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Recognition of a Subset of Signal Sequences by Ssh1p, a Sec61p-related Protein in the Membrane of Endoplasmic Reticulum of Yeast *Saccharomyces cerevisiae*

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Ssh1p of *Saccharomyces cerevisiae* is related in sequence to Sec61p, a general receptor for signal sequences and the major subunit of the channel that guides proteins across the membrane of the endoplasmic reticulum. The split-ubiquitin technique was used to determine whether Ssh1p serves as an additional receptor for signal sequences in vivo. We measured the interactions between the N_{ub}-labeled Ssh1p and C_{ub}-translocation substrates bearing four different signal sequences. The so-determined interaction profile of Ssh1p was compared with the signal sequence interaction profile of the correspondingly modified N_{ub}-Sec61p. The assay reveals interactions of Ssh1p with the signal sequences of Kar2p and invertase, whereas Sec61p additionally interacts with the signal sequences of Mfα1 and carboxypeptidase Y. The measured physical proximity between Ssh1p and the β-subunit of the signal sequence recognition particle receptor confirms our hypothesis that Ssh1p is directly involved in the cotranslational translocation of proteins across the membrane of the endoplasmic reticulum.

INTRODUCTION

In *Saccharomyces cerevisiae* signal sequence-bearing proteins can be targeted to the membrane of the endoplasmic reticulum (ER) either co- or posttranslationally (Hann and Walter, 1991). The signal recognition particle (SRP) interacts with a subset of signal sequences shortly after their synthesis and thereby initiates their cotranslational translocation across the ER membrane (Keenan *et al.*, 2001). Proteins bearing signal sequences that are not recognized by the SRP translocate at a significantly later state of their synthesis. The translocation of these proteins depends on the Sec62p/Sec63p complex, an assembly of four different proteins that is localized in the ER membrane (Deshaies *et al.*, 1991; Brodsky and Schekman, 1993; Panzner *et al.*, 1995). To distinguish whether a certain protein is targeted co- or posttranslationally the translocation of the protein is usually monitored in a strain harboring a mutation in one of the two targeting pathways. A hindrance of translocation in one of the two strains identifies the targeting pathway taken by this protein, provided that the protein cannot sidestep this obstruction by using the alternative way. According to this criterion the signal sequences of carboxypeptidase Y (CPY) and Mfα1 use the posttranslational mode, whereas the membrane proteins DPAP1 and Och1p strictly rely on the cotranslational targeting pathway (Ng *et al.*, 1996). The preference for either of the two pathways correlates with the hydrophobicity of the respective signal sequence. Comparatively low hydrophobicity selects the postranslational pathway, whereas stronger hydrophobic signal sequences prefer to translocate via the SRP (Bird *et al.*, 1987; Ng *et al.*, 1996; Martoglio and Dobberstein, 1998). However, the spectrum of signal sequences covers hydrophobicities that lie between those of CPY and DPAP1. Mutations in one of the two targeting pathways influence the translocation of these proteins to only a certain degree. The translocation of Kar2p and invertase, for example, is severely affected but not completely abolished in a strain carrying a deletion of the SRP (Hann and Walter, 1991; Johnsson and Varshavsky, 1994b; Ng *et al.*, 1996).

After being targeted to the ER membrane signal sequences are recognized by Sec61p (Jungnickel and Rapoport, 1995; Plath *et al.*, 1998). Sec61p is the major constituent of the channel that guides the proteins across the membrane (Rothblatt *et al.*, 1989; Görlich *et al.*, 1992; Hanein *et al.*, 1996; Beckmann *et al.*, 1997; Menetret *et al.*, 2000). Both targeting pathways converge at this point. The proteins targeted via the posttranslational pathway are translocated by the hep-

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tameric Sec-complex. This complex consists of the trimeric Sec61p and the tetrameric Sec62p/Sec63p complex (Deshaies *et al.*, 1991; Brodsky and Schekman, 1993; Panzner *et al.*, 1995). The cotranslational substrates are probably directly delivered to the trimeric Sec61-complex via SRP and the SRP receptor (SR) (Bacher *et al.*, 1999; Johnson and van Waes, 1999; Song *et al.*, 2000).

In yeast a second complex with high similarity to the trimeric Sec61p complex has been described previously (Finke *et al.*, 1996). Ssh1p is related to Sec61p and forms a trimeric complex with Sbh2p and Sss1p. Sbh2p shares sequence similarity with Sbh1p, the β-subunit of the Sec61p complex, and Sss1p is present in both trimeric complexes as the γ-subunit (Esnault *et al.*, 1993; Finke *et al.*, 1996). The functions of Ssh1p are not immediately evident. A strain carrying a deletion of *SSH1* shows no obvious translocation defects (Finke *et al.*, 1996; Ng *et al.*, 1996). However, a strain that combines a deletion of *SSH1* with the *sec61-2* temperature-sensitive allele is not viable at the permissive temperature for the *sec61-2* allele (Finke *et al.*, 1996). Furthermore, both Sec61p and Ssh1p bind to ribosomes and therefore seem to share a certain subset of functions (Prinz *et al.*, 2000). Although the nature of these functions remains unknown the presence of an alternative Sec-complex gives reason to the assumption that the entire range of signal sequences is not distributed between two but between three different channels: the trimeric Sec61p complex, the heptameric Sec-complex, and the trimeric Ssh1p complex.

In this study we tested this hypothesis by using the split-ubiquitin (split-Ub) technique to monitor the in vivo flux of signal sequences across the different channels in the membrane of the ER. Using this assay we show that Ssh1p in contrast to Sec61p exclusively recognizes proteins bearing signal sequences of stronger hydrophobic character.

MATERIALS AND METHODS

Construction of Fusion Proteins

The construction of the signal sequence bearing C_{ub} constructs of invertase and Mfα1 is described in Dünnwald *et al.* (1999). *CPY$_{30}$-CUB-Dha/URA3* was derived from construct XX of Johnsson and Varshavsky (1994a) and the *Mfα1$_{37}$-CUB* construct by replacing the *Cla*I-*Sal*I fragment containing the Mfα1$_{37}$ sequence by the corresponding sequence of the CPY gene (*PRC1*). To obtain *KAR2$_{20}$*- and *KAR2$_{40}$-CUB-URA3*, the Mfα1$_{37}$ sequence of the *Mfα1$_{37}$-CUB-URA3* was replaced by a *Cla*I *Sal*I cut polymerase chain reaction (PCR) fragment obtained from genomic DNA of the yeast strain JD53 and the appropriate PCR primers. The PCR primers for *KAR2$_{40}$* were as follows: 5′CCTCCATCGAT**ATG**TTTTTCAACAGACTAAG and 5′CCTCCGTCGACCC**AAT**TTCAGTCTTACCATTTTT. The PCR primers for KAR2$_{20}$ were as follows: 5′CCTCCATCGAT**ATG**TTTTTCAACAGACTAAG and 5′CCTCCCGTCGACCC**TCT**AACTAAAACATTGG. The underlined sequences are the *Cla*I and *Sal*I sites, respectively. Indicated in bold are the first or the last triplets from the sequence of *KAR2*. The expression of the Ura3p-based C_{ub} fusions was mediated by the P_{CUP1} promoter. The expression of the C_{ub}-dihydrofolate reductase fusions (DHFR-Dha) was mediated by the P_{ADH1} promoter. The construction of the N_{ub} fusions of *SEC61*, *SEC62*, *BOS1*, *STE14*, *SED5*, *SSH1*, *SEC22*, *TPI1* are described in Wittke *et al.* (1999). The N_{ub}-constructs of *UBC6* were assembled from the P_{CUP1}-N_{ub}-cassette and a PCR fragment containing the open reading frame (ORF) of *UBC6* and 188 nucleotides downstream of the STOP codon. A *Bam*HI site was used to bring the N_{ub} in frame with the PCR product. The linker between the last codon of N_{ub} (bold letters) and the second codon of *UBC6* (bold letters) reads GG ATC**CCT**GGGTCTGGG**GCT**. The *Bam*HI site is underlined. To insert the ha-epitope between N_{ub} and *SEC61* or *SSH1* we replaced the N_{ua} moiety in the corresponding N_{ua}-constructs by a newly created N_{ua}-ha module. *N_{ua}-HA* was constructed via PCR with *P_{CUP1}-N_{ua}* as a template and a PCR primer annealing to the sequence of the P_{CUP1} promoter and a primer annealing to the C-terminal coding sequence of *N_{ub}* and additionally harboring the sequence encoding the *HA* epitope. This primer reads 5′CCCCGGATCCC**AGCGTAATCTGGAACATCGTATGGGTA***CCCGATCCCTTCCTTGTCTTGAAT*. The BamHI restriction site is underlined, the *HA* coding sequence is shown in bold letters, and the *N_{ub}* sequence is shown in italic letters. The PCR product was ligated in front of the coding sequences of *SEC61* and *SSH1* by using the *Bam*HI restriction site. The obtained fusion products were integrated into the genome of the yeast JD53 as detailed in Wittke *et al.* (1999). The correct integration was verified by a diagnostic PCR. All N_{ub}-fusion proteins were expressed from the P_{CUP1} promoter in the pRS314 vector (Sikorski and Hieter, 1989).

SEC63-CUB-RURA3 was constructed using two primers to amplify the complete ORF of *SEC63* with genomic DNA as a template. The PCR product was cut with *Bam*HI and *Sal*I and inserted between the *CUB-RURA3* module and the P_{MET25} promoter in the vector pRS313 containing a CEN ARS element. The linker between the last codon (bold letters) of *SEC63* and the first codon of *CUB* (bold letters) reads **GAA**GGC GGG TCG ACC **GGT.** The *Sal*I site is underlined. The same PCR product was used to insert the ORF of *SEC63* between the P_{GAL1} promoter and the coding sequence of the ha-epitope to create *SEC63-ha* in the vector pRS424. The plasmids expressing Sec62p or Ste14-Dha from the P_{GAL1} promoter on a pRS313 vector are described in Wittke *et al.* (1999). *SRβ-CUB-RURA3* was constructed by PCR amplification of the last 479 base pairs of the coding sequence of *SRP102* not including the stop codon by using genomic DNA of *S. cerevisiae* as a template. The ends of the PCR product contained restriction sites to allow the in-frame fusion with the *CUB-RURA3* module located in the vector pRS303. The short linker sequence between the last codon of *SRP102* and the first codon of *CUB* reads **CTG** TCC GGG TCG ACC **GGT**. The last codon of *SRP102* and the first codon of *CUB* are in bold letters, and the *Sal*I site is underlined. The vector was cut at its unique *Sph*I site in the *SRP102*-containing sequence and transformed into the *S. cerevisiae* strain JD53 to yield, through homologous recombination, the integrated cassette that expressed SRβ-C_{ub}-RUra3p from the native promoter. Integration was confirmed by diagnostic PCR. The unmodified *SSH1* expressed from the P_{CUP1} promoter was obtained by PCR with genomic DNA as a template and two oligonucleotides priming at the start codon and 160 nucleotides downstream of the stop codon, respectively. The obtained fragment was cut with *Bam*HI and *Sal*I and inserted behind the P_{CUP1} promoter on a pRS315 vector.

Deletion of SSH1

The open reading frame of *SSH1* was replaced by the dominant kan MX marker essentially as described by Güldener *et al.* (1996). The PCR primers used for the construction of the kan MX disruption cassette were as follows: 5′ TTTAGCACATTTGCCCCCGCCACTCTCCATTGTTTTAGTACCAGCTGAAGCTTCGTACGC and 5′ TACGTATATAAATGCGCGTAGCAGAGAGAATTTGATCTTCTAGGCCACTAGTGGATCTG. Transformed yeast cells were selected for kan MX integration by Geneticin (Invitrogen, Paisley, Scotland), deletion was verified by diagnostic PCR, and the complementation of the small growth defect by the plasmid-borne *SSH1*.

Immunoblotting

Cell extraction for immunoblotting was performed essentially as described previously (Johnsson and Varshavsky, 1994b). All exper-

iments were performed without adding additional amounts of copper to the medium. Proteins were fractionated by SDS-12.5% PAGE and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), by using a semidry transfer system (Hoefer Pharmacia Biotech, San Francisco, CA). Blots were incubated with a monoclonal anti-ha antibody (Babco, Richmond, CA), or with the anti-Sec61p antibody. Bound antibody was visualized with horseradish peroxidase-coupled rabbit anti-mouse or goat anti-rabbit antibody, respectively (Bio-Rad, Hercules, CA), by using the chemiluminescence detection system (Pierce Chemical, Rockford, IL). The chemiluminescence was quantified with the aid of the lumi-imager system (Roche Applied Science, Mannheim, Germany). For comparing the amounts of expressed N_{ub}-ha-Sec61p and N_{ub}-ha-Ssh1p protein extracts were diluted with twofold PAGE sample buffer and heated for 20 min at 40°C before electrophoresis. The chemiluminescence was captured by a Hypofilm ECL (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, United Kingdom). The exposed film was scanned and quantified with the aid of the Aida/two-dimensional densitometry program (Raytest Isotopenmeβ, Straubenhardt, Germany).

Media and Interaction Assays

Yeast rich (YPD) and synthetic minimal media with 2% dextrose (SD) or 2% galactose (SG) followed standard recipes (Dohmen *et al.*, 1995).

For interaction assays, *S. cerevisiae* cells were first grown at 30°C in liquid selective media containing uracil to an OD_{600} of 1. 4 μl of these cultures, and serial 1:10 dilutions in water were spotted on agar plates selecting for the presence of the fusion constructs and lacking uracil. All experiments were performed without adding additional amounts of copper to the medium. The same dilutions were also spotted onto plates containing uracil to check for cell numbers. The plates were incubated at 30°C for 2–5 d.

RESULTS

Ssh1p Transiently Interacts with a Subclass of Signal Sequences

The split-Ub assay can detect the transient interactions that occur between a signal sequence-bearing protein and a component of the translocation machinery. For this application of the technique the N-terminal half of Ub (N_{ub}) is fused to the N terminus of a membrane protein of the translocation machinery and the C-terminal half of Ub (C_{ub}) is sandwiched between a signal sequence and a reporter protein. As soon as the signal sequence brings the attached C_{ub} into proximity of the N_{ub}, the two Ub halves will reconstitute the native-like Ub and the reporter will be cleaved off from the C terminus of C_{ub} by the ubiquitin-specific proteases (Ubps) (Figure 1) (Johnsson and Varshavsky, 1994a; Dünnwald *et al.*, 1999). Because the assay requires the cytosolic location of the reconstituted Ub only those interactions can be monitored that occur shortly before and during the translocation of the C_{ub} on the cytosolic face of the membrane. Sec61p and Ssh1p share a sequence identity of 34% across the entire lengths of the proteins. The N terminus of Sec61p points into the cytosol of the cell (Wilkinson *et al.*, 1996). The same topology was indirectly confirmed for Ssh1p (Wittke *et al.*, 1999). The N termini of both proteins were labeled with N_{ub} to directly compare their activities toward different signal sequence-bearing C_{ub} substrates. We knew from our previous studies that both N_{ub}-fusions displayed a comparable activity toward a C_{ub}-fusion of Ste14p. Ste14p does not interact with Sec61p nor Ssh1p (Wittke at al., 1999). Consequently, a measured difference in cleavage of a tested C_{ub}-fusion should indicate its specific interaction with either N_{ub}-Sec61p or N_{ub}-Ssh1p. We envision three scenarios if

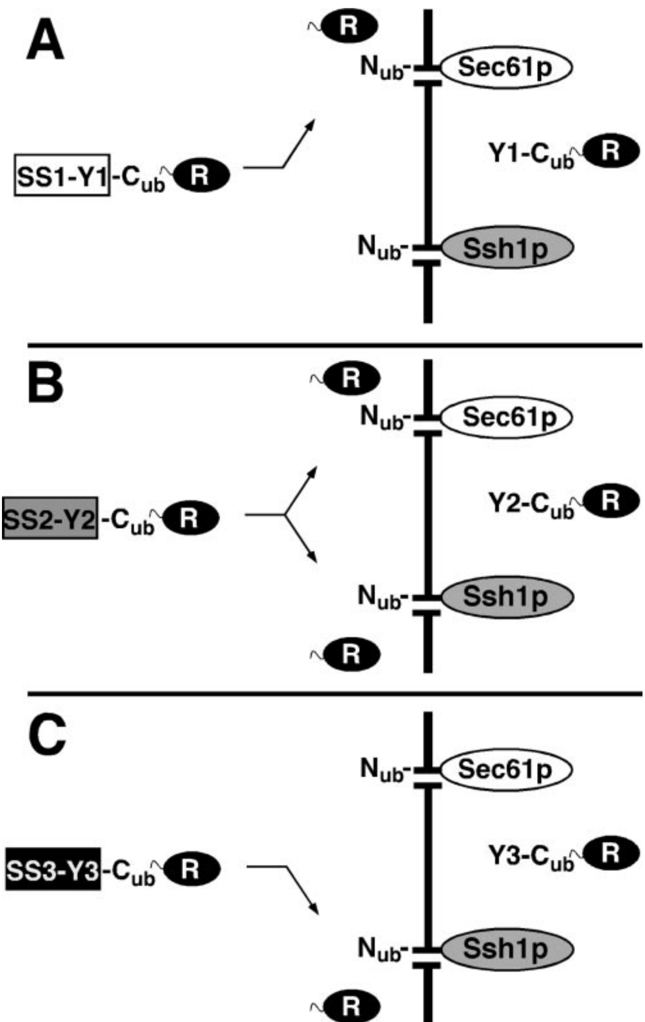

Figure 1. Split-Ub assay was used to measure the flow of a signal sequence-bearing C_{ub} fusion across Sec61p- or Ssh1p-translocation channels. Cells containing either N_{ub}-Sec61p or N_{ub}-Ssh1p were cotransformed with a signal sequence bearing SS-Y-C_{ub}-reporter fusion. SS indicates the signal sequence of a protein that is translocated in yeast. Y indicates the peptide that is derived from this protein to separate the hydrophobic core of the signal sequence from the Cub. The numbers 1–3 indicate three different proteins, each translocating across the membrane by using a different combination of channels. (A) If the signal sequence SS1 is translocated exclusively via Sec61p only the N_{ub}-moiety of the labeled Sec61p will get close to the C_{ub} of the translocation substrate. Consequently, the reporter activity (R) will be cleaved off by the ubiquitin-specific proteases and released into the cytosol of only those cells that contain N_{ub}-Sec61p. (B) Signal sequence SS2 that translocates via Sec61p or Ssh1p will induce the cleavage of the reporter in cells containing either N_{ub}-Sec61p or N_{ub}-Ssh1p. (C) Signal sequence SS3 that translocates exclusively via Ssh1p will induce cleavage of the attached C_{ub}-Reporter only in cells containing N_{ub}-Ssh1p but not in cells containing N_{ub}-Sec61p.

Ssh1p constitutes an independent translocation pore (Figure 1): 1) The C_{ub}-Reporter is attached to a signal sequence that translocates exclusively via Sec61p. Here cleavage of the reporter is observed in cells cotransformed with N_{ub}-Sec61p but not in cells carrying N_{ub}-Ssh1p (Figure 1A). 2) The C_{ub}-Reporter is attached to a signal sequence that translocates via Sec61p and Ssh1p. Here cleavage is observed in cells synthesizing either N_{ub}-Sec61p or N_{ub}-Ssh1p (Figure 1B). 3) The C_{ub}-Reporter is attached to a signal sequence that translocates exclusively via Ssh1p. Here cleavage is only observed in cells carrying N_{ub}-Ssh1p (Figure 1C). We chose the N-terminal sequences of the α-factor (Mfα1-), invertase (Suc2-), CPY-, and Kar2p (Kar2-) as the signal sequences of the C_{ub} constructs. The lengths of the peptides (spacer sequence) that separate the hydrophobic core of the signal sequences from the C_{ub} moiety are denoted as indices. We first used the ha-epitope–tagged mouse DHER (Dha) as a reporter protein. The Ubp-induced cleavage at the C_{ub}-Dha junction was detected by immunoblotting with the ha-antibody after cell extraction and denaturing gel electrophoresis. Cleavage is indicated by the presence of the free Dha of ~28 kDa. The translocated and uncleaved fraction of the C_{ub}-fusion protein gives rise to a second band with a higher molecular mass. However, due to differences in stability, glycosylation, and secretion, the fraction of the translocated proteins cannot be quantitatively compared among the different signal sequence bearing C_{ub}-fusions by Western blotting (Dünnwald *et al.*, 1999). Figure 2A shows that the coexpression of N_{ub}-Sec61p with the four different signal sequence-bearing substrates resulted in a significant accumulation of the cleaved Dha from Mfα1_{37}-C_{ub}-Dha, Suc2_{23}-C_{ub}-Dha, and CPY_{30}-C_{ub}-Dha. No cleavage was observed for Kar2_{40}-C_{ub}-Dha and only the uncleaved fraction of Kar2_{40}-C_{ub}-Dha could be detected on the Western blot. N_{ub}-Ssh1p induced significant cleavage of Suc2_{23}-C_{ub}-Dha only (Figure 2, A and B). No cleaved Dha was detectable in the extracts of the cells that contained Mfα1_{37}-C_{ub}-Dha or Kar2_{40}-C_{ub}-Dha. The slight accumulation of Dha observed upon coexpression of N_{ub}-Ssh1p with CPY_{30}-C_{ub}-Dha was below the background that was determined by the coexpression of the C_{ub}-translocation substrates with N_{ub}-Bos1p, a membrane protein of the ER that is not involved in translocation (Figure 2B) (Dünnwald *et al.*, 1999). To exclude that the different signal sequence interaction profiles of N_{ub}-Sec61p and N_{ub}-Ssh1p arise from a higher expression level of N_{ub}-Sec61p, we inserted an ha-epitope between the N_{ub} and the Sec61p- and Ssh1p coding sequence. After reducing the heterogeneity in the running behavior of N_{ub}-Ssh1p during SDS-PAGE the quantification of the Western blots of the cell extracts revealed a nearly identical amount of the two fusion proteins in the cells (Figure 2C).

The lack of cleaved Dha in cells coexpressing Kar2_{40}-C_{ub}-Dha and N_{ub}-Sec61p or N_{ub}-Ssh1p might indicate that Kar2p does not translocate via Sec61p or Ssh1p (Figure 2, A and B). We considered this very unlikely. As suggested by our experience with the invertase signal sequence, longer spacer sequences allow the still attached ribosomes to dock to the translocation channel before the C_{ub}-moiety of the fusion protein is translated. As a consequence the C_{ub} is not accessible for interactions with the N_{ub} in the cytosol of the cell (Johnsson and Varshavsky, 1994b; Dünnwald *et al.*, 1999). We therefore tested a second Kar2-C_{ub} construct (Kar2_{20}-

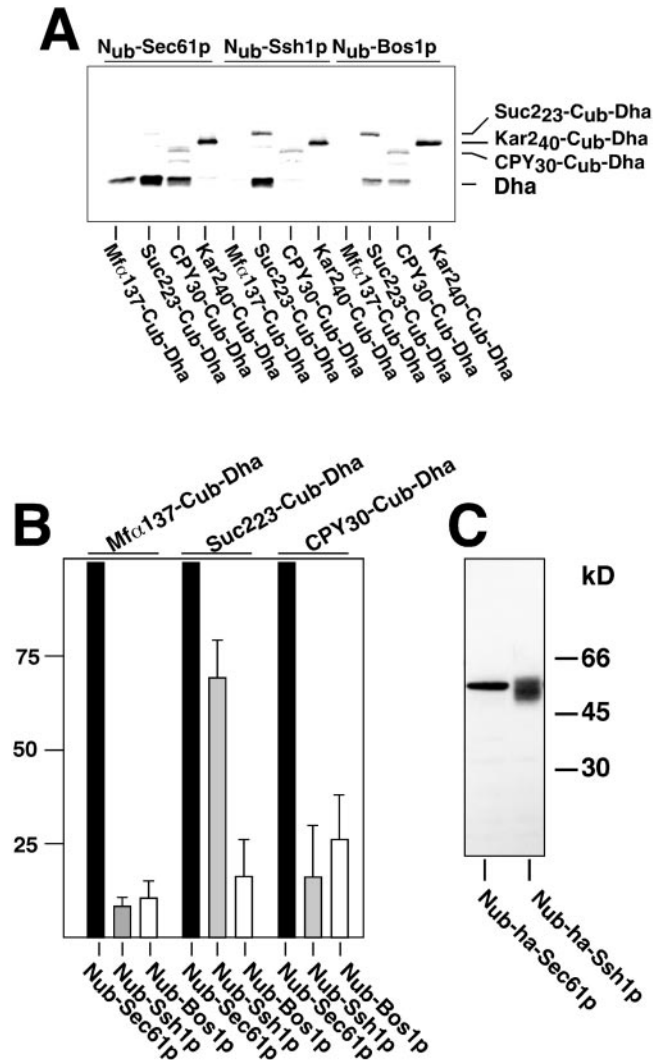

Figure 2. Signal sequence specificity of Ssh1p. (A) Immunoblot analysis of yeast cells containing either N_{ub}-Sec61p, N_{ub}-Ssh1p, or N_{ub}-Bos1p and expressing the four different signal sequence-bearing C_{ub} constructs. Uncleaved and cleaved Dha reporter was detected by an anti-ha antibody after SDS-PAGE and transfer onto nitrocellulose of whole cell extracts. (B) Quantification of three independent experiments is shown. In each experiment the amount of cleaved Dha that was induced by coexpressing N_{ub}-Sec61p was set to 100. The amount of cleaved Dha that was induced by N_{ub}-Ssh1p and N_{ub}-Bos1p was calculated in reference to the N_{ub}-Sec61p–induced cleavage for each of the signal sequence-bearing C_{ub}-Dha separately, except for Kar2_{40}-C_{ub}-Dha that yielded no cleavage with any of the N_{ub}-fusions. (C) Comparison of the expression levels of N_{ub}-ha-Sec61p and N_{ub}-ha-Ssh1p. Proteins extracts of yeast cells expressing N_{ub}-ha-Sec61p or N_{ub}-ha-Ssh1p were separated by SDS-PAGE and transferred onto nitrocellulose. The amounts of protein were estimated after anti-ha antibody treatment via the chemiluminescence generated by the conjugated second antibody. To better focus the N_{ub}-ha-Ssh1p during SDS-PAGE, the extracts were heated in sample buffer at 40°C for 20 min before loading on the gel.

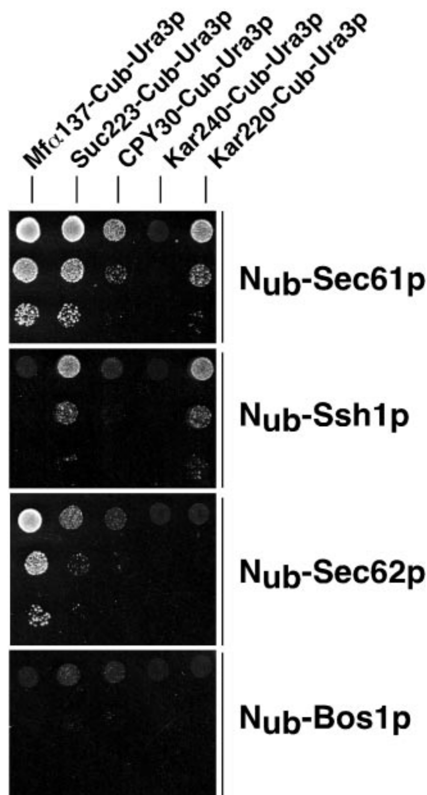

Figure 3. Ssh1p interacts with the signal sequences of invertase and Kar2p. Yeast cells that contained different signal sequence bearing C_{ub}-Ura3p and N_{ub}-Sec61p, N_{ub}-Ssh1p, N_{ub}-Sec62p, or N_{ub}-Bos1p were grown to an OD_{600} of 1. Then 4 μl of two serial 1:10 dilutions were spotted onto media lacking uracil and tryptophan and histidine to select for the presence of the plasmids expressing the fusion proteins. Growth was recorded after 3 d at 30°C. The growth of the cells is a measure of the interaction between the signal sequence of the C_{ub}-Ura3p and the cotransformed N_{ub}-fusion protein.

C_{ub}) that retained only 20 residues of the sequence of Kar2p between the hydrophobic core of the signal sequence and the C_{ub} moiety.

To avoid any artificial N_{ub}-C_{ub} reassociation and subsequent reporter cleavage after membrane rupture during protein extraction, we switched to the enzyme Ura3p as a reporter for the interaction assay. The localization of the enzyme Ura3p allows monitoring the interaction between translocation substrates and the components of the translocation machinery by a simple growth assay (Dünnwald *et al.*, 1999). On N_{ub}-induced cleavage, the Ura3p is released into the cytosol and enables the otherwise *URA3*-deficient cells to grow on medium lacking uracil (SD-Ura). We therefore cotransformed the corresponding signal sequence bearing C_{ub}-URA3 plus the newly constructed $Kar2_{20}$-C_{ub}-URA3 into cells expressing N_{ub}-Sec61p or N_{ub}-Ssh1p. The coexpression of the different C_{ub}-Ura3p constructs confirmed and extended the results obtained with the Dha reporter (Figure 3). Good growth of the N_{ub}-Sec61p–containing cells expressing $Mf\alpha1_{37}$-C_{ub}-Ura3p, $Suc2_{23}$-C_{ub}-Ura3p, and the weaker but still significant growth of the cells coexpressing CPY_{30}-C_{ub}-Ura3p indicated the proximity between these signal sequences and Sec61p during the process of translocation (Figure 3). As expected from the results with Dha as the reporter, $Kar2_{40}$-C_{ub}-Ura3p yielded no growth in the presence of N_{ub}-Sec61p. However, the coexpression of N_{ub}-Sec61p and $Kar2_{20}$-C_{ub}-Ura3p resulted in solid growth of the cells on SD-Ura. The specificity of each interaction signal was tested by comparing it with the growth of the cells coexpressing the signal sequence bearing C_{ub}-Ura3p and N_{ub}-Bos1p. None of the five different cotransformants yielded growth of the cells on SD-Ura (Figure 3).

N_{ub}-Ssh1p–containing cells grew on SD-Ura when coexpressing $Suc2_{23}$-C_{ub}-Ura3p and $Kar2_{20}$-C_{ub}-Ura3p (Figure 3). Interestingly, the interaction signal derived from $Suc2_{23}$-C_{ub}-Ura3p is less pronounced in the N_{ub}-Ssh1p than in N_{ub}-Sec61p–containing cells, whereas the signals derived from $Kar2_{20}$-C_{ub}-Ura3p are very similar in cells expressing N_{ub}-Ssh1p or N_{ub}-Sec61p (Figure 3). No interaction signals were detected upon coexpression of N_{ub}-Ssh1p and $Mf\alpha1_{37}$-C_{ub}-Ura3p, CPY_{30}-C_{ub}-Ura3p, or $Kar2_{40}$-C_{ub}-Ura3p (Figure 3). Because the well-established role of Sec61p in post- and cotranslational translocation of proteins across the ER membrane is reflected by its recognition of all four different signal sequences, we conclude that Ssh1p interacts only with the signal sequences of invertase and Kar2p. These two sequences display a higher hydrophobicity than the corresponding sequences of Mfα1 and CPY (Ng *et al.*, 1996; see DISCUSSION). A significant fraction of Kar2p and invertase is known to be targeted to the ER membrane via the SRP (Hann and Walter, 1991; Johnsson and Varshavsky, 1994b; Ng *et al.*, 1996). In contrast, Mfα1 and CPY are targeted via the tetrameric Sec62p/Sec63p complex. In this complex only Sec62p is known to be exclusively involved in the posttranslational translocation, whereas Sec63p fulfills a further role in the cotranslational translocation of proteins (Deshaies and Schekman, 1989; Ng *et al.*, 1996; Brodsky *et al.*, 1995; Young *et al.*, 2001). We coexpressed N_{ub}-Sec62p together with the different signal sequence bearing C_{ub}-Ura3p to compare the signal sequence interaction profiles of Sec62p and Ssh1p. N_{ub}-Sec62p revealed a strong interaction with $Mf\alpha1_{37}$-C_{ub}-Ura3p (Figure 3). No interactions were observed between Sec62p and the signal sequences of Kar2p and CPY. However, the weak interaction that is measured between N_{ub}-Sec62p and $Suc2_{23}$-C_{ub}-Ura3p is above the background that was determined by the growth of cells coexpressing $Suc2_{23}$-C_{ub}-Ura3p together with N_{ub}-Bos1p. This weak interaction was not detected by the growth assay in a previous study (Dünnwald *et al.*, 1999). Whereas Sec61p and Ssh1p showed an overlapping specificity toward the more hydrophobic signal sequences of invertase and Kar2p, none of the signal sequence bearing C_{ub} constructs that are recognized by Ssh1p induced a strong interaction signal with N_{ub}-Sec62p and vice versa.

Ssh1p Is in Vicinity of SRP-Receptor

The signal sequence interaction profile of Ssh1p suggests that Ssh1p might be involved in the cotranslational translocation of proteins across the ER membrane (Figure 3). To further substantiate this hypothesis, we measured the proximity between Ssh1p and the β-subunit of the SRP receptor (SRβ). Biochemical data showed that SRβ plays a role in coordinating the transfer of the signal sequence from the

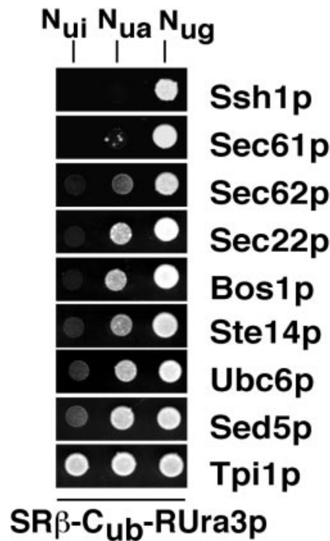

Figure 4. Ssh1p is close to the β-subunit of the SRP receptor. Cells expressing SRβ-C_{ub}-RUra3p from its own promoter were cotransformed with the N_{ui}-, N_{ua}-, and N_{ug}-fusions of the translocation components Sec61p, Sec62p, and Ssh1p. Cells were grown in media containing uracil to an OD_{600} of ~1. Then 4 μl of the cultures was spotted on plates lacking uracil. The plates were also lacking histidine and tryptophan to select for the presence of the plasmids expressing the fusion proteins. Growth was recorded after 5 d at 30°C. Nongrowth indicates interaction between the corresponding N_{ub}-fusion and SRβ-C_{ub}-RUra3p. Also included in this analysis were the N_{ub}-fusions of the ER membrane proteins Bos1p, Ste14p, Sec22p, and Ubc6p; the N_{ub}-fusion of the Golgi-protein Sed5p; and the cytosolic enzyme Tpi1p.

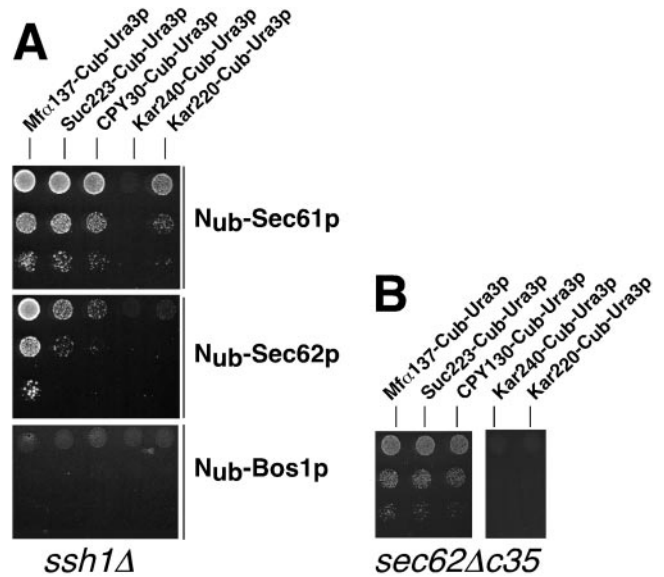

Figure 5. Flow of signal sequences is not redirected in *ssh1*Δ cells. (A) Analysis was as in Figure 3 but in cells containing a deletion of *SSH1*. (B) Translocation defect is detected by the signal sequence bearing C_{ub}-Ura3p fusions in cells carrying a *sec62* allele. The indicated C_{ub}-Ura3p fusions were expressed in cells carrying the *sec62ΔC35-DHA* allele expressed from the P_{CUP1} promotor. A partial translocation arrest is indicated by the growth of the cells on SD-Ura after 3 d at 30°C. The media were also lacking histidine to select for the presence of the plasmids.

SRP to the translocation channel (Fulga *et al.*, 2001). One consequence of this activity should be a physical proximity between SRβ and components of the channel. The N-terminal part of SRβ anchors the protein in the ER membrane and directs its C-terminal domain into the cytosol (Ogg *et al.*, 1998). If Ssh1p is indeed involved in cotranslational protein translocation it should ideally be as close to members of the SRP pathway as Sec61p. Furthermore, Ssh1p should be closer to SRβ than the proteins that are not involved in cotranslational protein translocation. We estimated the proximity between SRβ and Ssh1p by comparing the growth of cells containing SRβ-C_{ub}-RUra3p and N_{ub}-Ssh1p with the growth of cells containing SRβ-C_{ub}-RUra3p and a panel of other N_{ub}-labeled proteins. In this variation of the split-Ub assay the reporter RUra3p is immediately degraded by the enzymes of the N-end rule pathway after being cleaved from C_{ub} (Wittke *et al.*, 1999). Proximity between a pair of N_{ub}- and C_{ub}-labeled fusion proteins is therefore indicated by the nongrowth of the corresponding yeast transformants on SD-Ura. The N_{ub} mutants N_{ua} and N_{ug} have a lower affinity to C_{ub} than the wild-type N_{ub} (N_{ui}). An N_{ub}-fusion very close to a certain C_{ub}-fusion will therefore induce cleavage of the C_{ub}-linked reporter not only as its N_{ui}- but also as its N_{ua}- and potentially even as its N_{ug}-derivative (Wittke *et al.*, 1999). Figure 4 shows that N_{ub}-Ssh1p interacts with SRβ-C_{ub}-RUra3p as strongly as N_{ub}-Sec61p. According to this interaction assay both N_{ua}-fusion proteins display a physical proximity to SRβ-C_{ub}-RUra3p. Membrane proteins of the ER that are not involved in translocation, such as N_{ua}-Bos1p, N_{ua}-Ubc6p, N_{ua}-Ste14, and N_{ua}-Sec22p, are not close to SRβ-C_{ub}-RUra3p (Shim *et al.*, 1991; Sommer and Jentsch, 1993; Ballensiefen *et al.*, 1998; Romano and Michaelis, 2001). Importantly, N_{ua}-Sec62p as a component of the postranslational translocation pathway is more distant to SRβ than N_{ua}-Sec61p or N_{ua}-Ssh1p in our assay.

Flow of Translocated Proteins Is Not Measurably Changed in an ssh1Δ Strain

We constructed an *ssh1*Δ strain to ask whether the interactions between N_{ub}-Sec61p or N_{ub}-Sec62p and the signal sequence bearing C_{ub}-Ura3p are changed upon deleting *SSH1*. The *ssh1*Δ strain showed a slightly reduced growth at 17, 25, and 37°C but grew nearly indistinguishable from the wild-type JD53 at 30°C. The expression of a plasmid-borne N_{ub}-Ssh1p or the unmodified Ssh1p could compensate for the growth defect of this strain, thus proving the functionality of N_{ub}-Ssh1p (our unpublished observation). The signal sequence bearing C_{ub}-Ura3p constructs were coexpressed with N_{ub}-Sec61p, N_{ub}-Se62p, and N_{ub}-Bos1p in the *ssh1*Δ strain. The interaction assay between the N_{ub}-fusion proteins and the signal sequence bearing C_{ub}-Ura3p revealed no major differences between the wild-type and the *ssh1*Δ strain (Figure 5A). All four signal sequences still interact with N_{ub}-Sec61p, whereas N_{ub}-Sec62p strongly interacts with Mf$\alpha 1_{37}$-C_{ub}-Ura3p only. A slight increase in interaction between N_{ub}-Sec62p and $Suc2_{23}$-C_{ub}-Ura3p might be inferred from Figure 5A. However, the effect is very small.

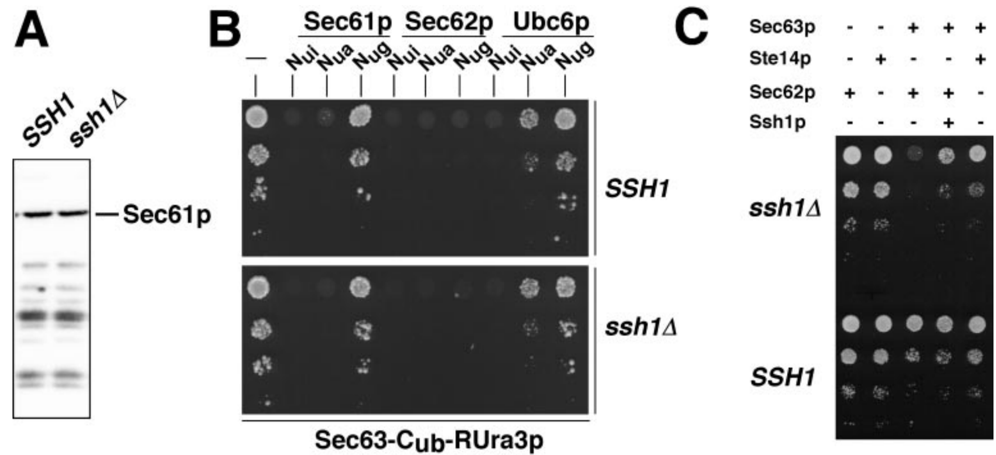

Figure 6. Concentration of Sec61p and its interaction with Sec63p are not measurably changed in *ssh1*Δ cells. (A) Whole cell extracts from a wild-type and an *ssh1*Δ strain were probed with an anti-Sec61p antibody after SDS-PAGE and transfer onto nitrocellulose. A representative blot from one of three experiments is shown. (B) Interactions between Sec63p and Sec61p, Sec62p and Ubc6p were analyzed with the split-Ub assay in wild-type and *ssh1*Δ cells. Cells containing Sec63-C_{ub}-RUra3p and coexpressing N_{ui}-, N_{ua}-, or N_{ug}-fusions of the indicated ER membrane proteins were grown in uracil-containing medium to an OD_{600} of ~1. Then 4 μl of these cultures and 4 μl of three serial 1:10 dilutions were spotted on plates lacking uracil and histidine and tryptophan to select for the plasmids. Growth was recorded after 3 d at 30°C. Nongrowth indicates proximity between the N_{ub}- and C_{ub}-labeled fusion proteins. (C) Overexpression of Sec62p and Sec63p is toxic in *ssh1*Δ cells. Cells containing the indicated combination of plasmid-borne proteins were grown to an OD_{600} of ~1 in media lacking leucine, histidine, and tryptophan to select for the presence of the plasmids and glucose to repress the expression of Sec62p, Sec63-ha, or Ste14-Dha. Then 4 μl of these cultures and 4 μl of three serial 1:10 dilutions were spotted on plates lacking leucine, histidine, and tryptophan to select for the plasmids and galactose to induce the P_{GAL1} driven expression of Sec62p, Sec63-ha, or Ste14-Dha. Growth was recorded after 3 days at 30°C. Cells were also spotted on glucose-containing medium to check for equal cell numbers (our unpublished observation).

Thus, the experiment does not detect a major rerouting from the co- to the posttranslational pathway of protein translocation upon deletion of *SSH1*. Interestingly, N_{ub}-Bos1p showed again no significant interaction with any of the signal sequence bearing C_{ub}-Ura3p. This observation implies that there is also no major translocation defect and consequently no cytosolic accumulation of any of the tested translocation substrates in the *ssh1*Δ strain. To prove that these artificial constructs can principally detect a defect in translocation, we expressed all five C_{ub}-Ura3p in a strain that harbors only a partially functional allele of Sec62p (*sec62ΔC35-Dha*) (Wittke *et al.*, 2000). A translocation defect will result in the cytosolic accumulation of Ura3p activity even in the absence of any coexpressed N_{ub} fusion. The cytosolic Ura3p fusion protein will enable the cells to grow on SD-Ura (Johnsson and Varshavsky, 1994b; Ng *et al.*, 1996). This effect is detected by the growth of the transformed strain on SD-Ura for the Mfα-, invertase-, and the CPY-signal sequence–bearing constructs (Figure 5B). The *sec62ΔC35-Dha* allele displayed no measurable defect in the translocation of the two Kar2-C_{ub}-Ura3p constructs (Figure 5B). We conclude that the deletion of *SSH1* did not cause a severe accumulation of any of the signal sequence-bearing C_{ub}-Ura3p in the cytosol of the cell, thus confirming the results derived from more direct translocation assays (Ng *et al.*, 1996).

Concentration of Sec61p Is Not Measurably Changed in an ssh1Δ Strain

The lack of significant defects in protein translocation in our *ssh1*Δ strain is the more surprising because we have shown that Ssh1p recognizes a certain subset of signal sequences (Figures 2 and 3). It is possible that the cell can compensate for the lack of Ssh1p in more than one way. One mechanism might include the up-regulation of Sec61p to provide more channels in the ER membrane. We tested this hypothesis by comparing the amount of Sec61p in a wild-type and an *ssh1*Δ strain. We found no significant difference between the two yeast strains (Figure 6A). An alternative mechanism of compensating for the loss of *SSH1* might use a readjustment in the composition of the Sec-complexes. Specifically, a weaker binding between Sec61p and the tetrameric Sec62p/Sec63p complex might free more Sec61p for the cotranslational way of protein translocation. Very similar to the measurements between SRβ-C_{ub}-RUra3p and the different N_{ub}-fusion proteins we compared the interaction of Sec63-C_{ub}-RUra3p with N_{ub}-Sec61p and also with N_{ub}-Sec62p in a wild-type and the *ssh1*Δ-strain (Wittke *et al.*, 1999). We monitored the interaction between the N_{ub}-modified Ubc6p and Sec63-C_{ub}-RUra3p as a pair of proteins whose proximity should be unaffected by a deletion of *SSH1*. Taking the growth on SD-Ura as a measure of interaction strength we found that our assay did not detect a difference between the *ssh1*Δ strain and the isogenic JD53 strain in the binding of Sec63-C_{ub}-RUra3p to N_{ub}-Sec61p or N_{ub}-Sec62p (Figure 6B). Specifically, the nongrowth of the wild-type and the *ssh*1Δ cells containing Sec63-C_{ub}-RUra3p and N_{ug}-Sec62p on SD-Ura indicates a very strong interaction between the two molecules. The nongrowth of wild-type and the *ssh*1Δ cells containing Sec63-C_{ub}-RUra3p and N_{ua}-Sec61p indicates an interaction that is weaker but still specific compared with the growth of the cells containing Sec63-C_{ub}-RUra3p and N_{ua}-Ubc6p (Figure 6C). Similar to N_{ub}-Ubc6p, N_{ub}-Ssh1p behaves as a typical ER membrane protein in this assay (Wittke *et al.*, 1999). However, because the measurements are purely qualitative, we cannot exclude that a slight but still signifi-

Table 1. Yeast strains

Strain	Relevant genotype	Source/comment
JD53	*MATαhis3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52*	Dohmen *et al*., 1995
NJY73-1	*MATαhis3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 NUI-BOS1::pRS304*	Derivative of JD53; this work
NJY73-A	*MAT his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 NUA-BOS1::pRS304*	This work
NJY73-G	*MATαhis3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 NUG-BOS1::pRS304*	This work
NJY61-1	*MATαhis3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 NUI-SEC61::pRS304*	Wittke *et al*., 1999
NJY61-A	*MATαhis3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 NUA-SEC61::pRS304*	Wittke *et al*., 1999
NJY61-G	*MATαhis3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 NUG-SEC61::pRS304*	Wittke *et al*., 1999
NJY78-1	*MATαhis3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 NUI-SSH1::pRS304*	Wittke *et al*., 1999
NJY78-A	*MATαhis3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 NUA-SSH1::pRS304*	Wittke *et al*., 1999
NJY78-G	*MATαhis3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 NUG-SSH1::pRS304*	Wittke *et al*., 1999
NJY144	*MATαhis3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 SRP102-CUB-RURA3:: pRS303*	Derivative of JD53; this work
NJY126Δ35	*MATαhis3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52 SEC62::KAN*$^{+}$ *PCUP1SEC62ΔC35:pRS314*	Wittke *et al*., 2000
NJY145	*MATαhis3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52* SSH1::KAN^{+}	This work

cant readjustment of the Sec-complex upon deleting *SSH1* might have gone unnoticed in our assay.

ssh1Δ Strain Is Less Resistant to Changes in Concentration of Components of Protein Translocation Machinery

If the cell does not react to the loss of Ssh1p by either increasing the amount of Sec61p or decreasing its association with the tetrameric Sec-complex, the correct balance between free and Sec62p/Sec63p-bound Sec61p might become more fragile in a cell lacking *SSH1*. To test this hypothesis we overexpressed Sec62p and an ha-tagged version of the Sec63p (Sec63-ha) in the wild-type and the *ssh1Δ* strain to limit the amount of free Sec61p in both cell types. Compared with the wild-type strain, the strain lacking *SSH1* did barely grow upon overexpression of SEC62p and Sec63-ha (Figure 6C). On introducing a plasmid-borne copy of *SSH1*, the *ssh1Δ* cells regained growth that was only slightly slower than the growth of the corresponding wild-type strain. The overexpression of Sec63-ha together with the membrane protein Ste14-Dha did affect the growth of the wild-type and the *ssh1Δ* cells less severely and to a similar extent (Figure 6C). Expression of Sec62p or Ste14-Dha alone had no effect on the growth of the cells (Figure 6C).

DISCUSSION

Sequence analysis of complete genomes revealed homologs of many known proteins whose functions are not immediately apparent in spite of their similarity. Ssh1p is related in sequence to Sec61p and is organized in a very similar trimeric complex. This, and its confirmed location in the ER membrane, led to the assumption that Ssh1p is involved in certain aspects of protein translocation (Finke *et al*., 1996). Surprisingly, a deletion of the gene did not reveal any severe translocation defects, and the hypothesis that Ssh1p interacts with signal sequences and is actively involved in translocation remained unproven (Finke *et al*., 1996; Ng *et al*., 1996). In this work we show in vivo that Ssh1p recognizes a subset of the signal sequences that are also recognized by Sec61p but not by Sec62p. Our data therefore provide direct evidence that Ssh1p is involved in the cotranslational mode of protein translocation across the membrane of the ER.

Ssh1p Recognizes Signal Sequences

We made use of the split-Ub technique to demonstrate proximity between signal sequence bearing C_{ub}-fusions and the N_{ub}-modified Ssh1p. The assay showed that Ssh1p is close to the signal sequence of invertase and Kar2p, whereas no interaction could be detected for the signal sequences of CPY and Mfα1 (Figures 2 and 3). The relevance, especially of the failure to detect interactions between Ssh1p and the signal sequences of CPY and Mfα1, is strengthened by the observation that all four signal sequences interact with Sec61p (Figures 2 and 3). Sec61p and Ssh1p, being closely related in sequence are organized in very similar trimeric complexes and have similar activities as N_{ub}-fusions toward unrelated C_{ub}-substrates (Wittke *et al*., 1999). We therefore interpret the finding that Sec61p interacts with the signal sequences of Mfα1 and CPY, whereas Ssh1p does not as a true reflection of the in vivo specificity of the two proteins toward these sequences. The signal sequences that are recognized by Ssh1p seem more hydrophobic than those that do not bind to Ssh1p. Counting the hydrophobic residues in a window of 11 we find that the signal sequence of Kar2p has an uninterrupted stretch of 11 hydrophobic amino acids. Invertase has a stretch of 10 hydrophobic residues that is interrupted by a glycine in position 8 of this stretch. Mfα1 has a stretch of nine hydrophobic residues that is interrupted by two serines in positions 7 and 8, and CPY has a stretch of six hydrophobic residues that is interrupted by two glycines and three hydroxylated residues in positions 3, 5, 8, 9, and 10. We conclude that the signal sequences of Mfα1 and CPY are less hydrophobic than the signal sequences of invertase and Kar2p. Ng *et al.* (1996) have convincingly shown that more hydrophobic signal sequences are targeted via SRP, whereas less hydrophobic signal sequences are targeted via the tetrameric Sec62p/Sec63p complex. Although the translocation of Kar2p and invertase are affected by a deletion of the SRP, the translocation of Mfα1 and CPY is not (Ng *et al*., 1996). The profile of signal sequences interacting with Ssh1p strongly suggests that the SRP and its receptors contact

Ssh1p during the targeting process. The physical proximity that we measured between Ssh1p and SRβ confirms this prediction (Figure 4).

The following simple model integrates the data of our study: Sec61p as the pore-forming subunit of the trimeric and heptameric Sec-complexes is involved in co- and posttranslational translocation and therefore recognizes all four different signal sequences (Deshaies and Schekman, 1987; Matlack *et al.*, 1998; Pilon *et al.*, 1998). Ssh1p has a more restricted specificity and is only involved in the SRP-dependent protein translocation. Consequently, Ssh1p only interacts with the signal sequences of invertase and Kar2p. Sec62p as part of the Sec62p/Sec63p complex that is exclusively involved in posttranslational translocation should therefore recognize the signal sequences that do not interact with Ssh1p. This prediction is fulfilled by our data concerning the Mfα1 signal sequence but not concerning the signal sequence of CPY (Figure 3). We can rationalize our failure to measure the postulated interaction between N_{ub}-Sec62p and CPY_{30}-C_{ub}-RUra3p in two ways. 1) The identity of the binding site(s) for signal sequences on the heptameric Sec-complex is still not completely defined. Sec62p might be responsible for the recognition of Mfα1, whereas a different component of the Sec-complex, for example, Sec72p, might be the primary acceptor site for CPY (Feldheim and Schekman, 1994; Matlack *et al.*, 1997). 2) We note that N_{ub}-Sec61p gives a weaker interaction signal with CPY_{30}-C_{ub}-RUra3p than with the corresponding Mfα1 construct (Figure 3). The relatively hydrophilic character of the signal sequence of CPY might cause a weaker interaction and a shorter residence time at the Sec-complex. As a consequence, the interaction between Sec62p and CPY might fall below the sensitivity of our assay.

On the Function of Ssh1p

The interaction of Ssh1p with a certain subset of signal sequences strongly suggests but does not unequivocally prove that Ssh1p is also directly involved in the translocation of those proteins across the membrane. Instead, Ssh1p might be an additional receptor that keeps the signal sequences bound to the membrane as long as no Sec61p is available for their translocation. These and related objections to Ssh1p being a channel withstanding, our data show that Ssh1p acts as an additional receptor in the cotranslational mode of protein translocation (Plath *et al.*, 1998). The question whether Ssh1p is a true channel can only be answered with the help of an in vitro system for cotranslational protein translocation in yeast.

A further unresolved issue is the lack of severe translocation defects in our *ssh1*Δ strain. Although initially surprising, the capacity of the SRP targeting pathway might suffice to overcome a shortage of Ssh1p-channels by pausing and thereby slowing down the translation of those proteins (Mason *et al.*, 2000). In support of this notion a very recent report by Wilkinson *et al.* (2001) demonstrated a genetic link between a mutation in one of the components of the yeast SRP and a deletion of *SSH1* leading to a synthetic lethality in the respective strain. The authors also demonstrate a remarkable capacity of *ssh1*Δ cells to adapt to and to suppress the initially observed translocation defects. These features might explain the lack of severe defects in our *ssh1*Δ strain. However, Wilkinson *et al.* (2001) noticed that adaptation correlates with the frequent occurrence of the petite phenotype in their W303 strain, whereas the *ssh1*Δ strain used in this study repeatedly grew well on glycerol or galactose-containing media (Figure 6C; our unpublished observation). The obvious difference between the two strains in responding to a deletion of *SSH1* might reflect subtle differences in the genotypes of the two different strains.

In discussing the consequences of deleting *SSH1* one has to be aware that the spectrum of proteins that were tested for translocation in an *ssh1*Δ strain still represents only a small fraction of all translocated proteins. Because it is now well established that different signal sequences show different requirements and kinetics for being targeted to the channel, it is possible that a still undiscovered fraction of proteins travels preferentially via Ssh1p across the membrane (Figure 1C). The slight growth defect of an *ssh1*Δ strain that is not completely cured by the ectopic overexpression of Sec61p hints at the existence of such a subset of Ssh1p-dependent translocation substrates (our unpublished data).

As more Sec61p and Ssh1p related proteins are identified in other organisms the flow of proteins across these different potential channels needs to be addressed. By allowing estimation of the contribution of the different components of the system, including its redundant members, the split-Ub technique can be used to analyze this flow in living cells.

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