Tail-Anchored Protein Insertion into Yeast ER Requires a Novel Posttranslational Mechanism Which Is Independent of the SEC Machinery†

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Received May 9, 2002; Revised Manuscript Received July 22, 2002

ABSTRACT: Tail-anchored or C-terminally-anchored proteins play many essential roles in eukaryotic cells. However, targeting and insertion of this class of membrane protein has remained elusive. In this study, we reconstitute insertion of tail-anchored proteins into microsomes derived from *Saccharomyces cerevisiae*. Using this approach, we are able to genetically manipulate the composition of the microsomes in order to address the question of which components of the endoplasmic reticulum (ER) are required for this process. We show that tail-anchored protein insertion is not dependent on the classical SEC translocation machinery but rather occurs via an ATP-dependent pathway involving at least one novel membrane protein factor. We further demonstrate that the specificity of this pathway is conserved between yeast and mammals.

Tail-anchored proteins encompass many essential proteins within eukaryotic cells including components of the ER[1] translocation machinery, mitochondrial import machinery, and vesicle trafficking SNARE proteins [for review, see reference (*1*)]. Some tail-anchored proteins are targeted directly to their destination membrane (*2*); however, the majority are initially inserted into the ER, and moved to their final destination via vesicular transport (*3*, *4*). Several studies have focused on the determinants within the pre-protein itself responsible for targeting to the ER. Domain swapping and mutagenesis of the transmembrane and surrounding domains have demonstrated that the length of the TM domain (*5*, *6*), and adjacent charged residues play a role in stabilizing the interaction with membranes (*7*); however, the underlying mechanism of targeting and insertion remains unknown.

Most integral membrane proteins with type I, type II, or polytopic topologies are targeted to the ER via a co-translational targeting reaction. This is mediated by an N-terminal signal sequence on the nascent polypeptide chain that interacts with signal recognition particle (SRP), which in turn binds to its cognate receptor molecule (SRP-receptor; SR) at the ER membrane. The binding of SRP to SR leads to a nucleotide-dependent transfer of the nascent chain to the Sec61-complex (or "translocon") which then provides the conduit through which the targeted nascent chain can be co-translationally translocated across the bilayer (*8*−*10*). However, tail-anchored proteins do not contain an N-terminal cleavable signal sequence; rather they have a single hydrophobic membrane anchor located within 50 residues of the C-terminus (*1*). This affords no opportunity for productive interaction of the nascent polypeptide with SRP since translation is complete before the hydrophobic membrane-anchor has emerged from the ribosome. Thus, targeting of tail-anchored proteins is independent of SRP and occurs posttranslationally (*1*).

Co-translational translocation has been reconstituted using mammalian membrane components and been shown to require the Sec61-complex (*11*). In contrast, membranes reconstituted following immunodepletion of the Sec61 complex remain competent for tail-anchored protein insertion, suggesting that co-translational translocation and tail-anchored protein insertion must occur by alternative mechanisms (*3*).

In *Saccharomyces cerevisiae*, a novel posttranslational pathway for the targeting and translocation of secretory proteins has been characterized. Full-length precursor polypeptides first interact with cytosolic chaperones which maintain the precursor in a translocation-competent state (*12*−*16*). Translocation across the bilayer again involves the trimeric Sec61-complex, but several additional components comprising the "Sec63-complex" are also required. The Sec63-complex comprises two essential integral membrane proteins (Sec62p and Sec63p), plus two nonessential proteins (Sec71p and Sec72p), and also interacts directly with Kar2p located in the ER lumen (*17*−*21*). Evidence from cross-linking studies suggests that the Sec62p component functions as a receptor for posttranslationally targeted precursors, and that precursors are then delivered to the Sec61-complex in an ATP-dependent reaction (*22*, *23*). Various lines of evidence then support the view that Sec63p and Kar2p interact to pull the precursor through the translocon (*19*, *23*−*27*). In addition, both Sec63p and Kar2p have subsequently been shown to be required for co-translational translocation (*28*), although their precise roles in this pathway have yet to be established.

† This work was supported by the MRC (G9722026 and 47969) and the Wellcome Trust (056084).

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[1] Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; CO_3, carbonate; Cyt*b5*, cytochrome *b*5; DTT, dithiothreitol; ER, endoplasmic reticulum; ECL, enhanced chemiluminescence; MSB, membrane storage buffer; NSF, *N*-ethylmaleimide sensitive protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SRP, signal recognition particle; SEC, secretory; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SNARE, soluble NSF attachment protein receptor; SR, SRP-receptor; TM, trans membrane; TCA, trichloroacetic acid; v-SNARE, vesicle-SNARE; YPD, yeast extract/peptone/dextrose.

10.1021/bi026105r CCC: $22.00
Published on Web 08/31/2002

Table 1: *Saccharomyces cerevisiae* Strains

strain	genotype	ref
CSY142	*MAT* α *leu2−3,112 pep4⁻::URA3*	
kar2−159	*MAT* α *ade2 ura3 kar2−159*	(*35*)
*sec61*Δ4−24	*MAT* α *ade2 leu2 his3 trp1 ura3 sec61::HIS3 [pCEN LEU2 sec61*Δ4−24]	this study
sec62−1	*MAT* α *his4 leu2−3, 112 ura3−52 sec62−1*	(*45*)
RC45a	*MAT* α *leu2 his3 trp1 ura3::TRP1 sec63−301*	(*28*)
FKY198	*MAT* α *sss1::URA3 pep4::LEU2 Leu2−3,112 ade2−1 ura3−1 his3−11,−15 trp1−1 can1−100* p[*GAL10::SSS1, ADE*2] p[*TPI::SUC2, TRP*1]	(*46*)
Δ*ssh*	*MAT* α *ade2 leu2 his3 trp1 ura3 ssh1::TRP1*	(*36*)

Kutay et al. (*3*) have shown that the Sec61-complex is not essential for tail-anchored protein insertion. However, the Sec63-complex remains an obvious candidate for a role in this posttranslational pathway. Moreover, recent studies in yeast have identified a second trimeric translocon, comprising Ssh1p, Sss1p, and Sbh2p (*29*), further raising the possibility that this "Ssh1-complex" might be involved in tail anchor insertion in yeast.

To test these hypotheses, we have first reconstituted tail-anchored protein insertion into yeast microsomes. Using this assay, we demonstrate the functional conservation of the insertion machinery between yeast and mammals. This assay further enabled us to test a variety of known ER components for any role in tail-anchor insertion. We confirm previous findings and show that insertion is independent of the Sec61p translocon complex. However, we go on to demonstrate that neither the Ssh1-complex nor the Sec63-complex is required for tail-anchored protein insertion in yeast. These results demonstrate that the insertion pathway is independent of both yeast translocons and is also independent of the posttranslational translocation pathway identified for secretory precursors. Our data thus require an entirely novel pathway for tail-anchored protein insertion.

EXPERIMENTAL PROCEDURES

Reagents used were from the following sources: enzymes for DNA manipulation from Roche Molecular Biochemicals; rabbit reticulocyte lysate from Promega Life Sciences; ^{35}S-methionine and ECL reagent from NEN Life Science Products. All other reagents were of analytical grade from Sigma.

Plasmids and Strains. Plasmids pEH3 (encoding prepro-alpha factor) and pSyb2 (encoding synaptobrevin 2) have previously been described (*30*, *31*). pGEM3-NYV1 was constructed as follows; the coding region of NYV1 was amplified from yeast genomic DNA by PCR using the following primers: 5′-CCATTTGGATCCATTTTGCATATG-GTCTTAGTAAGTTATGTG-3′ and 5′-TAATGTAAGCT-TCGTGGGACAGCTCCCCTT-3′ (encoding *Bam*HI and *Hin*dIII sites underlined). A 780 bp fragment was cloned into the corresponding sites of pGEM3 transcription vector (Promega). Yeast strains used in this study are listed in Table 1.

In Vitro Transcription and Translation. Messenger RNA was produced by in vitro transcription using Ribomax T3 or T7 polymerase as described by the manufacturer (Promega). Translation was performed in the presence of ^{35}S-methionine (1170 mCi/mL; NEN) using nuclease-treated rabbit reticulocyte lysates for 20 min at 30 °C. Translation was terminated by incubation for 10 min at 30 °C with 1 mM cycloheximide, before removal of ribosomes by ultracentrifugation (Beckman TLA 120.1 rotor; 85 000 rpm; 15 min; 4 °C).

Preparation of Yeast Microsomes and Liposomes. Preparation of a crude microsomal fraction from yeast has been described elsewhere (*31*); briefly, 200 mL of cells was grown in either YPD or selective media to an OD_{600} of between 1.0 and 1.5. Cells were spheroplasted and broken by glass bead lysis; unbroken cells were removed by low-speed centrifugation. Microsomes were isolated at 4 °C from the 17500*g* pellet, washed several times, and resuspended in membrane storage buffer (250 mM sorbitol, 20 mM Hepes, pH 7.4, 50 mM KOAc, 2 mM DTT) supplemented with protease inhibitors (1 μg/mL AEBSF, 2 μg/mL E64, 1 μg/mL aprotinin, 1 μg/mL pepstatin A, 1 μg/mL chymostatin) at 50 A_{280} units/mL, aliquoted, and stored at −80 °C. For liposome production, 5−10 A_{280} units of microsomes were extracted twice with chloroform/methanol (2:1), and the organic phase was further extracted with chloroform/methanol/0.9% NaCl 3 times. Solvent was evaporated from the organic phase under a stream of nitrogen gas, and the lipid film was reconstituted in the original volume of membrane storage buffer. Vesicles were prepared by sonication (30 min in a Branson 2210 sonicating water bath at full power) and analyzed using light scattering (Malvern, Zetasizer). Vesicles had a mean size of 374 ± 120 nm and behaved similarly to wild-type membranes in the insertion assay (96.5% recovery of phospholipid from the carbonate pellet). Phospholipid content was determined by forming a chloroform soluble complex with ammonium ferrothiocyanate and measuring the absorbance at 488 nm and comparing to standards using phosphatidylcholine.

Protease Treatment and ATP Depletion. Five A_{280} units of microsomes were incubated at 30 °C in the presence of 100 μg/mL proteinase K and trypsin for 20 min, microsomes were cooled on ice, and 1 mM AEBSF was added. Protease- or mock-treated membranes were isolated 3 times through a high-salt cushion [0.25 M sucrose, 0.5 M KOAc, 5 mM $Mg(OAc)_2$, 50 mM Hepes (pH 7.9)], supplemented with protease inhibitors. For translated precursor, nucleotides were removed by gel filtration using Biospin 6 minicolumns (BioRad). Microsomes were treated with hexokinase/glucose for 10 min at 30 °C immediately prior to the insertion assay.

Insertion/Translocation Assay. Twenty microliters of a standard translation reaction was incubated with 0.75 A_{280} unit of microsomes, or the equivalent amount of liposomes, in 50 μL final volume in membrane storage buffer supplemented with an ATP-regeneration cocktail [1 mM ATP, 40 mM creatine phosphate, 0.2 mg/mL creatine phosphokinase, 20 mM Hepes, pH 7.4, 150 mM KOAc, 250 mM sorbitol, 5 mM $Mg(OAc)_2$]. Insertion was allowed to proceed for 20 min at 30 °C. Then 15 μL aliquots were removed for fractionation by sedimentation through 150 μL high-salt sucrose cushions [250 mM sucrose, 500 mM KOAc, 5 mM $Mg(OAc)_2$, 50 mM Hepes, pH 7.9; 55 000 rpm; 10 min; 4 °C; Beckman TLA120.1 rotor]. Microsome pellets were resuspended in either high-salt sucrose buffer or 100 mM Na_2CO_3, incubated on ice for 30 min, and sedimented by ultracentrifugation, as above. Prepro-alpha factor translocation was carried out in an identical fashion except that the

50 μL reaction was split into two; half was immediately precipitated with 10% TCA, and the other was incubated with an equal volume of 1 mg/mL proteinase K for 1 h on ice, followed by precipitation with 10% TCA. Fractions were examined by SDS−PAGE and phosphorimage analysis. Quantitation was carried out using Advanced Image Data Analyzer software.

RESULTS

Tail Anchor Proteins Insert Posttranslationally. We investigated the insertion of two different tail-anchored proteins into yeast microsomes, namely, the yeast vacuolar Nyv1p and mammalian synaptobrevin Syb2. The yeast mating pheromone prepro-alpha factor was also used as a control for the posttranslational translocation via the classical SEC machinery. All precursors were translated using rabbit reticulocyte lysates in the presence of ^{35}S-methionine. Translation was terminated by the addition of cycloheximide, and ribosomes were removed by centrifugation. Posttranslational membrane insertion/translocation was then assayed by incubation of radiolabeled precursor with yeast microsomes, monitoring the amount of membrane association after sedimentation or flotation of membranes through alkaline sucrose gradients. After sedimentation analysis, approximately half of both Nyv1p and Syb2 were recovered in the membrane pellet fractions (Figure 1A, lanes 8 and 18). Approximately 50% of the precursor remains unbound (lanes 7 and 17); however, titration of microsomes (data not shown) indicates that 0.75 A_{280} unit of membranes (see Experimental Procedures) used in these experiments is not limiting. These findings suggest that a proportion of the translated protein is incompetent for insertion.

To determine whether membrane-bound material was stably inserted into microsomes, duplicate samples of membrane-bound material were resuspended in alkaline carbonate buffer (pH 11.5) that extracts peripherally associated proteins. For both tail-anchored precursors, greater than 90% of the membrane-bound material remained resistant to carbonate extraction (compare lanes 8 and 10, 18 and 20), confirming that Nyv1p and Syb2 are efficiently inserted into yeast microsomes. Quantitation of carbonate-resistant insertion activity is shown (Figure 1B).

Insertion-Competent Precursor Binds Efficiently to Yeast Microsomes. In the absence of yeast microsomes, less than 2% of Nyv1p or Syb2 was recovered in the pellet fractions (Figure 1A, lanes 3 and 13); this value was independently determined for each experiment and suggests a small degree of nonspecific aggregation. To rule out the possibility that the precursors were sedimenting due to microsome-induced aggregation, microsomes were recovered by flotation through an alkaline sucrose gradient. In agreement with sedimentation data, approximately 50% of both Nyv1p and Syb2 were recovered from the top fractions of the gradient (comigrating with Sec61p labeling; data not shown). This contrasts with the absence of microsomes where >90% of the precursor remains in the loading zone (data not shown).

Microsomes Enriched in ER Components Are Insertion-Competent. By using differential centrifugation and velocity sedimentation sucrose gradients, we have been able to fractionate yeast microsomes. Nyv1p insertion activity exactly comigrates with Sec61p labeling indicative of ER membranes (data not shown). We also confirmed the

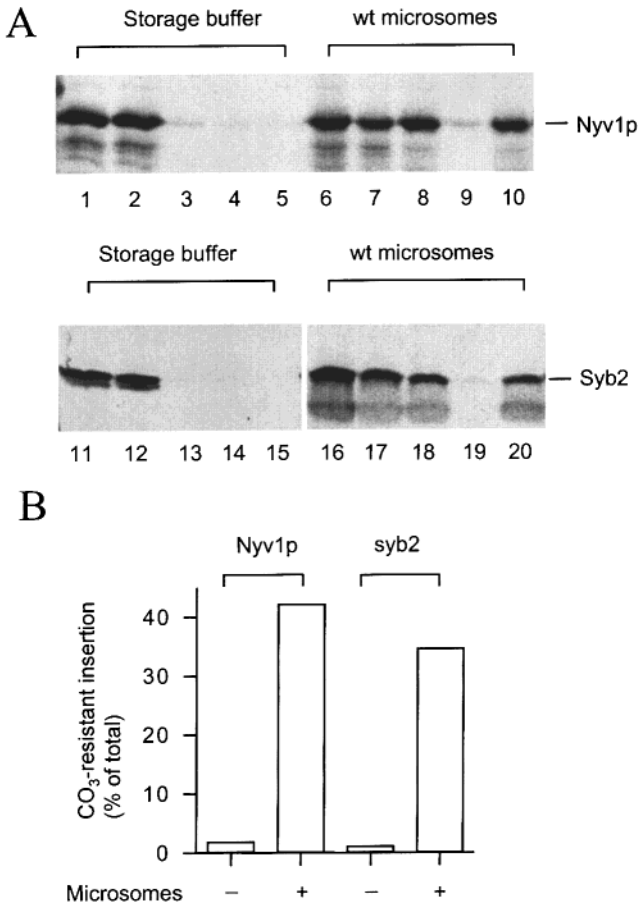

FIGURE 1: Stable insertion of tail-anchored proteins into yeast microsomes. (A) ^{35}S-labeled Nyv1p (lanes 1−10) or Syb2 (lanes 11−20) were incubated with membrane storage buffer (Storage buffer, lanes 1−5, 11−15) or yeast microsomes (lanes 6−10, 16−20), as described under Experimental Procedures. Membrane-associated material was sedimented by ultracentrifugation. Samples representing equal amounts of the total (lanes 1, 6, 11, and 16), soluble (lanes 2, 7, 12, and 17), membrane-bound (lanes 3, 8, 13, and 18), carbonate-soluble (lanes 4, 9, 14, and 19), and carbonate-resistant fractions (lanes 5, 10, 15, and 20) were analyzed by SDS−PAGE and phosphorimaging. (B) Quantitation of CO_3-resistant insertion of Nyv1p and Syb2 from lanes 5, 10, 15, and 20 is shown (expressed as a percentage of the total Nyv1p or Syb2 recovered).

posttranslational translocation of prepro-alpha factor into ER-derived microsomes using previously published methods (*32*). As previously reported (*47*), the soluble precursor, prepro-alpha factor, co-sediments in the membrane fraction presumably due to nonspecific aggregation (Figure 2A, lane 3). As this precursor undergoes a series of glycosylation steps upon insertion into the lumen of the ER, this can be used to quantify the amount of translocation. Unlike tail-anchored precursors, upon carbonate extraction, glycosylated prepro-alpha factor is released into the carbonate supernatant (Figure 2A, lane 4), and only a small proportion of unglycosylated species remains in the microsomal pellet (Figure 2A, lane 5). This material may be bound to the translocation machinery or may be due to aggregation. As expected, the glycosylated form of prepro-alpha factor is protected from proteolysis due to its sequestration within intact microsomes (Figure 2B, lane 4). In a typical experiment, approximately 40% of the prepro-alpha factor was glycosylated and protected from protease (Figure 2C), confirming efficient posttranslational translocation of this secretory precursor into wild-type membranes.

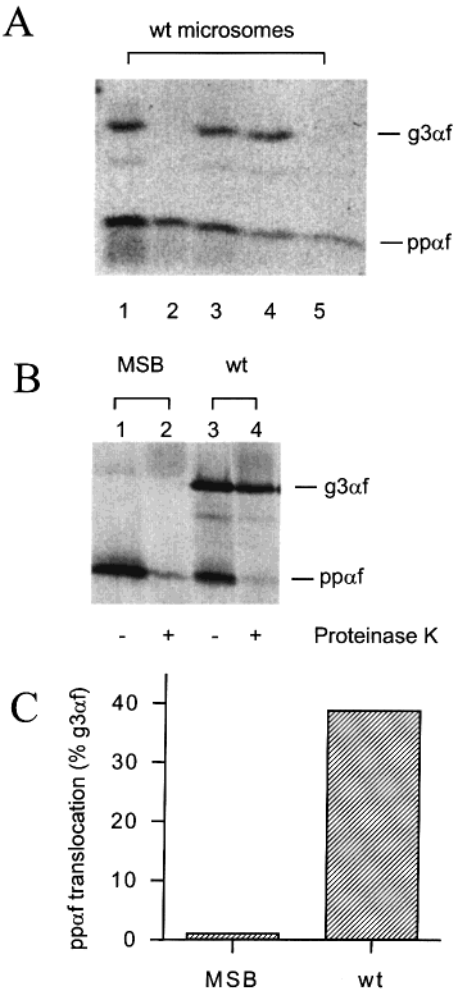

FIGURE 2: Prepro-alpha factor associates with microsomes and is translocated into the lumen of the ER. (A) ^{35}S-labeled prepro-alpha factor was incubated with yeast microsomes (lanes 1−5), as described under Experimental Procedures, and membrane-associated material was sedimented by ultracentrifugation. Samples representing equal amounts of the total (lane 1), soluble (lane 2), membrane-bound (lane 3), carbonate-soluble (lanes 4), and carbonate-resistant fractions (lane 5) were analyzed by SDS−PAGE and phosphorimaging. Positions of pre-pro form (ppαf) and triply glycosylated form (g3αf) are indicated. (B) ^{35}S-labeled prepro-alpha factor was incubated with membrane storage buffer (MSB, lanes 1 and 2) or yeast microsomes (wt, lanes 3 and 4), as described under Experimental Procedures. The reaction mixture was split into two and either immediately precipitated with 10% TCA (lanes 1 and 3) or incubated in the presence of 1 mg/mL proteinase K for 1 h on ice followed by precipitation with 10% TCA (lanes 2 and 4). Representative samples were analyzed by SDS−PAGE and phosphorimaging. Positions of pre-pro forms (ppαf) and triply glycosylated forms (g3αf) are indicated. (C) Quantitation of protease-protected forms of α-factor are shown (expressed as a percentage of the total).

Tail Anchor Insertion Requires a Protease-Sensitive Component. Previous studies have shown that Syb2 requires a proteinaceous receptor molecule for stable insertion into mammalian microsomes. Here we show that stable insertion of Nyv1p into yeast microsomes is sensitive to pretreatment of membranes with proteases. In this case, membranes were treated with 100 μg/mL trypsin and proteinase K and re-isolated on sucrose gradients in the presence of protease inhibitors. Substantial proteolysis of the cytoplasmic face of microsomes was observed, whereas the lumenal content remains intact (Figure 3D, compare lanes 1 and 2). After

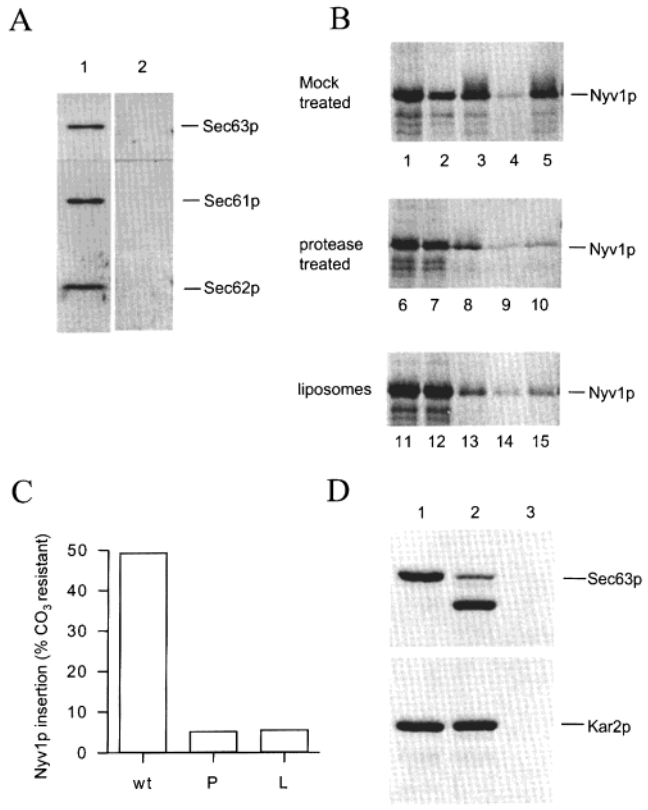

FIGURE 3: Efficient association of Nyv1p with membranes requires a protease-sensitive receptor. (A) 0.25 A_{280} unit (3.75 μg of phospholipid) of control membranes (lane 1) and the equivalent **amount** of protein-free liposomes (6.4 μg of phospholipid, lane 2) were run on 11% PAGE, transferred to nitrocellulose membranes, and probed for the indicated proteins by western blotting. Bands were visualized by ECL. (B) ^{35}S-labeled Nyv1p was incubated with mock-treated microsomes (lanes 1−5), protease-treated microsomes (lanes 6−10), or liposomes (lanes 11−15), as described under Experimental Procedures. Membrane-associated material was sedimented by ultracentrifugation. Samples representing equal amounts of the total (lanes 1, 6, and 11), soluble (lanes 2, 7, and 12), membrane-bound (lanes 3, 8, and 13), carbonate-soluble (lanes 4, 9, and 14), and carbonate-resistant fractions (lanes 5, 10, and 15) were analyzed by SDS−PAGE and phosphorimaging. (C) Quantitation of CO_3-resistant insertion of Nyv1p (expressed as a percentage of the total Nyv1p recovered) into mock-treated microsomes (wt), protease-treated (P), or liposomes (L). (D) 0.25 A_{280} unit of mock-treated membranes (lane 1), protease-treated membranes (lane 2), or protease-treated in the presence of 1% TX-100 (lane 3) was run on 11% PAGE, transferred to nitrocellulose membranes, and probed for the indicated proteins by western blotting. Bands were visualized by ECL.

protease treatment of microsomes, the amount of Nyv1p insertion was reduced, and approximately 5% of Nyv1p remained resistant to carbonate extraction (Figure 3B, lane 10), whereas mock-treated microsomes remained fully insertion-competent (Figure 3B, lane 5). After proteolysis of the membranes, a small but significant level of binding remains; this could be accounted for by binding to a protease-resistant receptor molecule or through direct interaction with membrane lipids. To distinguish between these possibilities, the interaction of Nyv1p directly with membrane lipids was investigated by measuring the insertion into liposomes. Liposomes were reconstituted from control membranes following protein removal (Figure 3A: compare lanes 1 and 2). Unlike Cyt*b5* that has been reported to efficiently bind to lipid (*33*), only 5% of Nyv1p bound to liposomes (Figure

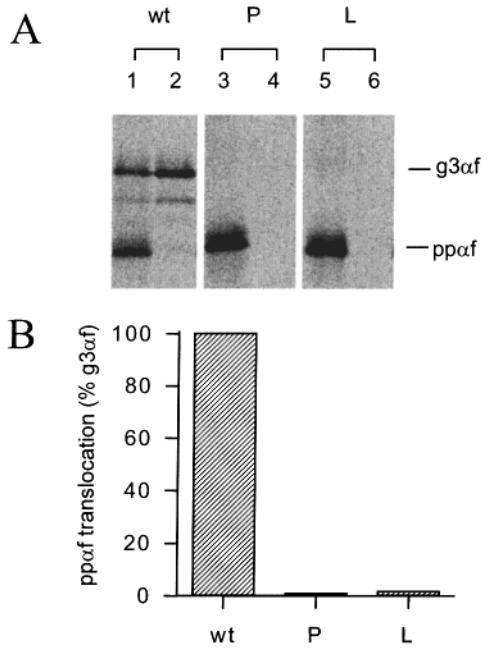

FIGURE 4: Efficient translocation of prepro-alpha factor requires a protease-sensitive receptor. (A) ^{35}S-labeled prepro-alpha factor was incubated with mock-treated microsomes (wt, lanes 1 and 2), protease-treated microsomes (P, lanes 3 and 4), or liposomes (L, lanes 5 and 6) as described under Experimental Procedures. The reaction mixture was split into two and either immediately precipitated with 10% TCA (lanes 1, 3, and 5) or incubated in the presence of 1 mg/mL proteinase K for 1 h on ice followed by precipitation with 10% TCA (lanes 2, 4, and 6). Representative samples were analyzed by SDS−PAGE and phosphorimaging. Positions of pre-pro forms (ppαf) and triply glycosylated forms (g3αf) are indicated. (B) Quantitation of protease-protected forms of α-factor is shown (translocation in wild-type expressed as 100%).

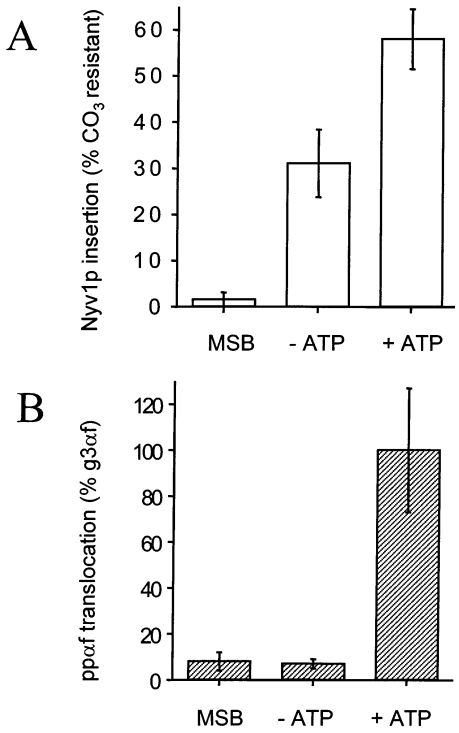

FIGURE 5: Efficient association of Nyv1p with membranes requires ATP. (A) ^{35}S-labeled Nyv1p was incubated with membrane storage buffer (MSB) or yeast microsomes, following pretreatment with hexokinase/glucose, re-isolation, and addition of either buffer control (−ATP) or ATP regeneration mix (+ATP) as described under Experimental Procedures. Membrane-associated material was sedimented by ultracentrifugation. Quantitation of CO_3-resistant insertion of Nyv1p (expressed as a percentage of the total Nyv1p recovered) is shown. (B) ^{35}S-labeled prepro-alpha factor was incubated with membrane storage buffer (MSB) or yeast microsomes following pretreatment with hexokinase/glucose, re-isolation, and addition of either buffer control (−ATP) or ATP regeneration mix (+ATP) as described under Experimental Procedures. Representative samples were analyzed by SDS−PAGE and phosphorimaging. (B) Quantitation of protease-protected forms of α-factor are shown (expressed as a percentage of the total).

3B, lanes 13 and 15), the same proportion that bound following protease digestion of membranes. This same level of background binding was also observed using synthetic liposomes (60% PC, 20% PE, 20% PS; data not shown), a composition that is similar to that of ER membrane (*34*). These findings confirm that interaction with membrane lipids is likely to account for the small amount of membrane insertion of Nyv1p into protease-treated yeast microsomes. We monitored the level of translocation of prepro-alpha factor into duplicate samples of microsomes and liposomes. After protease treatment or protein removal, prepro-alpha factor translocation is abolished (Figure 4A, lanes 4 and 6). In each case, translocation is less than 1% of the wild type (expressed as 100%).

ATP Stimulates Nyv1p Insertion. Next we tested whether the insertion reaction was required energy in the form of ATP. This was tested by depleting cytosol of endogenous ATP using hexokinase and glucose, or by removing nucleotides by gel filtration. Either treatment reduced Nyv1p insertion to approximately half of that found in untreated membranes (Figure 5A; −ATP). As a control, the translocation of prepro-alpha factor into duplicate samples of yeast microsomes was abolished by ATP depletion (Figure 5B). Both the insertion of Nyv1p and the translocation of prepro-alpha factor were restored by re-addition of an ATP-regeneration cocktail (+ATP). Our results indicate that tail anchor protein insertion into yeast membranes is stimulated by ATP. However, this reaction is less sensitive to ATP depletion than is the posttranslational translocation of prepro-alpha factor. These results suggest significant mechanistic differences between these two processes.

Tail Anchor Protein Insertion Is Independent of the Posttranslational Translocation Machinery. In addition to *SEC61*, posttranslational translocation of prepro-alpha factor requires the products of several other *SEC* genes. Four of these seven gene products are essential for protein translocation (*SEC61*, *SEC62*, *SEC63*, *SSS*1), with the remaining three being encoded by nonessential genes (*SEC71*, *SEC72*, and *SBH*1). A fifth essential gene, *KAR2*, encodes a lumenal chaperone also known to be required for protein translocation into the ER. To determine whether tail-anchored protein insertion might also be dependent on these same essential gene products, we used membranes derived from various mutant strains with severe constitutive defects in posttranslational translocation. Additionally, we analyzed the insertion of Nyv1p into microsomes derived from *kar2−159* strain that show irreversible translocation defects after shift to their restrictive temperature (*35*). Microsomes prepared from *sec61*Δ4−24, Δ*ssh* (see below), *sss1*, *sec62−1*, *sec63−301*, and *kar2−159* mutant strains all exhibited severe defects in the posttranslational translocation of prepro-alpha factor (Figure 7B). Similarly, membranes prepared from cells depleted of Sss1p following repression of the regulatable

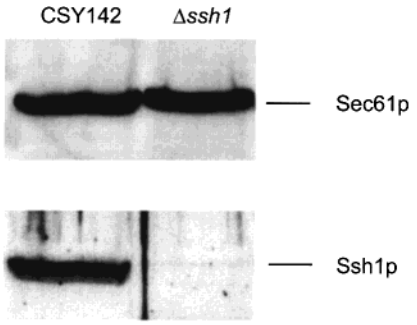

FIGURE 6: Western blot analysis of *ssh*1 membranes. 0.25 A_{280} unit of wild-type membranes (CSY142) or *ssh1* null membranes (Δ*ssh1*) was run on 11% PAGE, transferred to nitrocellulose membranes, and probed for the indicated proteins by western blotting. Bands were visualized by ECL.

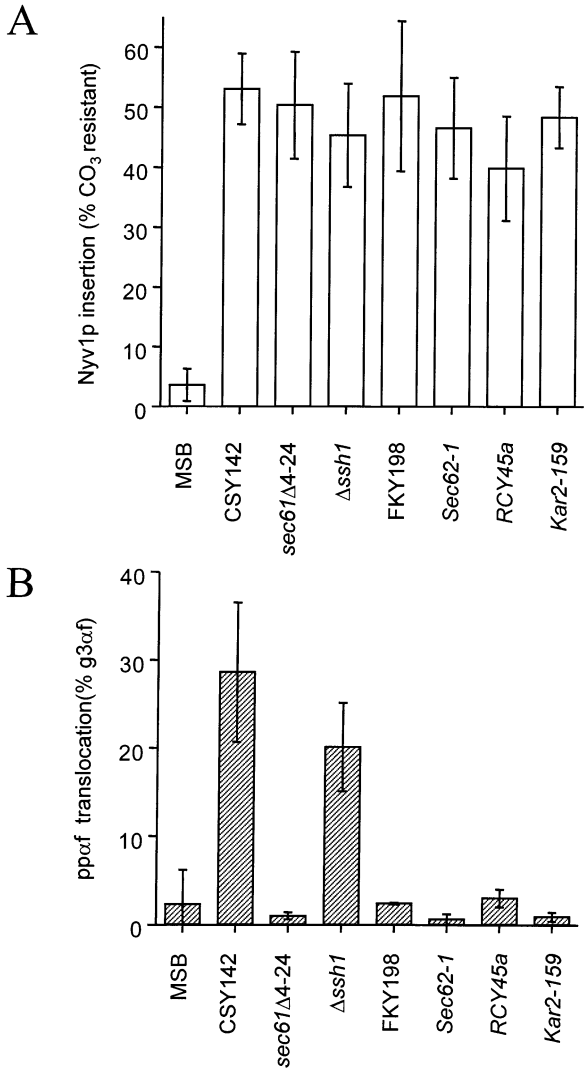

FIGURE 7: Nyv1p inserts into various mutant membranes. (A) ^{35}S-labeled Nyv1p was incubated with microsomes prepared from various strains as described under Experimental Procedures; in each case, the level of carbonate-resistant insertion was calculated (expressed as a percentage of the total). (B) ^{35}S-labeled prepro-alpha factor was incubated with duplicate samples of microsomes. Quantitation of protease-protected forms of α-factor is shown (translocation in wild-type expressed as a 100%).

Gal1 promoter were also severely defective in prepro-alpha factor translocation (Figure 7B). In contrast, Nyv1p insertion into duplicate membranes was unaffected in *sec61*Δ4−24, Δ*ssh* (see below), *sss1*, *sec62*−*1*, *sec63*−*301*, and *kar2*−*159* membranes (Figure 7A). From these results, we conclude that none of the essential genes required for secretory protein translocation into the yeast ER play a major role in Nyv1p insertion. In all cases, insertion into mutant membranes remains sensitive to protease treatment, and to ATP-depletion, from which we conclude that the insertion pathway is entirely unaffected by the mutations (data not shown).

Tail-Anchored Protein Insertion Is Independent of Ssh1p. Ssh1p was first identified by virtue of its sequence similarity to the Sec61 protein (*29*). Further studies have shown that *ssh1* mutant cells are substantially defective in protein translocation into the ER (*36*). We therefore decided to examine the effects of Ssh1p on tail-anchored protein insertion. Membranes were first prepared from an *ssh1* deletion mutant strain, and the absence of Ssh1p was confirmed by western blotting (Figure 6). These membranes were only slightly reduced in their capacity to translocate prepro-alpha factor, confirming that the Sec61p translocon represents the major posttranslational protein translocation channel in yeast membranes (*36*). However, these mutant membranes were fully competent for Nyv1p insertion, indicating that the Ssh1 protein is not essential for this process. Importantly, we have also tested *sec61*, *ssh1* double mutant membranes and have found that these are also fully competent for insertion (data not shown) from which we conclude that neither translocon is involved in tail-anchored protein insertion. These data clearly demonstrate that prepro-alpha factor and Nyv1p use different mechanisms for insertion and that tail-anchored protein insertion does not require either Sec61p or Ssh1p. Additionally, strains deleted in the nonessential components of the translocons, Δ*sbh1*, Δ*sbh2*, or Δ*sbh1*Δ*sbh2*, displayed no defect in either Nyv1p insertion or prepro-alpha factor translocation (data not shown).

DISCUSSION

The vacuolar v-SNARE, Nyv1p, used in this study is localized to vacuoles where it is required for vacuolar vesicle fusion (*37*). We show that, like other SNARE proteins (*3*, *38*), Nyv1p can insert into ER-enriched microsomes, before being transported to its cellular destination. Insertion of tail-anchored proteins was originally thought to be determined by hydrophobic interactions such as those observed for binding of Cyt*b5* and Bcl-2 (*39*, *40*). However, the findings presented here indicate that only a small proportion of insertion of Nyv1p (5%) is directly to membrane lipids, suggesting that hydrophobic interactions only play a minor role in tail-anchored protein insertion.

Stable insertion of Syb2 into mammalian dog pancreas microsomes occurs via an ATP-dependent and protease-sensitive receptor-mediated process (*3*, *38*). In this study, we demonstrate similar requirements for the insertion mechanism into yeast membranes. Interestingly, Nyv1p insertion shows only a partial ATP-dependence which is similar to that previously described for import of tail-anchored proteins into mitochondria and which may reflect the activity of cytosolic chaperones interacting with precursors prior to insertion (*41*). Furthermore, we find that the mammalian Syb2 protein inserts into yeast membranes with similar efficiency to Nyv1p, indicating substantial functional conservation of the insertion mechanism from yeast to mammals.

These findings suggest that common receptor molecules may be responsible for membrane insertion of this class of tail-anchored proteins in yeast and mammalian cells.

In mammalian cells, depletion of the trimeric Sec61-complex had no effect upon tail-anchored protein insertion. We demonstrate that, in yeast, the Sec61-complex (Sec61p, Sbh1p, Sss1p) is not required for tail-anchored protein insertion and extend these findings to include the Ssh1p-complex (Ssh1p, Sbh2p, Sss1p). These findings suggest that an aqueous pore-forming translocon is not required for the integration of tail-anchored proteins into the yeast ER. The fact that only a very few residues of any tail-anchored protein are required to cross the bilayer may explain the lack of a requirement for the translocon complexes.

Tail anchor protein insertion has been shown to occur posttranslationally, at least in vitro, and our study demonstrates that this is also the case in yeast. Previous studies have demonstrated a role for the Sec63-complex (Sec62p, Sec63p, Sec71p, Sec72p) in a posttranslational translocation mechanism for secretory precursors (*17*−*21*), with Sec62p suggested to be required for membrane targeting of precursors (*42*). The essential components of this complex have been shown to have homologues in mammalian ER although their function(s) has (have) yet to be established (*43*, *44*). To test whether this posttranslational targeting and insertion machinery was involved in tail-anchored protein insertion, we prepared membranes from various mutant strains of yeast. We can confirm that prepro-alpha factor translocation is dependent upon Sec61p, Sec62p, Sec63p, Sss1p, and Kar2p (Figure 7B). In contrast, Nyv1p insertion into duplicate membranes occurs with similar efficiency to wild-type microsomes (Figure 7A). These results conclusively demonstrate that tail-anchored protein targeting and/or insertion occurs independently of these mutations and so must occur via a different mechanism from that involved in the translocation of prepro-alpha factor.

This study has examined in detail the mechanism of insertion of tail-anchored precursors into yeast ER and defined a posttranslational insertion process that involves components distinct from the classical SEC machinery.

ACKNOWLEDGMENT

We thank Barrie Wilkinson and Martin Willer for providing strains and reagents and Nick Bryan for use of the Zetasizer 3000 HSa.

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BI026105R