The EMBO Journal vol.15 no.7 pp.1482–1494, 1996

A second trimeric complex containing homologs of the Sec61p complex functions in protein transport across the ER membrane of *S.cerevisiae*

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Yeast microsomes contain a heptameric Sec complex involved in post-translational protein transport that is composed of a heterotrimeric Sec61p complex and a tetrameric Sec62–Sec63p complex. The trimeric Sec61p complex also exists as a separate entity that probably functions in co-translational protein transport, like its homolog in mammals. We have now discovered in the yeast endoplasmic reticulum membrane a second, structurally related trimeric complex, named Ssh1p complex. It consists of Ssh1p (Sec sixty-one homolog 1), a rather distant relative of Sec61p, of Sbh2p, a homolog of the Sbh1p subunit of the Sec61p complex, and of Sss1p, a component common to both trimeric complexes. In contrast to Sec61p, Ssh1p is not essential for cell viability but it is required for normal growth rates. Sbh1p and Sbh2p individually are also not essential, but cells lacking both proteins are impaired in their growth at elevated temperatures and accumulate precursors of secretory proteins; microsomes isolated from these cells also exhibit a reduced rate of post-translational protein transport. Like the Sec61p complex, the Ssh1p complex interacts with membrane-bound ribosomes, but it does not associate with the Sec62–Sec63p complex to form a heptameric Sec complex. We therefore propose that it functions exclusively in the co-translational pathway of protein transport.

Keywords: endoplasmic reticulum/protein translocation/Sec61/yeast

Introduction

Protein translocation across the endoplasmic reticulum (ER) membrane can occur by two different pathways, a co- and a post-translational pathway, which are mechanistically distinct. In the co-translational pathway, the translating ribosome makes a tight linkage with the ER membrane (Connolly *et al.*, 1989; Crowley *et al.*, 1993, 1994) and the growing polypeptide chain is transferred directly from the channel in the ribosome through a protein-conducting channel in the ER membrane (Blobel and Dobberstein, 1975; Connolly *et al.*, 1989; Simon and Blobel, 1991; Görlich *et al.*, 1992; Görlich and Rapoport, 1993). Thus, there is probably no pushing or pulling machinery involved; the growing polypeptide chain simply has only one way out of the extended channel. In the post-translational pathway, the driving force for the transport process must be provided in a different way. Here, the lumenal chaperone BiP (called Kar2p in yeast) appears to function as a molecular ratchet (Simon *et al.*, 1992) by binding to the translocating polypeptide as it emerges at the lumenal side of the membrane, thus preventing its retrograde movement through the protein-conducting channel (Sanders *et al.*, 1992; Brodsky and Schekman, 1993; Scidmore *et al.*, 1993; Panzner *et al.*, 1995).

The co-translational translocation pathway in mammals can be reproduced with reconstituted proteoliposomes containing only three membrane protein components: the SRP receptor, the Sec61p complex and the TRAM protein (Görlich and Rapoport, 1993). The trimeric Sec61p complex appears to be the core of the translocation machinery in the membrane and may be the major component of the protein-conducting channel; its multi-spanning α-subunit is in proximity to the polypeptide chain throughout its transfer through the membrane (High *et al.*, 1991, 1993; Kellaris *et al.*, 1991; Görlich *et al.*, 1992; Mothes *et al.*, 1994; Nicchitta *et al.*, 1995), and the Sec61p complex is responsible for the anchoring of the ribosome to the ER membrane (Görlich *et al.*, 1992; Kalies *et al.*, 1994; Jungnickel and Rapoport, 1995).

A trimeric Sec61p complex, composed of Sec61p, Sbh1p and Sss1p, is also found in *Saccharomyces cerevisiae* (Panzner *et al.*, 1995). Sec61p and Sss1p are homologous to the α- and γ-subunits of the mammalian Sec61p complex, respectively (Görlich *et al.*, 1992; Hartmann *et al.*, 1994). They are also structurally related to the bacterial proteins SecYp and SecEp, respectively, that are key components of the prokaryotic protein export apparatus and are part of the heterotrimeric SecYEG complex (for review, see Schatz and Beckwith, 1990; Ito, 1995). The yeast component Sbh1p is a structural homolog of the β-subunit of the mammalian Sec61p complex (Panzner *et al.*, 1995) but neither is related to the third subunit of the bacterial complex, SecGp (Nishiyama *et al.*, 1993). The yeast Sec61p complex is found associated with ribosomes upon solubilization of microsomes in detergent (Panzner *et al.*, 1995) and is therefore believed to be involved in the co-translational pathway of translocation, like its counterpart in mammals (Görlich *et al.*, 1992; Görlich and Rapoport, 1993).

The post-translational translocation pathway in *S.cerevisiae* can be reproduced with reconstituted proteoliposomes containing a purified complex of seven ER membrane proteins, the Sec complex, which consists of the trimeric Sec61p complex and a tetrameric Sec62–Sec63p complex

composed of Sec62p, Sec63p, Sec71p and Sec72p (Panzner *et al.*, 1995). Efficient post-translational protein transport into reconstituted proteoliposomes also requires the presence of the lumenal chaperone Kar2p (BiP) and ATP (Panzner *et al.*, 1995); Kar2p is likely to exert its function via an interaction with a lumenal domain of Sec63p (Brodsky and Schekman, 1993; Scidmore *et al.*, 1993).

Most of the translocation components mentioned have been identified in *S.cerevisiae* in genetic screens for mutants defective in translocation. Conditional alleles of *SEC61*, *SEC62* and *SEC63* exhibit severe translocation defects at elevated temperatures, as indicated by the accumulation of precursor polypeptides in the cytoplasm (Deshaies and Schekman, 1987; Rothblatt *et al.*, 1989; Stirling *et al.*, 1992). All three genes are essential for cell viability. *SSS1* has been found as a suppressor of temperature-sensitive (ts) *sec61* mutations, and is also an essential gene (Esnault *et al.*, 1993). Depletion of Sss1p from yeast cells leads to severe translocation defects. Sec71p and Sec72p were found both in genetic screens (Green *et al.*, 1992; Kurihara and Silver, 1993) and in biochemical experiments as proteins associated with Sec62p and Sec63p (Deshaies *et al.*, 1991; Feldheim *et al.*, 1993; Feldheim and Schekman, 1994). Neither of the corresponding genes is essential; the deletion of *SEC71* leads to the simultaneous absence of Sec71p and Sec72p, and results in temperature-sensitive growth and translocation defects (Fang and Green, 1994). The absence of *SEC72* alone does not cause a growth phenotype but leads to the accumulation of some precursor polypeptides (Fang and Green, 1994; Feldheim and Schekman, 1994). Sbh1p, the β-subunit of the Sec61p complex in yeast and a constitutent of the Sec complex, was identified biochemically (Panzner *et al.*, 1995). It has not been detected in genetic screens and its function *in vivo* is therefore uncertain.

In the process of analyzing the function of Sbh1p in yeast cells, we have discovered that more translocation components exist than previously assumed. Yeast microsomes contain a structural homolog of Sbh1p, called Sbh2p. Single deletions of *SBH1* and *SBH2* do not result in a growth phenotype, but the deletion of both genes causes the accumulation of precursor polypeptides and leads to growth defects at elevated temperatures. Microsomes isolated from the double mutant show a reduced activity for post-translational protein translocation *in vitro*. Yeast microsomes also contain a homolog of Sec61p, named Ssh1p. The latter, in contrast to Sec61p, is not essential for cell viability, but it is required for normal growth rates. Both novel components are constituents of a trimeric complex, similar to the Sec61p complex, that contains Sss1p as the third subunit common to both complexes. The two trimeric complexes are distinct and not functionally equivalent; the Ssh1p complex is bound to membrane-bound ribosomes, like the Sec61p complex, but it is not found associated with the Sec62–Sec63p complex to form the heptameric Sec complex presumed to function in post-translational translocation. Our data suggest, therefore, that this complex is exclusively involved in the co-translational pathway of protein transport across the ER membrane.

Results

Sbh1p and Sbh2p are involved in protein translocation in yeast cells

To determine whether Sbh1p plays a role in protein translocation across the ER membrane *in vivo*, we tested a deletion mutant for growth and translocation defects. The mutant was constructed by replacing most of the coding region of *SBH1* with the *HIS3* gene (Δ*sbh1::HIS3*) in a diploid strain. After sporulation and tetrad dissection, four viable spores were obtained. Δ*sbh1* mutants grow at rates identical to the wild-type strain at all temperatures tested, both on plates (Figure 2A) and in liquid culture (not shown). Thus, in contrast to the other components of the trimeric Sec61p complex (Sec61p and Sss1p), Sbh1p is not essential for the growth of yeast cells.

Since the absence of a phenotype of deletion mutants in yeast may be caused by the presence of a second gene with overlapping function, we searched the data bank for homologs of Sbh1p. Indeed, an open reading frame in the genome of *S.cerevisiae* was found that encodes a protein of ~9.6 kDa with ~50% of its amino acids identical with Sbh1p (Figure 1A). This protein, which we named Sbh2p (Sec61 beta homolog 2), shares ~23% identical amino acids with the β-subunit of the mammalian Sec61p complex, which is approximately the same degree of similarity as between Sbh1p and Sec61β (Panzner *et al.*, 1995). The Sbh2 protein, like Sbh1p and Sec61β, is predicted to span the membrane once with a hydrophobic segment near its C-terminus (double underlined). As with Sec61β (Hartmann *et al.*, 1994), the N-terminal parts of Sbh1p and Sbh2p are probably located in the cytoplasm.

As with Sbh1p, deletion of the gene encoding Sbh2p, constructed by replacing its coding region by the *ADE2* gene (Δ*sbh2::ADE2* mutant), did not result in any obvious growth defect (Figure 2A). However, a double mutant lacking both Sbh1p and Sbh2p showed a strong temperature-sensitive growth phenotype: it grew normally at 30°C but barely at 37°C (Figure 2A).

We next tested whether the deletion mutants accumulate unprocessed precursors of exported proteins. The secretory protein α-factor and the lumenal protein Kar2p were analyzed. Yeast cells were pulse-labeled with [^{35}S]methionine and an extract was prepared and subjected to immunoprecipitation with specific antibodies (Figure 2B). As a control, the temperature-sensitive mutant *sec61-2* (Deshaies and Schekman, 1987) was included in the analysis. Accumulation of the unprocessed precursor of Kar2p was seen in the mutant lacking both Sbh1p and Sbh2p (lane 4), although to a lesser extent than in the *sec61-2* mutant (lane 5). Precursor accumulation was seen at the non-permissive temperature of 37°C (Figure 2B) as well as at the permissive temperature of 30°C (data not shown). No precursor of Kar2p was seen in the single deletion mutants at any temperature (Figure 2B, lanes 2 and 3, and data not shown). In the case of α-factor, no protein could be immunoprecipitated from wild-type cells (lane 1) in which the precursor form of α-factor (prepro-α-factor; ppαF) is transported and processed rapidly. However, in the mutant lacking both Sbh1p and Sbh2p, significant amounts of ppαF were detected (lane 4), albeit not as much as in *sec61-2* cells (lane 5). Interestingly, the single deletion of *SBH1*, but not of *SBH2*, also resulted in some accumulation of ppαF (lane 2 versus 3).

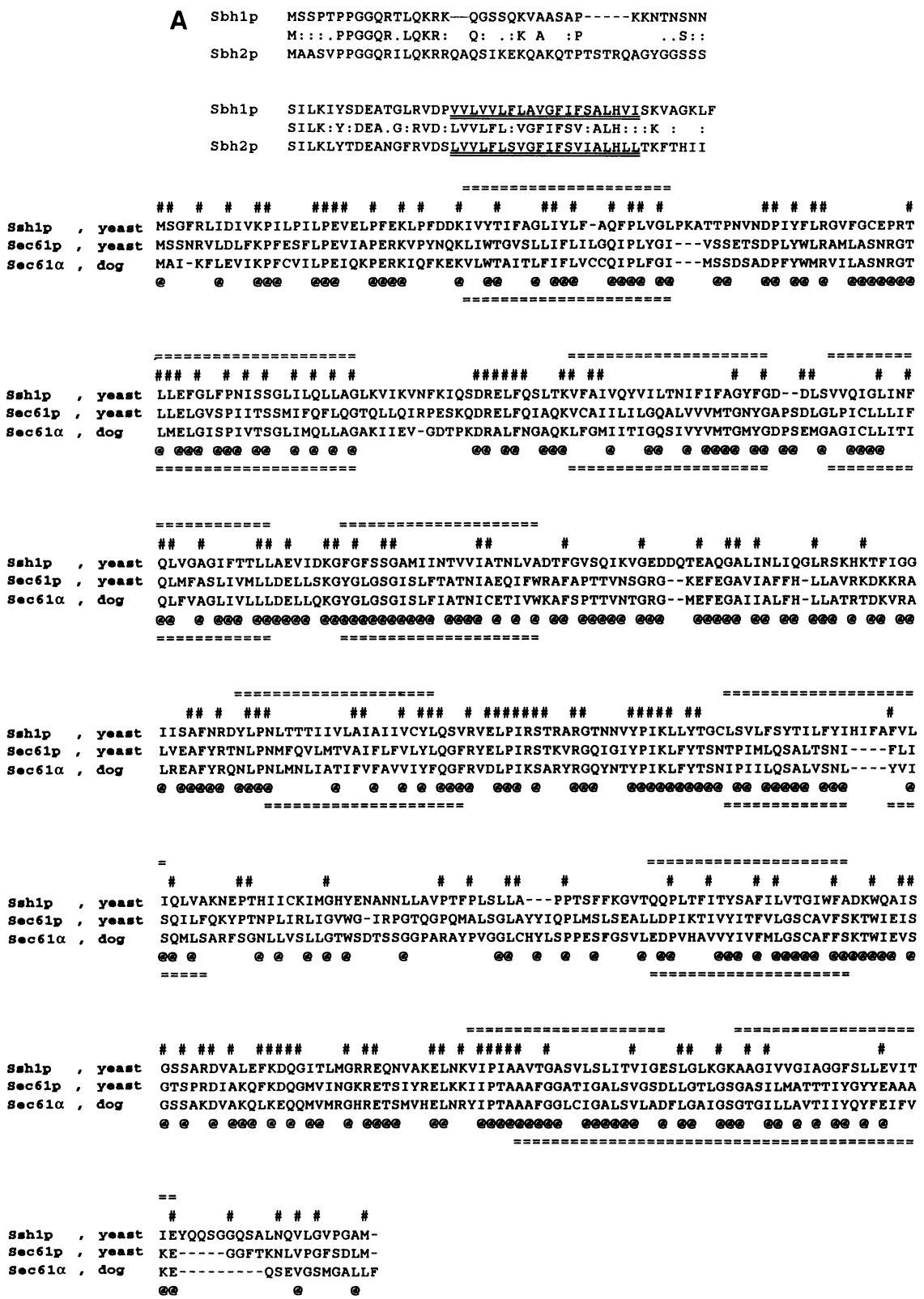

Fig. 1. Comparison of amino acid sequences. (**A**) Comparison of Sbh1p and Sbh2p from *S.cerevisiae*. A single letter between the sequences indicates identical amino acid residues between the two proteins; colons and dots indicate strong and weak similarities, respectively. (**B**) Comparison of Ssh1p and Sec61p from *S.cerevisiae* and Sec61α from dog pancreas. The # and @ signs above and below the sequences indicate identical residues between yeast Ssh1p and yeast Sec61p, and between yeast Sec61p and canine Sec61α, respectively. The double lines above or below the sequences indicate the positions of putative membrane anchors.

We also tested the deletion mutants for defects in post-translational protein translocation *in vitro*. Microsomes were isolated from wild-type (wt) and mutant cells (grown at the permissive temperature) and tested for the translocation of ppαF and of the bacterial protein proOmpA, both synthesized in an *in vitro* translation system (Figure 2C).

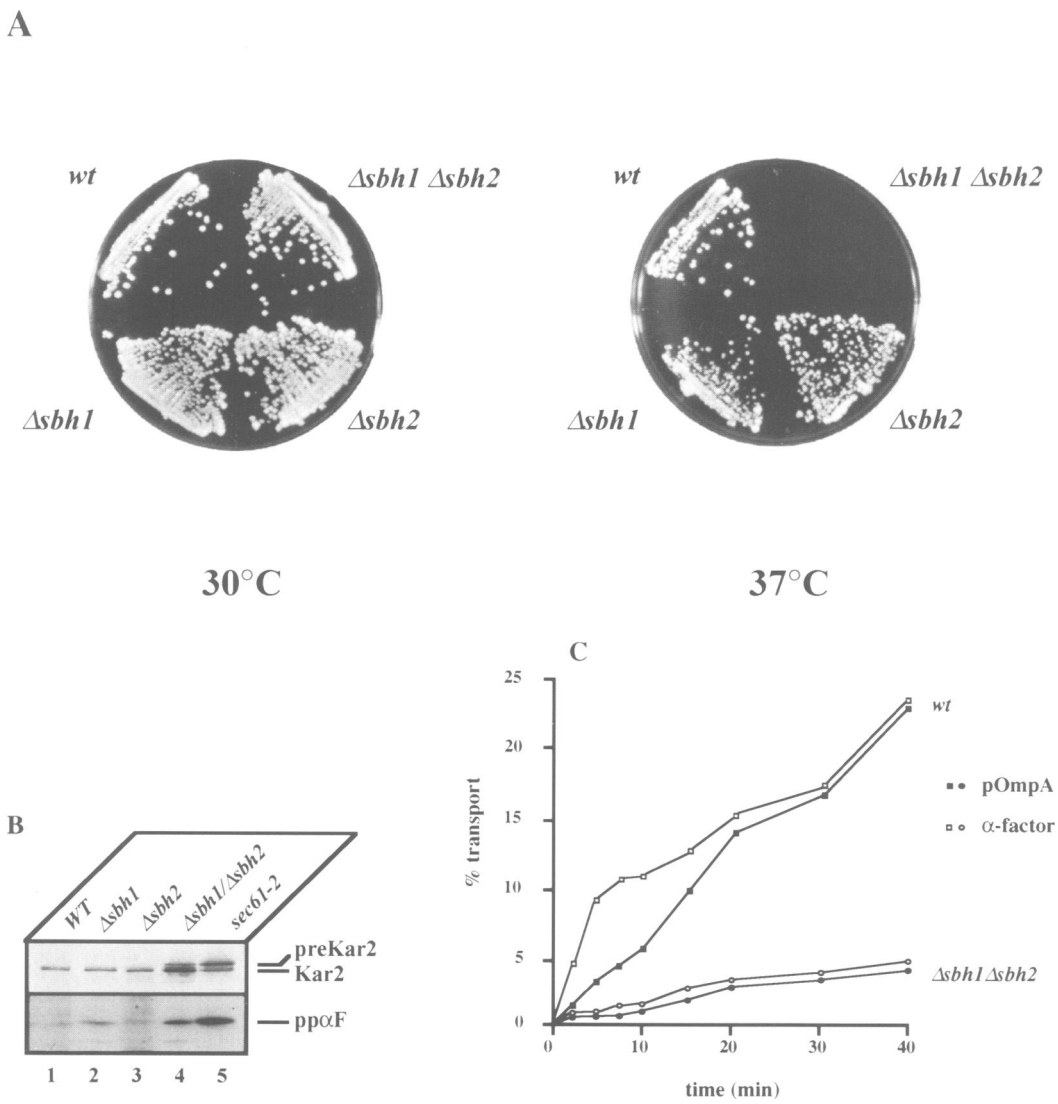

Fig. 2. Test of deletion mutants of Sbh1p and Sbh2p for growth and translocation defects. (**A**) Cells from a wild-type strain and from the deletion mutants Δ*sbh1*, Δ*sbh2* and Δ*sbh1*Δ*sbh2* were tested for growth at 30 and 37°C on plates containing rich medium. (**B**) To test for precursor accumulation, cells from a wild-type strain and from the deletion mutants were grown to exponential phase at 30°C and then shifted to 38°C for 2 h before labeling with [^{35}S]methionine for 10 min. Cell extracts were prepared and subjected to immunoprecipitation with antibodies to Kar2p or α-factor. The immunoprecipitated material was analyzed by SDS–PAGE and fluorography on 10% (Kar2p) and 15% (α-factor) acrylamide gels. preKar2 and ppαF are precursors of Kar2p and α-factor, respectively. (**C**) Post-translational translocation of ppαF and proOmpA was tested in *in vitro* microsomes isolated from wild-type cells or mutant cells lacking both Sbh1p and Sbh2p. The translocation substrates were synthesized in a reticulocyte lysate system in the presence of [^{35}S]methionine and their import into microsomes was tested by treatment with proteinase K followed by SDS–PAGE. The ratio of the radioactivity in the protease-protected material and the input radioactivity, both determined with a phosphorimager, is defined as percent transport and plotted against the incubation time. The amount of membranes added to the reaction mixtures was chosen to be below the saturation level and was normalized on the basis of the amount Sec62p present in the microsome preparation (estimated by quantitative immunoblotting).

In these experiments, non-saturating, equal amounts of microsomes were used (Sec62p was used as a reference and quantitated in immunoblots), and the amount of protease-protected and thus translocated protein was determined as a function of time. Microsomes from the Δ*sbh1*Δ*sbh2* double mutant transported both proteins with a reduced rate compared with microsomes from wild-type cells (Figure 2C). The reduction was 2- to 5-fold in various experiments and became insignificant with saturating membrane concentrations.

Taken together, these results show that Sbh1p and Sbh2p are involved in, but not essential for, protein transport across the ER membrane.

Ssh1p, a homolog of Sec61p with partially overlapping function

In addition to the Sbh1p homolog, yeast cells contain a homolog of the large subunit of Sec61p. This homolog was identified recently in the *S.cerevisiae* sequencing project (Feldmann *et al.*, 1994) and is called Ssh1p (for Sec sixty one homolog 1).

Ssh1p has ~30% amino acid identity with both yeast Sec61p and mammalian Sec61α (Figure 1B; comparison between Ssh1p and Sec61a not shown). Ssh1p is less similar to canine Sec61α than is Sec61p (the latter share ~50% identical amino acids). Like its homologs (Görlich *et al.*, 1992; B.Wilkinson and C.Stirling, personal com-

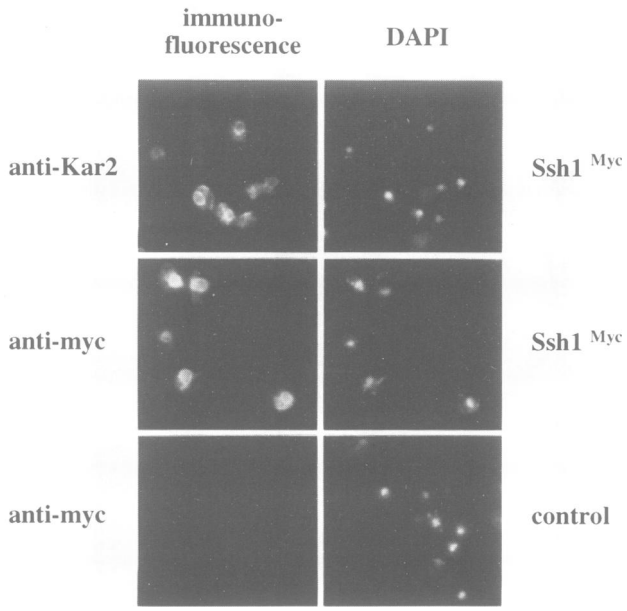

Fig. 3. Ssh1p is localized to the ER. Indirect immunofluorescence was carried out with haploid cells expressing either myc-tagged Ssh1p from a plasmid (Ssh1^{Myc}, two upper rows) or with cells containing a control vector (bottom row). As a control for ER staining, the cells in the first row were incubated with antibodies against the lumenal ER protein Kar2p. The cells of the two lower rows were incubated with antibodies specific for the c-myc epitope. In addition, all samples were stained with DAPI (right panels) to indicate the position of the nuclei.

munication), Ssh1p is predicted to span the lipid bilayer 10 times (hydrophobic segments are indicated by double lines in Figure 1B). Interestingly, the predicted lumenal domains and membrane anchors of the yeast proteins, Sec61p and Ssh1p, share only weak similarity, most of which is concentrated in the cytoplasmic loops (~40% identical amino acids), sometimes found in clusters of 5–7 residues. This pattern suggests that Ssh1p and Sec61p interact with a common cytoplasmic component. In contrast, when yeast Sec61p and mammalian Sec61α are compared, the cytosolic loops are slightly less conserved than the lumenal domains and membrane anchors.

Because of the evidence that suggested a role for Sbh2p in translocation and preliminary results that showed an association between Sbh2p and Ssh1p (see below), we wished to analyze the function of Ssh1p in more detail. We first confirmed that Ssh1p is located in the ER membrane. A tagged version of Ssh1p that carries a c-myc epitope at its C-terminus (Ssh1^{Myc}) was constructed and expressed in yeast cells via an ARS/CEN vector under the control of its normal promoter. The localization of the protein was determined by immunofluorescence microscopy with antibodies against the c-myc epitope. The Ssh1p staining pattern was indistinguishable from that of Kar2p, a lumenal ER protein used as a marker (Figure 3, compare top and middle rows). Staining was seen predominantly in a ring-shaped perinuclear region. For comparison, nuclei are shown stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; see right panels). Control cells, not expressing the tagged protein, showed virtually no staining with the c-myc-specific antibodies (bottom row). We therefore conclude that Ssh1p is localized to the ER.

To determine whether Ssh1p is required for cell viability, we constructed a deletion mutant by replacing most of the coding region of *SSH1* with a fragment containing the *LEU2* gene; the replacement was confirmed by Southern blotting (data not shown). As seen in Figure 4A, Ssh1p is not essential for cell viability, in contrast to its homolog Sec61p (Deshaies and Schekman, 1987). However, cells lacking Ssh1p grew more slowly, exhibiting a generation time of ~125–130 min compared with 90 min for the wild-type. Double deletions of *SSH1* and either *SBH1* or *SBH2* did not show reduced growth rates compared with the mutants lacking only *SSH1* (Figure 4A).

The identical cellular localization of Ssh1p and Sec61p and their similar structures raised the possibility of genetic interactions between them. Indeed, the deletion of *SSH1* and a ts mutation in *SEC61* (*sec61-2*) showed synthetic effects (Figure 5). We constructed a Δ*ssh1sec61-2* double mutant by introducing a Δ*ssh1::ADE2* deletion construct into the *sec61-2* strain. At 36°C, *sec61-2* cells are only marginally affected, but the Δ*ssh1sec61-2* strain showed a drastically impaired growth rate (Figure 5). Thus, Ssh1p and Sec61p must have at least partially overlapping functions under these conditions.

Ssh1p and Sbh2p are constituents of a trimeric complex that is similar to the Sec61p complex

As Sec61p and Sbh1p are constituents of a trimeric Sec61p complex, the possibility exists that their homologs, Ssh1p and Sbh2p, are also part of a trimeric complex in the ER membrane. To test this possibility, peptide-specific antibodies were generated against Ssh1p and Sbh2p. The specificity of the antibodies was tested in immunoblots with membranes prepared from either wild-type cells or from mutants carrying deletions of the corresponding genes (Figure 4B). The antibodies against Ssh1p recognized several closely spaced bands (lane 1) with electrophoretic mobilities similar to Sec61p. In strains carrying a deletion of *SSH1*, these bands were all missing (lanes 2, 5 and 6), indicating that they correspond to Ssh1p. The reason for the heterogeneity is presently unclear. The antibodies against Sbh2p recognized a band of ~10 kDa (lane 1) that was missing in strains in which the corresponding gene was deleted (lanes 4, 6 and 7). These antibodies were specific for Sbh2p, in contrast to those against Sbh1p (Panzner *et al.*, 1995) which showed some cross-reactivity with Sbh2p (lanes 1, 3 and 5; the cross-reacting band is indicated by a dot).

The immunoblot analysis also revealed that the deletion of *SSH1* results in drastically decreased levels of Sbh2p (lane 2), suggesting that the interaction with Ssh1p stabilizes Sbh2p. The reverse was not true: the deletion of *SBH2* did not alter the levels of Ssh1p (lane 4). Interestingly, when *SSH1* and *SBH1* were both deleted, Sbh2p remained at high levels (lane 5). As shown below, under these conditions, the protein appears to be stabilized by an interaction with Sec61p. Both Sec61p and Sbh1p remained unchanged in strains carrying deletions of *SSH1* or *SBH2* (lanes 2, 4 and 6).

The antibodies were then used in co-immunoprecipitation experiments (Figure 6A). Yeast microsomes were solubilized in digitonin and, after centrifugation, the extract was incubated with antibodies against Sec61p, Sbh1p, Sss1p, Sec62p and Sec71p, as well as against the novel

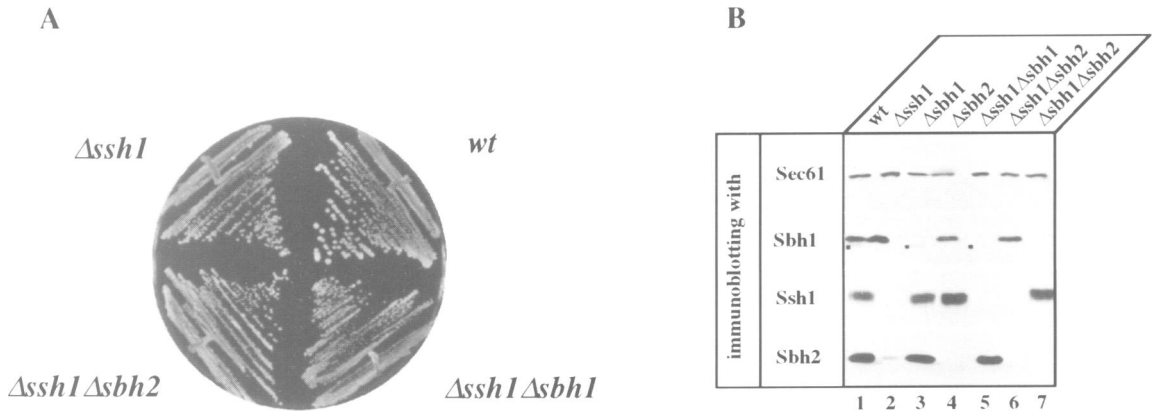

Fig. 4. Analysis of deletion mutants of *SSH1*. (**A**) Cells from a wild-type strain or from the mutants Δ*ssh1*, Δ*ssh1*Δ*sbh1* and Δ*ssh1*Δ*sbh2* were tested for growth at 30°C on plates containing rich medium. (**B**) The levels of Sec61p, Ssh1p, Sbh1p and Sbh2p were determined by immunoblotting in wild-type cells as well as in various deletion mutants. Membranes were prepared from exponentially growing cells and the proteins were separated by SDS–PAGE (13% polyacrylamide gels) before immunoblotting with specific antibodies. The dots show the position of Sbh2p that cross-reacts with the Sbh1p antibodies.

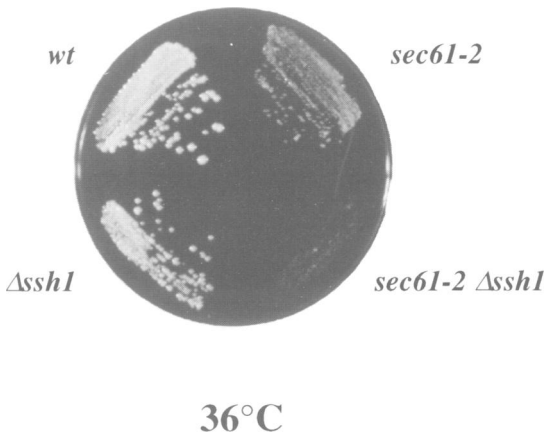

Fig. 5. Genetic interactions between *SEC61* and *SSH1*. Cells from a wild-type strain, from a ts mutant in *SEC61* (*sec61-2*) and from a deletion mutant of *SSH1* (Δ*ssh1*), as well as from a double mutant (Δ*ssh1sec61-2*) were tested for growth at 36°C on plates containing rich medium.

components Ssh1p and Sbh2p. The immunoprecipitated material was subsequently separated in SDS gels and probed in immunoblots with various antibodies. As expected, antibodies against Sec61p, Sbh1p or Sss1p brought down all three proteins as subunits of the Sec61p complex (lanes 1–3). Sbh1p and Sss1p antibodies also co-precipitated Sec62p, Sec71p and Sec72p (lanes 2 and 3), other components of the heptameric Sec complex (Panzner *et al.*, 1995). Conversely, antibodies against Sec62p or Sec71p co-precipitated all components of the trimeric Sec61p complex as well as the other subunits of the Sec complex (lanes 6 and 7). The fact that antibodies against Sec61p did not bring down Sec62p, Sec71p or Sec72p (lane 1) may perhaps be explained by assuming that they block a site in Sec61p that is involved in association with the tetrameric Sec62–Sec63p complex.

Antibodies against either of the novel components Ssh1p and Sbh2p precipitated both proteins (Figure 6A, lanes 4 and 5). Moreover, Sss1p was co-precipitated by both antibodies (lanes 4 and 5) and, conversely, antibodies against Sss1p brought down both Ssh1p and Sbh2p (as well as the components of the Sec61p and Sec complexes) (lane 3). These data indicate that Ssh1p, Sbh2p and Sss1p are contained in a complex. The latter appears to be distinct from the Sec61p complex since Ssh1p and Sbh2p were not co-precipitated by antibodies to Sec61p (lane 1). Antibodies to Sbh1p did precipitate them to a small extent (lane 2), but this is probably caused by their cross-reaction with Sbh2p (see above). Indeed, when the experiment was done in the opposite way, using antibodies against Ssh1p or Sbh2p, neither Sec61p nor Sbh1p was co-precipitated (lanes 4 and 5). Antibodies to Ssh1p or Sbh2p did not co-precipitate Sec62p, Sec71p or Sec72p (lanes 4 and 5) and, conversely, antibodies to Sec62p or Sec71p did not bring down Ssh1p or Sbh2p (lane 6). Taken together, these results indicate that Ssh1p, Sbh2p and Sss1p form a trimeric complex (named Ssh1p complex) that is distinct from the Sec61p complex and does not form a stable heptameric Sec complex.

The fact that two of the subunits of the Ssh1p complex are structurally related to the Sec61p complex raised the possibility that the third subunit may be a homologous protein distinct from Sss1p that cross-reacts with our Sss1p antibodies. We tested this possibility by immunoprecipitating proteins from microsomes of a yeast strain in which a deletion of *SSS1* is compensated for by the expression of the mammalian Sec61γ protein (Hartmann *et al.*, 1994). No reactivity with the Sss1p-specific antibody was observed (data not shown), indicating that the third subunit of the Ssh1p complex must be Sss1p itself.

To determine whether the Ssh1p complex contains additional subunits, we purified the complex by ion exchange chromatography (Figure 6B and C). A digitonin extract was prepared from yeast microsomes, and subjected to chromatography on a Q-Sepharose column. This column retains the heptameric Sec complex whereas both the Sec61p and Ssh1p complexes flow through. The latter were then bound to SP-Sepharose and eluted with a salt gradient. The trimeric Sec61p complex eluted mainly in fractions 10 and 11 (arrow 1), as demonstrated by blotting with antibodies directed against its subunits Sec61p, Sbh1p and Sss1p (Figure 6C). The Coomassie-stained gel shows that these fractions contained additional proteins which,

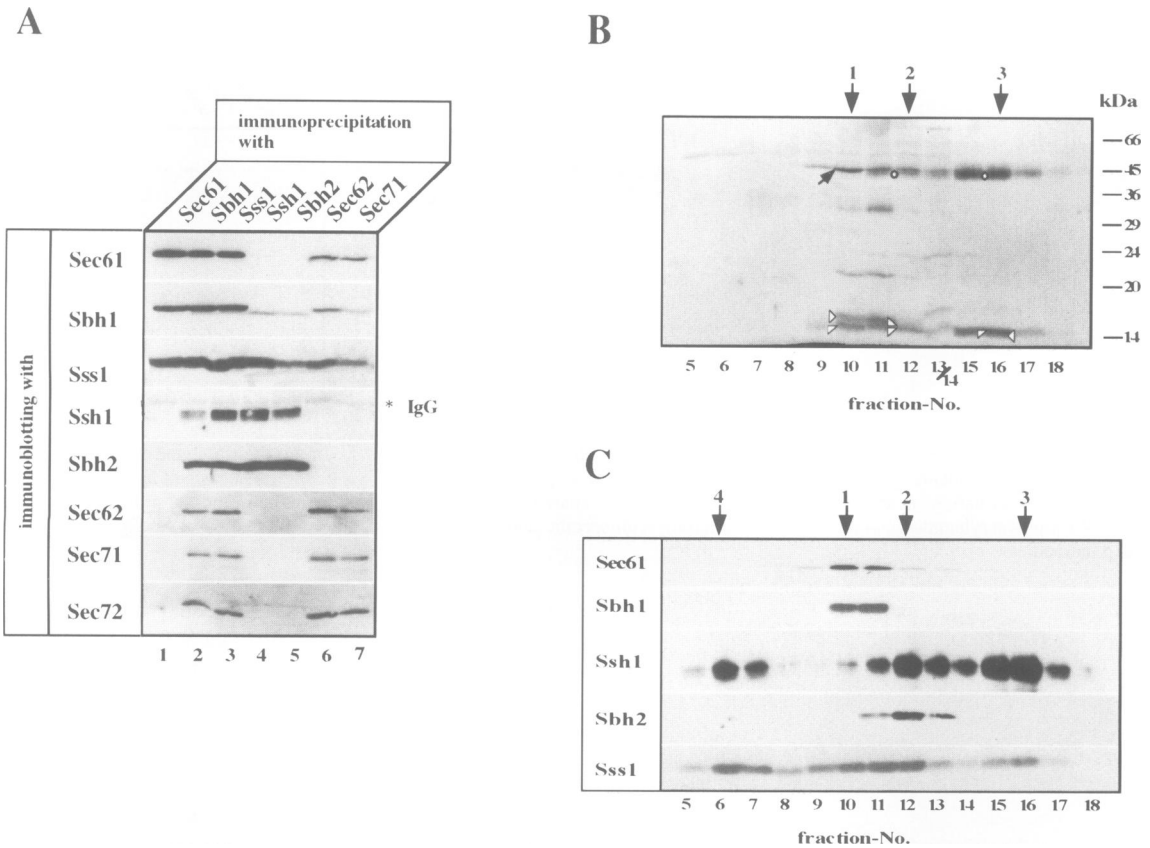

Fig. 6. Identification of a distinct Ssh1p complex. (**A**) Test for association of translocation components by co-immunoprecipitation. Microsomes from wild-type cells were solubilized in digitonin and aliquots of the detergent extract corresponding to 10 equivalents of microsomes were subjected to immunoprecipitation with various antibodies. The immunoprecipitated material was separated in SDS gels and proteins co-precipitated were analyzed by immunoblotting with various antibodies. The asterisk on the right side of the blots indicates the position of the immunoglobin heavy chains which were often detected in the immunoblots. (**B**) Separation of Sec61p and Ssh1p complexes. Shown is the last chromatographic step in the purification procedure in which the proteins were separated on a SP-Sepharose column (see Materials and methods for details). The proteins in the various fractions, corresponding to 1000 equivalents of microsomes, were separated in an SDS gel and stained with Coomassie Blue. The numbered arrows above the gel indicate the positions of the different complexes: arrow 1, trimeric Sec61p complex, consisting of Sec61p (small arrow), Sbh1p (middle triangle) and Sss1p (lower triangle); arrow 2, trimeric Ssh1p complex, consisting of Ssh1p (circle), Sbh2p and Sss1p (triangles, both proteins co-migrate); arrow 3, Ssh1p complex, comprised of Ssh1p (circle), Sss1p (left triangle, the upper band), and an N-terminally truncated form of Sbh2p (right triangle, the lower band; the N-terminal sequence of this form was determined to be RRQAQSIKEKQAKQTPT which corresponds to the sequence of Sbh2p starting with position 16). (**C**) Immunoblot analysis of the fractions shown in (B) using antibodies against various proteins. The samples correspond to 20 equivalents of microsomes. The numbers above the gels again show the position of the different complexes. Arrow 4 is a dimeric assembly of Ssh1p and Sss1p present in low amounts [it is not visible in the Coomassie-stained gel shown in (B)].

however, did not exactly co-elute (Figure 6B). Ssh1p eluted in three different peaks (Figure 6C). The peak in fraction 12 (arrow 2) is authentic Ssh1p complex that contains Ssh1p, Sbh2p and Sss1p. The peak in fractions 15 and 16 (arrow 3) contains Ssh1p, Sss1p and a degraded form of Sbh2p in which the N-terminus of the protein is lacking (determined by sequencing, see legend of Figure 6), and which therefore cannot react with the antibodies against Sbh2p. Both Sbh2p complexes did not contain any additional major protein that is detected by Coomassie Blue (see Figure 6B), indicating that the Ssh1p complex consists only of the three identified subunits. Some Ssh1p eluted in fractions 6 and 7 (arrow 4; Figure 6C), but this represented a minor fraction that could not be seen in the Coomassie-stained gel (Figure 6B). This fraction represents a dimeric complex of Ssh1p and Sss1p (see below).

Although Sbh1p and Sbh2p are found in distinct complexes in wild-type cells, it seemed possible that they could replace each other under conditions where one of them is absent. We therefore tested whether in single deletion mutants of *SBH1* or *SBH2* dimeric complexes would be found or if the remaining subunit would now associate with both Sec61p and Ssh1p. To this end, digitonin extracts from microsomes of the single deletion mutants were prepared and subjected to immunoprecipitation with various antibodies. Immunoblotting was used to detect proteins (Figure 7A and B). The results show that antibodies to Sec61p do not bring down Sbh2p if Sbh1p is absent (Figure 7A, lane 1) and, vice versa, antibodies to Ssh1p do not precipitate Sbh1p if Sbh2p is absent (Figure 7B, lane 4). Controls showed that the remaining Sbh subunit was associated with its usual partner proteins. Sec62p did not associate with the components of the Ssh1p complex, as in wild-type cells. These data indicate that even if only one Sbh subunit is present in the cell, it does not associate with the large subunit (Sec61p or Ssh1p) that lacks its partner. These results were confirmed by co-fractionation of the proteins on SP-Sepharose (Figure 7D and E). In extracts of microsomes

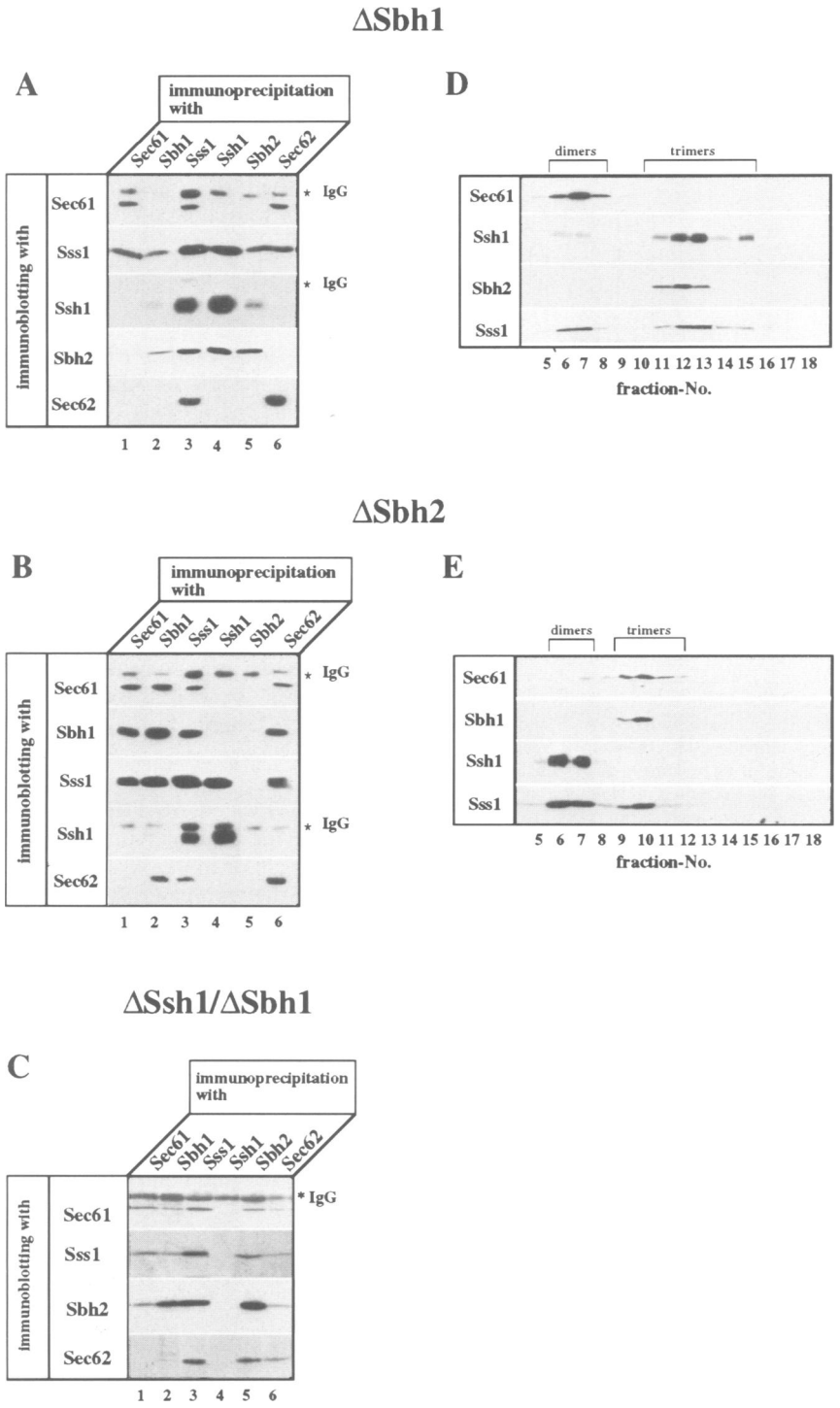

Fig. 7. Association of translocation components in mutant cells. (**A–C**) Co-immunoprecipitation of translocation components. Microsomes from mutant cells lacking either Sbh1p (A), Sbh2p (B) or Ssh1p and Sbh1p (C) were solubilized in digitonin and aliquots of the detergent extract corresponding to 10 equivalents of microsomes were subjected to immunoprecipitation with various antibodies. The immunoprecipitated material was separated in SDS gels and co-precipitated proteins were analyzed by immunoblotting with various antibodies. The asterisks on the right side of the blots indicate the position of the immunoglobin heavy chains which were often detected in the immunoblots. (**D** and **E**) Separation of translocation complexes on SP-Sepharose. Microsomes from mutant cells lacking either Sbh1p (D) or Sbh2p (E) were solubilized in digitonin. A fraction containing the Sec61p and Ssh1p complexes was subjected to chromatography on SP-Sepharose. The fractions eluted with a salt gradient were analyzed by SDS–PAGE and immunoblotting with various antibodies. Dimeric complexes elute in fractions 6–8, trimeric ones in fractions 9 or later. Material eluting in fraction 15 of (D) is Ssh1p complex containing a degraded form of Sbh2p (see Figure 6).

from the Δ*sbh1* strain, the dimeric complex of Sec61p and Sss1p eluted in fractions 6–8 (Figure 7D), distinctly earlier than the wild-type trimeric Sec61p complex (see Figure 6B and C), but the elution of the Ssh1p complex remained unaltered. Conversely, when *sbh2* was deleted, the dimeric complex of Ssh1p and Sss1p was found in

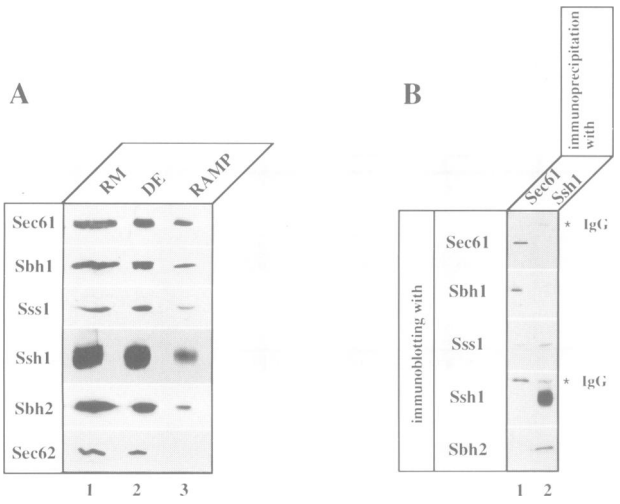

Fig. 8. The Ssh1p complex is found in association with membrane-bound ribosomes. (**A**) Yeast microsomes (RM) were washed with a buffer containing a high concentration of potassium acetate (0.8 M) to remove peripheral membrane proteins, and were then solubilized with digitonin. The ribosomes and associated membrane proteins were sedimented by centrifugation yielding a digitonin extract (DE) and a pellet from which the membrane proteins were released by treatment with puromycin/high salt (RAMP). Aliquots corresponding to 10 equivalents of microsomes were analyzed by SDS–PAGE and immunoblotting with various antibodies. (**B**) The RAMP fraction (corresponding to 20 equivalents of microsomes) was incubated with Sec61p or Ssh1p antibodies to test for the co-precipitation of other proteins. The immunoprecipitates were analyzed by SDS–PAGE and immunoblotting with various antibodies. The star indicates the position of the immunoglobulin heavy chain.

the early fractions, whereas the Sec61p complex was eluted as in extracts from wild-type cells (Figure 7E). In the *Δsbh1Δsbh2* mutant, both dimeric complexes eluted in the early fractions (not shown). Taken together, these data again indicate that distinct dimeric and trimeric complexes can co-exist in a cell and that the Sbh proteins do not normally switch partners.

We next tested whether Sbh2p would associate with Sec61p under conditions where both lacked their original partner proteins. Indeed, when detergent extracts from the double mutant *Δssh1Δsbh1* were subjected to immunoprecipitation, antibodies against Sbh2p co-precipitated Sec61p (Figure 7C, lane 5), and antibodies against Sec61p precipitated Sbh2p (lane 1).

To investigate whether the Ssh1p complex is associated with membrane-bound ribosomes, like the homologous Sec61p complex, rough microsomes from exponentially growing yeast cells were solubilized with digitonin, and the extract was centrifuged to obtain a supernatant fraction (DE, for digitonin extract) and a ribosome pellet. Membrane proteins associated with the ribosomes were then released by treatment with puromycin and high salt concentrations (referred to as RAMP, for ribosome-associated membrane proteins). Both the DE and RAMP fractions were probed in immunoblots with various antibodies (Figure 8A). As expected from previous results (Panzner *et al.*, 1995), Sec62p, representative of the heptameric Sec complex, was found almost exclusively in the DE, whereas the constituents of the trimeric Sec61p complex (Sec61p, Sbh1p and Sss1p) were found in both the DE and the RAMP fraction. Ssh1p and Sbh2p behaved similarly to the constituents of the trimeric Sec61p complex in that they were also in part associated with the ribosomes.

When the RAMPs were subjected to immunoprecipitation with antibodies against Sec61p or Ssh1p, only the components of the respective trimeric complex were precipitated (Sbh1p and Sss1p, or Sbh2p and Sss1p, respectively; see Figure 8B). Since the two trimeric complexes were not co-precipitated, they must bind ribosomes independently of each other.

Discussion

We have discovered a trimeric complex of membrane proteins in the ER of *S.cerevisiae*, composed of Ssh1p, Sbh2p and Sss1p. This Ssh1p complex is structurally related to the Sec61p complex that is believed to play a role in protein translocation. The largest, α-subunit (Ssh1p), is a rather distant homolog of Sec61p and is, in contrast to Sec61p, not essential for cell viability although it is required for normal cell growth. The β-subunit, Sbh2p, is more similar to its homolog, Sbh1p, of the Sec61p complex. Deletion of either *SBH1* or *SBH2* does not affect the growth of the cells, and leads to only mild translocation defects *in vivo*, providing an explanation for these components having escaped detection in genetic screens. The smallest, γ-subunit Sss1p, is common to both trimeric complexes.

We initially considered the possibility that the Ssh1p complex is located in a different organelle from the Sec61p complex; for example, in a lysosomal or pre-lysosomal compartment or in peroxisomes. However, the co-localization of Ssh1p with an ER marker in immunofluorescence experiments, the observed genetic interactions between Sec61p and Ssh1p, the fact that Sss1p is a common subunit of both complexes and the accumulation of precursors of secretory proteins in mutant cells lacking both Sbh1p and Sbh2p, argue that both trimeric complexes are located in the ER membrane.

Our results suggest that the Ssh1p complex is involved in protein transport across the ER membrane. While the deletion of *SBH1* alone has only minor effects on protein transport, the simultaneous deletion of both *SBH1* and *SBH2* leads to severe defects. Thus, Sbh2p must contribute to the translocation capacity of the cell. Furthermore, if *Δssh1* cells carry, in addition, a ts mutation in *SEC61* (*sec61-2* allele), their growth is impaired at a temperature at which *sec61-2* cells are barely affected (synthetic phenotype). Recent results have shown that the phenotype of the *sec61-2* mutant is caused by degradation of Sec61p (Sommer and Jentsch, 1993; Esnault *et al.*, 1994; Biederer *et al.*, 1996). The easiest interpretation of the observed synthetic phenotype is therefore that, at limiting concentrations of Sec61p, the contribution of the Ssh1p pathway becomes essential for the cell.

Although Ssh1p is clearly required for normal growth rates of the cells, it must have a more limited role in protein translocation than the Sec61p complex, despite the fact that they are both of about equal abundance in yeast microsomes (as judged from Coomassie-stained gels after their partial purification; see Figure 6B and unpublished data). This may be explained by our observation that only the Sec61p complex can associate with the Sec62–Sec63p complex to form a heptameric Sec complex;

the importance of the latter is indicated by the fact that both Sec62p and Sec63p are encoded by essential genes (Deshaies and Schekman, 1989; Sadler *et al.*, 1989) and that they both are almost exclusively found in this complex (Panzner *et al.*, 1995). The different behavior of the Sec61p and Ssh1p complexes in associating with the Sec62–Sec63p complex must be due to structural features of Ssh1p and Sec61p, since the difference persisted even in the absence of the β-subunits. Since only the heptameric Sec complex, and not the trimeric Sec61p complex, had the capacity to transport proteins post-translationally into reconstituted proteoliposomes (Panzner *et al.*, 1995), one may postulate that the trimeric Ssh1p complex also cannot function in the post-translational pathway of protein transport. Consistent with this hypothesis, in Δ*sbh2* cells, no accumulation of ppαF, a protein that can be transported post-translationally, was observed. In Δ*sbh1* cells, on the other hand, in which both the Sec61p and the Sec complexes lack a β-subunit, some defects in the translocation of ppαF were seen.

Rather than in the post-translational pathway, the Ssh1p complex may play a role in the co-translational mode of protein transport. It was found associated with ribosomes following the solubilization of rough microsomes in detergent, and it is released from them upon treatment with puromycin at high salt concentrations. These properties are identical to those of the Sec61p complex from yeast and mammals (Görlich *et al.*, 1992; Panzner *et al.*, 1995). Since the mammalian Sec61p complex has been demonstrated to function in reconstituted systems in co-translational protein transport (Görlich and Rapoport, 1993), we hypothesize that both trimeric yeast complexes also function in this pathway. A role for Sbh2p in the co-translational pathway would explain why cells lacking both Sbh1p and Sbh2p are more affected than those lacking only Sbh1p. It would also explain why the only obvious sequence homology between Sec61p and Ssh1p is found in their cytosolic loops that presumably have to interact with the ribosome. Interestingly, the lumenal domains are very different, suggesting that an interaction with a common lumenal protein is not required for the co-translational mode of protein transport. It should be noted that direct proof for a function of either the Sec61p or the Ssh1p complex in co-translational protein transport has to await the establishment of a reconstituted and SRP-dependent yeast system.

Taken together, the available evidence suggests a simple model: the Sec61p complex has a role in both co- and post-translational translocation pathways, as a separate entity and as a constituent of the heptameric Sec complex, respectively, and the Ssh1p complex functions only in the co-translational pathway. Why such a second co-translational transport system should exist is not entirely clear. One possibility is that the two trimeric complexes transport distinct protein substrates. In this case, one would have to assume that the Ssh1p complex only transports proteins that are not essential for cell viability or that the distinction is not absolute. One would also have to postulate two different targeting pathways that lead to either the Sec61p or the Ssh1p complex. Perhaps a more attractive idea is that the existence of a second co-translational system would allow the cell to regulate co- and post-translational pathways independently of each other. Obviously, if the Ssh1p complex did not exist, any change in the Sec61p complex would have an effect on both pathways simultaneously. The Ssh1p complex could therefore serve to change the level of co-translational transport under conditions where the post-translational pathway remains constant or, conversely, it could serve to maintain the flux through the co-translational pathway when the post-translational one is up- or down-regulated. Although the post-translational pathway could conceivably be regulated by the Sec62–Sec63p complex, its components may have other functions, such as in karyogamy or nuclear protein import (Kurihara and Silver, 1993; P.Walter, personal communication), thus preventing its free variation. An independent regulation of both translocation pathways may be particularly important for yeast cells that encounter vastly different growth conditions. Mammalian cells also have two *SEC61* genes but these are almost identical to each other (95% of the predicted amino acids are identical; Görlich *et al.*, 1992) and it is therefore uncertain whether they have the same relationship as the Sec61p–Ssh1p pair in yeast.

Our results also shed light on the role of the two newly discovered β-subunits (Sbh1p and Sbh2p). Since even the double deletion mutant has no growth phenotype at moderate temperatures, the β-subunits cannot be absolutely essential for the function of either the co- or post-translational translocation apparatus. Dimeric assemblies of Sec61p or Ssh1p with Sss1p must therefore be functional and can indeed be purified as stable complexes. Interestingly enough, even though the two β-subunits are quite similar in sequence, they do not appear to replace each other in their respective complexes as long as their genuine α-subunit partner is present. However, Sbh2p can associate with Sec61p if they both lack their original partners. This fact explains why the level of Sbh2p is not reduced in the double mutant Δ*ssh1*Δ*sbh1*, in contrast to the situation in the Δ*ssh1* single mutant. Apparently, the association of Sbh2p with one or the other α-subunits is sufficient for its stabilization. The undiminished levels of Sbh2p in Δ*ssh1*Δ*sbh1* cells also provide an explanation as to why this mutant does not show a ts phenotype comparable with that of the double deletion of *SBH1* and *SBH2*.

The situation with the β-subunits in yeast is reminiscent of that in *Escherichia coli*, in which the intermediate sized subunit SecGp of the homolog of the Sec61p complex, the SecYEGp complex, is not essential (Nishiyama *et al.*, 1993, 1994). SecYp and SecEp alone are also sufficient for translocation of proteins into reconstituted proteoliposomes, although the reaction is greatly stimulated by the addition of SecGp (Akimaru *et al.*, 1991; Nishiyama *et al.*, 1993). Taking further into account that there is no structural similarity between Sbh1p or Sbh2p and SecGp, it appears that the β-subunits generally have a less fundamental role.

Materials and methods

Strains and general methods

The yeast strains used in this study are listed in Table I. Genetic experiments and methods employing molecular biology were carried out as described (Ausubel *et al.*, 1992). The accumulation of precursor proteins in mutant cells was tested as described by Rothblatt *et al.* (1989). Immunoblots were visualized by Enhanced Chemiluminescence (ECL, Amersham Corporation).

Table I. Yeast strains

Strain	Genotype	Source or reference
YFP338	*matα, sec61-2, leu2-3,-112, ura3-52, ade2-3, pep4-3*	Rose *et al.* (1989)
YTX69	*mata/α*, homozygous *his3-11,-15, leu2-3,-112, trp1-1, ura3-1, ade2-1, can1-100*	Hartmann *et al.* (1994)
YTX83	*mata/α, Δssh1::LEU2/SSH1, homozygous his3-11,-15, leu2-3,-112, trp1-1, ura3-1, ade2-1, can1-100*	this study
YTX84	*matα, Δssh1::LEU2, his3-11,-15, leu2-3,-112, trp1-1, ura3-1, ade2-1, can1-100*	this study
YTX87	*matα, his3-11,-15, leu2-3,-112, trp1-1, ura3-1, ade2-1, can1-100*	this study
YKF5	*matα, sec61-2, Δssh1::ADE2, leu2-3,-112, ura3-52, ade2-3, pep4-3*	this study
YKF7	*mata/α, Δsbh1::HIS3/SBH1, Δsbh2::ADE2/SBH2*, homozygous *his3-11,-15, leu2-3,-112, trp1-1, ura3-1, ade2-1, can1-100*	this study
YKF8	*matα, Δsbh1::HIS3, his3-11,-15, leu2-3,-112, trp1-1, ura3-1, ade2-1, can1-100*	this study
YKF9	*matα, Δsbh2::ADE2, his3-11,-15, leu2-3,-112, trp1-1, ura3-1, ade2-1, can1-100*	this study
YKF16	*matα, Δsbh1::HIS3, Δsbh2::ADE2, his3-11,-15, leu2-3,-112, trp1-1, ura3-1, ade2-1, can1-100*	this study
YKF19	*matα, Δssh1::LEU2/Δsbh2::HIS3, his3-11,-15, leu2-3,-112, trp1-1, ura3-1, ade2-1, can1-100*	this study
YKF20	*mata/α, Δssh1::LEU2/SSH1, Δsbh2::ADE2/SBH2*, homozygous *his3-11,-15, leu2-3,-112, trp1-1, ura3-1, ade2-1, can1-100*	this study
YKF24	*mat?, Δssh1::LEU2/Δsbh1::HIS3, his3-11,-15, leu2-3,-112, trp1-1, ura3-1, ade2-1, can1-100*	this study

Antibodies and immunoprecipitation

Antibodies were raised against peptides corresponding to the C-terminus of Ssh1p (NQVLGVPGAM) and the N-terminus of Sbh2 (AASVPPGGQRI) with additional Cys residues at the N- and C-termini, respectively. Affinity purification and immobilization of the antibodies were carried out as described (Görlich *et al.*, 1992; Görlich and Rapoport, 1993). Affinity-purified antibodies against Sec61p, Sbh1p, Sss1p and Sec62 used in this study are described by Panzner *et al.* (1995). 9E10 antibodies specific for the c-myc epitope were purified from the supernatant of the hybridoma cell line GE10 (a kind gift of Dr J.Behrens) on a protein G–Sepharose column (Protein G Sepharose Fast Flow, Pharmacia). Antibodies specific for Kar2p and α-factor were kindly supplied by Drs M.Rose and R.Schekman, respectively. Immunoprecipitations were done as described (Görlich *et al.*, 1992).

Cloning and deletion of SSH1, SBH1 and *SBH2*

Clones for *SSH1*, *SBH1* and *SBH2* were isolated from a genomic library (cloned into the plasmid pSEY8) by hybridization with oligonucleotides. *SSH1* was found by screening with the oligonucleotide 5′-GCGCGTAGCAGAGAGAATTTGATC-3′, which corresponds to the sequence of the 5′ end of the clone S77888 in the gene bank. *SBH1* was found by screening with the oligonucleotide 5′-ACTCTACCTCCTACCATACTCC-3′ which corresponds to the 5′-non-translated region of the clone SCE9747 in the gene bank. *SBH2* was found by screening with the degenerate oligonucleotide 5′-GA(AG)AA(AG)CA(AG)GCNAA(AG)CA(AG)AC-3′ that is derived from the peptide sequence EKQAKQT determined by protein sequencing of Sbh2p. The complete nucleotide sequences of positive clones were determined. While this work was in progress, the sequence of *SSH1* was published by Feldmann *et al.* (1994).

For construction of a null allele of *SSH1*, a 1756 bp *Nhe*I–*Sna*BI fragment from a genomic library clone was subcloned into vector pUC19. Subsequently, a 649 bp *Eco*RI–*Bgl*II fragment of the coding region was replaced by a 2.2 kb fragment containing either the *LEU2* or the *ADE2* marker gene. For construction of *Δsbh1::HIS3*, a 410 bp fragment corresponding to the 5′-flanking region (5′FR, nucleotides −387 to +23; position +1 refers to the A residue of the ATG start codon) and a 361 bp fragment corresponding to the 3′-flanking region (3′FR, nucleotides +224 to +584) of *SBH1* were amplified by PCR from a genomic library clone. The *HIS3* marker gene was cloned between the two flanking regions of the *SBH1* gene. Disruption of the *SBH2* gene was performed in an analogous manner. The 5′FR included the nucleotides −215 to +17, the 3′FR the nucleotides +227 to +637. The *ADE2* or *HIS3* marker genes were placed between the flanking regions, resulting in plasmids pKF20 and pKF21, respectively. All deletion constructs were introduced into the diploid wild-type strain YTX69. Double mutants were generated by a second round of transformation with a deletion construct (YKF16 and YKF19) or by crossing of the single mutants and subsequent tetrad dissection (YKF24). A double mutant carrying a ts allele of *SEC61* and a deletion of *SBH1* was constructed by transformation of the *sec61-2* strain (YFP338) with a *Δssh1::ADE2* construct. The resulting strains were verified by immunoblotting blotting and, in some cases, by Southern analysis.

Epitope tagging of SSH1 and immunofluorescence

Ssh1p was tagged with the myc epitope at its C-terminus. The gene was modified by mutagenesis of the nucleotides 13 and 16 upstream of the stop codon to introduce a *Kpn*I restriction site. This site was used for the insertion of a linker consisting of two oligonucleotides encoding the myc epitope (sense: 5′-CAGGTGCTATGGAACAAAAGTTGATCTCTGAAGAAGACTTGTAAGCATGCGTAC-3′ and antisense: 5′-GCATGCTTACAAGTCTTCTTCAGAGATCAACTTTTGTTCCATAGCACCTGGTAC-3′). The myc-tagged version of *SSH1* under the control of its native promotor was subcloned into the ARS/CEN vector pRS414 (Ausubel *et al.*, 1992), resulting in plasmid pKF15, which was used for transformation of the *Δssh1::LEU2* strain (YTX84). Cells were processed for indirect immunofluorescence as described (Guthrie and Fink, 1991). Primary antibodies were used at a concentration of 1:5000 for rabbit anti-Kar2p and 1:20 for mouse anti-c-myc. As secondary antibodies either 1:100 goat anti-rabbit–fluorescein 5-isothiocyanate (FITC) or 1:100 goat anti-mouse–FITC were used. The mounting medium was supplemented with DAPI for staining of the nuclei. Slides were examined at 1000× magnification in a Zeiss Axiophot microscope. Images were recorded on Ilford HP5 film.

Isolation of membranes

For the immunoblot analysis of total membrane proteins, microsomes were isolated from 10 A_{600} nm units of exponentially growing yeast cells. The cells were washed once with 10 mM azide and resuspended in 200 ml of homogenization buffer (50 mM Tris pH 7.5, 10 mM EDTA) containing 10 mg/ml leupeptin, 5 mg/ml chymostatin and 5 mg/ml pepstatin. They were homogenized with 1 vol of glass beads by four repeated cycles of mixing with a vortex for 30 s at maximum speed, interrupted by 30 s incubations on ice. The cell lysates were removed from the glass beads with 1 ml of homogenization buffer containing 1 mg/ml leupeptin, 0.5 mg/ml chymostatin and 0.5 mg/ml pepstatin and centrifuged at 400 *g* for 10 min at 4°C. The supernatants were subjected to centrifugation for 10 min at 12 000 *g* at 4°C and the resulting membrane pellets were resuspended in 100 ml of SDS sample buffer containing 50 mM dithiothreitol (DTT).

For most experiments, microsomes were isolated on a larger scale as described (Panzner *et al.*, 1995). Eighty grams of yeast cells yielded ~75 000 eq of rough microsomal membranes [1 equivalent (eq) of membranes is defined as 1 ml of a membrane suspension with an absorption at 280 nm of 50]. The microsomes were washed with a buffer containing 0.8 M potassium acetate and then solubilized in 3% digitonin in the same buffer, as described (Panzner *et al.*, 1995). After centrifugation, a digitonin extract and a ribosome pellet were obtained. From the latter, RAMPs were released by treatment with puromycin/high salt as described (Panzner *et al.*, 1995). Both fractions were subjected to immunoprecipitation with various antibodies.

Purification of the Ssh1p complex

A digitonin extract of yeast microsomes (Panzner *et al.*, 1995) was diluted with LD buffer (20 mM HEPES/KOH pH 7.5, 10% glycerol, 1% digitonin, 2 mM DTT) to a final salt concentration of 150 mM. After incubation with SP-Sepharose (1 ml per 40 A_{280} nm units), the resin was washed with a buffer consisting of a mixture of 20% HD buffer (50 mM HEPES/KOH pH 7.5, 1.2 M potassium acetate, 10% glycerol, 1% digitonin, 2 mM DTT) and 80% LD buffer. The proteins were eluted with 80% HD/20% LD buffer. After dialysis against 20 mM HEPES/KOH pH 7.5, 1 mM magnesium acetate, 400 mM sucrose and

2 mM DTT, the solution was passed through a 40 ml column of Q-Sepharose Fast Flow equilibrated in LD buffer. The flow-through fractions were bound to a 1 ml SP-Sepharose column (HiTrap SP, Pharmacia) and eluted with 30 ml of a linear gradient from 15 to 65% of HD/LD buffer. Fractions of 1.5 ml were collected.

In vitro translocation assays

For *in vitro* translocation, ppαF and proOmpA were synthesized in the reticulocyte lysate in the presence of [^{35}S]methionine. After translation, cycloheximide was added and the ribosomes were sedimented before addition of the microsomes (Panzner *et al.*, 1995). The samples were incubated at 22°C in the presence of 1 mM ATP and an energy-regenerating system (10 mM creatine phosphate and 1 mg/ml creatine kinase) for different periods of time. Translocated material was assayed after incubation with proteinase K by SDS–PAGE and analysis with a phosphorimager (Panzner *et al.*, 1995).

Acknowledgements

We are grateful to Drs M.Rose and R.Schekman for gifts of antibodies and yeast strains and to K.Verhey, B.Jungnickel, M.Rolls and S.M.Rapoport for critical reading of the manuscript. This work was supported by grants to T.S. from the Deutsche Forschungsgemeinschaft (So 271/2-1), and to E.H. and T.A.R. from the Deutsche Forschungsgemeinschaft, the BMFT, the EC and the Human Science Frontier Program.

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Received on October 20, 1995; revised on November 24, 1995

Note added in proof

Toikkanen *et al.* [(1996) *Yeast*, **12**, in press] report the cloning of *SEB1* and *SEB2* genes in *S.cerevisiae* which are identical with *SBH1* and *SBH2*, respectively, described here.