomes in the presence of the luminal chaperone Kar72p (9).

Two other protein complexes that are part of the translocation site modify the nascent chain while being translocated, the oligosaccharyl transferase complex and the signal peptidase complex (SPC). The SPC cleaves the signal peptides of most secretory and many membrane proteins as soon as the luminal domain of the translocating polypeptide is large enough to expose its cleavage site to the enzyme (10). Mammalian signal peptidase has been purified from dog pancreas microsomes as a complex of five different polypeptide chains (11). The cDNAs of all five subunits have been cloned and sequenced (12–15). Remarkable differences exist between the topologies of these proteins within the ER membrane. Three of them, SPC18, SPC21, and the glycoprotein SPC22/23 are single-spanning membrane proteins with their amino termini facing the cytosol. The major part of these proteins is located within the lumen of the ER, and they all contain a second only moderately hydrophobic sequence close to their carboxyl termini (11, 16). SPC18 and SPC21 show high homology to each other (12). Moreover, their sequences are related to leader peptidases (17), the enzyme responsible for signal sequence cleavage during translocation of proteins across the plasma membrane of bacteria (18). Therefore, these polypeptides may function as catalytic subunits. The functions of the SPC22/23, however, remains unclear.

In contrast, SPC12 and SPC25 contain two membrane spanning segments with their amino and carboxyl termini facing the cytosol and almost no residues in the lumen of the ER. Therefore, it has been speculated, that SPC12 and SPC25 are involved in processes other than substrate binding or the actual enzymatic reaction. This assumption was supported by the fact that an active signal peptidase that comprises only an SPC18 homolog and an SPC22/23 homolog could be purified from hen oviduct (19, 20).

In yeast, the SPC contains four subunits (21, 22). Three of them have been characterized to date. The Sec11p is homologous to SPC18 and SPC21 (23) as well as to leader peptidases of bacteria (17, 24). SEC11 is essential for cell viability. The essential nature of Sec11p is seen when haploid cells containing a temperature-sensitive allele of SEC11 are shifted to non-permissive temperature. These cells rapidly accumulate precursors of secretory proteins and stop growing (25). Spc1p and Spc2p, the other two subunits that were analyzed until now, are homologs of the canine SPC12 and SPC25, respectively. Both yeast proteins are nonessential for cell viability and signal peptide cleavage under normal growth conditions (22, 26). However, they modulate the activity of yeast SPC as overproduction of Spc1p suppresses the sec11 temperature-sensitive phenotype and depletion of Spc2p results in a defect in signal transduction of Spc1p. The Sec12p is homologous to SPC12 and SPC25 (27, 28).

In eukaryotic cells signal sequences of secretory and membrane proteins are cleaved by the signal peptidase complex during their transport into the lumen of the endoplasmic reticulum. The signal peptidase complex in yeast consists of four subunits. To date, three of these subunits have been functionally characterized. One of them, the Sec11p, is essential for viability of yeast cells. It shows significant homology to the mammalian SPC18 and SPC21 as well as to bacterial leader peptidases. Two other subunits, Spc1p and Spc2p, have been shown to be homologous to mammalian SPC12 and SPC25, respectively, and are not essential for protein translocation or signal peptide cleavage.

We have purified and analyzed the fourth subunit of yeast signal peptidase, Spc3p. The protein is essential for viability of yeast cells. Depletion of SPC3 leads to accumulation of precursors of secretory proteins in vivo and to the loss of the signal peptidase activity in vitro. Therefore, in contrast to the bacterial leader peptidases, yeast signal peptidase requires a second subunit for its function.
peptidase activity in cells incubated at high temperature.

In this paper, we analyze the function of the fourth subunit of yeast SPC, the Spc3p, which is revealed as the homolog of the canine SPC22/23. SPC is essential for cell viability. Its depletion leads to accumulation of precursors of secretory proteins in vivo and to the loss of the signal peptidase activity in vitro. Overexpression of Sec11p does not suppress this phenotype. Therefore, in contrast to bacterial leader peptidases, the yeast enzyme requires a second subunit for its function.

**EXPERIMENTAL PROCEDURES**

**General Methods**—Media and cell growth conditions have been described elsewhere (9, 27). Specific modifications to these procedures are described elsewhere (9, 27). Specific modifications to these procedures are described elsewhere (9, 27).

**Sequence Analysis**—Sequence analysis was performed as described previously (26). The identity of the Spc3p was confirmed by Edman degradation of the purified protein.

**N-Glycosidase F Assay**—15 μl of purified signal peptide was de-natured for 5 min at 62 °C in 1% SDS, 85 μl of buffer F (2 ml Hepes, pH 7.6, 12 ml EDTA, 1% Triton X-100, 4 ml β-mercaptoethanol) and 1 unit of N-glycosidase F (Boehringer Mannheim) was added. The sample was incubated at 37 °C for 8 h, separated by SDS-PAGE, and analyzed by immunoblotting.

**In Vivo Assay for Signal Peptidase Activity**—Aliquots of cells growing in SD minimal medium were removed at distinct time points and were labeled with 200 μCi of [35S]methionine for 10 min at 30 °C (pulse). After incubation, the cells were stopped with 20 mM sodium azide, washed, and homogenized with glass beads in 100 μl of lysis buffer (1% SDS, 50 mM Tris, pH 7.5, 10 μM leupeptin, 5 μM chymostatin) using a Vortex mixer. The homogenate was diluted with 4 volumes of IP dilution buffer (1.25% Triton X-100, 190 mM NaCl, 60 mM Tris, pH 7.4, 6 mM EDTA, 10 μM leupeptin, and 5 μM chymostatin), and labeled proteins were precipitated with anti-Kar2p antibodies. The immunoprecipitated material was analyzed by SDS-PAGE and fluorography. The ratio of the radioactivity in the preKar2p and Kar2p bands was determined by a phosphoimager (Fuji Bas 2000).

**In Vitro Assay for Signal Peptidase Activity**—The assay was performed according to YaDeau et al. (21) with the following modifications. Yeast cells of induced cultures were shifted to glucose (YPF or SD minimal medium) and were grown for various times. After harvesting the cells, membranes were isolated. To obtain comparable amounts of membranes, the samples were normalized to the same amounts of Sec62p. Digitonin extract corresponding to 10 eq (membrane equivalent, see Ref. 3) membranes were prepared, and 210 μl of buffer was added. The final conditions of the assay were 50 mM triethanolamine, pH 8.0, 1.1 mM diithiothreitol, 1 mg/ml phenylmethylsulfonyl fluoride, 0.4 mg/ml phosphatidylcholine, 0.01% SDS (from the SDS treatment of the prep-o-factor), 120 mM KCN, and 0.5% digitonin. The added prep-o-factor was synthesized in a reticu-lose system in the presence of [35S]methionine. After 2 h at 25 °C, the samples were precipitated and separated by SDS-PAGE and then analyzed by a phosphoimager (Fuji Bas 2000).

**Antibodies, Immunoblots, and Immunoprecipitation**—Antibodies against the amino termini of Sec11p and Spc1p as well as the antibody against the carboxyl terminus of Spc2p have been described earlier (26). Antibodies against the amino termini of Sec11p and Spc1p as well as the antibody against the carboxyl terminus of Spc2p have been described earlier (26). Anti-Spc2p antibodies were raised by the same methods using a recombinant human protein as antigen. Immunoblot preparations were performed as described earlier (9) using the ECL system (Amersham Corp.).

For immunoprecipitation, digitonin extracts corresponding to 50 eq of membranes were used and diluted with LD buffer (9) to a final concentration of 1.5% digitonin, 100 mM KAc, 50 mM Hepes, pH 7.6, 2 mM MgAc, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 5 μM chymostatin. 5 to 10 μl of purified antibodies were added and incubated overnight at 4 °C in a rotary shaker. After washing the beads, the proteins were eluted with 50 μl of SDS-sample buffer.
RESULTS

A Protein with Homology to Canine SPC22/23 Is Part of the Yeast Signal Peptidase Complex—To identify a possible yeast homolog of canine SPC22/23, we searched the GenBank™ data base for similar proteins. In addition to the already described chicken gp23 (20), we identified open reading frames in C. elegans, S. pombe, and S. cerevisiae (Fig. 1) as well as expressed sequence tags of human, mouse, Drosophila melanogaster, Brugia malayi, Schistosoma mansoni, and Arabidopsis thaliana with significant homology to SPC22/23. Analysis of the yeast gene, hereafter designated as SPC3, with different computer programs predicted that it codes for a glycoprotein with 26% identity to canine SPC22/23. Spc3p has a membrane anchor close to its amino terminus like the other proteins of the family (Fig. 1). Because Spc3p is a glycoprotein (see below) that has potential sites for N-linked glycosylation only in the carboxyl-terminal part (Fig. 1), it is very likely that Spc3p has the same membrane topology as canine SPC22/23.

To determine whether Spc3p is part of the yeast signal peptidase, we purified the SPC from S. cerevisiae as described earlier (26). Antibodies raised against the luminal part of Spc3p recognize a protein on Western blots that co-purifies with the already known yeast SPC subunits, Sec11p, Spc1p, and Spc2p (Fig. 2A). This immunoreactive band corresponds to a prominent protein in a fraction highly enriched in yeast SPC (Fig. 2B). To confirm its identity with Spc3p, the purified protein was subjected to Edman degradation. The obtained peptide sequence matches perfectly with the predicted protein sequence (Fig. 1).

To demonstrate the integrity of the complex by an independent method, we performed immunoprecipitation experiments with anti-Spc1p antibodies using yeast membranes solubilized in digitonin as starting material. The analysis of the precipitates by immunoblotting showed that under these conditions, Spc2p, Sec11p, and Spc3p are co-precipitated but the control protein Sec62p was not (Fig. 2C, lanes 1–3).

Spc3p contains two potential sites for N-linked glycosylation (see Fig. 1). To determine whether Spc3p is indeed a glycopro-
tetrade analysis of this strain revealed that the function of "Experimental Procedures." The yeast strain DF5 according to the protocol described under "Experimental Procedures." In different experiments, we cultivated the rescued strain in liquid culture and determined for various times and then analyzed for the occurrence of uncomplete digestion.

The wild-type (wt) membranes were isolated from the diploid strain DF5. Digitonin extract corresponding to about 20 eq membranes was used for prepro-protease, a homolog of bacterial leader peptidase) remains present in the samples (e.g. the mitochondrial inner membrane protease, a homolog of bacterial leader peptidase) appears to stabilize at a lower expression level. It should be noted that we never observed a complete loss of signal peptidase activity. Whether this is due to residual amounts of SPC that could not be detected by our antibodies or whether another protease with a substrate specificity similar to SPC was present in the samples (e.g. the mitochondrial inner membrane protease, a homolog of bacterial leader peptidase) remains open.

Phenotypes Caused by the Depletion of Spc3p Cannot Be Rescued by Sec11p Overexpression—Sec11p is known to be an essential yeast protein. Therefore, one may speculate that the loss of signal peptidase activity in response to the down-regulation of Spc3p is mainly due to the simultaneous loss of Sec11p. To test this, we tried to rescue the Δspc3 mutant by overexpression of Sec11p. SEC11 was put under control of the Met3 promoter and introduced into an HMY1 yeast strain, which contained the plasmid pH1 with SPC3 under control of a Gal10 promoter. As a control, the Met3Sec11 containing plasmid was also introduced into the Δspc3-deficient strain SEC11. The strain Δscp3 -pHM1 containing Sec11p decreased below the detection level. The content of Spc2p decreased also, but in contrast to the other subunits, the amount of Spc2p appears to stabilize at a lower expression level. It should be noted that we never observed a complete loss of signal peptidase activity. Whether this is due to residual amounts of SPC that could not be detected by our antibodies or whether another protease with a substrate specificity similar to SPC was present in the samples (e.g. the mitochondrial inner membrane protease, a homolog of bacterial leader peptidase) remains open.

FIG. 3. Effect of the Spc3p depletion on preprotein processing in vivo. A, growth curves of strain Δscp3 -pHM1 (pGal10SPC3) during Spc3p depletion. Cells were incubated at 30 °C in minimal medium containing 2% sucrose and 0.5% galactose, and a portion was later shifted to SD minimal medium to repress Spc3p expression. B, prepro-protein processing of strain Δscp3 -pHM1 during Spc3p depletion. Aliquots of cells growing in SD minimal medium were removed at 0, 4, 8, or 12 h after the shift and were labeled with [35S]methionine for 10 min. Cell extracts were prepared, and labeled proteins were precipitated with anti-Kar2p antibodies. The immunoprecipitated material was analyzed by SDS-PAGE and fluorography. "preKar2p" is the precursor of Kar2p.

FIG. 4. Effect of the Spc3p depletion on SPC stability and signal peptidase activity in vitro. The strain Δscp3 -pHM1 was preincubated in YPgal medium and then shifted to YPD medium. Aliquots of cells were removed at 0, 6, 9, 12, and 15 h after the shift, membranes were prepared, and samples were normalized by immunoblotting to about the same amount of Sec62p. The wild-type (wt) membranes were isolated from the diploid strain DF5. Digitonin extract corresponding to about 20 eq membranes was used for prepro-α-factor cleavage. After 3 h at 25 °C, the samples were precipitated and separated by SDS-PAGE and then analyzed by a phosphoimager. A, result of the cleavage assay. pp-α-f indicates the prepro-α-factor; p-α-f, indicates the pre-α-facto. B, relative amount of SPC subunits. The amount of the SPC subunits in the samples was monitored by immunoblotting with antibodies against Spc1p, Spc2p, Spc3p, and Sec11p.

To analyze the function of Spc3p in more detail, we then tested the effect of the "Experimental Procedures." As depicted in Fig. 2D, lane 1, a concanavalin A-binding protein co-migrates with Spc3p. To confirm this result and check whether both potential sites of Spc3p might be used, purified yeast SPC was treated with the N-glycosidase F and then analyzed by immunoblotting (Fig. 2D, lane 3). Only Spc3p, but not Spc1p showed a shift in mobility during SDS-PAGE compared with mock-treated membranes (Fig. 2D, lanes 2 and 3). The size of the mobility shift of about 5 kDa indicates that both glycosylation sites are likely to be used. The double band in lane 3 is probably a result of incomplete digestion.

Spc3p Is Essential for Cell Viability and Signal Peptidase Activity—Next we tested the effect of the in vivo depletion of Spc3p. First, we disrupted one allele of SPC3 in the diploid yeast strain DF5 according to the protocol described under "Experimental Procedures." Tetrad analysis of this strain revealed that the function of SPC3 is essential for cell viability. The cells could be rescued by introducing a plasmid that expresses Spc3p under control of the Gal10 promoter in the presence of galactose, confirming that the loss of SPC3 function is the reason for the observed phenotype.

To analyze the function of Spc3p in more detail, we then cultivated the rescued strain in liquid culture and determined its growth rate under conditions that either induce (presence of galactose) or repress (presence of glucose) the promoter (see "Experimental Procedures"). In different experiments, we found that the growth rate of the repressed culture starts to decrease after about 10 h (Fig. 3A).

In a second set of experiments, cells were grown in glucose for various times and then analyzed for the occurrence of unprocessed Kar2p after 10 min of labeling in vivo (Fig. 3B). After an 8-h repression of SPC3, precursors of Kar2p accumulated in the cells. As this strong defect in protein processing is preceding the decrease in growth rate, it is likely to be a direct effect of Spc3p depletion.

To test this assumption further, we analyzed cells harvested at different time points after shifting to glucose for signal peptidase activity using an in vitro assay (see "Experimental Procedures"). The amount of material used for the assay was calibrated by immunoblotting using anti-Sec62p-antibodies. Signal peptidase activity started decreasing after 6 h, a time point where repressed cells still have about the same growth rate as the control. After 15 h, signal peptidase activity of the repressed cells was about 10 times lower than that of DF5 control cells (Fig. 4A). In addition, the integrity of the yeast SPC during the Spc3p depletion was monitored by immunoblotting (Fig. 4B). Surprisingly, we found that, parallel to the loss of Spc3p also, the amount of Spc1p and Sec11p decreased below the detection level. The content of Spc2p decreased also, but in contrast to the other subunits, the amount of Spc2p appears to stabilize at a lower expression level. It should be noted that we never observed a complete loss of signal peptidase activity. Whether this is due to residual amounts of SPC that could not be detected by our antibodies or whether another protease with a substrate specificity similar to SPC was present in the samples (e.g. the mitochondrial inner membrane protease, a homolog of bacterial leader peptidase) remains open.

To test this assumption further, we analyzed cells harvested at different time points after shifting to glucose for signal peptidase activity using an in vitro assay (see "Experimental Procedures"). The amount of material used for the assay was calibrated by immunoblotting using anti-Sec62p-antibodies. Signal peptidase activity started decreasing after 6 h, a time point where repressed cells still have about the same growth rate as the control. After 15 h, signal peptidase activity of the repressed cells was about 10 times lower than that of DF5 control cells (Fig. 4A). In addition, the integrity of the yeast SPC during the Spc3p depletion was monitored by immunoblotting (Fig. 4B). Surprisingly, we found that, parallel to the loss of Spc3p also, the amount of Spc1p and Sec11p decreased below the detection level. The content of Spc2p decreased also, but in contrast to the other subunits, the amount of Spc2p appears to stabilize at a lower expression level. It should be noted that we never observed a complete loss of signal peptidase activity. Whether this is due to residual amounts of SPC that could not be detected by our antibodies or whether another protease with a substrate specificity similar to SPC was present in the samples (e.g. the mitochondrial inner membrane protease, a homolog of bacterial leader peptidase) remains open.

Phenotypes Caused by the Depletion of Spc3p Cannot Be Rescued by Sec11p Overexpression—Sec11p is known to be an essential yeast protein. Therefore, one may speculate that the loss of signal peptidase activity in response to the down-regulation of Spc3p is mainly due to the simultaneous loss of Sec11p. To test this, we tried to rescue the Δspc3 mutant by overexpression of Sec11p. SEC11 was put under control of the Met3 promoter and introduced into an HMY1 yeast strain, which contained the plasmid pH1 with SPC3 under control of a Gal10 promoter. As a control, the Met3Sec11 containing plasmid was also introduced into the SEC11-deficient strain HMY2. Growth of these strains was then tested under conditions that induce the Met-promotor of Sec11p on agar plates either containing galactose or glucose (Fig. 5A). We found, that Sec11p alone is not able to rescue the depletion of
Spc3p although Sec11p is strongly overproduced in these cells (Fig. 5B).

Another possibility is that Sec11p alone is sufficient to function as signal peptidase. In this scenario, Sec11p cannot suppress the depletion of Spc3p because Spc3p performs an essential function, different from the processing of signal peptides. To investigate this possibility, we set up the following experiment. HMY1 cells that contain the two plasmids pHM1 (pGal10Spc3) and pHM3 (pMet3Sec11) were grown in SGal minimal medium, with galactose as the carbon source, to repress the Gal10 promoter. At various time points, samples were taken and analyzed by immunoblotting (see “Experimental Procedures”). As a control, the strain Δsec11+pHM4 was used. Strains were plated on SD or SGal minimal medium and incubated at 30 °C for 2 or 3 days. The diagram at the top right depicts the arrangement of the strains under study: B, cells of the strain Δsec11+pHM1 with pHM3 were grown in SGal minimal medium, harvested at 0.6 A600, and membranes were prepared. The amounts of Spc3p, Spc2p, and Sec11p were analyzed by immunoblotting. Wild-type membranes serving as a control were prepared from a diploid strain DF5.

Fig. 5. A, overexpression of Sec11p does not complement Spc3p depletion. Cells of the strain Δspc3+pHM1 (pGal10Spc3) were transformed with pHM3 (pMet3Sec11). As a control, the strain Δsec11+pHM4 was used. Strains were plated on SD or SGal minimal medium and incubated at 30 °C for 2 or 3 days. The diagram at the top right depicts the arrangement of the strains under study: B, cells of the strain Δsec11+pHM1 with pHM3 were grown in SGal minimal medium, harvested at 0.6 A600, and membranes were prepared. The amounts of Spc3p, Spc2p, and Sec11p were analyzed by immunoblotting. Wild-type membranes serving as a control were prepared from a diploid strain DF5.

Fig. 6. Overexpression of Sec11p does not rescue loss of signal peptidase activity \textit{in vitro}. HMY1 (Δspc3) cells containing the plasmids pHM1 (pGal10Spc3) and pHM3 (pMet3Sec11) were grown in SGal minimal medium (2% sucrose, 1% galactose, −trp, −ura, −met) and afterwards shifted to SD minimal medium (−trp, −ura, −met). After different time points (0, 5, 10, and 15 h), aliquots of cells were removed and analyzed as described in Fig. 4A. A, result of the cleavage assay. \textit{pp-α-f}, indicates the pre-pro-α-factor; \textit{p-α-f}, indicates the pre-α-factor. B, relative amounts of SPC subunits.

DISCUSSION

We have identified the yeast homolog of canine SPC22/23, Spc3p, which is identical with the last uncharacterized subunit of yeast SPC. Homologs of the other subunits of canine SPC have been identified in yeast earlier (22, 25, 26), and it was shown that they are part of a heterotetrameric complex (21, 22). Therefore, the principle structure of the eucaryotic SPC turns out to be conserved from yeast to mammals.

Spc3p is essential for cell viability. Its depletion results in accumulation of precursors of secretory proteins \textit{in vivo} and loss of signal peptidase activity \textit{in vitro}, a phenotype that is very similar to that observed in Δsec11 mutants (25). Moreover, the depletion of Spc3p destabilizes the SPC. While the Spc1p and the Sec11p are not detectable in the ER in the absence of Spc3p, Spc2p seems to be more stable.

The Spc3p may be directly involved in the cleavage of signal peptides. This is mainly indicated by the tight coupling of the loss of signal peptidase activity with the loss of Spc3p. Although Spc3p depletion leads to a simultaneous loss of Sec11p, the overexpression of Sec11p is not able to rescue Δspc3 cells. Moreover, depletion of Spc3p in a strain overexpressing Sec11p revealed that, even in the presence of normal amounts of Sec11p, cells lose signal peptidase activity if Spc3p is absent. Therefore, it is unlikely that the decrease in signal peptidase activity observed during Spc3p depletion is caused by the parallel loss of Sec11p.

It should be noted, that a simultaneous overexpression of Sec11p and Spc3p did not result in a significantly higher signal peptidase activity as measured \textit{in vitro}. Although Spc1p and Spc2p are not essential \textit{in vivo}, a limiting amount of one or both of these proteins could be the reason for this observation, as the
dimeric subcomplex between Spc3p and Sec11p might be less stable under the conditions used for the in vitro assay than the complete SPC. Alternatively, it may well be that other parts of the translocon are needed for a correct assembly and for stabilization of the SPC.

These findings shed a new light on the question of to what extent the eucaryotic signal peptidase of the ER and bacterial leader peptidase may be similar to each other. Signal peptidase activity of the bacterial plasma membrane is comprised of a single polypeptide, the leader peptidase. It has been shown that in contrast to this, the eucaryotic signal peptidase consists of several subunits that substantially differ from each other. However, the identification of regions in the bacterial leader peptidase and the eucaryotic SPC18, SPC21, and Sec11p, which display primary sequence similarity, raised the question of whether all of the eucaryotic subunits are directly involved in the cleavage of signal peptides (17, 24). This notion has been supported by two other facts. 1) Enzymatically active signal peptidase may be purified from hen ovoduct as a complex of only two subunits (SPC18 and gp23) (19), and 2) neither the SPC12 homolog, Spc1p, nor the SPC25 homolog, Spc2p, is essential for viability or signal peptidase activity in S. cerevisiae. The identification of Spc3p as a second essential component of yeast SPC described above demonstrates that there must be indeed a substantial difference in the mode of signal peptide cleavage in eucaryotes and prokaryotes.

What could be the precise function of Spc3p in this process? It could well be, that the protein provides only a backbone for Sec11p to fold correctly. However, Spc3p could also be more directly involved in the proteolytic process. First, experiments indicate that the hydrophobic region at the carboxyl terminus that is conserved among all SPC22/23 homologs may be essential for the in vivo function of Spc3p.2 This domain could be involved in substrate binding, either alone or in combination with the similarly located second hydrophobic domain of the Sec11p/Spc3p subcomplex.

Why did this complex structure of SPC evolve in eucaryotes? One possibility is that the ER signal peptidase has a broader substrate specificity than the bacterial enzyme. For instance, in contrast to data reported for E. coli leader peptidase (33), we did not find that yeast SPC activity is blocked in vivo if the substrate contains a proline immediately after the cleavage site.3 Another reason could be that the SPC is involved in other proteolytic processes like the digestion of signal peptides (34) or the degradation of membrane proteins (35). It is also possible that protein translocation across the ER is more regulated than the related process in bacteria and that some SPC subunits are involved in this function. In this regard, it will be of interest to investigate also Archea signal peptidase as the possible ancestor to eucaryotic signal peptidase. To date, a gene having higher homology to eucaryotic SPC18/SPC20/Sec11p than to bacterial leader peptidases has been identified in Methanococcus jannaschii (36), and whether homologs of the other SPC subunits are also present in Archea is still open.

Acknowledgments—We thank Francoise Kepes, Thomas Sommer, and Steffen Panzer, for providing antibodies, strains, and plasmids, Brigitte Nentwig and Angelika Wittruck, for technical assistance, and T. Sommer and the members of the Hartmann lab, for critical reading of the manuscript.

REFERENCES
1. Blobel, G., and Dobberstein, B. (1975) J. Cell Biol. 67, 835–851
2. Rapoport, T. A., Jungnickel, B., and Kutsay, U. (1996) Annu. Rev. Biochem. 65, 271–203
3. Görlich, D., and Rapoport, T. A. (1993) Cell 75, 615–630
4. Meng, W., Prehn, S., and Rapoport, T. A. (1994) EMBO J. 13, 3937–3982
5. Kalies, K.-U., Görlich, D., and Rapoport, T. A. (1994) J. Cell Biol. 126, 925–934
6. Jungnickel, B., and Rapoport, T. A. (1995) Cell 82, 261–270
7. Höhfeld, D., Metlack, K. E., Stein, H., Kalies, K.-U., Miller, K. R., Rapoport, T. A., and Ackey, C. W. (1996) Cell 87, 721–732
8. Brudsky, J. L., and Schekman, R. (1993) J. Cell Biol. 123, 1355–1363
9. Panzer, S., Dreier, L., Hartmann, E., Kostka, S., and Rapoport, T. A. (1995) Cell 81, 561–570
10. Blobel, G., and Dobberstein, B. (1975) J. Cell Biol. 67, 852–862
11. Evans, E. A., Gilmore, R., and Blobel, G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 581–586
12. Shelnos, G. S., Kanwar, Y. S., and Blobel, G. (1988) J. Biol. Chem. 263, 17063–17070
13. Greenburg, G., Shelnos, G. S., and Blobel, G. (1989) J. Biol. Chem. 264, 15762–15765
14. Greenburg, G., and Blobel, G. (1994) J. Biol. Chem. 269, 23554–23558
15. Kalies, K.-U., and Hartmann, E. (1996) J. Biol. Chem. 271, 3925–3929
16. Shelnos, G. S., Lin, L., and Nichitta, C. V. (1995) J. Biol. Chem. 268, 5201–5208
17. van Dijl, J. M., de Jong, A., Vehmanen, J., Venema, G., and Bron, S. (1992) EMBO J. 11, 2019–2028
18. Zewinski, C., and Wickner, W. (1988) J. Biol. Chem. 255, 7973–7977
19. Baker, R. K., and Lively, M. O. (1987) Biochemistry 26, 8561–8567
20. Newhouse, A. L., McLean, J. W., and Lively, M. O. (1992) Biochem. J. 282, 447–452
21. YaDeau, J. T., Klein, C., and Blobel, G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 517–521
22. Fang, H., Panzer, S., Mullins, C., Hartmann, E., and Green, N. (1996) J. Biol. Chem. 271, 16460–16465
23. Shelnos, G. S., and Blobel, G. (1998) J. Biol. Chem. 273, 9512–9519
24. Dalley, R. E., and von Heijne, G. (1992) Trends Biochem. Sci. 17, 474–478
25. Bohni, P. C., Deshaies, R. J., and Schekman, R. W. (1988) J. Cell Biol. 106, 1035–1042
26. Mullins, C., Meyer, H.-A., Hartmann, E., Green, N., and Fang, H. (1996) J. Biol. Chem. 271, 29094–29099
27. Finke, K., Plath, K., Panzer, S., Prehn, S., Rapoport, T. A., Hartmann, E., and Sommer, T. (1996) EMBO J. 15, 1482–1494
28. Schneider, B. L., Seuflert, W., Steiner, B., Yang, Q. H., and Dutcher, A. B. (1995) Yeast 11, 1265–1274
29. Sikorski, R. A., and Hieter, P. (1989) Genetics 122, 19–27
30. Johnston, M., and Davis, R. W. (1984) Mol. Cell. Biol. 4, 1440–1448
31. Christiansen, T. W., Sikorski, R. A., Dante, M., Shero, J. H., and Hieter, P. (1992) Gene (Amst.) 110, 119–122
32. Chrest, H., Kerjan, P., and Surdin-Kerjan, Y. (1987) Mol. Gen. Genet. 210, 307–313
33. Niessen, L., and van Heijne, G. (1992) FEBS Lett. 299, 243–246
34. Lyko, F., Martoglio, B., Jungnickel, B., Rapoport, T. A., and Dobberstein, B. (1995) J. Biol. Chem. 270, 17139–17147
35. Mullins, C., Lu, Y., Campbell, A., Fang, H., and Green, N. (1995) J. Biol. Chem. 270, 17139–17147
36. Bult, C. J., Olsen, G. et al. (1996) Science 273, 1058–1073

3 K. Plath, and E. Hartmann, unpublished results.
4 E. Hartmann, unpublished results.