Interactions between Spc2p and Other Components of the Endoplasmic Reticulum Translocation Sites of the Yeast Saccharomyces cerevisiae*

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Wolfram Antonin‡§, Hellmuth-Alexander Meyer§, and Enno Hartmann¶

From the Abteilung Biochemie II, Zentrum Biochemie und Molekulare Zellbiologie, Universität Göttingen, Heinrich-Düker Weg 12, Göttingen 37073, Germany

In yeast, the endoplasmic reticulum membrane proteins Sec11p and Spc3p are essential for the cleavage of signal peptides of nascent polypeptide chains during their passage through translocation sites. Genetic and biochemical experiments demonstrate that Sec11p and Spc3p are tightly associated with two other proteins, Spc1p and Spc2p, whose functions are largely unknown. Using anti-Spc2p antibodies, we show here that this heterotetrameric complex associates with Sbh1p and Sbh2p, the β-subunits of the Sec61p complex and the Shs1p complex, respectively. Depletion of Spc2p decreased the enzymatic activity of the SPC in vitro, led to a loss of Spc1p, and led to a down-regulation of the amount of Sec11p and Spc3p in the endoplasmic reticulum. Moreover, the deletion of Spc2p also decreased the expression level of Shs2p. These data implicate that Spc2p not only enhances the enzymatic activity of the SPC but also facilitates the interactions between different components of the translocation site.

Transport of proteins across the translocation sites in the endoplasmic reticulum (ER) membrane is triggered by signal sequences that are usually located at the amino terminus of the polypeptide chain (for review, see Refs. 1 and 2). It is performed either co-translationally, with the ribosome tightly bound to the membrane, or post-translationally. In both cases the gene components of the translocation site.

In yeast, two different types of ribosome-bound Sec61 complexes exist, which most likely are involved in the co-translational pathway: the Sec61p complex and the Shs1p complex. The two complexes differ in both their α-subunits (Sec61p and Shs1p) and β-subunits (Sbh1p and Sbh2p), but contain the same γ-subunit (Sss1p) (3–5). In addition, yeast contains a Sec complex consisting of the Sec61p complex and the Sec62/63 subcomplex which is essential and sufficient for post-translational translocation (4). Deletion of SEC61, SSS1, SEC62, or SEC63 results in a massive accumulation of precursors of secretory proteins and is lethal (3, 6, 7). In contrast, the Shs1p complex is not essential for cell viability, but is required for a normal growth rate. Additionally, the β-subunits of the Sec61-like complexes, Sbh1p and Sbh2p, are not essential for the function of the respective translocation sites. However, cells lacking both proteins accumulate precursors of secretory proteins in the cytoplasm, and their growth at elevated temperatures is severely impaired (5).

Another component of the ER translocation site is the signal peptidase complex (SPC), which removes signal sequences from the nascent chains during the transport process. In yeast, the SPC consists of four different polypeptides named Spc1p, Spc2p, Spc3p, and Sec11p (8). Spc3p and Sec11p are single transmembrane spans with the majority of the protein being located in the ER lumen. Both proteins are essential for yeast viability. Deletion of either protein leads to the loss of signal peptidase activity both, in vivo and in vitro (9–11). Sec11p shows some homology to the bacterial leader peptidase LepB (12, 13) and site-directed mutagenesis experiments demonstrate that it forms the active center of the SPC (14).

Nothing is known so far about the function of Spc1p and of Spc2p. Their mammalian homologues Spc12 and Spc25 probably span the membrane twice with only very few residues facing the ER lumen. Based on this topology, it was suggested that these proteins may also perform functions independent from the actual proteolytic activity of the SPC (15). This idea is supported by the fact that chicken Spc12 and Spc25 are not needed for the proteolytic activity of the SPC in vitro (16). Additionally, yeast SPC1 and SPC2 are not essential for the survival of the cells at temperatures below 38 °C. However, at 42 °C Δspc1 mutant cells display an accumulation of precursors of secretory proteins in vivo and a reduced cell viability (17, 18).

We show here that Spc2p is required for full enzymatic activity of the SPC in vitro. In addition, depletion of Spc2p decreases the expression levels of other SPC components and of Shs2p. Immunoprecipitation also demonstrates that Spc2p is in complex with Shs1p and Shs2p, the β-subunits of Sec61-like complexes. Together, these data implicate that a major function of the Spc2p is to facilitate the interactions between different components of the translocation site.
**RESULTS**

**Deletion of Spc2p Reduces the Activity of the SPC in Vitro**—In *in vivo* analysis of Δspc2p strains showed an accumulation of precursors of secretory proteins at 42 °C (18). To investigate whether this effect of the SPC2 deletion is directly linked to the activity of the SPC, we set out to analyze the enzymatic activity of Spc2p-free SPC in *vitro*. Signal peptidase cleavage was assayed as described in Ref. 10 using digitonin solubilized membranes from the Δspc2 mutant and prepro-α-factor as a substrate. As a control we included membranes derived from a wild-type strain, a Δspc1 strain, and a strain with a Δspc1/Δspc2 double deletion. The amount of digitonin extract added was calibrated to the same amount of Sec11p and Spc3p. We found that the deletion of Spc1p had no effect on the signal peptidase activity (Fig. 1A). In contrast, deletion of Spc2p reduced the activity by a factor of 2. A Δspc1/Δspc2 double deletion resulted in no further reduction of the signal peptidase activity. We wondered whether the decreased signal peptide cleavage could be due to a direct effect on the catalytic center of the SPC or a diminished stability of the complex. To test whether the stability is altered in a Δspc1/Δspc2 strain, a digitonin extract was bound to concanavalin A. In wild-type cells yeast SPC does not dissociate into its subunits in the presence of digitonin (18). In addition to the glycosylated Spc2p, the bound material contained the nonglycosylated Sec11p, indicating that also in the absence of Spc1p and Spc2p the two remaining subunits did not dissociate (Fig. 1C). To confirm these results, we repeated the cleavage assay in the presence of saponin using membranes from wild-type cells and a Δspc1/Δspc2 mutant strain. These conditions are probably more gentle for the enzyme, because integral membrane proteins like the SPC are not released from the lipid bilayer. Again, the activity of the SPC from the double deletion strain was only half that of wild-type SPC (data not shown). Together these data indicate that Spc2p is modulating the peptidase activity of the yeast SPC.

**Influence of Spc2p on the Composition and the Steady State Level of Yeast SPC**—Next we wanted to analyze the SPC composition of the deletion strains in more detail. Membranes purified from a Δspc1 strain, a Δspc2 strain, a Δspc1/Δspc2 strain, and a corresponding wild-type strain were calibrated using to the same amount of Sec62p as a measure of the number of translocation sites. The amount of the different SPC subunits was analyzed by immunoblotting (Fig. 1D). We found...
that membranes of a Δspc2 strain did not contain Spc1p. Spc1p was also not detectable if complete cell lysates were analyzed. Nevertheless, there was no reduction in the expression of Spc1p mRNA detectable, indicating that the regulation was at the level of protein synthesis or degradation (data not shown). Moreover, we observed that strains depleted of Spc2p expressed a lower amount of Sec11p and Spc3p in relation to wt, Δspc2, Δspc1, and Δspc1/Δspc2 strain. Microsomes were purified from wt (SEY62.10), Δspc2, Δspc1, and Δspc1/Δspc2 strains and normalized to similar amounts of Sec62p. Digitonin extract corresponding to 20 and 40 eq of membranes were prepared and analyzed as indicated under "Experimental Procedures" with the exception that the assay was performed for 3 h at 25 °C. Samples were separated by SDS-PAGE and analyzed by a bioimager (Fuji Bas 2000). ppBF, prepro-a-factor; paF, pro-a-factor. B, expression level of Sec1p, Spc2p, Spc3p, and Sec11p. The relative amount of the different SPC subunits in the microsomes analyzed in A was determined by immunoblotting using 35S-labeled secondary antibodies and a bioimager (Fuji Bas 2000). C, microsomes from a wt and a Δspc1/Δspc2 strain were bound to concanavalin A-Sepharose. Unbound (U) and bound (B) material that corresponded to either 12 eq of membranes (Spc3p and Spc2p) or 30 eq of membranes (Sec11p) was processed by SDS-PAGE, and the presence of the different SPC subunits was determined by immunoblotting.

To confirm that the differences between the wild-type strain and the deletion strains were not due to differences in the genetic background, we repeated the experiment using a isogenic wild-type strain (HFY403) transformed with the plasmid pHM3 expressing Spc2p under control of a Gal10 promoter was preincubated in YPGal medium allowing the expression of Spc2p. The cells were then shifted to YPD medium to repress the Spc2p synthesis. After 10 h cells were shifted back to YPGal medium to reinroduce the Spc2p synthesis. Aliquots of cells were removed at the time points indicated, and microsomes were prepared. wt membranes were isolated from the SEY62.10 strain. A, signal peptidase activity of digitonin extracts corresponding to 40 eq of microsomes was analyzed. ppBF, prepro-a-factor; paF, pro-a-factor. B, amounts of Spc3p, Spc2p, and Spc1p were analyzed by SDS-PAGE and immunoblotting. Quantification of proteins with 35S-labeled secondary antibody was performed as described above (Fig. 1).

Fig. 1. SPC composition and signal peptidase activity of yeast strains deficient in Spc1p and Spc2p. A, signal peptidase activity of a wt, Δspc2, Δspc1, and Δspc1/Δspc2 strain. Microsomes were purified from wt (SEY62.10), Δspc2, Δspc1, and Δspc1/Δspc2 strains and normalized to similar amounts of Sec62p. Digitonin extract corresponding to 20 and 40 eq of membranes were prepared and analyzed as indicated under "Experimental Procedures" with the exception that the assay was performed for 3 h at 25 °C. Samples were separated by SDS-PAGE and analyzed by a bioimager (Fuji Bas 2000). ppBF, prepro-a-factor; paF, pro-a-factor. B, expression level of Sec1p, Spc2p, Spc3p, and Sec11p. The relative amount of the different SPC subunits in the microsomes analyzed in A was determined by immunoblotting using 35S-labeled secondary antibodies and a bioimager (Fuji Bas 2000). C, microsomes from a wt and a Δspc1/Δspc2 strain were bound to concanavalin A-Sepharose. Unbound (U) and bound (B) material that corresponded to either 12 eq of membranes (Spc3p and Spc2p) or 30 eq of membranes (Sec11p) was processed by SDS-PAGE, and the presence of the different SPC subunits was determined by immunoblotting.

Fig. 2. Analysis of the SPC composition and signal peptidase activity using a conditional spc2 mutant. A Δspc2 strain (HFY403) transformed with the plasmid pHM3 expressing Spc2p under control of a Gal10 promoter was preincubated in YPGal medium allowing the expression of Spc2p. The cells were then shifted to YPD medium to repress the Spc2p synthesis. After 10 h cells were shifted back to YPGal medium to reinroduce the Spc2p synthesis. Aliquots of cells were removed at the time points indicated, and microsomes were prepared. wt membranes were isolated from the SEY62.10 strain. A, signal peptidase activity of digitonin extracts corresponding to 40 eq of microsomes were analyzed. ppBF, prepro-a-factor; paF, pro-a-factor. B, amounts of Spc3p, Spc2p, and Spc1p were analyzed by SDS-PAGE and immunoblotting. Quantification of proteins with 35S-labeled secondary antibody was performed as described above (Fig. 1).
depleted. Therefore, we repeated the experiment and assayed the amount of other components of the translocation apparatus in the ER by immunoblotting (Fig. 3A). As for Sec62p, the amount of Sec72p, another component of the Sec62/63 complex, did not change in response to alterations of the Spc2p level. Similarly, the amount of components of the Sec61p complex Sec61p and Sbh1p, and the amount of Ssh1p, the α-subunit of the Ssh1 complex, did not change. However, the level of Sbh2p (the β-subunit of the Ssh1p complex) decreased to about one fifth following depletion of Spc2p and returned to the wild-type level, after re-initiation of synthesis of Spc2p (Fig. 3A).

These differences in the expression level of Sbh2p were also detected if membranes of a Δspc2 strain that carried no SPC2 plasmid were compared with membranes of an isogenic wild-type strain (Fig. 3B). Deletion of SPC1 did not decrease the Sbh2p level (Fig. 3B).

**Sbh1p and Sbh2p Are in Proximity to the SPC**—The observed effect of Spc2p on the stability of Sbh2p could be explained by a physical interaction of these two proteins in the membrane. In order to test this hypothesis, we performed immunoprecipitation experiments. Yeast rough membranes were solubilized with digitonin and depleted of ribosome-associated Sec61-like complexes by centrifugation. In addition to the components of the SEC61 complex, antibodies raised against Sec61p were able to precipitate both Sbh2p and Sbh1p from this extract (Fig. 4). The amount of Sbh1p and Sbh2p molecules associated to the SPC under these conditions was in the order of 5–10% of the whole population. Other components of the translocation machinery like Sec62p or Sec61p were not precipitated under these conditions. The same result was obtained using antibodies raised against Spc1p. No precipitation was observed if membranes of a Spc2p deletion strain were used. This shows that the SPC is in a complex with Sbh1p and Sbh2p.

**DISCUSSION**

Several data indicate that the mammalian SPC is located close to ribosome-associated translocation channels that are active in co-translational protein translocation. Signal peptides of nascent chains are cleaved off by the SPC subunits while being translocated (24). Chemical cross-linking experiments have revealed that mammalian SPC25 is in the vicinity of Sec61p in the presence of translocating ribosomes (25). However, so far, no complex between the SPC and components of the Sec61 complex has been purified. It was therefore possible that the observed proximity between SPC and the Sec61 complex is only mediated by a simultaneous interaction of both complexes with membrane-bound ribosomes. We now show that in yeast the SPC is associated with the β-subunits of the Sec61 complex in the absence of ribosomes. We could not demonstrate that the SPC-associated β-subunits are also associated to Sec61p or Ssh1p. Moreover, antibodies raised against the amino termini of Ssh1p and Sbh2p immunoprecipitate Sec61-like complexes but not SPC components. Besides the possibility of technical limitations, these findings could also indicate that the population of Ssh1p and Sbh2p that is associated with the SPC is different from that found in the Sec61 complex. In contrast to Ssh1p, Sbh2p becomes unstable in vivo, when not able to associate with an α-subunit such as Ssh1p or Sec61p (5, 26). Therefore, the fact that the depletion of Spc2p results in a comparable decrease in the steady state level of Sbh2p confirms the existence of complexes between the SPC and Sec61p. Moreover, it indicates that interaction between the SPC and the β-subunits of the Sec61 complex is mediated by the Spc2p. Cross-linking experiments in mammals also point toward a direct interaction between SPC25 and Sec61p (25). We speculate that Sec2p and Sbh1p or Sbh2p form an interface for the association between the SPC and the Sec61 complex.

The Spc1p/SPC12 and Spc2p/SPC25 are components of the signal peptidase that are not found in procaryotic organisms. Although chicken SPC depleted of SPC12 and SPC25 has signal peptidase activity in vitro, the in vivo analysis of a SPC2 deletion mutant showed a weak phenotype resulting in an
accumulation of precursors of secretory proteins at 42 °C (18). Protease protection assays revealed that the precursors are located within the ER lumen, indicating that the accumulation is not caused by a lower translocation efficiency (e.g. due to the lower amount of Sbh2p in these cells) (data not shown). The biochemical analysis of these mutants now demonstrates that the in vivo phenotype is probably a result of two effects. First, the depletion of Spc2p significantly reduces the amount of SPC per translocation site in the ER membrane. Second, SPCs that have no Spc2p and no Spc1p show a diminished cleavage activity in vitro at least for the substrate used in the assay. Deletion of Spc1p alone has no influence on the activity of the SPC. Interestingly, the analysis of the conditional spc2 mutant indicates that SPCs which are only depleted of the Spc2p show also no decreased activity (Fig. 3A; compare 4 and 6 h after shift to glucose). To this end, it is not clear how the presence of Spc2p (and perhaps also Spc1p) enhances the activity of the catalytic subunit Sec11p. One possibility is that the membrane-spanning regions of Spc1p and Spc2p help to form the binding pocket for the hydrophobic substrate of the protein. Thus, they may maintain structural flexibility or stability of SPC needed for effective processing of signal peptides.

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