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Preferential Targeting of a Signal Recognition Particle-dependent Precursor to the Ssh1p Translocon in Yeast

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Protein translocation across the endoplasmic reticulum membrane occurs via a "translocon" channel formed by the Sec61p complex. In yeast, two channels exist: the canonical Sec61p channel and a homolog called Ssh1p. Here, we used trapped translocation intermediates to demonstrate that a specific signal recognition particle-dependent substrate, Sec71p, is targeted exclusively to Ssh1p. Strikingly, we found that, in the absence of Ssh1p, precursor could be successfully redirected to canonical Sec61p, demonstrating that the normal targeting reaction must involve preferential sorting to Ssh1p. Our data therefore demonstrate that Ssh1p is the primary translocon for Sec71p and reveal a novel sorting mechanism at the level of the endoplasmic reticulum membrane enabling precursors to be directed to distinct translocons. Interestingly, the Ssh1p-dependent translocation of Sec71p was found to be dependent upon Sec63p, demonstrating a previously unappreciated functional interaction between Sec63p and the Ssh1p translocon.

Import or integration of most newly synthesized proteins into the endoplasmic reticulum (ER)[2] occurs through channel complexes called translocons. Targeting to the translocon can occur either co- or post-translationally. The co-translational pathway involves recognition of the signal sequence on a nascent polypeptide by a signal recognition particle (SRP) and subsequent targeting of the ribosome-nascent chain complex to the ER membrane via the SRP receptor. The targeted precursor then engages the Sec61p translocon complex, allowing the growing polypeptide to be delivered directly into the translocation channel (1, 2). For the post-translational pathway, the nascent protein can be fully translated in the cytosol, where it requires cytosolic chaperones to maintain an unfolded conformation prior to its targeting to Sec61p via the Sec62p receptor (reviewed in Ref. 3).

The Sec61p complex functions in both co- and post-translational translocation pathways and comprises the pore-forming protein Sec61p together with Sss1p (Sec sixty-one suppressor 1 protein) and Sbh1p (Sec61 beta homolog 1 protein) (4). In yeast, the core Sec61p complex functions as part of two larger complexes that are required for translocon function *in vivo*. For post-translational translocation, the larger complex takes the form of the heptameric "SEC complex," comprising the trimeric Sec61p complex plus Sec62p, Sec63p, Sec71p, and Sec72p (5, 6). Sec63p is an essential membrane protein involved in both co- and post-translational reactions (7–9), whereas Sec62p is involved in post-translational signal sequence recognition, and Sec71p and Sec72p have unknown nonessential roles in the post-translational reaction (10). Co-translational translocation does not require Sec62p (11) but does appear to require the hexameric SEC′ complex, containing all of the SEC proteins except Sec62p (9, 12).

Yeast cells possess a second translocon complex comprising the Ssh1p (Sec sixty-one homolog 1 protein) plus Sbh2p and Sss1p (which is common to both complexes) (11). It has recently been confirmed that Ssh1p is indeed structurally similar to Sec61p (13), and a variety of data provide supporting evidence suggesting a role in translocation (11, 14–16). However, it does not appear to form larger complexes similar to either SEC or SEC′ that are characteristic of the Sec61p complex (11, 17). The absence of any detectable interaction with Sec62p has led to the suggestion that Ssh1p might be specific to co-translational translocation. However, the lack of any interaction with Sec63p appears inconsistent with a translocase function given that Sec63p is essential for both co- and post-translational translocation pathways (8, 9). Moreover, unlike Sec61p, Ssh1p has not been shown to directly interact with a translocating polypeptide chain, and so its role as a translocase remains speculative.

Here, we used cross-linking of trapped translocation intermediates to demonstrate that Ssh1p functions as a translocon channel. Having identified that insertion of the SRP-dependent membrane protein Sec71p appears to be Sec61p-independent *in vivo*, we used stalled translation intermediates to monitor the local environment of its signal anchor domain by chemical cross-linking during both targeting and translocation. We found the signal anchor domain to interact first with the Srp54p and Sec65p components of SRP in yeast cytosol. Following membrane targeting, we found that the precursor became cross-linked to Ssh1p, providing clear evidence that Ssh1p constitutes a protein-conducting channel in a manner similar to Sec61p. Remarkably, we found that the co-translational integration of Sec71p occurred exclusively via the Ssh1p complex. This is the first example of a nascent polypeptide targeting preferentially to Ssh1p, and so we conclude that specific substrates

S The on-line version of this article (available at http://www.jbc.org) contains supplemental "Methods," Tables S1 and S2, and Fig. 1.
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[2] The abbreviations used are: ER, endoplasmic reticulum; SRP, signal recognition particle; DPAPB, dipeptidyl aminopeptidase B; RNC, ribosome-nascent chain complex.

are sorted to distinct translocons at the ER membrane. Furthermore, we identify a role for Sbh2p in this preferential targeting process, as specific targeting to Ssh1p is lost in *sbh2Δ* membranes. Having identified a direct role for Ssh1p in translocation of Sec71p, we went on to examine whether this might also involve Sec63p. Our data confirm that Sec63p is essential for Ssh1p-dependent translocation and so demonstrate a general role for Sec63p in co-translational translocation.

EXPERIMENTAL PROCEDURES

Media and Growth Conditions—Yeast strains were grown at 24 or 30 °C in 1% yeast extract and 2% peptone containing 20 mg/liter adenine and either 2% glucose or 2% glycerol and 2% ethanol or in minimal medium (0.67% yeast nitrogen base with ammonium sulfate) with 2% glucose or with 2% glycerol and 2% ethanol plus appropriate supplements for selective growth. Solid media were supplemented with 2% Bacto agar. All media were from Difco Laboratories.

Yeast Strains and Plasmids—The *Saccharomyces cerevisiae* strains used in this study are listed in supplemental Table S1. Strains and plasmid constructions are described under supplemental "Methods," and the oligonucleotides used are listed in Table S2.

Radiolabeling and Immunoprecipitations—Preparation of [^{35}S]methionine-labeled yeast cell extracts and immunoprecipitation were carried out as described previously (16). Immunoprecipitations from *in vitro* reactions were performed following the addition of 1% SDS.

In Vitro Insertion Assay—Microsome preparation and *in vitro* translations were carried out as described previously (7). For transcription/translation of Sec71p-HA, mRNA was transcribed from PvuI-linearized pMPS32 with RiboMAX SP6 RNA polymerase (Promega) according to the manufacturer's instructions. mRNA was added to nuclease-treated cytosol at a concentration of 40 ng/μl. Translations/translocations were carried out in the presence of L-[^{35}S]methionine, and microsomes (50 (A_{280})/ml) were added to a concentration of 10%.

Purification of Yeast Ribosome-Nascent Chain Complexes and in Vitro Targeting Assay—Truncated Sec71p mRNA comprising 91 codons of Sec71p-HA but lacking a stop codon was translated using BamHI-linearized pMPS46. Truncated DHC-αF (α-factor with signal sequence replaced with the dipeptidyl aminopeptidase B (DPAPB) signal anchor domain) mRNA was transcribed from a PCR-amplified fragment of pMPS53 generated using oligonucleotides SP6 and aF92R, resulting in a sequence encoding a 92-amino acid DHC-αF protein. Transcription and translation were carried out as described previously (7), and the translation reaction was stopped by the addition of 10 μg/ml cycloheximide. The translation reaction was then layered onto 800 μl of a 1 M sucrose cushion (5 mM MgOAc, 1 M sucrose, 25 mM HEPES-KOH (pH 7.4), 500 mM KOAc, and 10 μg/ml cycloheximide). Ribosome-nascent chain complexes (RNCs) were pelleted by centrifugation at 85,000 rpm in a Beckman MLA130 rotor for 40 min at 4 °C. Ribosome pellets were resuspended in 100 μl of buffer containing 25 mM HEPES-KOH (pH 7.4), 5 mM MgOAc, and 5 μg/ml cycloheximide, to which microsomes (50 (A_{280})/ml) were added to a concentration of 10%. This mixture was incubated for 20 min at 20 °C, and the membrane fraction was isolated by centrifugation at 14,000 rpm for 10 min at 4 °C. For cross-linking, the membranes were resuspended in 50 μl of membrane storage buffer (250 mM sorbitol, 20 mM HEPES-KOH (pH 7.4), 50 mM KOAc, and 2 mM MgOAc), followed by the addition of 500 μM *o*-phenylenedimaleimide. Cross-linking was performed at 20 °C for 40 min and then quenched by the addition of 2 mM DTT.

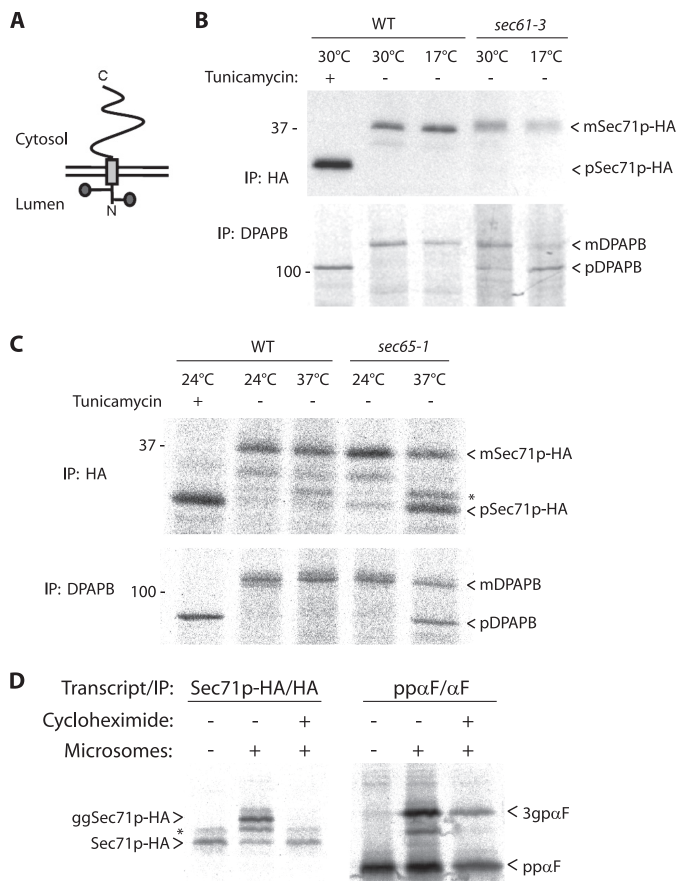

FIGURE 1. **Sec71p insertion *in vivo* is SRP-dependent and co-translational.** *A*, diagram showing the topology and glycosylation sites in Sec71p. Glycosylation sites are represented by *circles*. *B*, Sec71p insertion in *sec61-3* yeast. Wild-type and *sec61-3* yeast strains expressing Sec71p-HA were grown at 30 °C and then shifted to 17 °C for 90 min before being pulse-labeled with L-[^{35}S]methionine, and extracts were immunoprecipitated (*IP*) with anti-HA or anti-DPAPB antibodies and resolved by 10.5 and 8% SDS-PAGE respectively. Translocated (*mSec71p-HA* and *mDPAPB*) and untranslocated (*pSec71p-HA* and *pDPAPB*) forms are indicated. *C*, wild-type or temperature-sensitive *sec65-1* cells expressing Sec71p-HA were grown at 24 °C and shifted to 37 °C for 1 h before radiolabeling and were then immunoprecipitated as described for *B*. A species that precipitated with anti-HA antibody following incubation at 37 °C (but is not Sec71p-3-HA) is marked with an *asterisk*. *D*, Sec71p insertion occurs co-translationally. Either Sec71p-HA or prepro-α-factor (*ppαF*) RNA was translated *in vitro* using SRP^{+} yeast cytosol (7) and L-[^{35}S]methionine in the presence (co-translational) or absence (post-translational) of microsomes. For the post-translational reaction, protein synthesis was stopped by the addition of cycloheximide prior to the addition of microsomes. The positions of glycosylated (*ggSec71p-HA* and *3gpαF*) and unglycosylated (*Sec71p-HA* and *ppαF*) Sec71p-HA and prepro-α-factor, as well as the unglycosylated species immunoprecipitated by anti-HA antibody (*), are indicated.

RESULTS

Sec71p Integration Is Co-translational but Not Sensitive to the sec61-3 Mutation—Sec71p is a type III ER membrane protein with a non-cleavable signal anchor domain whose insertion

into the bilayer requires the translocation of a small 28-residue N-terminal region that is glycosylated in the ER lumen (Fig. 1*A*) (18). To monitor Sec71p insertion, we examined the glycosylation state of a C-terminally 3-HA-tagged form of Sec71p *in vivo* by pulse labeling and immunoprecipitation. We found efficient glycosylation of Sec71p-HA in wild-type cells, consistent with its expected topology (Fig. 1*B*, *second lane*). We then used various yeast mutants to determine the requirements for Sec71p-HA insertion compared with the well established co-translational model membrane protein DPAPB. As expected, *sec61-3* cells exhibited a minor defect in the co-translational translocation of DPAPB at 30 °C but were substantially defective at the restrictive temperature of 17 °C (Fig. 1*B*) (19). However, we found no detectable defect in the insertion of Sec71p-HA under either condition, suggesting that insertion was refractory to the *sec61-3* mutation. The only other yeast ER membrane proteins known to behave in this way are the C-terminal anchor proteins whose insertion is post-translational and independent of SRP (20, 21). We therefore examined the SRP dependence of Sec71p-HA insertion in the temperature-sensitive *sec65-1* mutant (22). As expected, these mutant cells accumulated an untranslocated form of the SRP-dependent substrate DPAPB at 37 °C that was not evident at 24 °C or in the wild-type controls. We also observed a substantial accumulation of an unglycosylated form of Sec71p-HA under the same conditions (Fig. 1*C*), indicating that, like DPAPB, Sec71p insertion is SRP-dependent.

It has previously been suggested that SRP might be involved in the post-translational targeting of the C-terminal anchor protein Sec61-β (23). To exclude the possibility that Sec71p insertion is SRP-dependent but post-translational, we examined insertion *in vitro*. Labeled Sec71p-HA was translated in yeast cytosol in the presence or absence of yeast ER-derived membranes (Fig. 1*D*). When translated in the presence of microsomes, Sec71p-HA was efficiently inserted (Fig. 1*D*, *second lane*). However, when translation was terminated with cycloheximide before the addition of microsomes, we observed no microsome-dependent glycosylation of Sec71p-HA (Fig. 1*D*, *third lane*). This was in contrast to the glycosylation of the post-translationally translocated substrate prepro-α-factor (Fig. 1*D*, *sixth lane*). Therefore, this is the first substrate observed whose insertion is both strictly co-translational (Fig. 1, *C* and *D*) and apparently Sec61p-independent (Fig. 1*B*).

Identification of Intermediates in Sec71p-HA Insertion by Cross-linking—To further investigate the mechanism of this Sec61p-independent insertion, we next exploited truncated mRNAs to create stable RNCs with which to probe the environment of a translocation intermediate (24, 25). A derivative of *SEC71* containing a single cysteine codon (Fig. 2*A*) was transcribed to produce a truncated mRNA encoding a 91-residue form of Sec71p-HA lacking a stop codon. This was translated in yeast cytosol, and ribosomes were collected by centrifugation, whereupon a substantial proportion of the labeled translation product was found in the ribosome pellet, confirming the generation of yeast RNCs. When the resuspended RNCs were added to wild-type yeast membranes, a proportion of Sec71p-HA became glycosylated (Fig. 2*B*), indicating that the N-terminal region of some nascent Sec71p-HA peptides was able to access the lumen of the ER. We next sought to identify the protein components in close proximity to the signal anchor region of nascent Sec71p-HA by using the disulfide cross-linker *o*-phenylenedimaleimide. This cross-linker has a spacer arm of 7.7–10.5 Å and so will form disulfide bridges between cysteine residues within this distance of one another.

When the cross-linker was added to RNCs in the absence of membranes, we observed two major adducts at 70 and 45 kDa, with a further minor adduct at 35 kDa (cross-links 1–3) (Fig. 2*B*, *third* and *fourth lanes*). The ~70-kDa species (cross-link 1) could be immunoprecipitated with both anti-HA and anti-SRP54 antibodies and so represents a cross-link between 14-kDa Sec71p-S27C and 60-kDa Srp54p (Fig. 2*C*, *lane 2*). Interestingly, our analysis revealed that the Sec71p-S27C precursor also cross-linked to the 31-kDa Sec65p subunit of yeast SRP because the 45-kDa adduct (cross-link 2) was found to immunoprecipitate using anti-Sec65p antibodies (Fig. 2*C*, *third lane*). As might be expected, the observed interactions with SRP subunits were reduced following the addition of membranes, whereupon two novel adducts appeared at ~55 and ~40 kDa (cross-links 4 and 5) (Fig. 2*B*, *fourth lane*). To determine whether cross-link 4 represents a genuine insertion intermediate, we treated our stalled insertion reactions with puromycin. This attaches to the nascent chain and terminates translation, releasing the nascent polypeptide from the ribosome and allowing it dissociate from the translocon and enter the ER (25, 26). Importantly, we found that cross-link 4 was absent when membrane-targeted RNCs were treated with puromycin prior to the addition of the cross-linker (Fig. 2*D*), demonstrating that this adduct represents a genuine insertion intermediate. Under these conditions, a 40-kDa cross-link (Fig. 2*D*, ●) was formed, and this may represent an interaction between fully integrated Sec71p-S27C and some unidentified binding partner.

Sec71p Preferentially Inserts via the Ssh1p Complex—The ~55-kDa species identified as cross-link 4 would be predicted to correspond to a cross-link between the single cysteine in Sec71p-S27C and a cysteine-containing protein with a relative gel mobility of ~35–40 kDa. Both Sec61p and its homolog Ssh1p migrate at approximately this size, but because the Sec71p-HA insertion is Sec61p-independent *in vivo* (Fig. 1*B*), we tested whether Sec71p-HA cross-links to Ssh1p. To do this, we performed cross-linking using membranes containing a Myc-tagged version of Ssh1p, enabling us to immunoprecipitate Ssh1p-Myc. We found that the 55-kDa adduct (cross-link 4) formed with wild-type membranes appeared to migrate more slowly when using Ssh1p-Myc membranes (Fig. 3*A*, *sixth lane*). Moreover, this shifted adduct could be specifically immunoprecipitated with anti-Myc antibodies (Fig. 3*B*, *sixth lane*), showing that the Sec71p-HA RNC does indeed cross-link to Ssh1p-Myc.

Our finding that Sec71p is inserted by the Ssh1p translocon demonstrates that Ssh1p functions as a protein channel for this particular substrate. However, this did not rule out the possibility that it is targeted to Sec61p as well as to Ssh1p because the location of the cysteines in native Sec61p might simply not favor cross-linking to our substrate. This possibility was excluded by the emergence of a novel cross-link in *ssh1*Δ membranes that was identified as containing Sec61p by virtue of its

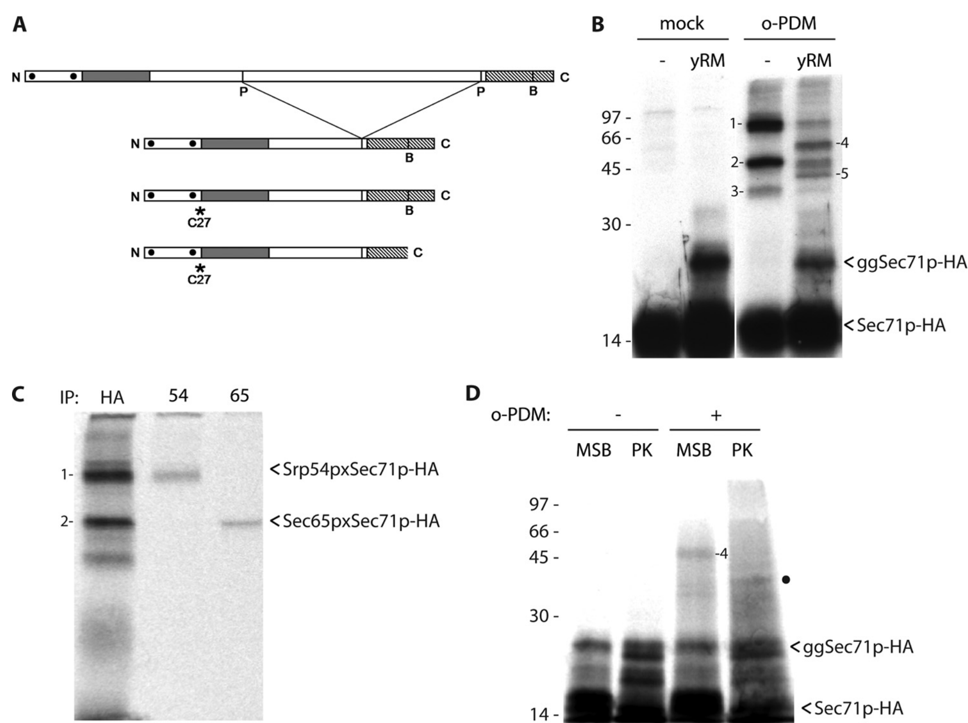

FIGURE 2. **Probing the molecular environment of the Sec71p transmembrane domain.** *A*, diagram showing the creation of truncated Sec71p-S27C. The *top line* shows full-length Sec71p-HA, with glycosylation sites indicated (●). The transmembrane region is shown as a *gray box*, and the HA tag as a *hatched box*. *P*, PacI restriction sites; *B*, BamHI site. The *second line* shows the removal of sequence from the cytosolic region of Sec71p-HA by digestion with PacI and religation. The *third line* shows the cysteine (*) introduced at position 27. The *fourth line* shows the generation of a stop codon-lacking 91-amino-acid-encoding construct by removal of the 3′-end of the sequence with BamHI. *B*, Sec71p-S27C RNCs were prepared, and RNCs were precipitated and added to rough microsomes prepared from W303 yeast (*yRM*) or to membrane storage buffer (−), followed by incubation with and without the cross-linker *o*-phenylenedimaleimide (*o-PDM*). HA immunoprecipitations were carried out, and samples were resolved by 12.5% SDS-PAGE. The positions of unglycosylated (*Sec71p-HA*) and glycosylated (*ggSec71p-HA*) Sec71p-HA are indicated. The positions of cross-linker-dependent species are numbered from *1* to *5*. *C*, identification of cross-link 1 as Srp54p×Sec71p-HA and cross-link 2 as Sec65p×Sec71p-HA. Parallel immunoprecipitations were carried out on cross-linker-treated Sec71p-S27C RNCs. Samples were immunoprecipitated (*IP*) with anti-HA, anti-Srp54p (*54*), or anti-Sec65p (*65*) antibody and resolved by 9% SDS-PAGE. The positions of cross-link product 1 (Srp54p×Sec71p-HA) and cross-link product 2 (Sec65p×Sec71p-HA) are indicated. *D*, cross-link 4 is puromycin-sensitive. Sec71p-S27C RNCs were incubated with wild-type microsomes, and following harvesting of membranes, pellets were resuspended in membrane storage buffer (*MSB*) or 1 mM puromycin and 600 mM potassium acetate (*PK*). Cross-linking was performed as described above, and samples were immunoprecipitated using anti-HA antibody and resolved by 12.5% SDS-PAGE. The positions of cross-link 4 and glycosylated (*ggSec71p-HA*) and unglycosylated (*Sec71p-HA*) Sec71p-HA are shown. An additional cross-link of ~40 kDa (●) was observed that is similar in size to that identified as adduct 5 in *B*.

precipitation with anti-Sec61p antibodies (Fig. 4*A*). Quantification of the relative levels of this Sec61p×Sec71p-S27C in wild-type *versus ssh1*Δ membranes, using a longer exposure of the same gel, is shown in the *right panel*. Almost no trace of the Sec61p×Sec71p-S27C adduct could be seen in immunoprecipitations from wild-type membranes. Moreover, when Sec61p was present but not able to cross-link to Sec71p-HA (*sec61nocys*), the cross-linking profile was identical to wild-type membranes. Therefore, in the presence of Ssh1p, the vast majority of Sec71p-S27C did not interact with Sec61p but readily did so when Ssh1p was absent. This finding demonstrates that Sec71p is preferentially targeted to Ssh1p in wild-type membranes.

In contrast to Ssh1p, Sec61p has been shown to assemble into a complex containing the mature Sec71 protein (11). As before, we used puromycin release of the nascent chain to determine whether or not the observed Sec61p cross-link represents a biosynthetic insertion intermediate. Once again, we found that the observed Sec61p×Sec71p-S27C cross-link was lost upon puromycin treatment (Fig. 4*B*), indicating that this cross-link represents a genuine insertion intermediate.

DPAPB Is Not Preferentially Targeted—To exclude the possibility that preferential targeting to Ssh1p is simply a characteristic of this particular *in vitro* assay, we used DPAPB as a comparison. We know that, unlike Sec71p-HA, DPAPB accumulates precursor in a *sec61* mutant (Fig. 1*B*), so it should therefore show targeting to Sec61p *in vitro*. To investigate this, we used the well characterized model substrate DHC-αF, which is based on the signal anchor domain of DPAPB (see Fig. 1) (7, 27). We inserted a cysteine residue at position 20 of the DHC-αF sequence, which is at the lumenal end of the transmembrane domain (Fig. 5*A*), and translated a truncated form of this protein in yeast cytosol to generate RNCs as described above. These RNCs were then added to microsomes prepared from either wild-type or *ssh1*Δ yeast, followed by the addition of the cross-linker (Fig. 5*B*). In contrast to the Sec71p-HA substrate shown in Figs. 2–4, a cross-link between DHC-αF and Sec61p could be clearly observed in wild-type microsomes (Fig. 5*B*, *third lane*). The intensity of this adduct was increased by ~2-fold in *ssh1*Δ microsomes. To confirm DHC-αF interaction with Ssh1p, we incubated DHC-αF RNCs with microsomes containing Myc-tagged Ssh1p (Fig. 5*C*). A cross-link was formed that could be immunoprecipitated with anti-Myc antibody (Fig. 5*C*, *second lane*), confirming that DHC-αF targets to both Ssh1p and Sec61p. Thus, preferential sorting to Ssh1p is not a general feature of our *in vitro* assay.

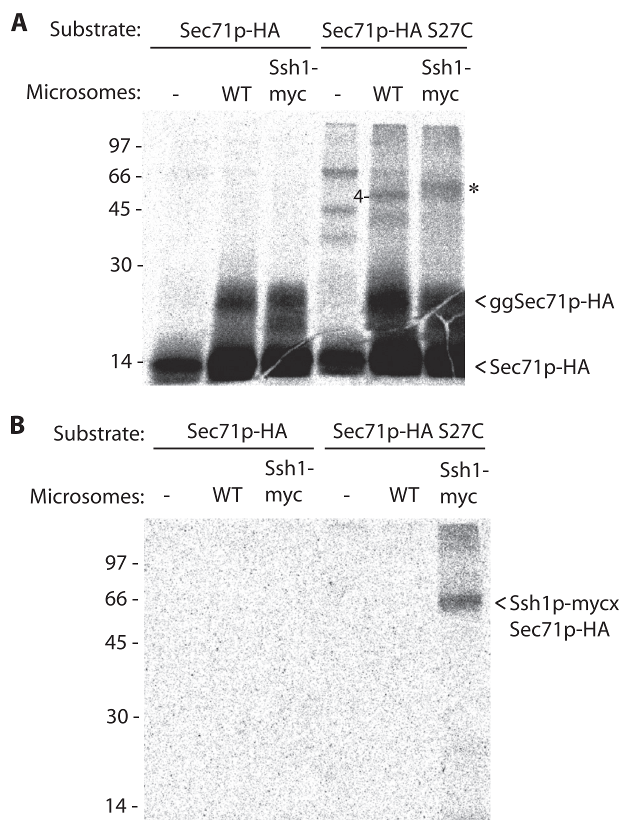

FIGURE 3. **Sec71p is targeted to the Ssh1p translocon.** *A*, Sec71p-HA cross-linking in the presence of Ssh1p-Myc membranes. Sec71p-HA or Sec71p-S27C RNCs were incubated with membrane storage buffer (−), with microsomes prepared from wild-type yeast, or with microsomes prepared from yeast containing Ssh1p-Myc. All samples were treated with the cross-linker and subjected to immunoprecipitation using anti-HA antibodies. Samples were resolved by 12.5% SDS-PAGE, and the positions of the potential Ssh1p×Sec71p-HA adduct (cross-link 4) and the potential Ssh1p-Myc×Sec71p adduct (*) are indicated. *ggSec71p-HA*, glycosylated Sec71p-HA; *Sec71p-HA*, unglycosylated Sec71p-HA. *B*, Sec71p-HA cross-links to Ssh1p-Myc. Targeting and cross-linking reactions were performed as described for *A* but were immunoprecipitated using anti-Myc antibodies. The position of the Ssh1p-Myc×Sec71p-HA adduct is indicated.

Preferential Targeting to the Ssh1p Complex Requires Sbh2p—To further investigate the requirements for this preferential targeting to Ssh1p, we next tested whether the translocon β-subunits might be required. It has previously been demonstrated that these play a role in co-translational targeting through interaction with the SRP receptor (15, 28). Therefore, if Sbh2p is the link between the RNC and Ssh1p, deletion of the Ssh1p β-subunit might alter the targeting profile for Sec71p-HA. To test whether this is the case, we performed the Sec71p-HA RNC targeting reaction with microsomes prepared from either *sbh1*Δ or *sbh2*Δ yeast (Fig. 6*A*), and we observed that cross-linking of Sec71p-HA to Sec61p was greatly increased in the *sbh2*Δ mutant. To examine the effect of *sbh* deletions on Ssh1p targeting, we performed the same reactions in microsomes containing the Myc-tagged variant of Ssh1p (Fig. 6*B*). We found that targeting of Sec71p-HA to Ssh1p-Myc in the *sbh1*Δ mutant (Fig. 6*B*, *fifth lane*) was similar to wild-type membranes but was almost completely absent in the *sbh2*Δ mutant (*sixth lane*). This reduced targeting to Ssh1p and increased targeting to Sec61p were not due to reduced levels of Ssh1p, as it has previously been shown that Ssh1p is stable in *sbh2*Δ yeast (11). We therefore conclude that Sbh2p is required for the preferential targeting of precursor to the Ssh1p translocon.

Ssh1p-dependent Translocation of Sec71p-HA Requires Sec63p—The specific targeting of Sec71p to Ssh1p during its insertion into the ER membrane allowed us to determine whether Sec63p is required for this process. We chose to study Sec63p, as Sec63p is required for the co-translational insertion of DPAPB (8), but a stable complex between Ssh1p and Sec63p has not yet been detected (11). To test the requirement for Sec63p for Ssh1p-mediated insertion *in vivo*, we used two alleles of *sec63* that had specific domains deleted: *sec63*Δ*brl*, which has the cytosolic “Brr2-like” domain deleted and does not form SEC′ or SEC complexes, and *sec63*Δ*J*, which lacks the lumenal J-domain. Both of these domains of Sec63p have been shown to be required for DPAPB insertion (12). Both mutations also disrupted Sec71p-HA insertion, causing accumulation of the Sec71p-HA precursor at the same level as the DPAPB precursor (Fig. 7; see supplemental Fig. 1 for the levels of mutant Sec63p). This requirement for Sec63p shows that insertion by Ssh1p is clearly dependent on Sec63p *in vivo*, indicating a universal role for Sec63p in co-translational translocation.

DISCUSSION

A number of studies have implicated the Sec61p homolog Ssh1p in ER translocation in yeast. However, although mutant cells lacking Ssh1p exhibit translocation defects, the fact that they remain viable has led to the conclusion that Ssh1p makes a relatively minor contribution compared with the essential Sec61p (16). Using a “split-ubiquitin” approach, it has also been shown that the signal sequences of Kar2p and Suc2p are in close proximity to Ssh1p *in vivo*, but these interactions are not specific to Ssh1p because the same substrates are also associated with Sec61p (14). Most recently, detergent-solubilized Ssh1p was found to bind to purified RNCs (13). Our results confirm that this interaction is functional by demonstrating the close proximity of Ssh1p and a translocating polypeptide chain.

We have used the co-translationally inserted protein Sec71p as a model for membrane protein integration. Insertion of this protein *in vivo* was not sensitive to *sec61* mutation, so to elucidate the mechanism of Sec71p integration, we generated stalled translation intermediates *in vitro* in a fashion similar to that described previously (25). These intermediates allowed us to use chemical cross-linking to determine which proteins are in the local environment of Sec71p during its translation and integration. The formation of a membrane-dependent cross-link that failed to immunoprecipitate with anti-Sec61p antibodies was in contrast to previous studies that showed that several stalled translation intermediates can be photo-cross-linked or chemically cross-linked to mammalian Sec61 (29, 30), including a synthetic protein, with the same topology as Sec71p, generated from a modified *Escherichia coli* gene (25), and that post-translationally targeted prepro-α-factor can be photo-cross-linked to yeast Sec61p (10). Our subsequent determination that the cross-link contained Ssh1p, rather than Sec61p, is the first time that a translocating substrate has been cross-linked to the Ssh1p translocon.

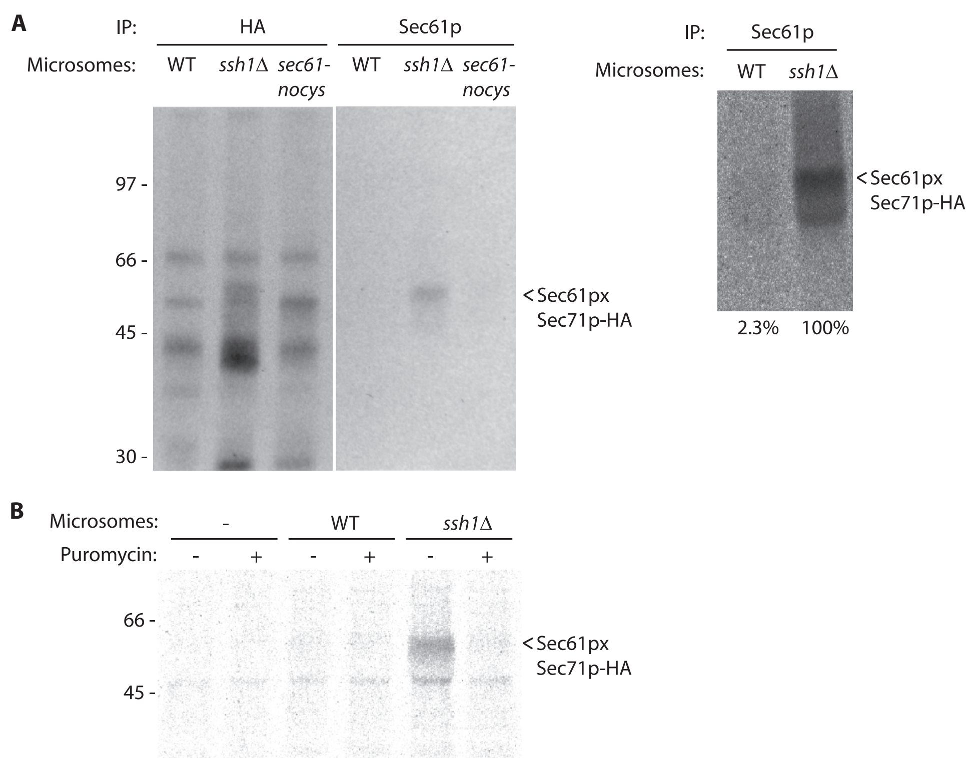

FIGURE 4. **Sec71p-HA cross-links to Sec61p in the absence of Ssh1p.** *A*, *left panel*, Sec71p-S27C RNCs were incubated with microsomes prepared from wild-type (W303), *ssh1*Δ (BWY464), or *sec61nocys* (CMY8) yeast, followed by cross-linking. Membrane fractions were recovered by centrifugation, split into two, subjected to immunoprecipitations (*IP*) using either anti-HA or anti-Sec61p antibodies, and resolved by 9% SDS-PAGE. The position of the novel Sec61p×Sec71p-HA cross-link is indicated. *Right panel*, a longer exposure is shown, from which the relative intensities of Sec61p×Sec71p-S27C cross-links in wild-type and *ssh1*Δ membranes have been quantified. *B*, the Sec61p×Sec71p-HA adduct is puromycin-sensitive. Sec71p-S27C RNCs were incubated with membrane storage buffer, wild-type microsomes, or microsomes prepared from *ssh1*Δ yeast, followed by the addition of puromycin. Cross-linking was performed, and samples were immunoprecipitated using anti-Sec61p antibody and resolved by 8.5% SDS-PAGE. The position of the Sec61p×Sec71p-HA adduct is indicated.

Not only was Sec71p targeted to the Ssh1p translocon, it was targeted to this translocon in preference to Sec61p. This was in contrast to the membrane protein DHC-αF, which utilized both translocons under wild-type conditions in the same RNC targeting assay. The specific targeting for Sec71p is also in contrast to the previous observation that Kar2p and Suc2p show no preference between Ssh1p and Sec61p (14), meaning that, among co-translationally translocated substrates tested so far, Sec71p is unique in showing a preference between Ssh1p and Sec61p. Our data demonstrate that a translocation substrate can be preferentially targeted to the Ssh1p translocon. However, it is difficult to predict how many other proteins besides Sec71p might use the Ssh1p complex preferentially. Earlier reports have demonstrated relatively minor growth defects in an *ssh1*Δ knock-out mutant (11, 33), suggesting that the Ssh1p complex is not required for translocation of any essential proteins. However, we have shown that an Ssh1p-dependent substrate can be redirected to Sec61p in *ssh1*Δ membranes. Interestingly, there are very strong synthetic growth defects observed when a conditional *sec61* mutation is combined with *ssh1*Δ (33). Such effects have previously been interpreted as indicating some "backup" role for Ssh1p in the context of a partially disabled Sec61p. However, our data raise the possibility that the converse may also prove to be the case. Whether such mutual redundancy might be sufficient to explain the presence of two distinct translocons in yeast remains a matter for speculation. Alternatively, it may be that the two translocons offer the potential for differential regulation that might favor the translocation of distinct subsets of precursors under certain physiological conditions.

This leads us to the question of how a particular precursor is targeted to Ssh1p or Sec61p, and our data reveal a novel mechanism that enables specific precursors to be targeted to a particular translocon complex. This mechanism likely comprises two parts: a feature of the Sec71p RNC that is recognized at the membrane and an ER membrane component that directs RNCs to the Ssh1p channel. At the membrane, we have identified a role for the translocon β-subunit because deletion of Sbh2p results in redirection of Sec71p to the Sec61p translocon, suggesting a direct role for Sbh2p in promoting differential targeting to Ssh1p. This would be consistent with a proposed role for Sbh1p/Sbh2p in promoting translocon interaction with the SRP receptor (15). However, our data allow us to extend this hypothesis such that Sbh2p also recognizes a substrate-specific feature of the targeted RNC to promote differential sorting. No alternative isoforms have yet been discovered for either SRP or the SRP receptor, so this substrate-specific feature might be the signal anchor sequence *per se* or possibly some conformational feature of the RNC induced by the specific nature of the signal anchor sequence. It has been shown that the ribosome exit tunnel is able to recognize transmembrane helices (32), so it is possible that an Ssh1p-targeting signal might also be recognized within the ribosome, causing conformational changes that result in the RNC having higher affinity for Ssh1p than for

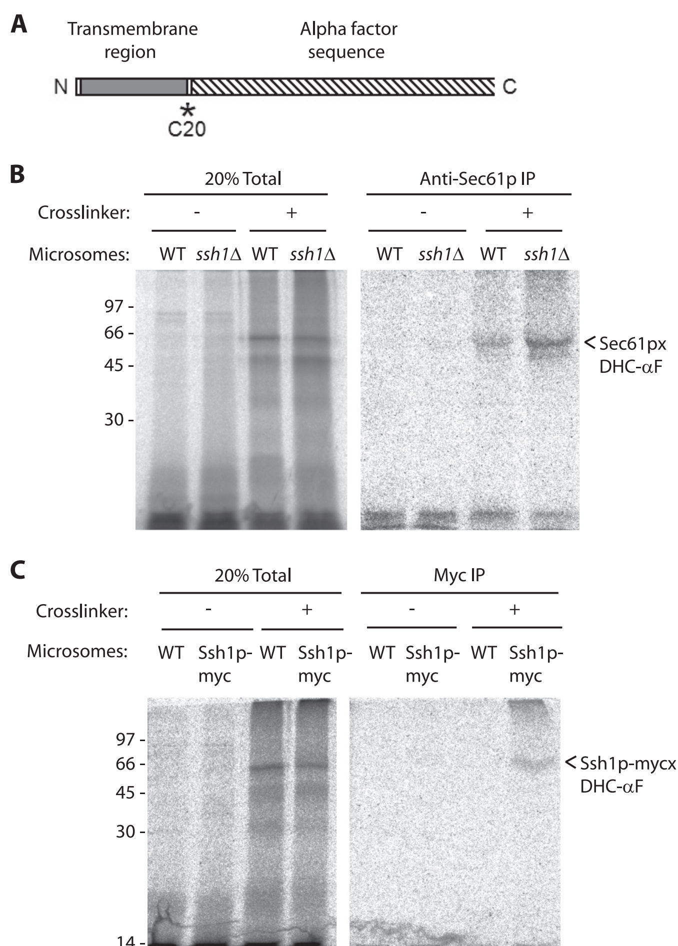

FIGURE 5. **DHC-αF can be targeted to either Sec61p or Ssh1p.** *A*, schematic showing the 92-amino acid chimeric DHC-αF protein, composed of the hydrophobic core of the DPAPB transmembrane domain (*gray box*) and a portion of α-factor protein (*hatched box*). The position of the inserted cysteine residue is indicated (*). *B*, formation of a Sec61p$\times$DHC-αF adduct. DHC-αF RNCs with a cysteine residue at position 20 were incubated with either wild-type or *ssh1*Δ microsomes, followed by the addition of the cross-linker. Samples, were subjected to immunoprecipitation (*IP*) using anti-Sec61p antibody and resolved by 12.5% SDS-PAGE, alongside 20% of the total cross-linking reaction. The position of the Sec61p$\times$DHC-αF adduct is indicated. *C*, formation of an Ssh1p-Myc$\times$DHC-αF adduct. DHC-αF RNCs with a cysteine residue at position 20 were incubated with either wild-type or Ssh1p-Myc microsomes, followed by the addition of the cross-linker. Samples were treated as described for *B*, except that immunoprecipitation was performed using anti-Myc antibody.

Sec61p. This could lead to an RNC with a higher affinity for Ssh1p/Sbh2p than for Sec61p/Sbh1p. Alternatively, it might be the particular topology of Sec71p that could cause it to be targeted to the Ssh1p complex.

Our Sec71p substrate was unusual in another sense in that it could be cross-linked to the SRP component Sec65p, prior to targeting to Ssh1p. Sec65p has not previously been shown to contact nascent protein, although a model of the mammalian SRP molecule puts the Sec65p homolog SRP19 in close proximity to the N-terminal end of the nascent signal sequence (33). Our results support this model, although whether Sec65p is simply in close proximity due to a structural role or whether there is any connection between the interaction of nascent Sec71p with Sec65p and its subsequent targeting to Ssh1p is not known.

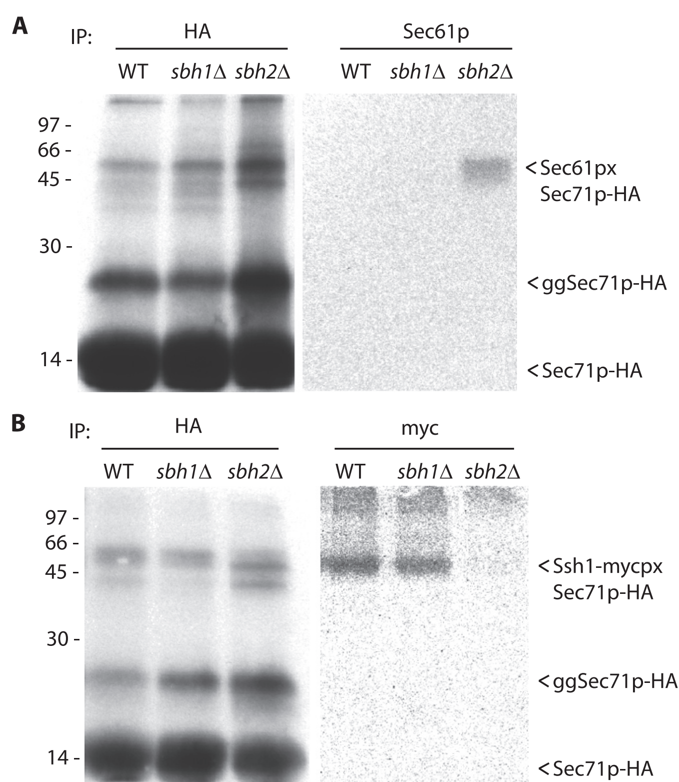

FIGURE 6. **Role of the β-subunits in preferential targeting.** *A*, Sec71p-S27C RNCs were incubated with microsomes prepared from wild-type (W303), *sbh1*Δ (BWY594), or *sbh2*Δ (BWY596) yeast, followed by cross-linking as described in the legend to Fig. 3. Membrane fractions were recovered by centrifugation, subjected to immunoprecipitations (*IP*) using either anti-HA or anti-Sec61p antibodies, and resolved by 12% SDS-PAGE. *ggSec71p-HA*, glycosylated Sec71p-HA; *Sec71p-HA*, unglycosylated Sec71p-HA. *B*, Sec71p-S27C RNCs were incubated with microsomes prepared from *ssh1*Δ (BWY464), *ssh1*Δ/*sbh1*Δ (MPSY24), or *ssh1*Δ/*sbh2*Δ (MPSY26) yeast, all of which also carried the Ssh1p-Myc-encoding plasmid pPR14, followed by cross-linking as described in the legend to Fig. 3. Membrane fractions were recovered by centrifugation, subjected to immunoprecipitations using either anti-HA or anti-Myc antibodies, and resolved by 12% SDS-PAGE.

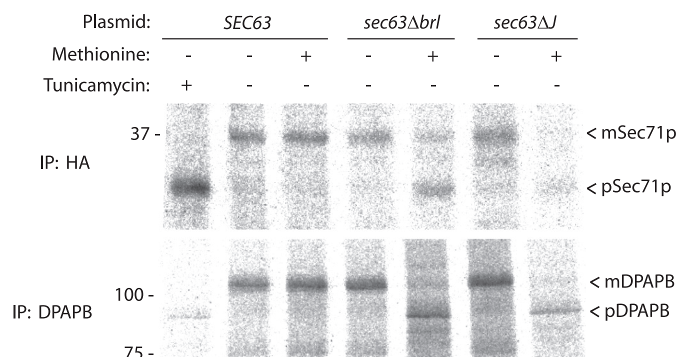

FIGURE 7. **Sec71p-HA insertion requires functional Sec63p.** The insertion of Sec71p-HA was measured in *sec63Δbrl* or *sec63ΔJ* cells. Yeast carrying plasmids expressing *SEC63*, *sec63Δbrl*, or *sec63ΔJ*, as well as Sec71p-3-HA, had transcription of the genomic copy of *SEC63* repressed with methionine. Cultures were harvested and pulse-labeled, and samples were split and immunoprecipitated (*IP*) with anti-HA or anti-DPAPB antibodies. Samples were resolved on 10.5 and 8.5% gels, respectively. The precursor and mature forms of Sec71p-HA (*pSec71p* and *mSec71p*) and DPAPB (*pDPAPB* and *mDPAPB*) are indicated.

The requirement for Sec63p in Ssh1p-mediated insertion is interesting in light of earlier reports that Ssh1p does not interact with Sec63p (4). We have previously shown that depletion

or mutation of Sec63p results in a failure to correctly insert the membrane protein DPAPB (7, 8). Depletion of Sec63p results in loss of the SEC′ complex in yeast (made of the Sec61p and Sec63p complexes, but with no Sec62p), but even in the *sec63ΔJ* mutant, which forms the SEC′ complex, translocation of DPAPB is blocked (12). In this study, we have shown that Sec63p, including its J-domain, is required for integration of a co-translationally inserted membrane protein via the Ssh1p translocon. The known function of the Sec63p J-domain is the recruitment and activation of the lumenal chaperone Kar2p (34, 35). For membrane protein insertion, the mammalian homolog of Kar2p, BiP, has been shown to gate the lumenal end of the translocon channel, whereas transmembrane segments are integrated into the bilayer (36). The requirement for the Sec63p J-domain in Sec71p and DPAPB integration may reflect an important role for the J-domain in gating yeast translocons, although the effects of an ungated translocon on the processes of translocation and integration are not known. The development of a cross-linking-based assay for investigating yeast co-translational translocation, as described in this study, may help to answer this and other questions about the mechanism of the translocation process.

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