Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 8468–8472, December 1987
Cell Biology

Sec53, a protein required for an early step in secretory protein processing and transport in yeast, interacts with the cytoplasmic surface of the endoplasmic reticulum

(secretory mutant/*Saccharomyces cerevisiae*)

HANNELE RUOHOLA AND SUSAN FERRO-NOVICK*

Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510

Communicated by George E. Palade, August 10, 1987

ABSTRACT **The *sec53* mutant is a conditional lethal yeast secretory mutant. At 37°C, precursors to exported proteins become firmly attached to the endoplasmic reticulum membrane and are not released into the lumen in a soluble form. The accumulated precursors are insoluble in the detergent Triton X-100; however, urea, a known protein denaturant, solubilizes them. Using antibody directed against the Sec53 protein, we found that a substantial portion of the Sec53 protein is associated with the cytoplasmic surface of the endoplasmic reticulum membrane. Membrane-bound Sec53 protein is largely insoluble in Triton X-100, but the protein is effectively released from the membrane by urea. We propose that the Sec53 protein may be a member of a complex of proteins required for an early step in protein processing and transport.**

A biochemical dissection of heterologous *in vitro* translocation systems in higher eukaryotes has led to the identification of two components that play a role in targeting proteins to the endoplasmic reticulum (ER): signal recognition particle and docking protein, also called the signal recognition particle receptor (1–3). Based on *in vitro* findings the signal recognition particle has been proposed to recognize the signal sequence of exported proteins and target them to the signal recognition particle receptor, which resides on the ER membrane. Translocation across the bilayer is believed to occur through an aqueous tunnel (4).

Genetic studies in yeast have identified two genes, *SEC53* and *SEC59*, whose products have been implicated in protein translocation across the ER membrane (5, 6). These mutants rapidly and dramatically block the transit of all major cell-surface proteins at the restrictive temperature of 37°C, but not at the permissive temperature of 24°C. DNA sequence analysis has shown that the *SEC53* gene product is a hydrophilic protein; hydropathic analysis has not revealedany hydrophobic regions long enough to span the lipid bilayer (7). In this report we show that two pools of Sec53 protein (Sec53p) exist in wild-type cells, a soluble fraction and a membrane-bound fraction. Membrane-bound Sec53p is attached to the cytoplasmic surface of the ER membrane. Our data suggest that this localization is mediated by a protein–protein interaction. The precursors to exported proteins that accumulate in the *sec53* mutant at 37°C also appear to adhere to the ER membrane as a result of a protein–protein interaction.

MATERIALS AND METHODS

Buffers. Phosphate-buffered saline (PBS) is 0.2 M sodium chloride/12.5 mM potassium phosphate, pH 7.6. Dilution buffer is PBS with 2% Triton X-100 plus aprotinin (100 units/ml). Spheroplast medium is 1.4 M sorbitol/50 mM potassium phosphate, pH 7.5/10 mM sodium azide/56 mM β-mercaptoethanol containing zymolyase (1 mg/ml). Sorbitol cushion is 1.7 M sorbitol/20 mM sodium phosphate, pH 7.5. Lysis buffer is 0.3 M mannitol/10 mM Mops, pH 7.0, containing *N,N'*-diphenyl-*p*-phenylenediamine (0.1 μg/ml), chymostatin (1 μg/ml), and aprotinin (100 units/ml).

Antibody to the *SEC53* Gene Product. The *SEC53* gene was cloned from a CEN library by complementation of the *sec53* growth defect. A 1.7-kb *Bgl* II–*Bgl* II fragment containing the *SEC53* gene was subcloned into pRB307 (Ura^+ 2μ Amp^r) to yield pNCW-E1 (Ura^+ 2μ Amp^r *SEC53*). A *SEC53–trpE* gene fusion was constructed by cloning the *Kpn* I–*Sca* I fragment of pNCW-E1 into the pATH3 (*trpE, bla*) vector (T. J. Koerner and A. Tzagoloff, unpublished results). To obtain anti-Sec53p antibody, the hybrid protein was purified (8) and injected into the popliteal lymph nodes of New Zealand White rabbits (9).

Cell Fractionation. Wild-type yeast grown at 24°C were labeled with [^{35}S]sulfate for 6 hr at 24°C as described (7). Radiolabeled cells (100 OD_{599} units) were centrifuged at 4°C and washed once with 10 mM sodium azide. Pellets were resuspended in 1 ml of spheroplast medium and converted to spheroplasts during a 1-hr incubation at 38°C. The spheroplast mixture was layered onto a sorbitol cushion (3 ml) and centrifuged for 10 min at 5110 × *g* in a SS34 rotor. The spheroplasts were resuspended in lysis buffer (4 ml per 100-OD_{599} unit cell equivalents) with a Pasteur pipette, homogenized five times using a 2-ml Wheaton tissue grinder, and centrifuged at 3000 × *g* for 6 min. To obtain a good yield of ER membranes, this low-speed spin had to be done immediately after lysis. The low-speed pellet (LSP) was resuspended in 60% (wt/wt) sucrose (3 ml per 100-OD_{599} unit cell equivalents) and homogenized six times as described above. Membranes were subfractionated in a sucrose gradient using a modification of the procedure described by Knipe *et al.* (10). The LSP in 60% sucrose (2.5 ml) was placed at the bottom of an SW41 ultracentrifuge tube and overlaid with the following sucrose solutions: 1 ml of 55% sucrose; 1.5 ml each of 50%, 45%, 40%, and 35% sucrose; and 2 ml of 30% sucrose. All sucrose solutions were buffered with 10 mM Hepes, pH 7.2. The gradient was centrifuged at 3°C for 12 hr at 170,000 × *g* to reach isopycnic conditions. A total of 23 fractions, each ≈500 μl, were collected using an autodensi-flow instrument (Buchler Instruments Div., Nuclear-Chicago, Fort Lee, NJ). $NaDodSO_4$ was added (final concentration 1%) to a portion of each fraction, and the samples were heated for 5 min at 100°C. The $NaDodSO_4$ boiled samples were used to immunoprecipitate Sec53p. The density of sucrose in each fraction was determined by refractometry.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ER, endoplasmic reticulum; Sec53p, Sec53 protein; LSP, low-speed pellet; LSS, low-speed supernatant.
*To whom reprint requests should be addressed.

Immunoprecipitation and Electrophoresis. Aliquots of radiolabeled samples boiled in 1% NaDodSO$_4$ were diluted 50- to 100-fold with 1 ml of dilution buffer and centrifuged for 15 min in an Eppendorf microfuge at 4°C. The supernatant (0.9 ml) was removed, and one of the following antibodies was added: 2 μl of invertase antiserum per 1-OD$_{599}$ unit cell equivalent; or 3 μl of anti-Sec53p antibody per 0.2-OD$_{599}$ unit cell equivalent; or 1 μl of carboxypeptidase Y antiserum per 0.4-OD$_{599}$ unit cell equivalent. Samples were incubated at 0°C for 12–16 hr, and then Protein A-Sepharose (80 μl of a 10% solution per 2 μl of serum) was added to precipitate immune complexes. After a 90-min incubation at 4°C, the Protein A-Sepharose was washed as described (5). Samples were subjected to NaDodS$_4$/gel electrophoresis (5, 11), fluorographed, and quantitated by densitometric scanning.

Enzyme Assays and Other Procedures. NADPH-cytochrome-*c* reductase was assayed as described by Kreibich *et al.* (12). Vanadate-sensitive ATPase was assayed as described (13). Cytochrome *c* oxidase, with 0.3% Lubrol WX, was assayed as described by Mason *et al.* (14). Invertase was assayed as previously described (15). The assay for α-mannosidase was based on the method of Tulsiani *et al.* (16) with the following modifications: the substrate concentration was 0.8 mM, and the buffer was pH 6.5, as described by Opheim (17) for yeast α-mannosidase. Protein was measured using the fluorescamine protein assay (18); bovine serum albumin was used as a standard. Cytoplasmic invertase was purified as described (15), and antibody was produced (9).

RESULTS

A Significant Portion of the *SEC53* Gene Product Is Membrane Bound. To begin our analysis of the *SEC53* gene product we produced antibody to Sec53p and localized it in wild-type cells. To do this cells were labeled with [^{35}S]sulfate for 6 hr, converted to spheroplasts, and lysed osmotically. The lysate was centrifuged at 100,000 × *g* for 1 hr, and the distribution of Sec53p in the lysate, soluble, insoluble (membrane), and periplasmic fractions was determined by quantitative immunoprecipitation. Our findings indicate that the 29-kDa Sec53p is present in both the soluble and insoluble fractions (Fig. 1*B*). None is present in the periplasmic fraction (Fig. 1*A*), indicating that this protein is not secreted. Densitometric scanning of these samples indicates that a substantial amount of the Sec53p (≈80%) is in the insoluble (membrane) fraction; the remaining portion (≈20%) is in the soluble fraction. We have repeated this fractionation many times and always find ≈50–80% of the Sec53p in the insoluble fraction and 20–50% in the soluble fraction. When the soluble fraction is analyzed on a sucrose velocity gradient, most of the Sec53p migrates with the approximate S value expected for the soluble form of this protein (unpublished results). Thus, the majority of Sec53p in the 100,000 × *g* supernatant is truly soluble and unlikely to have resulted from membrane fragmentation. If cells are pulse labeled for 2 min and the same fractionation experiment is done, Sec53p distribution is found to be approximately the same as in the steady-state situation (data not shown). Thus, when Sec53p is quantitatively immunoprecipitated from wild-type cell fractions, we always find a significant portion in the insoluble (or membrane) fraction.

Intracellular Location of the Sec53p. We developed a cell-fractionation protocol to determine the intracellular location of the Sec53p in wild-type cells. This protocol consists of four steps: (*i*) enzymatic removal of the yeast cell wall; (*ii*) osmotic lysis of spheroplasts; (*iii*) low-speed centrifugation; and (*iv*) centrifugation in a sucrose step gradient.

Radiolabeled yeast cells were converted to spheroplasts and lysed osmotically. The lysate was spun at 3000 × *g* to yield a low-speed pellet (LSP) and low-speed supernatant (LSS). The distribution of marker enzymes was monitored in these two fractions (Table 1). In addition, aliquots of the lysate, LSS, and LSP were treated with NaDodSO$_4$ and subjected to immunoprecipitation with anti-Sec53p antibody and invertase antibody (Table 1). Cytoplasmic invertase was used as a cytoplasmic marker protein. Yeast cells synthesize two forms of invertase—a constitutively expressed cytoplasmic form and a secreted glycosylated form that is under hexose repression (19). Cytoplasmic invertase was only detected in the soluble fraction. Most of the cellular protein, the majority of the plasma membrane-marker enzyme (vanadate-sensitive ATPase), and vacuolar membrane-marker enzyme (α-mannosidase) were also recovered in the LSS. The ER membrane-marker enzyme and the mitochondrial-marker enzyme (cytochrome *c* oxidase) were enriched in the LSP. Also present in the LSP was a substantial portion of the Sec53p (60%).

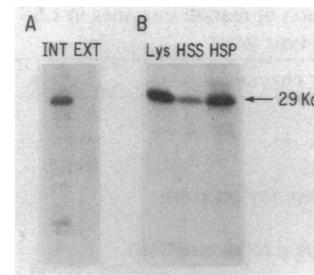

FIG. 1. Sec53p is predominantly membrane-bound. (*A*) Wild-type cells, SFNY33-2C (MAT*a ura3-52 his4-619*), carrying a multicopy invertase-encoding plasmid (19), pRB58 (Ura$^+$ 2μ Amp^r *SUC2*), were radiolabeled with [^{35}S]sulfate for 6 hr at 24°C and converted to spheroplasts. The spheroplasts were spun and divided into two fractions: internal fraction, INT (contents retained in the cell); and external fraction, EXT (contents secreted into the periplasm). Sec53p was immunoprecipitated from each fraction, and solubilized immunoprecipitates were electrophoresed in a 12.5% NaDodSO$_4$/polyacrylamide slab gel (11) and fluorographed. (*B*) Wild-type cells were radiolabeled, converted to spheroplasts, and lysed. The lysate was spun at 100,000 × *g* for 1 hr, and the pellet was resuspended in the original volume. Sec53p was quantitatively immunoprecipitated with anti-Sec53p antibody from three different fractions: Lys, lysate or total sample; HSS, high-speed supernatant; and HSP, high-speed pellet. Solubilized immunoprecipitates were electrophoresed and fluorographed. When wild-type cells are transformed with a 2μ plasmid containing *SEC53* gene at high copy, a protein of the same molecular weight is overproduced.

To determine whether the ER-marker enzyme cofractionates with Sec53p, the LSP was subfractionated in a sucrose density gradient. Fig. 2 shows that the Sec53p and NADPH-cytochrome *c* reductase activity migrate as peaks that are coincident. The mitochondrial-marker enzyme and the plasma membrane ATPase are clearly separated from the NADPH-cytochrome *c* reductase activity and Sec53p (Fig. 2). Therefore, by this criterion membrane-bound Sec53p is specifically associated with the ER membrane and not with other intracellular membranes.

We have also performed the same fractionation experiment with the *sec53* mutant. Less Sec53p appears to be present in the mutant at the restrictive temperature than in wild type, and nearly 100% is reproducibly found in the membrane fraction. Subfractionation of membranes has revealed that the density of mutant ER is altered at 37°C (significantly less dense than wild-type ER), while the distribution of other membranes is unchanged. Both Sec53p and the ER-marker enzyme continue to cofractionate with each other in a sucrose density gradient. At the permissive temperature the distribution of Sec53p is the same as in wild-type cells (data not shown).

Table 1. Distribution of marker enzymes in LSS and LSP fractions from wild-type yeast

Marker enzyme	Lysate	LSS	LSP
Protein,			
mg per fraction	24.7	23.2	2.3
% of lysate	100	94	9
Cytoplasmic invertase (cytoplasm),			
% of lysate*	100	119	ND
NADPH-cytochrome *c* reductase (ER),			
units†	0.422	0.097	0.303
% of lysate	100	23	72
Vanadate-sensitive ATPase (PM),			
units‡	2.855	1.514	1.224
% of lysate	100	53	43
Cytochrome *c* oxidase (mitochondria),			
units§	398	22	368
% of lysate	100	6	92
α-Mannosidase (vacuole),			
units¶	2.540	1.749	0.879
% of lysate	100	69	35
Sec53p,			
% of lysate*	100	40	60

ND, not detected; PM, plasma membrane. Data represent results from three separate experiments. The distribution of Sec53p has been measured in 17 separate experiments (mean is 50% in LSS and 50% in LSP).

Units of activity for each enzyme are expressed as follows: *Levels of cytoplasmic invertase and Sec53p were determined by immunoprecipitation and densitometric scanning of autoradiograms. †NADPH-cytochrome *c* reductase: μmol of cytochrome *c* reduced per min per fraction. ‡Vanadate-sensitive Mg^{2+} proton transporting ATPase: μmol of phosphate produced per min per fraction. §Cytochrome *c* oxidase: nmol of cytochrome *c* oxidized per min per fraction. ¶α-Mannosidase: nmol of *p*-nitrophenyl α-D-mannopyranoside hydrolyzed per min per fraction.

Topology of Membrane-Associated Sec53p. Membrane-associated Sec53p may be located on the cytoplasmic or luminal surface of the ER membrane. Protease protection experiments were done to address this question. To do these experiments we needed a soluble ER protein to assess the integrity of the ER membrane during cell lysis, membrane isolation, and proteolysis treatment. For this reason the *sec18* mutant, rather than wild type, was used. At 37°C the *sec18* mutation blocks protein transport from the ER to the Golgi complex. As a result, the secreted glycoprotein invertase accumulates within the lumen of the ER (20). The accumulated invertase was used as a luminal marker.

A LSP fraction was prepared as described in the legend for Fig. 3. Distribution of Sec53p in the LSS and LSP fractions is the same in *sec18* and wild-type cells (data not shown). The LSP was treated with trypsin with or without detergent. Aliquots were removed to measure invertase activity, and the remaining portion was processed for immunoprecipitation using anti-Sec53p antibody. No invertase activity was lost during protease treatment, and most of the invertase (62 ± 7%) that accumulated within the lumen of the ER was not accessible to substrate (sucrose) before or after trypsin treatment. Thus, during proteolysis with trypsin (10–500 μg/ml), most microsomes were intact, and luminal contents were not accessible to trypsin. Although most accumulated invertase was sequestered within the lumen of the ER during proteolysis, all Sec53p was susceptible to degradation by protease. Sec53p was effectively degraded by 200 μg/ml of trypsin (Fig. 3), and the pattern of digestion was the same with or without detergent. Control experiments indicated that the degradation of Sec53p was dependent on trypsin (Fig. 3). Thus, it appears that the Sec53p is associated with the cytoplasmic surface of the ER membrane.

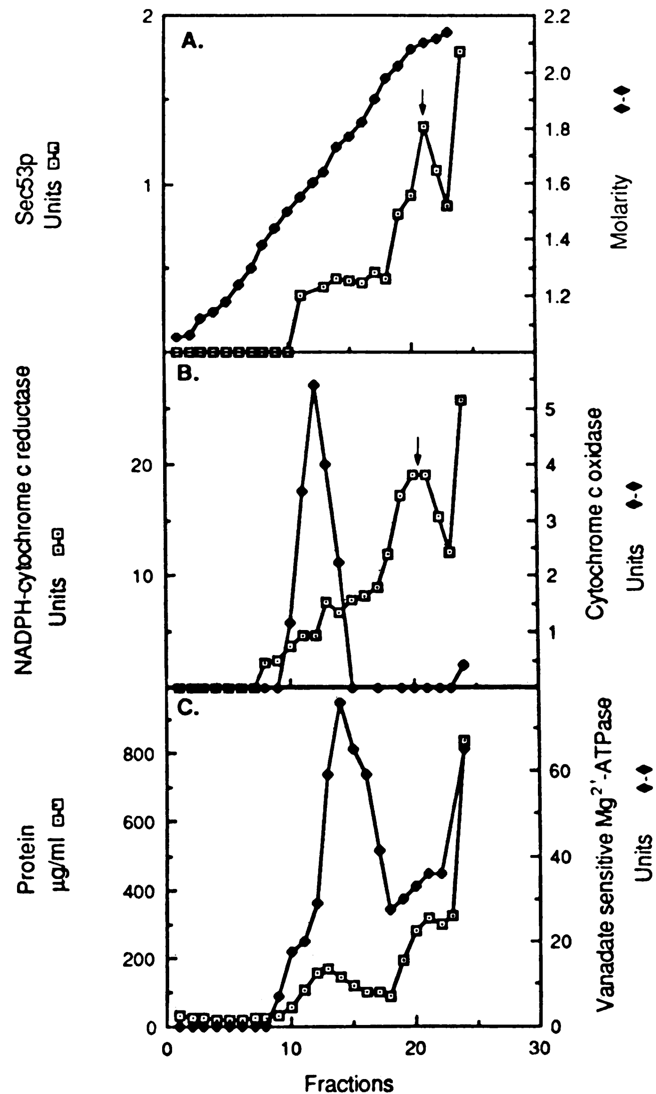

FIG. 2. Sec53p copurifies with NADPH–cytochrome *c* reductase activity. Wild-type cells (SFNY33-2C/pRB58) were radiolabeled and fractionated as described in *Materials and Methods*. Units are expressed as follows: the concentration of Sec53p was determined by densitometric scanning of autoradiograms and is expressed in arbitrary units; molarity of sucrose; NADPH–cytochrome *c* reductase (μmol of cytochrome *c* reduced per min per ml); cytochrome *c* oxidase (nmol of cytochrome *c* oxidized per min per ml); vanadate-sensitive Mg^{2+} proton transporting ATPase (μmol of phosphate produced per min per 0.7 ml).

Solubilization of Membrane-Associated Sec53p. To investigate the interaction between Sec53p and the ER membrane, various reagents were used to solubilize this protein. Wild-type cells were radiolabeled, converted to spheroplasts, and lysed osmotically. A LSP fraction was generated and incubated with different reagents. Following treatment, samples were spun at 100,000 × *g* to generate a soluble and insoluble fraction. Anti-Sec53p antibody was used to quantitatively immunoprecipitate Sec53p from these fractions. Because the membranes are not washed before treatment with these reagents, the small amount that is recovered in the soluble fraction may represent soluble Sec53p that is loosely attached to the surface of the ER membrane. After treatment with either low or high salt (either KCl or NaCl), Triton X-100, or deoxycholate, the majority of the Sec53p was recovered in the membrane fraction (Fig. 4 and Table 2). The Sec53p was not solubilized by Triton X-114 either; although sodium

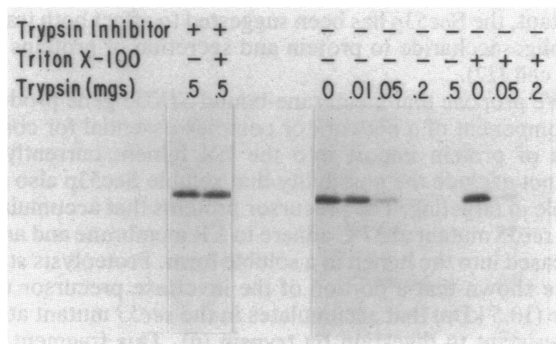

FIG. 3. Sec53p is on the cytoplasmic surface of the ER membrane. *sec18* mutant cells, NY 431 (MAT*a ura3-52 sec18-1*), were radiolabeled with [^{35}S]sulfate for 6 hr at 24°C. At the end of the incubation, cells were spun, resuspended in prewarmed minimal medium containing 0.1% glucose (to derepress the synthesis of invertase), 25 μM ammonium sulfate, [^{35}S]sulfate, and the incubation was continued for 1 hr at 37°C. Cells were converted to spheroplasts, lysed osmotically, and a LSP was prepared. Samples ($\approx$0.7 mg/ml of protein) were treated with varying concentrations of trypsin (0.01–0.5 mg/ml) for 1 hr on ice, with or without Triton X-100 (0.1%) or trypsin inhibitor (0.9 mg/ml). At the end of incubation, trypsin inhibitor was added; aliquots were removed for invertase assays, and the remaining portion of the sample was boiled with 1% $NaDodSO_4$. Immunoprecipitation, with anti-Sec53p antibody, $NaDodSO_4$/gel electrophoresis, and fluorography were done.

carbonate, pH 11.5, was tested, degradation of the protein prevented any conclusions (data not shown). The combination of Triton X-100 and high salt, however, released a substantial portion of this protein into the 100,000 × *g* supernatant; urea, a protein denaturant that is known to destroy protein–protein interactions, released most Sec53p into the supernatant (Fig. 4 and Table 2).

Solubilization of Accumulated Precursors in the *sec53* Mutant. Transported proteins that accumulate in the *sec53* mutant at 37°C are firmly associated with the ER membrane. They are not extracted by high salt (H.R. and S.F.-N., unpublished results) and are largely insoluble in Triton X-100 (6). As discussed above, Sec53p has similar solubility properties (Fig. 4 and Table 2). We determined whether urea could release invertase and carboxypeptidase Y from the ER of *sec53* mutant cells.

The *sec53* mutant accumulates a heterogeneous form of invertase that has an apparent mass of 60 to 64.5 kDa (5). This form of invertase is present in the LSP fraction (Fig. 5*A*). The constitutively synthesized cytoplasmic form of invertase is

Table 2. Solubilization of the Sec53p from the LSP

	S, %	P, %
No salt	16	84
Low salt (0.1 M KCl)	8	92
High salt (0.5 M KCl)	20	80
Triton X-100 (1%)	29	71
Triton X-100 + high salt	60	40
Deoxycholate (1%)	28	72
Urea (5 M)	93	7

The level of Sec53p in the supernatant (S) and pellet (P) fractions was determined by quantitative immunoprecipitation and densitometric scanning of autoradiograms. SD for the LSP fractions is ±15%. Data represent results from four separate experiments.

only found in the LSS (Fig. 5*A*). The zymogen form of the vacuolar protease carboxypeptidase Y (5, 21) is also found in the LSP (Fig. 5*B*). The LSP was treated with 5 M urea, spun to separate soluble and insoluble subfractions, and invertase and carboxypeptidase were immunoprecipitated from each fraction. Urea solubilized the invertase and carboxypeptidase Y that accumulated in the LSP fraction (Fig. 5 *A* and *B*). Thus, although the precursors to exported proteins that accumulate in the *sec53* mutant at 37°C are largely Triton insoluble, urea effectively releases them from the ER membrane.

DISCUSSION

Precursors to exported proteins accumulate in the *sec53* mutant at 37°C. These precursors are firmly attached to the ER membrane and are not released into the lumen. Solubilization (ref. 6 and Fig. 5) studies suggest this attachment is a result of a protein–protein interaction. When the *sec53* mutant is shifted to the permissive temperature, in the presence of cycloheximide, the accumulated precursors are secreted (6). The reversible nature of this secretion defect implies that the *sec53* block is a functional intermediate on the secretory pathway.

Using antibody directed against the Sec53p we found two pools of Sec53p in wild-type cells: soluble and membrane-bound fractions (Fig. 1*B*). Membrane-bound Sec53p is attached to the cytoplasmic surface of the ER membrane (Fig. 3); our data suggest this attachment is mediated by a protein–protein interaction (Fig. 4 and Table 2). In the *sec53* mutant, protein transport is blocked at the restrictive temperature and the soluble pool of Sec53p is largely depleted.

Because Schekman and colleagues (7) previously reported that the *SEC53* gene product is a cytoplasmic protein, we

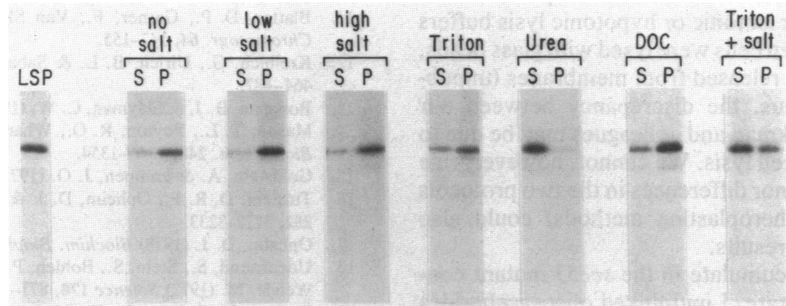

FIG. 4. Solubilization of the Sec53p from the LSP. Wild-type cells were radiolabeled, converted to spheroplasts, lysed, and spun to generate a LSP ($\approx$0.1 mg/ml of protein). Membranes were resuspended in lysis buffer and aliquoted into several different tubes. Each tube contained 250 μl of membranes and an equal volume of the following reagents [prepared in 10 mM Mops, pH 7.0, containing chymostatin (1 μg/ml) and aprotinin (100 units/ml)]: Mops buffer/0.2 M KCl/1 M KCl/2% Triton X-100/10 M urea/2% deoxycholate (DOC); 2% Triton X-100 plus 1 M KCl. Samples were mixed, incubated on ice for 10 min, spun at 100,000 × *g* for 1 hr, and separated into two fractions: S, supernatant; and P, pellet. The pellet was resuspended in the original volume of solubilization buffer, $NaDodSO_4$ was added to all fractions (final concentration of 1%), and samples were heated to 100°C for 5 min. Sec53p was immunoprecipitated from each fraction, and solubilized immunoprecipitates were electrophoresed and fluorographed. No salt, Mops buffer; low salt, 0.1 M KCl; and high salt, 0.5 M KCl.

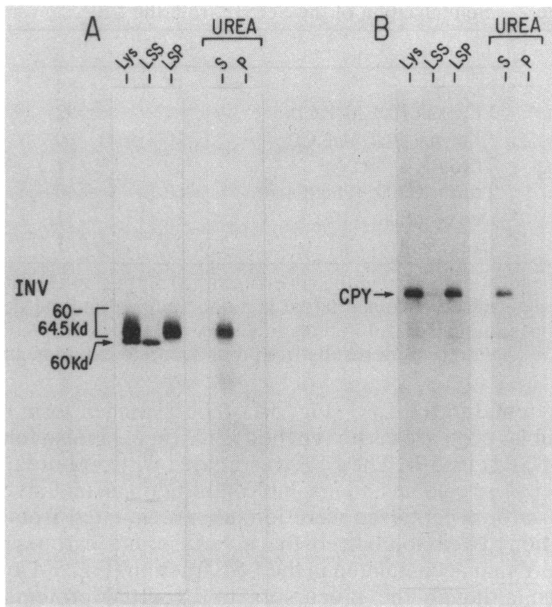

FIG. 5. Precursors that accumulate in the *sec53* mutant are solubilized by urea. (*A*) *sec53-6* mutant cells, SFNY28-13B (MAT*a* *ura3-52 his4-619 sec53-6*) carrying pRB58, were labeled at 37°C as described (5). Cells were converted to spheroplasts, lysed, and spun to generate two fractions, LSS and LSP. Anti-invertase antibody was used to immunoprecipitate invertase (INV) from the lysate Lys, LSS, and LSP. The LSP was treated with urea as described in the legend for Fig. 4. Samples were spun at 100,000 × *g* for 1 hr and separated into two fractions: S, supernatant; and P, pellet. Anti-invertase antibody was used to immunoprecipitate invertase from each fraction. Solubilized immunoprecipitates were electrophoresed in a 10% $NaDodSO_4$/polyacrylamide slab gel (5) and fluorographed. (*B*) Conditions are the same as in Fig. 5*A* except that anti-carboxypeptidase Y (CPY) antibody was used to immunoprecipitate CPY from the Lys, LSS, LSP, high-speed supernatant (S), and high-speed pellet (P) fractions.

repeated their fractionation protocol to assess this discrepancy. Although their protocol is similar to ours in several ways, there are some significant differences: (*i*) in the earlier work, before membrane-fraction isolation, a low-speed spin was done to remove unbroken cells, and that pellet was discarded; (*ii*) an isotonic lysis buffer was used; (*iii*) spheroplasts were lysed by mixing with glass beads on a Vortex mixer. When we repeated their fractionation protocol, most Sec53p was in the soluble fraction. In addition, with our fractionation protocol the distribution of Sec53p was identical, regardless of whether isotonic or hypotonic lysis buffers were used. However, when cells were lysed with glass beads, Sec53p was more readily released from membranes (unpublished observations). Thus, the discrepancy between our results and those of Schekman and colleagues may be due to the different methods of cell lysis. We cannot, however, rule out the possibility that minor differences in the two protocols (for example, in the spheroplasting methods) could also account for the differing results.

The precursors that accumulate in the *sec53* mutant contain little or no carbohydrate. Lipid-linked oligosaccharides are synthesized in this mutant at the restrictive temperature; yet, the pattern is somewhat aberrant (22). Because *sec53* mutant also blocks the export of proteins whose transport is not dependent on the addition of carbohydrate (5), the effects on protein export are unlikely to result from a failure to glycosylate proteins. Given the phenotype of the *sec53* mutant, the Sec53p has been suggested to affect both transfer of oligosaccharide to protein and secretion of proteins from the cell (22).

We propose that membrane-bound *SEC53* gene product is a component of a network or complex essential for completion of protein import into the ER lumen; currently, we cannot exclude the possibility that soluble Sec53p also plays a role in targeting. The precursor proteins that accumulate in the *sec53* mutant at 37°C adhere to ER membrane and are not released into the lumen in a soluble form. Proteolysis studies have shown that a portion of the invertase precursor molecule (10.5 kDa) that accumulates in the *sec53* mutant at 37°C is resistant to digestion by trypsin (6). This fragment sediments with yeast microsomes (S.F.-N., unpublished results) and may represent the region of the protein that holds invertase onto the membrane. These studies have not revealed the exact topology of the precursors that accumulate in the *sec53* mutant (6, 7). The precise role of the *SEC53* gene product also awaits definition.

Note Added in Proof. It was recently reported (23) that precursors accumulated in the *sec53* mutant are protease resistant. Because these precursors are not soluble (6) several interpretations of these data are possible. *In vitro* translocation studies may allow a more reliable conclusion.

We thank Nancy Walworth and Joe Liu for cloning the *SEC53* gene; Peter Novick for NY431 and the *CEN* library; Alex Tzagoloff for the pATH3 vector; Joel Rothman and Tom Stevens for providing anticarboxypeptidase antibody; and Anna Newman, Nancy Walworth, and Jaakko Saraste for stimulating discussions. We also thank Ari Helenius for advice, and we especially thank George Palade for many helpful discussions and his support and encouragement during the course of this work. This work was supported by National Institutes of Health Grant GM 35421-02 and by Biomedical Research Support Grant RR 05358 awarded to S.F.-N. from the Biomedical Research Support Grant Program, Division of Resources, National Institutes of Health. H.R. is a recipient of a Nordic Yeast Research Program Predoctoral Fellowship; S.F.-N. is a recipient of a Swebilius Cancer Research Award.

1. Walter, P., Gilmore, R. & Blobel, G. (1984) *Cell* **38**, 5–8.
2. Walter, P. & Lingappa, V. R. (1986) *Annu. Rev. Cell Biol.* **2**, 499–516.
3. Hortsch, M. & Meyer, D. I. (1984) *J. Cell Biol.* **52**, 1–8.
4. Gilmore, R. & Blobel, G. (1985) *Cell* **42**, 497–505.
5. Ferro-Novick, S., Novick, P., Field, C. & Schekman, R. (1984) *J. Cell Biol.* **98**, 35–43.
6. Ferro-Novick, S., Hansen, W., Schauer, I. & Schekman, R. (1984) *J. Cell Biol.* **98**, 44–53.
7. Bernstein, M., Hoffmann, W., Ammerer, G. & Schekman, R. (1985) *J. Cell Biol.* **101**, 2374–2382.
8. Dieckman, C. L. & Tzagoloff, A. (1985) *J. Biol. Chem.* **260**, 1513–1520.
9. Louvard, D., Reggio, H. & Warren, G. (1982) *J. Cell Biol.* **92**, 92–107.
10. Knipe, D. M., Baltimore, D. & Lodish, H. F. (1977) *J. Virol.* **21**, 1128–1139.
11. Blatter, D. P., Garner, F., Van Slyke, K. & Bradley, A. (1972) *J. Chromatogr.* **64**, 147–155.
12. Kreibich, G., Ulrich, B. L. & Sabatini, D. D. (1978) *J. Cell Biol.* **77**, 464–487.
13. Bowman, B. J. & Slayman, C. W. (1979) *J. Biol. Chem.* **254**, 2928–2934.
14. Mason, T. L., Poyton, R. O., Wharton, D. C. & Schatz, G. (1973) *J. Biol. Chem.* **248**, 1346–1354.
15. Goldstein, A. & Lampen, J. O. (1975) *Methods Enzymol.* **42**, 504–511.
16. Tulsiani, D. R. P., Opheim, D. J. & Touster, O. (1977) *J. Biol. Chem.* **252**, 3227–3233.
17. Opheim, D. J. (1978) *Biochim. Biophys. Acta* **524**, 121–130.
18. Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W. & Weigle, M. (1972) *Science* **178**, 871–872.
19. Carlson, M. & Botstein, D. (1982) *Cell* **28**, 145–154.
20. Schauer, I., Emr, S., Gross, C. & Schekman, R. (1985) *J. Cell Biol.* **100**, 1664–1675.
21. Stevens, T., Esmon, B. & Schekman, R. (1982) *Cell* **30**, 439–448.
22. Huffaker, T. & Robbins, P. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 7466–7470.
23. Feldman, R. I., Bernstein, M. & Schekman, R. (1987) *J. Biol. Chem.* **262**, 9332–9339.